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1 **HDV RNA replication is associated with HBV repression and interferon-**  
2 **stimulated genes induction in super-infected hepatocytes**

3  
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30 **List of abbreviations:**

31 ADAR, adenosine deaminase acting on RNA; aa, amino acid; cccDNA, circular  
32 covalently closed DNA; CHD, chronic hepatitis delta; dHepaRG, differentiated HepaRG;

33 HBV, hepatitis B virus; HDV, hepatitis D virus; HDAg, Hepatitis delta antigen; HIV, human  
34 immunodeficiency virus; hNTCP, human sodium taurocholate cotransporting polypeptide;  
35 IFN, interferon; ISG, interferon stimulated genes; LR- $\beta$  lymphotoxin receptor- $\beta$ ; MOI,  
36 multiplicity of infection; n.s., non-significant; PAMP, pathogen-associated molecular  
37 pattern; pgRNA, pregenomic RNA; PHH, primary human hepatocytes; p.i., post-infection;  
38 PRR, pathogen recognition receptor; PEG, polyethylene glycol; SRB, sulforhodamine B;  
39 TLR ,toll-like receptor; VGE, virus genome equivalent; WHV, woodchuck hepatitis virus.

40

41 **Running title**

42 HDV/HBV interplay in innate immune-competent hepatocytes

43

44 **Key Words:**

45 Hepatitis D virus; hepatitis B virus; viral interference; IFN response; Interferon stimulated  
46 genes

47

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49 No conflict of interest to declare on this work.

50

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65

66

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- 68 - Study concept and design: DA, JL, FZ, PD and DD
- 69 - Acquisition of data: DA, MM, NAS, JL
- 70 - Analysis and interpretation of data: DA, JL, PD and DD
- 71 - Writing of the manuscript: DA, CS, FZ, JL, PD and DD
- 72 - Statistical analysis: DA, BT
- 73 - Technical or material support: JCC and CS

74

## HIGHLIGHTS

75

- A model of super-infection with HDV on HBV-infected hepatocytes was established;

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- HDV infection induces a strong IFN response in these immune-competent hepatocytes;

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- In this model, HDV infection is associated with HBV inhibition, thus access to recapitulating *in vivo* viral interference;

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- This super infection model is also suitable for the evaluation of novel drugs/antivirals, including immune-modulators.

82

83

84 **ABSTRACT**

85 Hepatitis D virus (HDV) super-infection of Hepatitis B virus (HBV)-infected patients is the  
86 most aggressive form of viral hepatitis. HDV infection is not susceptible to direct anti-HBV  
87 drugs, and only suboptimal antiviral responses are obtained with interferon (IFN)-alpha-  
88 based therapy. To get insights on HDV replication and interplay with HBV in  
89 physiologically relevant hepatocytes, differentiated HepaRG (dHepaRG) cells, previously  
90 infected or not with HBV, were infected with HDV, and viral markers were extensively  
91 analyzed. Innate and IFN responses to HDV were monitored by measuring pro-  
92 inflammatory and interferon-stimulated gene (ISG) expression. Both mono- and super-  
93 infected dHepaRG cells supported a strong HDV intracellular replication, which was  
94 accompanied by a strong secretion of infectious HDV virions only in the super-infection  
95 setting and despite the low number of co-infected cells. Upon HDV super-infection, HBV  
96 replication markers including HBeAg, total HBV-DNA and pregenomic RNA were  
97 significantly decreased, confirming the interference of HDV on HBV. Yet, no decrease of  
98 circular covalently closed HBV DNA (cccDNA) and HBsAg levels was evidenced. At the  
99 peak of HDV-RNA accumulation and onset of interference on HBV replication, a strong  
100 type-I IFN response was observed, with interferon stimulated genes, *RSAD2* (Viperin)  
101 and *IFI78* (MxA) being highly induced. We established a cellular model to characterize in  
102 more detail the direct interference of HBV and HDV, and the indirect interplay between  
103 the two viruses via innate immune responses. This model will be instrumental to assess  
104 molecular and immunological mechanisms of this viral interference.

## 106 Introduction

107 Chronic hepatitis delta (CHD) affects 15-20 million people worldwide (5-10% of the  
108 hepatitis B virus (HBV)-infected patients) (1). It is considered to be the most aggressive  
109 form of chronic viral hepatitis, with an accelerated progression towards fibrosis and  
110 cirrhosis and an increased risk of liver disease decompensation, hepatocellular  
111 carcinoma and premature death (2). Pegylated-alpha interferon (Peg- $\alpha$ IFN) remains the  
112 sole therapeutic option for these patients, leading to a low virological response rate (<  
113 30%) at 24 weeks post-treatment and high rate (>50%) of late relapse (3). The overall  
114 long-term sustained virologic response (SVR) rate is therefore very low in the clinical trial  
115 setting, and is even lower in the “real-life” clinical management (4). HBV-reverse  
116 transcriptase inhibitors have no effect on hepatitis D virus (HDV) replication. The pipeline  
117 of investigational drugs against HDV infection remains limited due to the fact that i) HDV  
118 does not encode enzymatic activities and uses cell DNA-dependent RNA polymerases  
119 (particularly RNA pol II) for its replication, ii) there are remaining gaps in the knowledge of  
120 the viral life-cycle, and iii) no appropriate *in vitro* model of this satellite co- or super-  
121 infection exists to screen antiviral drugs. Amongst few others, Myrcludex (a viral entry  
122 inhibitors) and farnesyl transferase (i.e. Lonafarnib) inhibitors are in early clinical trial  
123 evaluation (5, 6).

124 HDV is a subviral agent satellite of HBV, and its genome, the smallest known among  
125 mammalian viruses, has similarities to plant viroids. To ensure propagation, HDV relies  
126 on HBV, as HDV ribonucleoproteins are surrounded by HBV envelope-embedded  
127 glycoproteins. Furthermore, HDV entry into human hepatocytes is mediated through the  
128 large HBV envelope protein (L-HBsAg) interaction with the recently discovered cell

129 surface HBV receptor, *i.e.* the human sodium taurocholate cotransporting polypeptide  
130 (hNTCP) (7).

131 HDV genome is a single-stranded circular RNA of ~1680 bp, with high intra-molecular  
132 base pairing, allowing a rod-like structure folding. Its complementary 'antigenomic' strand  
133 encompasses the *SHD* gene that codes a single protein, the small, 24 kDa, HD protein  
134 (S-HDAg), which is essential for HDV RNA replication. At a later phase of the HDV  
135 replication cycle, *SHD* stop codon editing, catalyzed by Adenosine Deaminase acting on  
136 RNA-1 (ADAR-1), leads to the synthesis of a 19-20 amino-acid (aa) carboxy-terminal  
137 extended isoform of HDAg; this large, 27kDa, protein (L-HDAg), thwarts HDV RNA  
138 replication and, in its farnesylated form, is involved in particle assembly (8, 9).

139 Both clinical and experimental data support the existence of viral interference between  
140 HDV and HBV. In the clinical setting, most patients infected with both HBV and HDV  
141 feature a pattern of HDV dominance, with a significant decrease in HBV-DNA viral load,  
142 when compared to mono-infected patients (10–12). Moreover, studies on liver biopsies  
143 from chronically HDV-infected patients have shown a decreased level of HBV replicative  
144 intermediates in the liver (13). Finally, this negative interference has been confirmed *in*  
145 *vivo*, in super-infection conditions, using HBV-infected chimpanzees, woodchuck  
146 hepatitis virus (WHV)-infected woodchucks, and more recently HBV-infected humanized  
147 mice (14–17).

148 To understand the molecular basis of HDV interference on HBV, relevant infection-based  
149 *in vitro* models are essential. Viral interference has been observed in Huh7 cells by  
150 transfection of DNA vectors expressing HBV and HDV (or either HDAg isoforms) (18).  
151 Direct inhibition of HBV enhancer-1 and activation of *MxA* gene, an interferon-stimulated  
152 gene (ISG) known to suppress HBV replication, have been documented in the same cell



153 line (19). However, transfection models with cDNAs expressing HDV genome have  
154 limitations and protein overexpression may lead to inaccurate assumptions. To explore  
155 HBV/HDV interference, the access to a cell culture model featuring both cccDNA  
156 formation and a competent innate immunity would be instrumental. Until recently, the  
157 knowledge on innate immune response related to HDV infection remained scarce. After  
158 *in vitro* studies suggesting a modulation of the IFN response (20, 21), recent data from  
159 mouse models (both the humanized uPA-SCID and the hNTCP transgenic mice)  
160 revealed a strong induction of the intra-hepatocyte ISG expression (22, 23). Further  
161 knowledge on the interactions between HDV and the innate immune system could be  
162 invaluable to get insights on the interplay between HDV and its helper, as well as to  
163 identify novel therapeutic strategies.

164 The aim of this study was to establish a novel cellular model of HDV super-infection, and  
165 characterize HBV/HDV interactions via direct viral interference mechanisms or through  
166 hepatocyte innate immune response to infection. This model could furthermore allow an  
167 evaluation of novel drugs on HDV replication. Using the differentiated HepaRG  
168 (dHepaRG) cells, which are immune-competent (24), we confirmed an efficient  
169 suppression of HBV replication (i.e. inhibition of intracellular HBV RNA and DNA  
170 accumulation, as well as HBeAg secretion), with no detectable effect on cccDNA nor  
171 HBsAg expression, and showed that HDV infection is associated with induction of ISGs,  
172 but not with induction of NF-kappaB regulated genes. Finally, we demonstrate the  
173 usefulness of this model with respect to antiviral discovery, by studying the antiviral  
174 activity of interferon alpha, specific anti-HBV and investigational specific anti-HDV drugs.

## 175 **Material and Methods**

### 176 ***Production of HBV and HDV virions***

177 High-titer HBV particles were retrieved from HepG2.2.15 cells supernatant as previously  
178 described (25). HDV particles were produced by Huh7 cotransfection of a trimer HDV-1  
179 prototype replication-competent plasmid (pSVLD3) and an HBsAg-encoding plasmid  
180 (pT7HB2.7) according to Sureau et al. (26) (see also **Sup. Fig. 1**). Both HBV and HDV  
181 supernatants were concentrated with 8% PEG 8000 (Sigma-Aldrich). All virus  
182 preparations were tested for the absence of endotoxin (Lonza).

### 183 ***Cell culture and infection***

184 The human liver progenitor HepaRG cells were cultured, differentiated using DMSO and  
185 infected using PEG4% overnight with either HBV or HDV as previously described (24, 25,  
186 27). For super-infection experiments, cells already infected with HBV for 6 days (100 viral  
187 genome equivalents [vge]/cell, unless otherwise indicated) were exposed to HDV  
188 overnight (100 vge/cell, unless otherwise specified). Primary human hepatocytes (PHH)  
189 were isolated and infected as previously reported (25, 26). HepG2-NTCP were kindly  
190 provided by Dr Stephan Urban (Univ. Heidelberg, Germany); there were cultivated in  
191 10%-FCS supplemented DMEM (4.5 g/L glucose) without DMSO until confluency and  
192 with 2% DMSO after and infected as for HepaRG.

### 193 ***Nucleic acid quantification: qPCR, RT-qPCR and Northern Blot***

194 For HBV titration, DNA was extracted with the QiAmp Ultrasens Virus kit (Qiagen) and  
195 submitted to qPCR. HDV was titrated by qRT-PCR after RNA extraction with the  
196 NucleoSpin RNA Virus kit (Macherey-Nagel) and digestion with DNase I (Life  
197 Technologies) 1h at 37°C, followed by 20 min at 70°C, to eliminate residual plasmid DNA.

198 Supernatants from infected-dHepaRG cells were used for viral particle RNA and DNA  
199 quantification. In order to remove free nucleic acid, clarified supernatants were submitted  
200 to DNase and RNase digestion (Roche), followed by overnight precipitation with 8%  
201 PEG 8000 (Sigma-Aldrich). After centrifugation, pellets were suspended in PBS 1X and  
202 nucleic acids were extracted with the Nucleospin 96 Virus kit (Macherey Nagel). The  
203 resultant nucleic acids were quantified using qPCR and RT-qPCR for HBV and HDV,  
204 respectively.

205 For HBV intracellular total DNA quantification, DNA extraction was performed using the  
206 Master Pure Complete DNA and RNA extraction kit (Epicentre), or the Nucleospin 96  
207 tissue kit (Macherey Nagel). Intracellular total RNA was extracted with the NucleoSpin  
208 RNA kit (Macherey Nagel), which includes a DNase digestion step.

209 All primers and probes are listed in **Supplementary Table 1**. HDV quantification was  
210 performed by one-step RT-qPCR (Express One-Step SYBR Greener, Life Technologies)  
211 using the primers described by Scholtès & colleagues (28), and the following cycling  
212 conditions: 50°C for 20 min (retro-transcription - RT), 95°C for 5 min and then 40 cycles  
213 of 95°C for 30 s, 60°C for 20 s, and 72°C for 20 s. PCR was run in the Roche LightCycler  
214 480. Serial dilution of quantified full length HDV-1 RNA (obtained from *in vitro*  
215 transcription of a pCDNA-3-derived plasmid containing a monomeric full length HDV-1  
216 cDNA insert) was used as a quantification standard (**Sup. Fig. 2**).

217 HBV DNA/RNA and innate immune gene expression were performed as previously  
218 described (25). For cccDNA quantification, total DNA was submitted to digestion with  
219 plasmid-safe DNase (Epicentre) for 4hours at 37°C, followed by 30 minutes of heat  
220 inactivation. Quantification was performed by FRET-based qPCR as previously described  
221 (29). Beta globin was used as a house-keeping gene. For all intracellular gene

222 expression analysis, the comparative cycle threshold (Ct) method was applied and  
223 results displayed as a ratio, to a control sample (described for each experiment) (30).

224 Northern blot for HDV and HBV RNA detection was essentially performed as previously  
225 described (26, 31). Briefly, purified RNA was denatured at 50°C for one hour with glyoxal  
226 (Life Technologies), subjected to electrophoresis through a phosphate 1.2% agarose gel  
227 and transferred to a nylon membrane (Amersham N+, GE). Membrane-bound RNA was  
228 hybridized to <sup>32</sup>P-labeled full HDV genome or DIG-labeled HBV-specific probes.  
229 Quantitative analysis of HDV RNA was achieved by phosphorimager scanning (Typhoon  
230 Fla 9500, GE); 18S and/or 28S rRNA quantification was used as loading control.  
231 Quantitative analysis of HBV RNAs was achieved using “ImageLab software” (Bio-Rad).

### 233 ***Elisa, immunofluorescence and western blotting***

234 Commercial immunoassay kits (Autobio Diagnostics Co., China) were used for HBsAg  
235 and HBeAg quantification in the cell culture supernatant. Results are presented as a ratio  
236 to a control sample, described for each experiment. Cut-offs for these ELISA were 1  
237 NCU/mL (i.e. 1 NCU ≈ 13 ng) for HBeAg and 2.5 ng/mL for HBsAg. Human IP-10  
238 cytokines were detected in the supernatants using the DuoSet® ELISA kit according to  
239 the manufacturer (R&D Systems). Analysis of Secreted Type I Interferon was performed  
240 as described previously (24).

241  
242 To perform immunofluorescence, cells were fixated with paraformaldehyde 4% and  
243 permeabilized by Triton 0.3%. Labeling was done using the following antibodies: HBcAg  
244 – monoclonal mouse antibody from Abcam (Ab-8637 – 1/200 dilution); HDAg – polyclonal

245 in-house rabbit antibody (kind gift from Alan Campbell Kay; 1/200 dilution). Secondary  
246 labeling was performed with Alexa Fluor fluorescent antibodies (wavelengths 555 and  
247 488) and cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). All images  
248 were obtained by epifluorescence microscopy (Nikon eclipse TE2000-E; Nikon) and  
249 processed with ImageJ software. Labeling was quantified by a combination of automatic  
250 nuclei counting provided by the software and manual counting of labeled cells. Displayed  
251 results correspond to the average of at least three fields (200x magnification).

252 For Western blots, cell lysis was performed with M-PER reagent (Pierce) in the presence  
253 of protease inhibitors. Western blots were performed with standard procedures using in-  
254 house polyclonal rabbit anti-HDAg antibodies and anti-tubulin mouse monoclonal  
255 antibody (Sigma Aldrich). Detection was performed with Gel Doc XR+ System (BioRad)  
256 and images were analyzed with ImageJ software

### 257 ***Antiviral treatment***

258 IFN $\alpha$  (Roche, used at 1000 UI/mL), tenofovir (Gilead Sciences, used at 10  $\mu$ M), the  
259 farnesylation inhibitor FTI-277 (Sigma Aldrich, used at 10  $\mu$ M) and Myrcludex $\text{\textcircled{R}}$  (Kind gift  
260 of Dr. Stephan Urban, used at 100 nM) were evaluated of their antiviral effect on an  
261 established HDV infection. dHepaRG cells were infected with HBV and super-infected  
262 with HDV as previously described and treated at days 3 and 7 and 11 post-HDV  
263 infection. Myrcludex $\text{\textcircled{R}}$  was further evaluated for its effect on HDV entry, by treatment 2  
264 hours before and during HDV inoculation. For all conditions, at day 14 post-HDV  
265 infection, supernatants were collected for cytotoxicity evaluation, ELISA and viral nucleic  
266 acid extraction and cells were lysed for RNA extraction.

### 267 ***Cell viability and cytotoxicity evaluation***

268 Apolipoprotein B was quantified in cell culture supernatants using the total human  
269 Apolipoprotein B ELISA assay (Alerchek), according to the manufacturer's  
270 recommendations. Lactate deshydrogenase release was quantified by colorimetric assay  
271 (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega), according to the  
272 manufacturer's protocol. Neutral red uptake assay and Sulforhodamine staining to  
273 estimate cell viability/cytotoxicity were performed as previously described (38). To  
274 functionally assess the cytotoxic effect of HDV, HDV-infected dHepaRG cells were  
275 treated with the apoptosis inhibitor QVD-OPH (Sigma-Aldrich) for 12 days. As a control,  
276 dHepaRG were treated with different concentration of the apoptosis inducer  
277 Staurosporine (Sigma-Aldrich) for 16h in combination with different concentration of  
278 QVD-OPH.

279

### 280 ***Statistical analysis***

281 Results were computed with Microsoft Office Excel and Prisma Graph Pad softwares.  
282 Sample groups were first evaluated for the presence of outliers with Dixon test. Statistical  
283 analysis was subsequently performed with Mann-Whitney test for single comparisons  
284 and Kruskal-Wallis test with Dunns correction for multiple comparisons. The  $p$ -values are  
285 represented according to the following convention:  $p>0.05$  (non-significant, n.s);  $p<0.05$   
286 (\*);  $p<0.01$  (\*\*);  $p<0.001$ (\*\*\*).

## Results

### ***In a mono-infection setting, dHepaRG cells support a strong, yet transient, HDV replication, associated with a strong expression of ISGs***

To assess the conditions of HDV inoculation, dHepaRG cells were either mock-infected or infected with HDV at multiplicities of infection (MOI) ranging from 1 to 500 vge/cell. At day-6 post-infection (p.i.), intracellular HDV RNA could be detected by RT-qPCR from the lowest MOI tested (1 vge/cell), with a linear increase up to 50 vge/cell, reaching a plateau for higher MOIs, up to 500 vge/cell (**Fig. 1A**). Northern blot analysis confirmed RT-qPCR findings and, using a genomic sense probe, indicated *de novo* formation of HDV replicative antigenomic RNA through the initiation of a replicative cycle (**Fig 1B**). This was further confirmed in western blot and IF analyses, showing a plateau of HDV protein expression for MOIs higher than 100 vge/cell and a number of infected cells of no more than 5% of the monolayer (**Fig. 1C and S3**). Importantly, the level of HDV replication in dHepaRG was as high as that observed in HepG2-NTCP cells (40), but 10x lower than that obtained in infected PHH (**Fig. S4**). For the experiments performed later, HDV MOIs of 10 or 100 vge/cell were mainly used for an optimal viral stock management.

To get insights on infection kinetics, dHepaRG cells were infected with HDV and total RNA and proteins were collected sequentially. As a negative control, cells were treated with the entry inhibitor Myrcludex®, from 2 hours before infection up to the end of viral inoculation, as shown in **Fig. 2A**. A steep rise in HDV intracellular RNA accumulation was detected from day 2 p.i. by RT-qPCR, reaching a peak at day 6 and a subsequent decrease (**Fig. 2B and 2C**). At later time point p.i., HDV RNA remained detectable (**Fig. 2A and 2B**), indicating a residual accumulation of replicative intermediates and/or a low replication persistence. As expected, no significant increase in HDV intracellular RNA

311 occurred in the Myrcludex®-treated control (**Fig. 2**), highly suggesting that the replication  
312 detected in the assay occurred after hNTCP receptor-mediated specific entry process.  
313 Increase in HDV RNA levels was associated with an increase in the expression of HDAg  
314 (**Fig. 2C and S3**), with both forms (S-HDAg and L-HDAg) being clearly detectable from  
315 day 3 p.i. (**Fig. 2C**). The pattern of expression was slightly delayed as compared to HDV  
316 RNA, but followed the same bell-shaped curve. At later time points both HDAg isoform  
317 signals decreased but remained detectable (**Fig. 2C and S3**). A very slight impairment of  
318 hepatocyte viability was associated with HDV infection, as documented by levels of  
319 secreted ApoB throughout time, neutral-red staining, and sulforhodamine assays (**Fig.**  
320 **S6A, S6B and S6C**). However this weakly measurable toxicity did not parallel the kinetic  
321 of replication of HDV, as it was constant over time. Moreover, the use of an apoptosis  
322 inhibitor (i.e. QVD-OPH) did not modify the level of HDV replication, therefore confirming  
323 that HDV do not induce a specific death of infected dHepaRG cells (**Fig. S6D and S6E**).

324 In contrast to hepatoma cells, dHepaRG cells express functional innate immune sensors,  
325 namely pathogen recognition receptors (PRRs), and, therefore may be relevant to study  
326 antiviral response in hepatocytes (24). We aimed to decipher IFN response to HDV  
327 infection in this model. Upon HDV mono-infection, increased expression of several  
328 representative *ISGs* could be detected. Interestingly, *ISGs* expression peaked at day-6  
329 p.i. and correlated with HDV RNA replication kinetics (**Fig. 3A**). No *ISGs*' induction  
330 occurred in the presence of Myrcludex®, excluding a non-specific stimulation by the viral  
331 inoculum. Furthermore, no induction was detected during the first 3 days p.i., which may  
332 suggest that the IFN response matched HDV RNA replication and HDV RNA neo-  
333 synthesis, rather than the incoming viral RNA material. In comparison to non-infected  
334 cells, highest expressions were detected for *RSAD2* (i.e. *VIPERIN*; mean fold change  
335 289) and *IFI78* (i.e. *MXA*; mean fold change 143,2). Other evaluated genes included



336 *ISG15* (mean fold change 36,2), *OAS1* (mean fold change 21,2), *DDX58* (i.e. *RIGI*; mean  
337 fold change 20,7), *MDA5* (mean fold change 9) and *IFN-β* (mean fold change 4,4). For all  
338 studied time points, non-significant and less than 2 fold differences in expression were  
339 found respectively for *IFN-α* and *IL-6* between HDV infected cells and mock or  
340 Myrcludex® treated controls. In addition, we observed an increased secretion of IP-10  
341 and type-I IFN that paralleled the increase of HDV RNA observed with the different  
342 amounts of HDV particles used for infection (**Fig. 3B**).

343

344 ***During super-infection, secretion of infectious HDV particles demonstrates the***  
345 ***existence of HBV/HDV co-infected cells.***

346 To set up the super-infection model, dHepaRG cells were first inoculated with HBV (100  
347 or 500 vge/cell), and, at the plateau of HBV replication (i.e. day-6) (32), cells were  
348 inoculated with HDV. In this setting, three infected cell populations could be identified,  
349 upon labeling with anti-HBcAg and anti-HDAg antibodies: HBcAg positive/HDAg negative  
350 cells, HBcAg negative/HDAg positive cells and HBcAg positive/HDAg positive cells (**Fig.**  
351 **4A**). This suggested that cells were either mono-infected by either HBV or HDV, or by  
352 both viruses, respectively. The same observation was obtained when HBsAg  
353 immunostaining was used instead of HBcAg labeling (**data not shown**). The proportion  
354 of infected cells expressing either antigen remained below 5% for either HBV or HDV  
355 markers, whereas co-labeling occurred in approximately 1-2% of the total dHepaRG  
356 cells.

357 Interestingly, despite the low number of detectable co-labeled cells, quantification of HDV  
358 RNA reached  $1.3 \times 10^7$  vge/mL in the supernatant of HBV-HDV super-infected cells for the

359 best condition. This result was obtained without detectable cell toxicity (**data not shown**),  
360 suggesting the secretion of viral particles. As expected, HDV intracellular RNA levels  
361 increased significantly with HDV MOI, but non-significantly with HBV MOI (**Fig. 4B**),  
362 whereas HDV secretion was proportional to both HDV and HBV MOIs (**Fig. 4C**). Of note,  
363 infections of HepG2-NTCP cells with concentrated HDV particles secreted from our  
364 super-infection experiments (called HDV-2P, for second passage) were as efficient as  
365 primary HDV inoculum (using equivalent MOI). This was demonstrated both by  
366 intracellular HDV RNA quantification and HDAg IF staining (**Fig. 4D**). Despite the low  
367 number of HBcAg/HDAg-positive cells, and likely due to the high efficiency of HDV-  
368 replication per cell (33), in the dHepaRG HBV/HDV super-infection setting, supernatant  
369 RNA associated from infectious HDV particles could be easily quantified, demonstrating  
370 that some dHepaRG cells can be infected by both viruses and are hence a suitable  
371 model for the evaluation of HDV-HBV interactions and the selection of drug resistant  
372 HDV variants.

373 ***In HBV-infected cells, HDV super-infection is associated with a MOI-dependent***  
374 ***induction of ISGs and decreased HBV replication***

375 The expression of innate immune related genes was evaluated at day-15 post-HBV  
376 infection (day-9 post-HDV infection). As previously determined (34), HBV alone did not  
377 induce any innate gene expression at this time point (**Fig. 5**). In contrast, HDV infection  
378 was clearly associated with a strong induction of all studied *ISGs*, which was HDV MOI-  
379 dependent but HBV-independent. Consistent with HDV mono-infection, super-infection,  
380 induced preferentially *RSAD2* and *MXA*, with a respective 83.5 and a 48.6 fold increased  
381 gene expression (at HBV 100 vge/mL and HDV 100 vge/mL). Finally, no induction of NF-  
382  $\kappa$ B induced genes was identified in HDV infections, as exemplified for *IL-6* (**Fig. 5**), *IL-8*

383 and *IL-1 $\beta$*  (**data not shown**). Collectively, these data indicate that in dHepaRG cells,  
384 HDV infection induces a strong IFN response at the peak of RNA replication,  
385 independently of both HBV infection and NF- $\kappa$ B pathway.

386 To investigate viral interference in the same setting, replication parameters were  
387 analyzed at day-15 post-HBV inoculation (9 days post-HDV super-infection). A significant  
388 decrease in both HBeAg secretion and intracellular HBV DNA accumulation was  
389 observed upon HDV super-infection of HBV-infected cells and the decrease was more  
390 pronounced with increasing HDV MOIs (**Fig. 6A and 6E**). Concomitantly, HBV-DNA level  
391 decreased in the supernatant when cells were super-infected by HDV (**Fig. 6D**). In such  
392 conditions, no reduction of HBsAg was observed (**Fig. 6B**), and no variation of cccDNA  
393 levels occurred (as measured by specific qPCR; **Fig. 6C**). Considering HBV MOI  
394 condition of 100 vge/cell, the decrease of HBV pgRNA correlated with the HDV MOI  
395 (25% reduction for HDV MOI 100 vs HBV mono-infection;  $p < 0.05$ ) (**Fig. 6G and 6H**). In  
396 contrast, no variation in the amount of total HBV RNA was observed even at high HDV  
397 MOI (**Fig. 6F and 6H**).

398 To further characterize this viral interference, HBeAg and HBsAg secretion were followed  
399 throughout time in dHepaRG inoculated, or not, with HBV (100 vge/cells) and  
400 superinfected, or not, with HDV (**Fig. 7A and 7B**). Compared to HBV-mono-infected  
401 cells, HDV-super-infected cells displayed a significant decrease of HBeAg secretion  
402 (34% decrease,  $p < 0.01$ ) (**Fig. 7A**). Increasing HDV MOI further inhibited HBeAg  
403 secretion (**Fig. 7B and 7C**) and decreased the number of HBcAg positive cells (**Fig. 7C**)  
404 suggesting that viral interference was dependent of HDV-MOI. In contrast, there was no  
405 significant difference in HBsAg secretion levels between HBV-mono-infected and HDV-  
406 super-infected cells (**Fig. 7A and 7B**).

407

408 ***Study of various drugs for their anti-HDV efficiency***

409 In order to further validate this HBV-HDV super-infection model, we aimed to explore the  
410 inhibitory effect of different molecules that could interfere with different steps of HBV and  
411 HDV life cycles in infected dHepaRG cells. Besides approved compounds such as IFN $\alpha$   
412 and tenofovir di-fumarate (TDF), we also verified the potential action of the entry inhibitor  
413 Myrcludex® and lonafarnib, a farnesyl transferase inhibitor (FTI), that have recently  
414 entered in phase II clinical trials for chronic HBV/HDV liver disease indication. Doses and  
415 treatment schedules were selected based on previously published data (35–38).

416 As expected, upon treatment with the HBV-polymerase inhibitor tenofovir, a significant  
417 decrease was observed in the amount of secreted HBV DNA (70%;  $p < 0,0001$ ), but not in  
418 secreted antigens or intracellular RNA levels (**Fig. S7A**). No effect was documented on  
419 HDV replication or viral secretion (**Fig. 8A**). IFN $\alpha$  treatment led to an important reduction  
420 of both HBV and HDV replicative parameters (**Fig 8B and S7B**). Unlike the other drugs,  
421 IFN $\alpha$  treatment was associated with a significant decrease in Apolipoprotein B secretion  
422 (67%;  $p < 0,0001$ ). As no increased LDH release was observed, such finding may be  
423 related to hepatocyte de-differentiation rather than cytotoxicity (**Fig. S8**).

424 Prenylation inhibitors have been shown both *in vitro* and *in vivo* to impact HDV  
425 envelopment and secretion without having a direct effect on viral replication. By treating  
426 HBV/HDV super-infected cells with FTI-277, we could observe a modest, albeit non-  
427 significant, reduction of HDV secretion into the supernatant (40%,  $p = 0,16$ ), which,  
428 interestingly, was associated with an increase of intracellular HDV RNA levels (2 fold  
429 increase;  $p < 0,05$ ) (**Fig. 8C**). As expected, FTI-277 treatment had no effect on HBV

430 replicative markers (**Fig. S7C**). Treatment with combinations of IFN $\alpha$  and tenofovir or  
431 FTI-277 and IFN $\alpha$  did not evidence a further decrease of neither HBV nor HDV  
432 parameters compared to single drug treatments (**Fig. 8D, 8E, S7D and S7E**). In this  
433 model, we could also confirm a suppression of HDV entry by Myrcludex® treatment  
434 previous and during HDV inoculation, while excluding a post-entry effect on both HBV  
435 and HDV replications (**Fig. 8F and S7F**). Overall, these results validate this model for the  
436 evaluation of both immune modulatory and direct-acting antiviral compounds acting on  
437 both HBV and HDV and at different steps of the viral life cycles.

## 439 Discussion

440 Despite leading to the most severe form of chronic viral hepatitis and infecting 15 to 20  
441 million of HBV-positive people worldwide, HDV remains a neglected pathogen. Getting  
442 more fundamental knowledge on HBV/HDV co-infections and viral interference may  
443 ultimately translate into the development of much needed new therapeutic strategies  
444 against HDV.

445 One aim of this work was to implement a relevant cell culture model to study this viral  
446 interplay, taking into account a subcellular innate immunity component. PHH are  
447 considered as the gold standard to perform *in vitro* studies on HBV and by extension on  
448 HDV. However, the low accessibility of fresh human liver resections, as well as the  
449 quality and variability of individual preparations limit their use. Interestingly, similarly to  
450 PHH, and in contrast to widely-used HepG2 and Huh7 cells, HepaRG cells functionally  
451 express most of innate immunity sensors (24) and are therefore considered as immune-  
452 competent (39). Despite their lower susceptibility to HBV and HDV infection, dHepaRG  
453 are the best alternative to PHH cultures to study HBV infection, as a full replication cycle  
454 can be obtained without the need of ectopically expressed hNTCP (32). Moreover,  
455 cccDNA can be detected in infected HepaRG cells, and has been shown, in a proof of  
456 concept study, to be degradable in an APOBEC3A/B-dependent manner by activation of  
457 IFN- $\alpha$  or lymphotoxin receptor- $\beta$  (LR- $\beta$ ) response pathway(s) (29). Therefore, the  
458 HepaRG cell line represents a unique model to study the interplay between HBV/HDV  
459 and hepatocyte-specific innate immunity, as well as to explore new therapeutic  
460 developments. So far, regarding HDV biology, the HepaRG model has mostly been used  
461 for studying the entry step (26), its inhibition by Myrcludex®, a drug competing with

462 hNTCP viral attachment (40), thus confirming the relevance of this cellular receptor for  
463 HDV entry (7).

464 In mono-infection with HDV we found that, as expected, only a small percentage of  
465 dHepaRG were infected (< 5% in IF). But in contrast to what seen with HBV (32), the  
466 intracellular level of HDV replication was very high, and could be detected even without  
467 amplification, by northern blot. Notably, in a super-infection setting, despite the very low  
468 proportion of co-infected cells (1-2%), neo-produced infectious HDV particles were titered  
469 at  $10^7$  vge/mL in supernatant, thus reflecting again the very high efficiency of viral RNA  
470 replication (33). The rather low proportion of detectable infected cells could be due, at  
471 least in part, to cell polarization and accessibility of hNTCP in the basolateral membrane  
472 of hepatocytes (41). Interestingly, in both mono-infection and super-infection conditions,  
473 HDV replication seemed to decline after a peak of replication at day-6 post inoculation. A  
474 similar decrease over time has also been described in mice injected with a HDV cDNA  
475 construct, in the chimpanzee experimental model and, more recently, in the hNTCP  
476 transgenic mouse model (17, 23, 42). At least two hypotheses might explain such  
477 phenotype: the infection is limited in time either by the decrease of available factor  
478 needed for replication, such as S-HDAg or some proviral host factors, or by accumulation  
479 of inhibitors, such as L-HDAg or cellular negative factor(s). Alternatively, active antiviral  
480 innate immune response that was, in our experiments, found temporally related to the  
481 peak of RNA accumulation, could also contribute to such inhibition.

482 With respect to the former, it was suggested that, besides mediating virion assembly, L-  
483 HDAg could inhibit viral replication, and therefore play crucial role to switch life-cycle from  
484 replicative to morphogenetic phase (43). However in our model, L-HDAg may not play  
485 this role, as the ratio of S-HDAg and L-HDAg remained constant throughout the kinetics

486 of HDV RNA replication, and in the super-infection setting, in which HDV virion release is  
487 observed, the decline of HDV RNA signals after day-6 p.i. is still observed. Regarding the  
488 immune hypothesis, we showed that the induction of some *ISGs* expression occurs at the  
489 peak of HDV RNA accumulation, indicating that neo-synthesized HDV-replicative  
490 intermediates, rather than inoculum RNA, act as a pathogen-associated molecular  
491 pattern (PAMP). Whether the activation of IFN response could lead to the decline of HDV  
492 replication after day-6 post infection is still unknown in dHepaRG, but such an hypothesis  
493 was not confirmed in the transgenic hNTCP mouse model (23).

494 Interestingly, in the cellular super-infection setting, we were able to confirm that HDV can  
495 interfere with HBV replication. The observations that HDV super-infection is associated  
496 with a decrease of HBeAg, HBV virion secretion, intracellular HBV DNA and pgRNA,  
497 although not HBsAg, total HBV RNA or cccDNA, are in agreement with what has been  
498 described in HDV-infected patients (13). This is part of the originality of this satellite  
499 infection that may often overcome its helper replication, while maintaining its budding  
500 trans-complementation. A competition for viral egress through the HBsAg secretory  
501 pathway is unlikely, as HBsAg is produced in large excess leading to a high proportion of  
502 empty subviral particles, and both viruses may not have the same cellular egress  
503 pathway (45). Furthermore, this would not account for the specific diminution of the HBV  
504 pgRNA that might be due to a modulation of cccDNA transcriptional activity (13, 18). In  
505 reporter systems and exogenous expression of HD proteins, direct inhibition of both HBV  
506 enhancers, especially by L-HDAg has been previously suggested (19). In a previous  
507 pioneer work using Huh7 cell co-transfected by both HBV-expressing plasmid  
508 pA3HBV3.8 and pSVLD3 (or pSVL-HDAg), J.C. Wu and co-workers suggested a  
509 possible repressive effect on transcription of the 3.5 kb and the 2.1 kb transcripts of HBV  
510 by HDV-replication or HDAg coding gene expression (18). But this approach was less



511 physiological than that based on proper infections. We trust the cellular super-infection  
512 model described in this study may therefore further contribute to determine at which step  
513 (e.g. cccDNA transcription, viral mRNA export and/or stability), and with which kinetic, the  
514 HDV-induced HBV-inhibition may occur.

515 Another explanation of this viral interference may be linked to the HDV-induced IFN  
516 response. Our results indicate that an increase in HDV MOI was associated with a more  
517 significant decrease of HBV replicative intermediates and a dose-dependent increase in  
518 ISGs expression. Previous works on HBV have shown that type-I IFN response  
519 modulates transcriptional regulation of cccDNA, decreasing pgRNA synthesis through  
520 modifications in histone acetylation status and recruitment of chromatin modifying  
521 enzymes (46). Whether HDV super-infection could induce such an epigenetic negative  
522 regulation of cccDNA transcription remains to be further explored.

523 During HDV infection (with and without a previous infection by HBV), we identified a  
524 pattern of gene activation suggesting the induction of an IFN response, without any effect  
525 on NF- $\kappa$ B regulated genes. The induction of ISGs expression by HDV is fully in  
526 agreement with results obtained in in both the humanized and hNTCP transgenic mice  
527 (22, 23). Among the studied ISGs genes that match HDV replication, *RDSA2* was found  
528 to be the most activated one. Interestingly, it has been suggested that in the woodchuck  
529 hepatitis infection of woodchuck neonates, Viperin was found to be at a higher level in  
530 neonates that resolved their infection, than those who progress to chronic carriage (47).  
531 Another innate-immune mediated mechanism of HBV repression due to HDV super-  
532 infection might be linked to a counteract the inhibition of the *MxA* expression probably  
533 linked to the HBV capsid (48–50)

534 Finally, our results demonstrate that, unlike other cellular models, dHepaRG sequentially  
535 infected by HBV and HDV represent a relevant model for the evaluation of antiviral drugs.  
536 Nucleos(t)ide analogues, such as tenofovir, while widely used in the setting of chronic  
537 hepatitis B, have failed to show a beneficial effect on the treatment of HDV infected  
538 patients (51, 52). Our data support these findings, as no effect of tenofovir was observed  
539 on either HDV replication or HBsAg secretion. Whereas an antiviral effect of IFN $\alpha$  on  
540 HBV replication has been thoroughly studied (35), data obtained in cellular models have  
541 been conflicting regarding its mechanism of action on HDV. Indeed, no direct effect of  
542 interferon on HDV replication was previously demonstrated (20), and other mechanisms  
543 of action have been suggested (53, 54). Our findings, being consistent with a  
544 suppression of HDV replication by interferon, in the absence of cytotoxicity, are in line  
545 with the data obtained *in vivo*, supporting the notion that dHepaRG cells is a more  
546 pertinent model than hepatoma derived cells for the evaluation of immune-modulators.  
547 We also aimed to evaluate the effect of some investigational drugs, currently undergoing  
548 clinical trials. The HBV/HDV entry inhibition we could observe with Myrcludex $\text{\textcircled{R}}$  treatment  
549 confirmed previous data from other groups (15, 40). Although not reaching statistical  
550 significance, we reproduced a trend of decreased HDV secretion inhibition by the  
551 prenylation inhibitor FTI-277. The fact that this effect was less pronounced than  
552 previously described, may be associated with the small number of co-infected cells in the  
553 HepaRG cell model. Interestingly, and unlike previous studies, the treatment with FTI-277  
554 in our model was associated with an increased HDV RNA accumulation in the cells.  
555 These results, although unexpected, can be explained i) by a possible abrogation of the  
556 inhibitory effect of L-HDAg on HDV replication in the absence of prenylation or ii) a defect  
557 in assembly of HDV RNP with HBsAg (55).

558 In summary, we demonstrated here the usefulness of the HepaRG cell line model for the  
559 study of HDV infection, in mono- and super-infection settings and could show that a  
560 robust HDV replication occurs in these cells and is associated with a strong induction of  
561 ISG expression. Moreover, upon HDV-super-infection of HBV-infected cells, HDV/HBV  
562 viral interference contributing to lowering HBV expression and the production of  
563 infectious HDV particles could be confirmed.

## 564

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755

## 756 **Figure legends**

757 **Figure 1. MOI-dependent replication of HDV in dHepaRG cells in mono-infection**  
758 **setting.** dHepaRG were infected with HDV at different MOIs (ranging from 1 to 500  
759 vge/cell). At day-6 post-infection, (A) levels of intracellular HDV RNA were assessed by  
760 RT-qPCR or (B) northern blot analyses using a genomic probe for antigenome detection,  
761 (C) HDAg expression was evidenced by western blot. Data in (A) represent the mean +/-  
762 SEM of 3 independent experiments. *MOI*, multiplicity of infection; *AG*, antigenome; *L-*  
763 *HDAg*, large hepatitis delta antigen; *S-HDAg*, small hepatitis delta antigen; *n.s.*, non-  
764 significant.

765  
766 **Figure 2. Kinetics of HDV mono-infection in dHepaRG cells.** dHepaRG cells were  
767 infected with HDV at 10 vge/cell and viral parameters were followed over time. As  
768 controls, cells were treated or not with Myrcludex® at 100nM for 2h before and during  
769 HDV inoculation. At the indicated time, (A) levels of intracellular HDV RNA were  
770 assessed by RT-qPCR or (B) northern blot analyses using a genomic probe for  
771 antigenome detection, (C) HDAg expression was evaluated by western blot. Data in (A)  
772 represent the mean +/- SEM of 3 independent experiments. *Myr*, Myrcludex®; *AG*,  
773 antigenome; *L-HDAg*, large hepatitis delta antigen; *S-HDAg*, small hepatitis delta antigen.

774  
775 **Figure 3. Kinetics of *IL-6*, *IFNs*, and *ISGs* expression in HDV-infected cells & MOI-**  
776 **dependent secretion of IP10 and type-I *IFNs*.** (A) dHepaRG cells were inoculated with  
777 HDV at 10 vge/cell and cells were harvested at different time points post-inoculation. *IL-*  
778 *6*, *type-1 IFNs* and *ISG* expressions were evaluated by RT-qPCR. Controls included



779 mock-infected cells, and cells treated with Myrcludex® at 100nM for 2h before and during  
780 HDV inoculation. **(B)** dHepaRG cells were inoculated with HDV at indicated MOIs. Six  
781 days later, levels of intracellular HDV RNA were assessed by RT-qPCR and IP10  
782 secretion or type I IFN activity were respectively assessed by ELISA or reporter gene  
783 assay. Results are presented as ratio to the mock condition at each day and represent  
784 the mean +/- SEM of 3 independent experiments each performed in triplicate.

785  
786 **Figure 4. HDV super-infection of HBV infected dHepaRG leads to secretion of HDV**  
787 **infectious particles. (A, B, C)** dHepaRG cells were infected by HBV and super-infected  
788 by HDV at day-6 at indicated MOIs. Fourteen days post-HBV inoculation, **(A)** cells were  
789 labeled with anti-HBcAg or anti-HDAg antibodies (magnification 600X), **(B)** levels of  
790 intracellular or **(C)** secreted HDV RNA were assessed by qRT-PCR. Results are  
791 presented as ratio **(B)** to cells co-infected with HBV MOI 100 plus HDV MOI 100 or **(C)** to  
792 cells infected with HBV HDV MOI 100 and represent the mean +/- SEM of 2 independent  
793 experiments each performed in triplicate. **(D)** HepG2-NTCP cells were inoculated with  
794 HDV or concentrated supernatant from HBV/HDV co-infected dHepaRG cells (HDV-2P)  
795 at 10 vge/cell. As controls, cells were treated or not with Myrcludex® at 100nM for 2h  
796 before and during HDV inoculation. 6 days later, levels of intracellular HDV RNA and  
797 HDAg were respectively assessed by qRT-PCR or immunofluorescent staining followed  
798 by confocal microscopy analyses. Results are presented as ratio to HDV infected cells  
799 and represent the mean +/- SEM of one representative experiment performed in  
800 triplicate.

802 **Figure 5. ISG induction is also present in a super-infection setting.** dHepRG cells  
803 were either mock (i.e. 0 vge/mL) or infected with HBV at 100 vge/cell and, 6 days later,  
804 super-infected with HDV at 0, 10 or 100 vge/cell. *IL-6*, *type-1 IFNs* and *ISGs* expressions  
805 were evaluated by RT-qPCR at day-15 post-HBV infection. Results are presented as  
806 ratio to the mock condition at each day and represent the mean +/- SEM of 3  
807 independent experiments each performed in triplicate.

808  
809 **Figure 6. HDV super-infection of HBV-infected cells leads to inhibition of HBV**  
810 **replication.** Differentiated HepRG cells were either mock (i.e. 0 vge/mL) or infected with  
811 HBV at 100 vge/cell and, 6 days later, super-infected with HDV at 0, 10 or 100 vge/cell.  
812 At day-15 post-HBV infection, (A) HBeAg and (B) HBsAg secretion were assessed by  
813 ELISA, (C) HBV cccDNA, (D) HBV secreted DNA and (E) HBV total intracellular DNA  
814 were assessed by qPCR whereas (F, H) HBV total intracellular RNA or (G, H) HBV pre-  
815 genomic RNA (pgRNA) were assessed by (F, G) RT-qPCR and (H) northern blot  
816 analyses. Results are presented as ratio to HBV cells infected at MOI 100 and represent  
817 the mean +/- SEM of 3 independent experiments each performed in triplicate.

818  
819 **Figure 7. HDV super-infection affects HBeAg, but not HBsAg, secretions and**  
820 **intracellular HBcAg expression.** Differentiated HepaRG cells were either mock or  
821 infected with HBV at 100 vge/cell for 6 days and either mock- or super-infected with HDV  
822 at the indicated MOI. (A, B) At the indicated time, HBeAg and HBsAg secretion were  
823 assessed by ELISA. (C) At day 15 post HBV-infection, HBcAg and HDAG were detected  
824 by immunofluorescent specific staining and confocal microscopy analyses.

825

826 **Figure 8. Evaluation of the anti-HDV effect of approved and investigational**  
827 **molecules.** Differentiated HepaRG cells were infected with HBV at 100 vge/cell for 6  
828 days and super-infected with HDV at the indicated at 10 vge/mL. Three days post HDV  
829 infection, cells were treated with (A) Tenofovir, (B) IFN $\alpha$ , (C) FTI-277, (D) Tenofovir and  
830 IFN $\alpha$ , (E) FTI-277 and IFN $\alpha$  for 10 days. (F) Cells were treated with Myrcludex B (Myr)  
831 either 2 hours before and during HDV inoculation (Pre) or once the infection was  
832 established as described for the other drugs. Levels of intracellular (RNAic) or secreted  
833 (RNA SN) HDV RNA were assessed by qRT-PCR. Results are presented as ratio to the  
834 non treated condition and represent the mean +/- SEM of 6 independent experiments  
835 each performed in triplicate.

836

**Figure 1.**

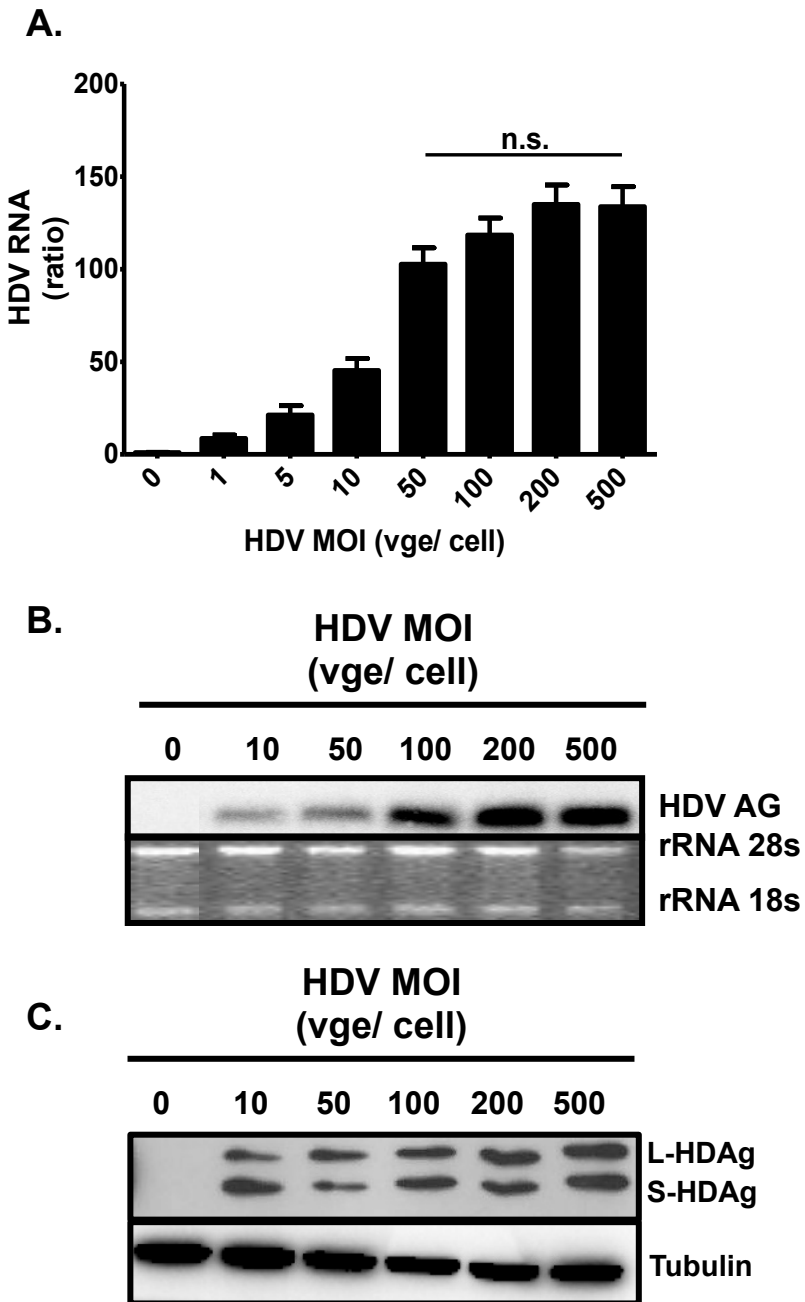
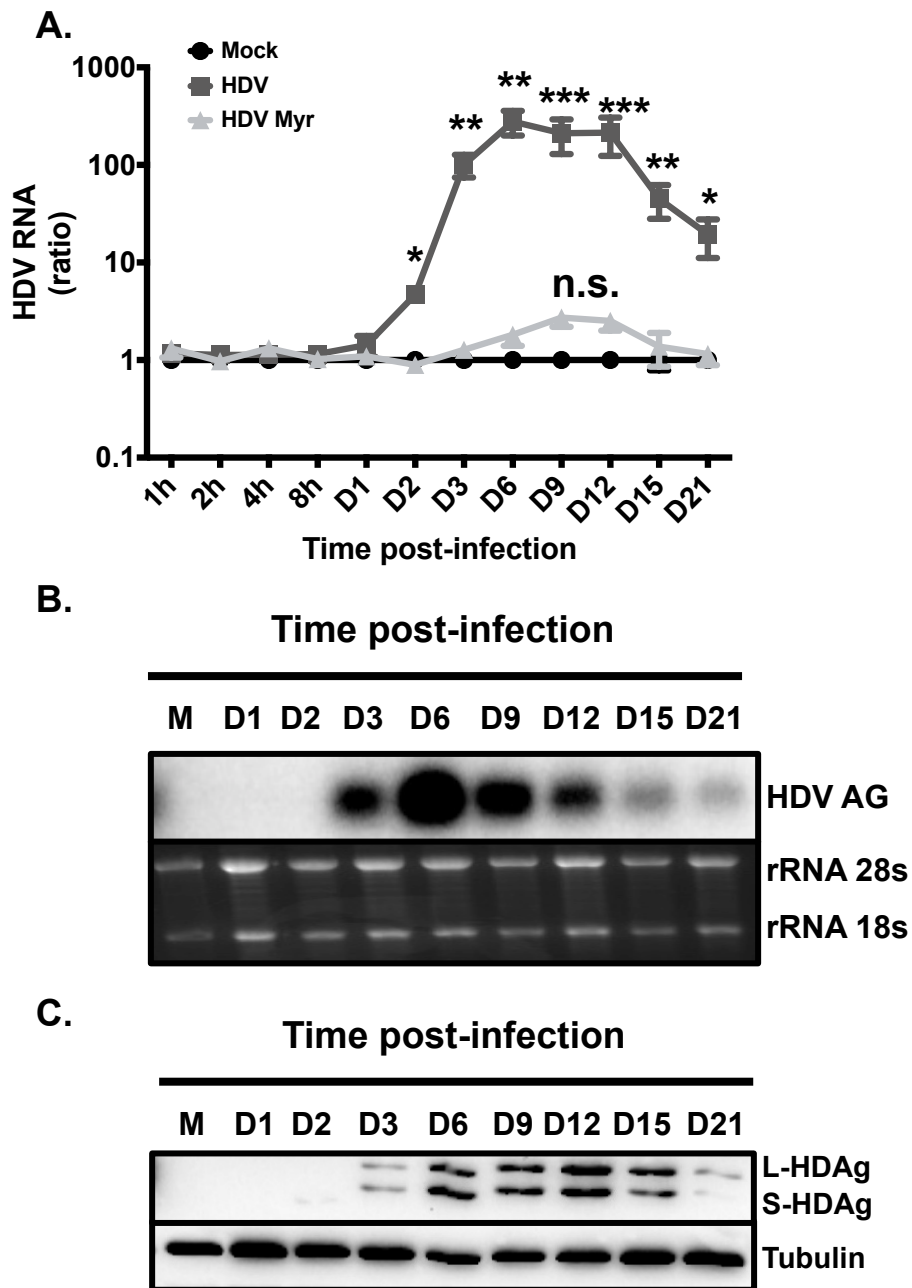
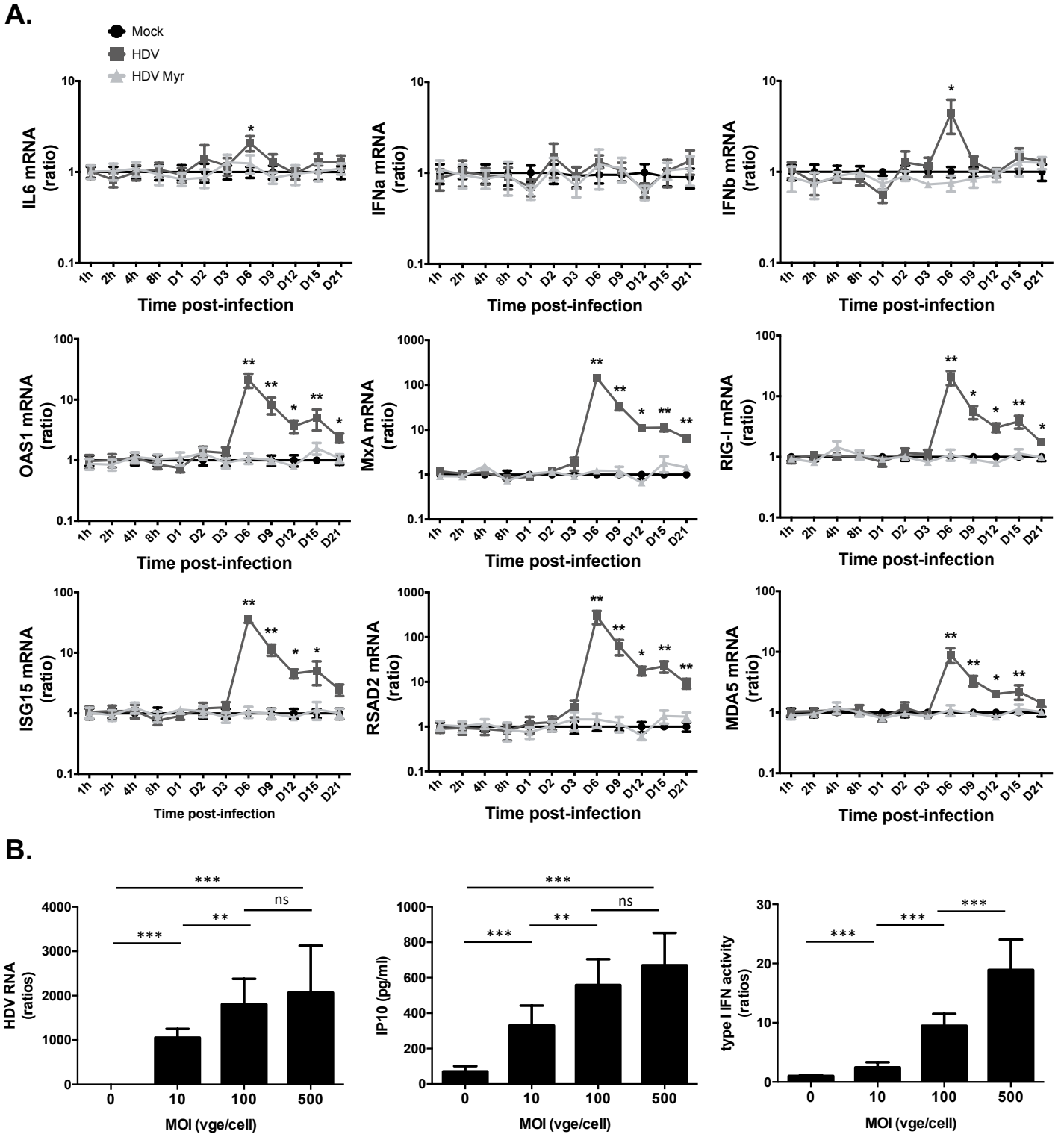


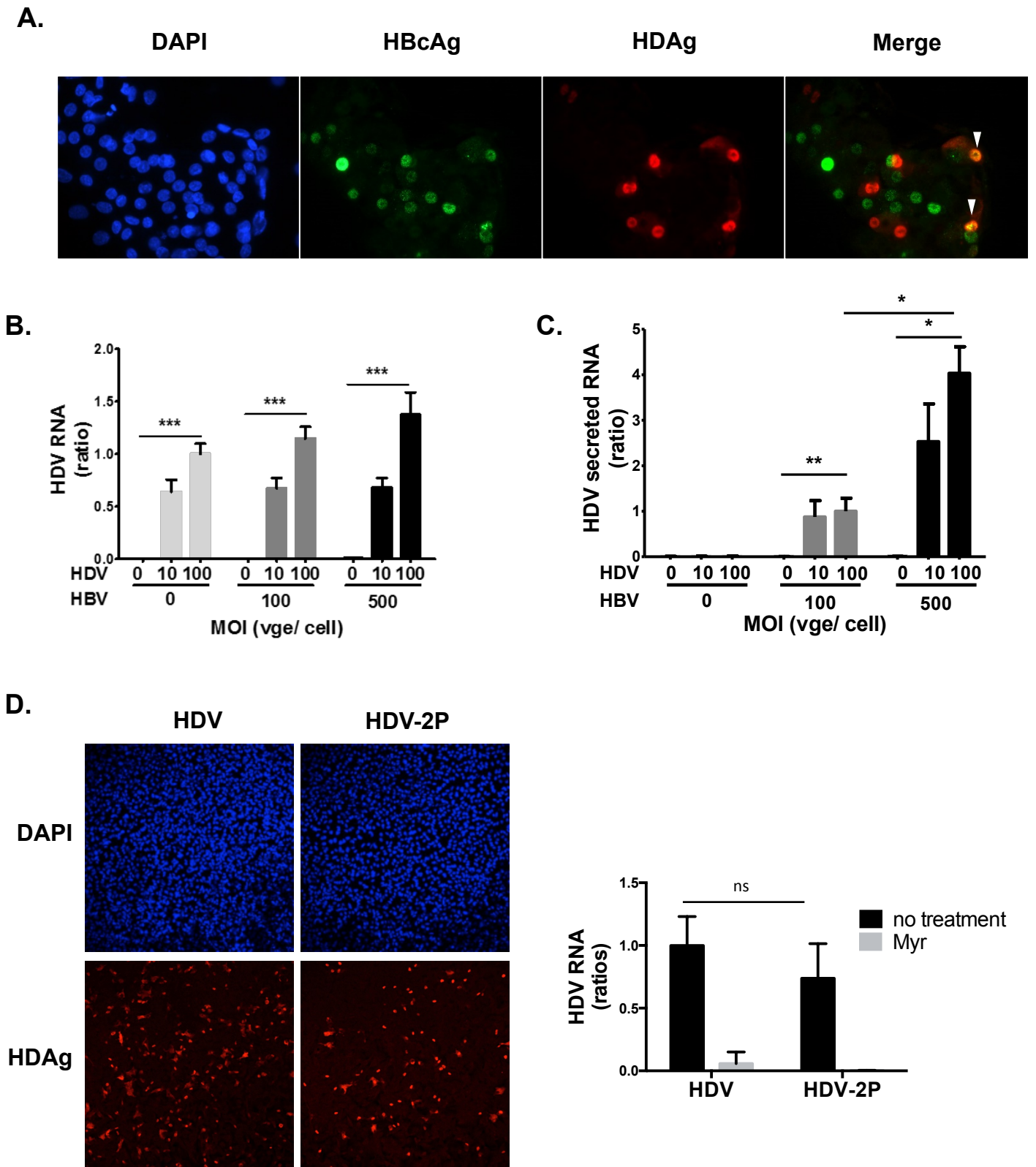
Figure 2.



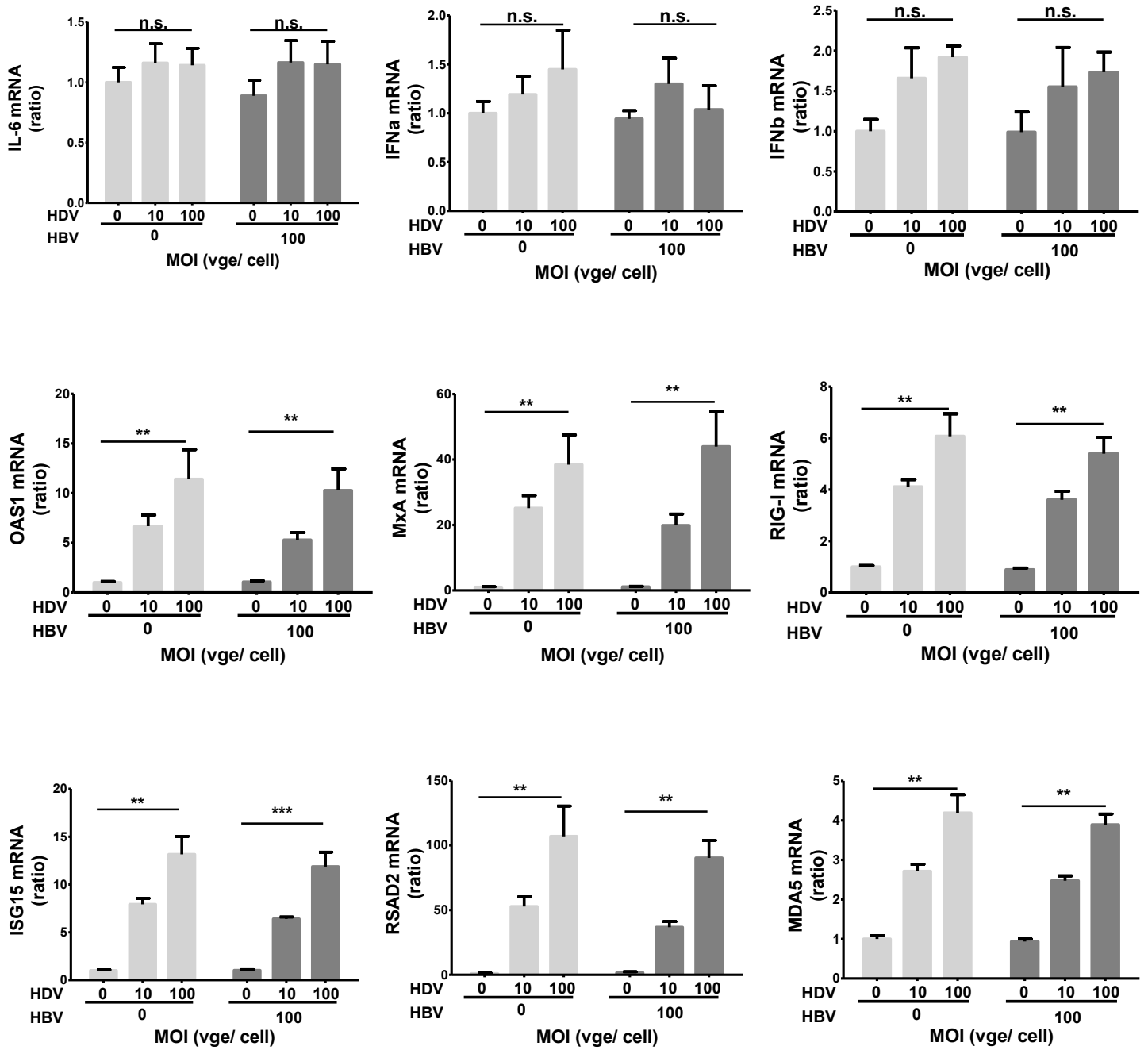
# Figure 3.



**Figure 4.**

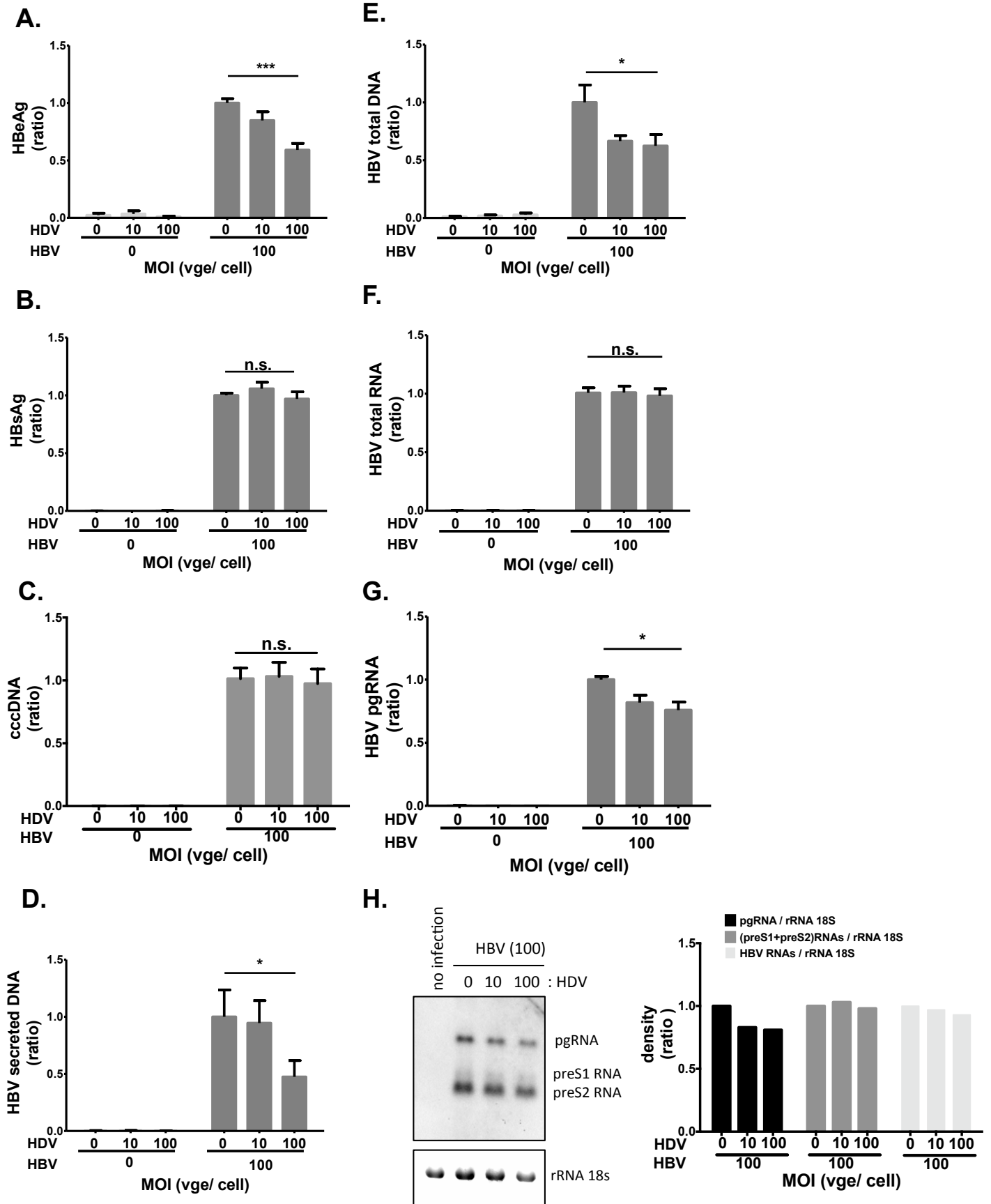


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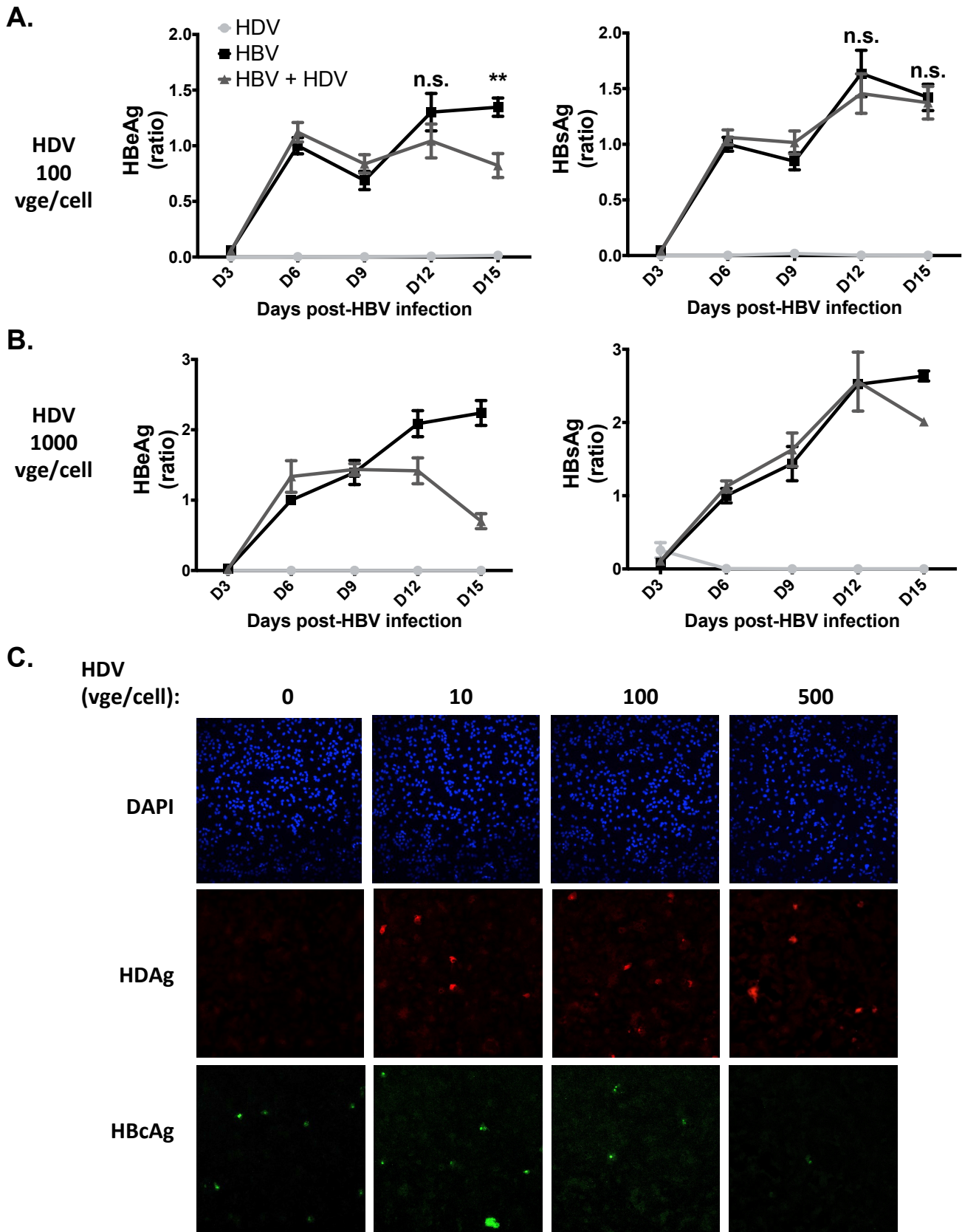




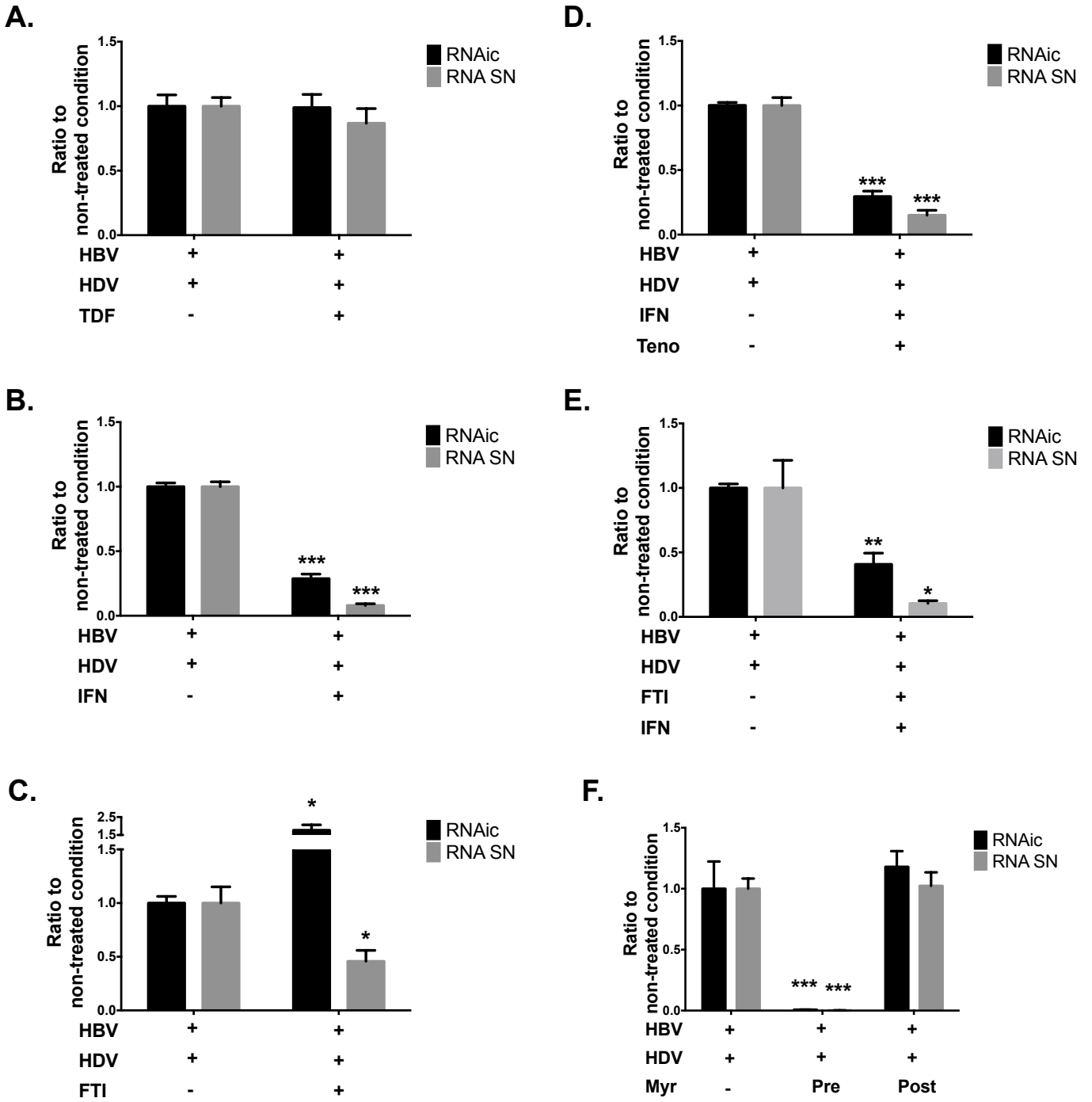
**Figure 6.**



**Figure 7.**



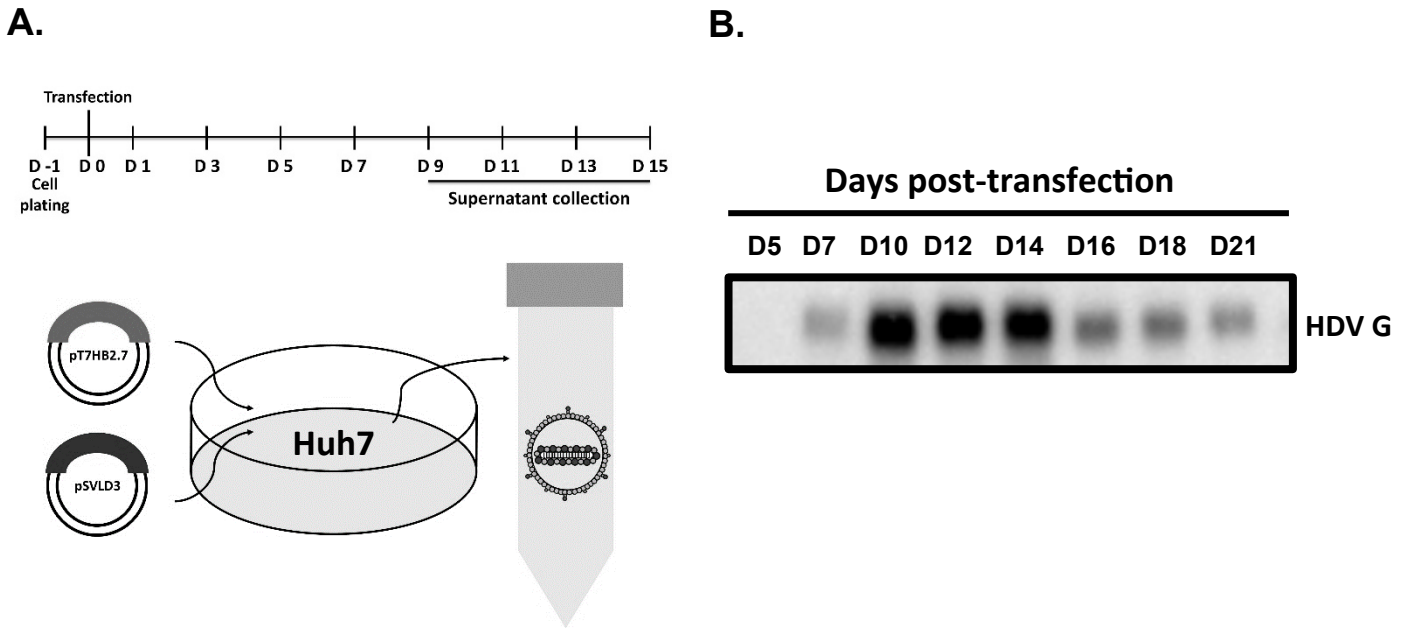
**Figure 8.**



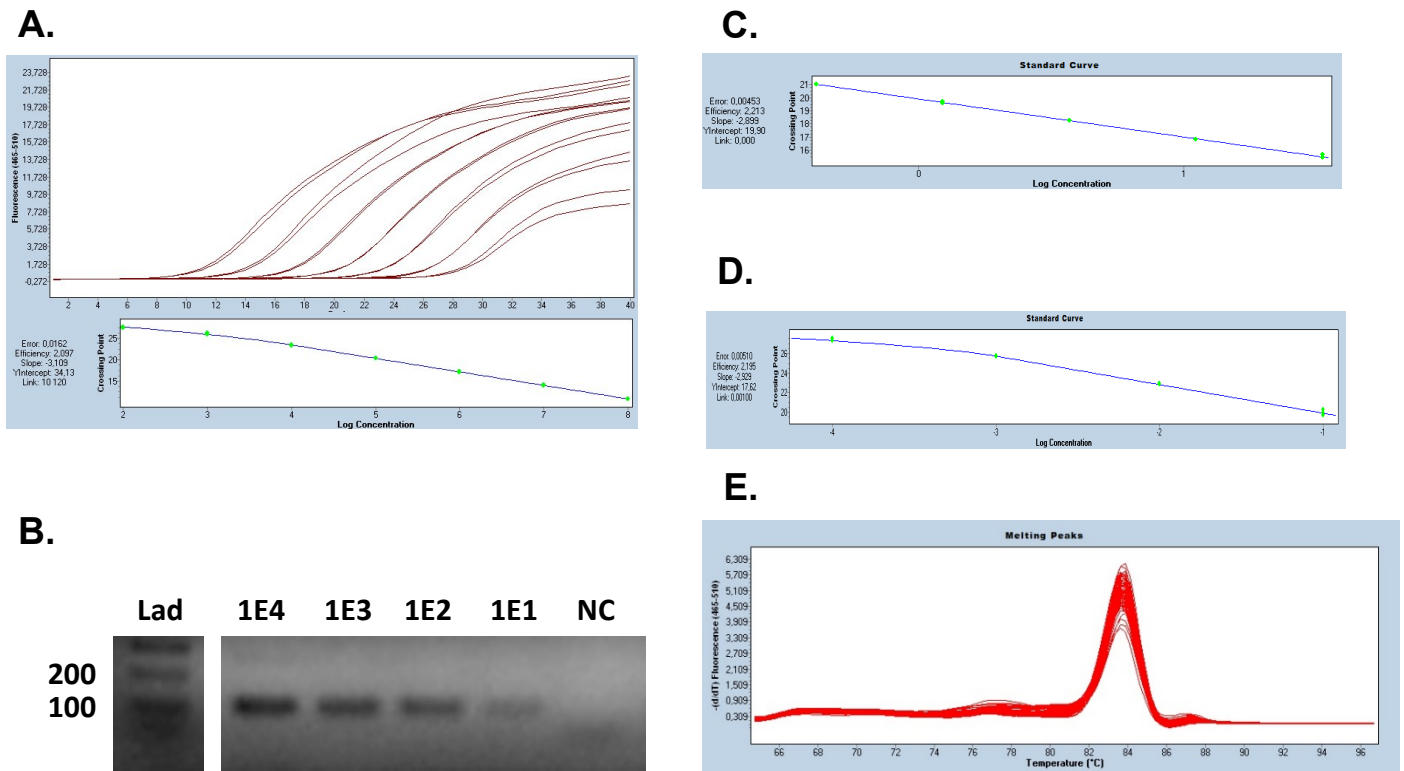
# **Supplementary table and figures**

<b>Designation</b>		<b>Sequence (5'-3')</b>
HDV	Forward Primer	CGGGCCGGCTACTCTTCT
	Reverse Primer	AAGGAAGGCCCTCGAGAACA
HBV total	Forward Primer	GCT GAC GCA ACC CCC ACT
	Reverse Primer	AGG AGT TCC GCA GTA TGG
HBV pgRNA	Forward Primer	GGA GTG TGG ATT CGC ACT CCT
	Reverse Primer	AGA TTG AGA TCT TCT GCG AC
HBV cccDNA	Forward Primer	CTC CCC GTC TGT GCC TTC T
	Reverse Primer	GCC CCA AAG CCA CCC AAG
	Probe	GTT CAC GGT GGT CTC CAT GCA ACG T
	Probe	AGG TGA AGC GAA GTG CAC ACG GAC C
GUS	Forward Primer	CGTGGTTGGAGAGCTCATTGGAA
	Reverse Primer	ATTCCCAGCACTCTCGTCGG
RPLP0	Forward Primer	CAC CAT TGA AAT CCT GAG TGA TGT
	Reverse Primer	TGA CCA GCC CAA AGG AGA AG
B-globin	Forward Primer	ACA CAA CTG TGT TCA CTA GC
	Reverse Primer	CAA CTT CAT CCA CGT TCA CC
	Probe	CAA ACA GAC ACC ATG GTG CAC CTG ACT CCT GAG GA
	Probe	AAG TCT GCC GTT ACT GCC CTG TGG GGC AA
IL6	Forward Primer	ACCCCTGACCCAACCACAAAT
	Reverse Primer	AGCTGCGCAGAATGAGATGAGTT
IFNa	Forward Primer	GTGAGGAAATACTTCCAAAGAATCAC
	Reverse Primer	TTCATGATTTCTGCTCTGACAA
IFNb	Forward Primer	GCCGCATTGACCATGTATGAGA
	Reverse Primer	GAGATCTTCAGTTTCGGAGGTAAC
OAS1	Forward Primer	AGGTGGTAAAGGGTGGCTCC
	Reverse Primer	ACAACCAGGTCAGCGTCAGAT
ISG15	Forward Primer	ATGGGCTGGGACCTGACG
	Reverse Primer	GCCAATCTTCTGGGTGATCTG
MxA	Forward Primer	GGTGGTCCCCAGTAATGTGG
	Reverse Primer	CGTCAAGATTCCGATGGTCCT
RSAD2	Forward Primer	CTTTGTGCTGCCCTTGAG
	Reverse Primer	TCCATACCAGCTTCCTTAAGCAA
RIG-I	Forward Primer	GCTGATGAAGGCATTGACATTG
	Reverse Primer	CAGCATTACTAGTCAGAAGGAAGCA
MDA5	Forward Primer	CCCATGACACAGAATGAACAAAA
	Reverse Primer	CGAGACCATAACGGATAACAATGT

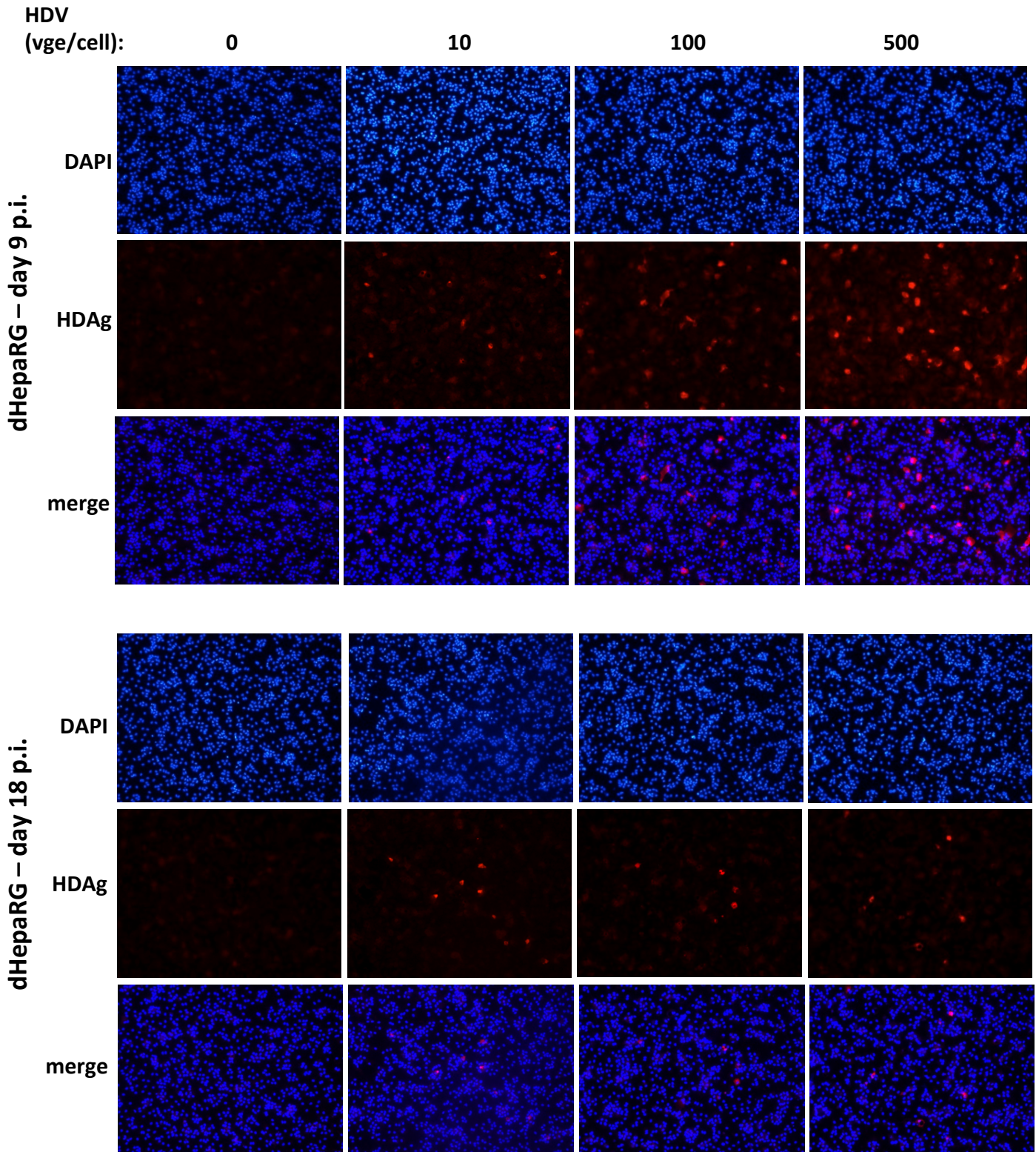
**Table S1. List of primers and probes used for qPCR**



**Figure S1. HDV viral inoculums production.** (A) HDV was produced in vitro by co-transfection of Huh7 cells with T7HB2.7 (coding for PreS1-PreS2-S from HBV) and pSVLD3 (containing a trimer of HDV genotype 1 genome). Supernatant was collected every other day from day-9 to day-15 post-transfection. (B) Northern blot analysis (with a full-length anti-genomic probe, for detection of HDV genome) of HDV RNA in the supernatant of transfected cells throughout time. G, genome.

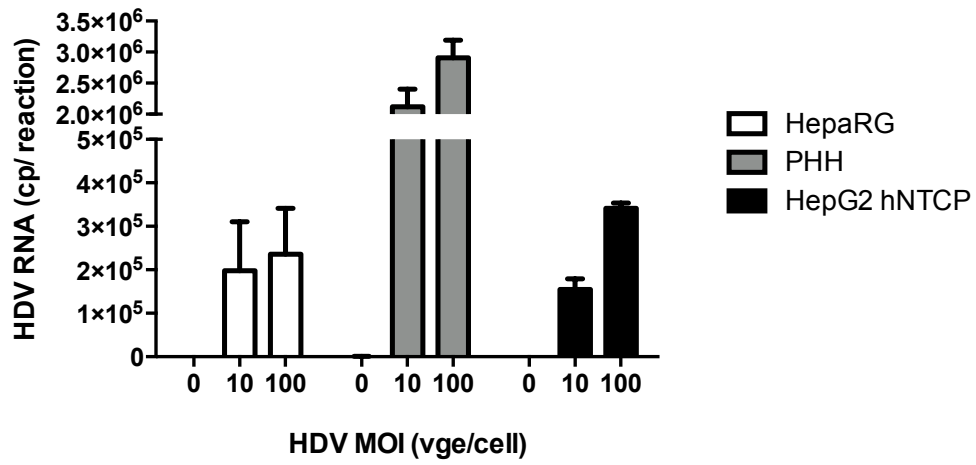


**Figure S2. Set up of the HDV RT-qPCR.** (A) Serial 10-fold dilutions of a full-length HDV-1 RNA genomic transcript are used as a quantification standard, confirm PCR linearity within a range of 10<sup>2</sup> to 10<sup>8</sup> copies/ reaction with a PCR efficiency of ~2,1. (B) Electrophoresis of PCR products evidences a unique band located between 100 and 200bp, consistent with the predicted 129bp amplicon. (C) Serial 10-fold dilutions of RNA extracted from cell culture supernatant, confirm PCR linearity between 6X10<sup>1</sup> and 6X10<sup>5</sup> copies/ reaction, with a PCR efficiency of ~2,2. (D) Serial 3-fold dilutions of intracellular total RNA confirm a linear HDV amplification within a range of 0.4-33,3 ng of total RNA per PCR reaction. (E) Melting curve plot of HDV infected samples, confirming one single T<sub>m</sub> peak consistently found at 84°C. Lad, DNA ladder (100bp, New England Biolabs); NC, negative control; T<sub>m</sub>, melting temperature.

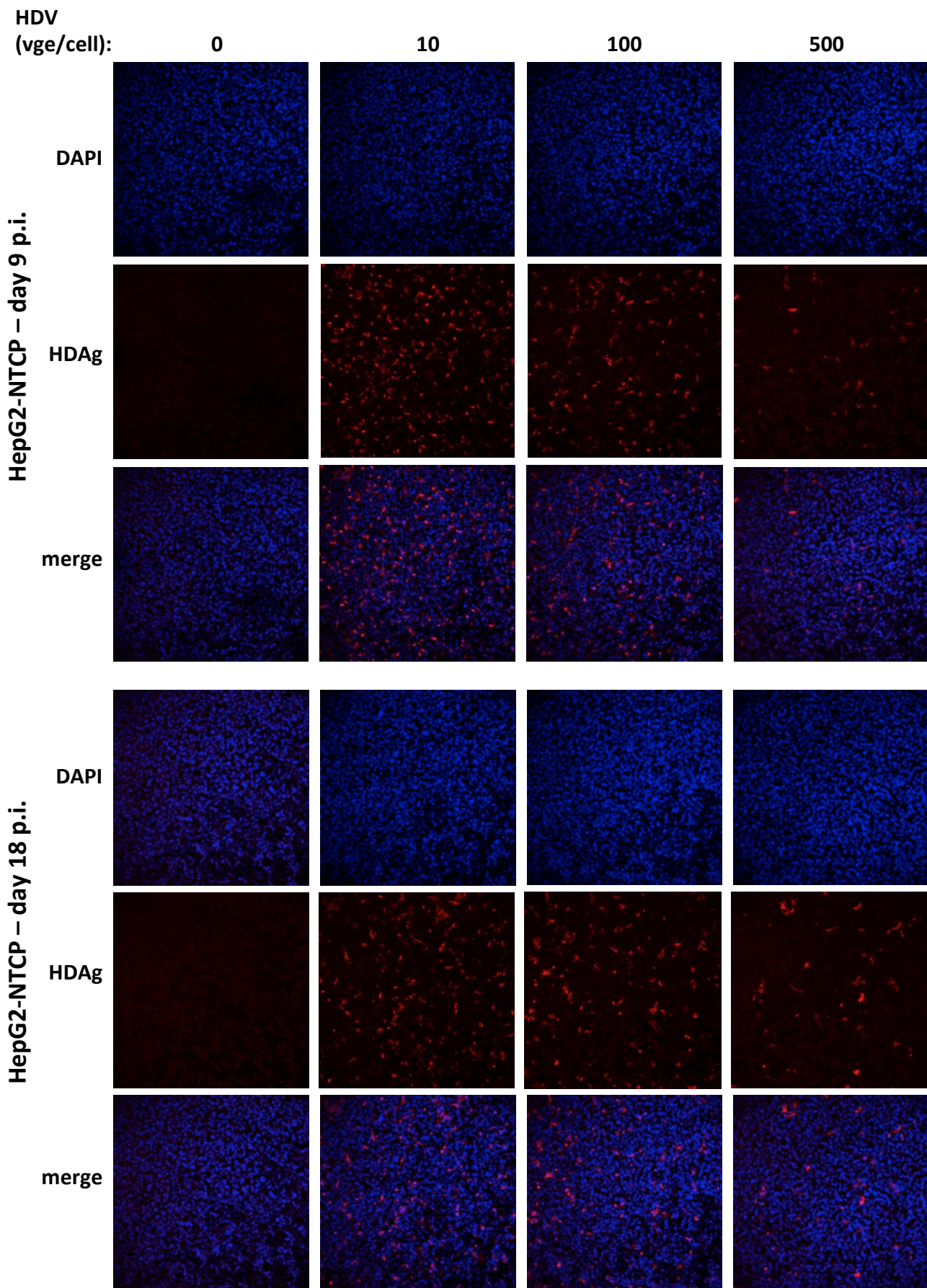


**Figure S3. Increasing MOI lead to increases of HDV infected dHepaRG.** dHepaRG cells were infected by HDV at the indicated MOI. At the indicated time, cells were fixed, permeabilized, labeled with anti-HDAg antibodies and analyzed by epifluorescence microscopy.

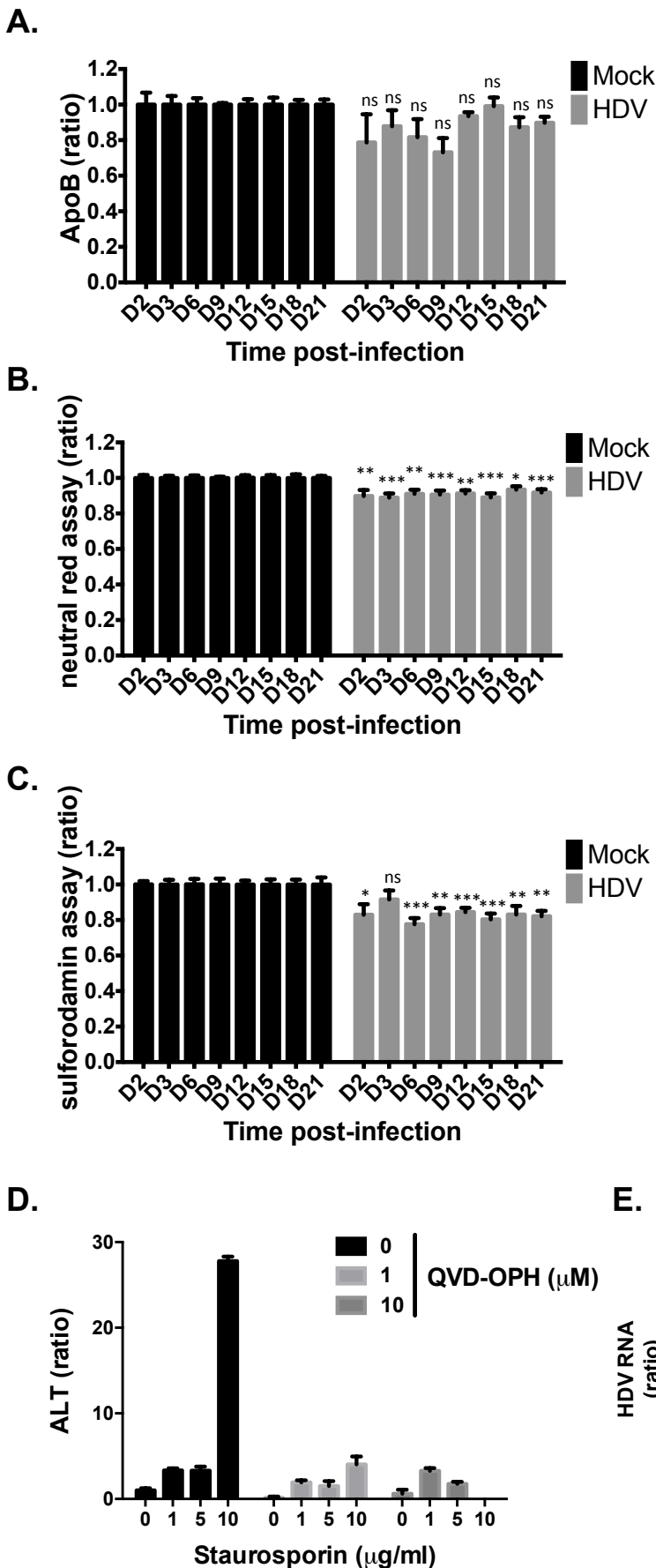




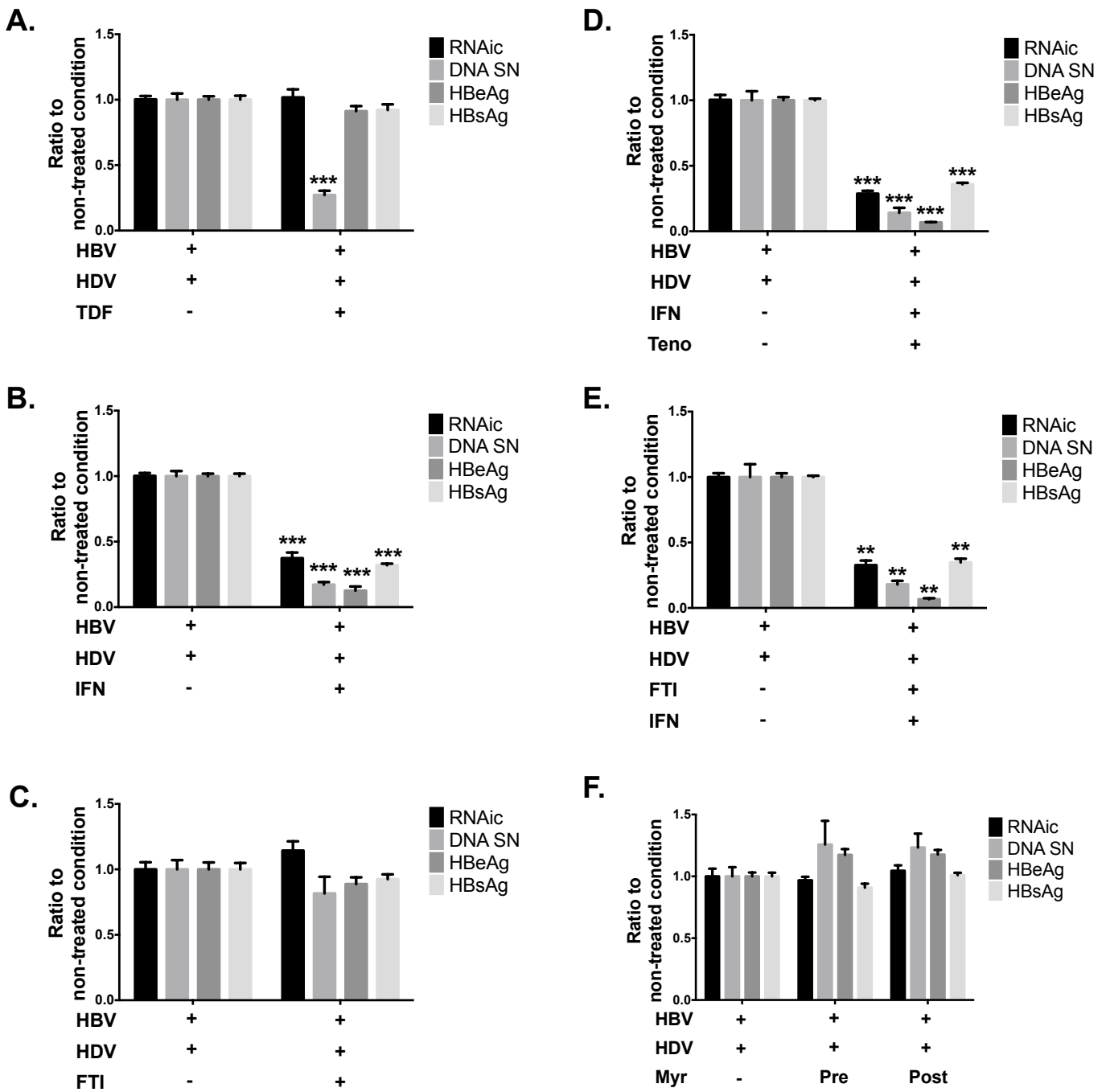
**Figure S4. HDV infection of different cells.** dHepaRG, PHH or HepG2-NTCP cells were infected by HDV at 10 vge/mL. 6 days later, levels of intracellular HDV RNA were assessed by RT-qPCR



**Figure S5. HDV infection of different cells.** HepG2-NTCP cells were infected by HDV at the indicated MOI. At the indicated time, cells were fixed, permeabilized and labeled with anti-HDAg antibodies and analyzed by confocal microscopy.

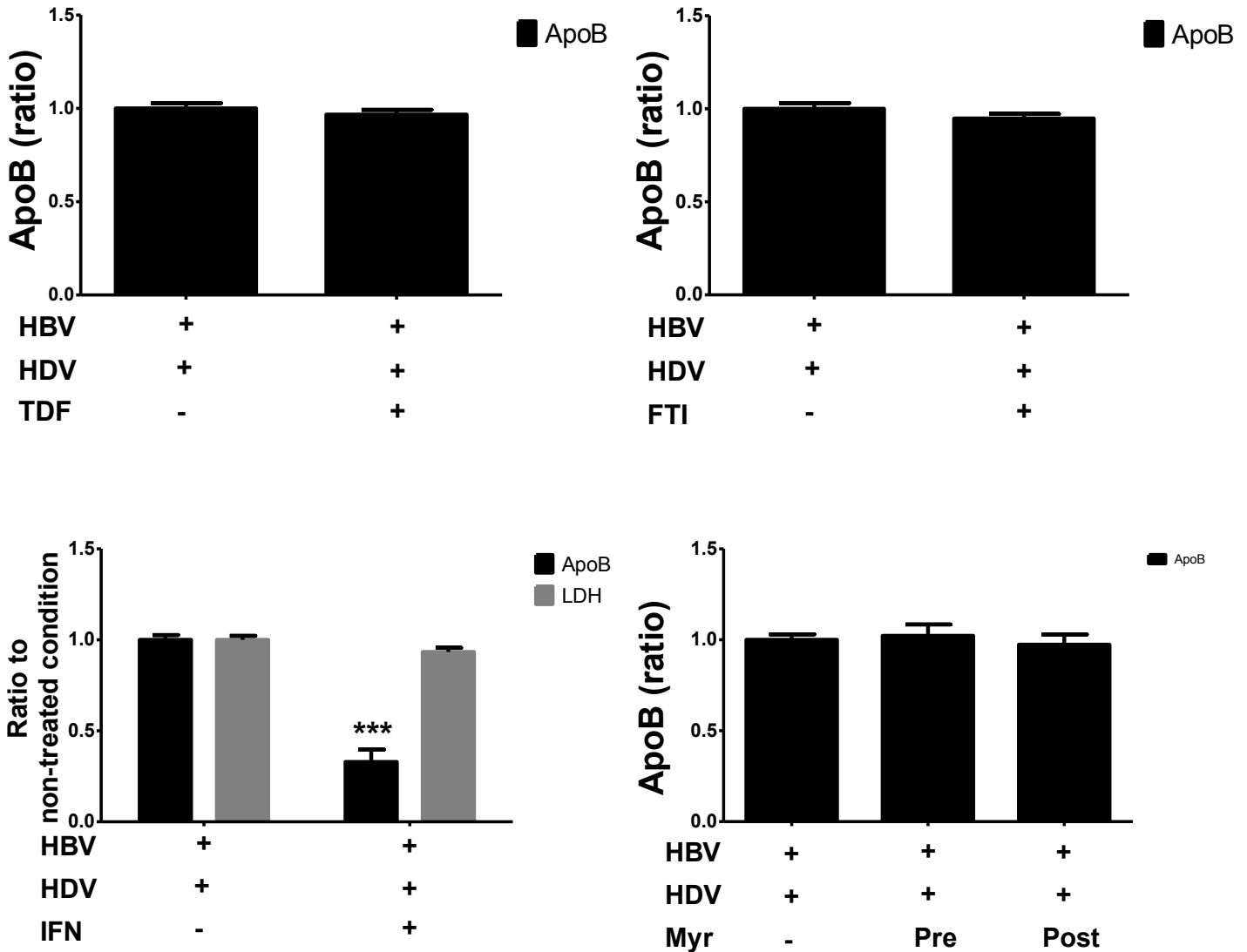


**Figure S6. HDV does not induce specific death of infected dHepaRG cells.** (A, B, C) dHepaRG cells were infected with HDV at 10 vge/cell and (A) Apolipoprotein B secretion as well as (B, C) cells viability were assessed by ELISA, neutral red or sulforodamin assays. Results are presented as ratio to non infected cells (mock) and represent the mean  $\pm$  SEM of 8 replicates (D) dHepaRG were treated with the indicated concentration of staurosporine and QVD-OPH for 16 hours and ALT activity were measured with colorimetric assays. (E) dHepaRG cells were infected by HDV and treated with QVD-OPH for 12 day. Levels of intracellular HDV RNA were assessed by RT-qPCR. Results are presented as ratio to the mock condition and represent the mean  $\pm$  SEM of 3 independent experiments each performed in triplicate.



**Figure S7. Evaluation of the anti-HBV effect of approved and investigational molecules.** dHepaRG cells were infected with HBV at 100 vge/cell for 6 days and super-infected with HDV at the indicated at 10 vge/mL. Three days post HDV infection, cells were treated with (A) Tenofovir, (B) IFNa, (C) FTI-277, (D) Tenofovir and IFNa, (E) FTI-277 and IFNa for 10 days. (F) Cells were treated with Myrcludex B (Myr) either 2 hours before and during HDV inoculation (Pre) or once the infection was established as described for the other drugs. Levels of intracellular HBV RNA (RNAiC) or secreted HBV DNA (DNA SN) were respectively assessed by qRT-PCR and qPCR. HBeAg and HBsAg secretion were assessed by ELISA. Results are presented as ratio to the non treated condition and represent the mean +/- SEM of at least 3 independent experiments performed in triplicate.

**Figure S8.**



**Figure S8. Evaluation of cell viability.** dHepaRG cells were infected with HBV at 100 vge/cell for 6 days and super-infected with HDV at the indicated at 10 vge/mL. Three days post HDV infection, cells were treated with Tenofovir, IFNa or FTI-277 for 10 days. Cells were also treated with Myrcludex B (Myr) either 2 hours before and during HDV inoculation (Pre) or once the infection was established as described for the other drugs. Levels of secreted ApoB were assessed by ELISA. When a decrease was identified, lactate deshydrogenase (LDH) activity was measured. Results are presented as ratio to the non treated condition and represent the mean +/- SEM of 3 independent experiments performed in triplicate. TDF, tenofovir; IFN, interferon; Myr,myrclydex; Pre, treatment before and during inoculation; post, treatment after infection.

- A model of super-infection with HDV on HBV-infected hepatocytes was established;
- HDV infection induces a strong IFN response in these immune-competent hepatocytes;
- In this model, HDV infection is associated with HBV inhibition, thus access to recapitulating *in vivo* viral interference;
- This super infection model is also suitable for the evaluation of novel drugs/antivirals, including immune-modulators.