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Analysis of Host Responses to Hepatitis B and Delta Viral Infections in a Micro-scalable Hepatic Co-culture System

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Hepatitis B virus (HBV) remains a major global health problem with 257 million chronically infected individuals worldwide, of whom approximately 20 million are co-infected with hepatitis delta virus (HDV). Progress toward a better understanding of the complex interplay between these two viruses and the development of novel therapies have been hampered by the scarcity of suitable cell culture models that mimic the natural environment of the liver. Here, we established HBV and HBV/HDV co-infections and super-infections in self-assembling co-cultured primary human hepatocytes (SACC-PHHs) for up to 28 days in a 384-well format and highlight the suitability of this platform for high-throughput drug testing. We performed RNA sequencing at days 8 and 28 on SACC-PHHs, either HBV mono-infected or HBV/HDV co-infected. Our transcriptomic analysis demonstrates that hepatocytes in SACC-PHHs maintain a mature hepatic phenotype over time, regardless of infection condition. We confirm that HBV is a stealth virus, as it does not induce a strong innate immune response; rather, oxidative phosphorylation and extracellular matrix–receptor interactions are dysregulated to create an environment that promotes persistence. Notably, HDV co-infection also did not lead to statistically significant transcriptional changes across multiple donors and replicates. The lack of innate immune activation is not due to SACC-PHHs being impaired in their ability to induce interferon stimulated genes (ISGs). Rather, polyinosinic:polycytidylic acid exposure activates ISGs, and this stimulation significantly inhibits HBV infection, yet only minimally affects the ability of HDV to infect and persist. *Conclusion:* These data demonstrate that the SACC-PHH system is a versatile platform for studying HBV/HDV co-infections and holds promise for performing chemical library screens and improving our understanding of the host response to such infections. (Hepatology 2020;71:14-30).

Hepatitis B virus (HBV), which is part of the *Hepadnaviridae* family, has a compact 3.2-kb relaxed circular DNA (rcDNA) genome encoding four viral gene products: the viral polymerase, which also acts as a reverse transcriptase; the hepatitis B core antigen (HBcAg), which forms the nucleocapsid; the hepatitis B X protein (HBx), which is thought to epigenetically regulate the HBV covalently closed circular DNA (cccDNA); and the large (L), medium (M), and small (S) envelope proteins (HBsAgs).[1] HBV is considered a uniquely hepatotropic virus, entering its target cells, primary human

Abbreviations: ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; dpi, days post infection; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ETV, prophylactic entecavir; GO, gene ontology; HBcAg, hepatitis B core antigen; HBV, hepatitis B virus; HBVcc, cell culture–produced HBV; HBx, hepatitis B x protein; HDV, hepatitis delta virus; hNTCP, human sodium-taurocholate co-transporting polypeptide; HTS, high-throughput screening; IFN, interferon; ISGs, interferon stimulated genes; MOI, multiplicity of infection; MPCCs, micropatterned co-cultures; MyrB, Myrcludex B; PEG, polyethylene glycol; pgRNA, pregenomic RNA; PHHs, primary human hepatocytes; poly(I:C), polyinosinic:polycytidylic acid; rlog, regularized log_2-transformed; RNASeq, RNA sequencing; SACC-PHHs, self-assembling co-cultured primary human hepatocytes; vPLAYR/TSA, viral proximity ligation assay for RNA and tyramide signal amplification.

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hepatocytes (PHHs), by using the bile acid transporter human sodium-taurocholate co-transporting polypeptide (hNTCP).[2] The HBV rcDNA enters the nucleus, where it is repaired through host cell machinery to form cccDNA, the stable and persistent form of the HBV genome.[1]

A satellite virus of HBV and sole member of the *Deltavirus* genus, the enveloped hepatitis delta virus (HDV) has a 1,679-nt, negative-sense RNA genome encoding for two isoforms of the delta antigen.[3] HDV highjacks the HBsAgs (L, M, and S) for packaging infectious HDV virions and thus relies on co-infection with HBV.[3] Consequently, HDV also uses hNTCP as a receptor but replicates its genome independent of HBV.

In addition to a very limited tissue tropism, HBV and HDV exhibit a restricted host tropism, robustly infecting only human and chimpanzee hepatocytes.[4] However, HDV infection has been experimentally established in species susceptible to other hepadnaviruses related to HBV, such as woodchuck hepatitis virus (WHV) in woodchucks. Recently, HDV-like viruses have also been detected in birds and snakes.[5,6] However, these viruses significantly differ from human HDV in their cellular tropism and genomic sequence. Studying these two viruses has therefore historically been difficult. Transfections of plasmids encoding HBV over-length genomes have been used to analyze aspects of viral replication and gene transcription in human hepatoma cells.[7] However, the artificial nature of such systems fails to fully recapitulate the mechanisms observed under native infection conditions. The cell line HepaRG, derived from a hepatocellular carcinoma biopsy, is susceptible to HBV infection,[8] but only after prolonged re-differentiation, limiting its utility. With the discovery of hNTCP as a bona fide receptor for HBV and HDV, ectopic expression of this protein subsequently made human hepatoma cell lines, such as HepG2 and Huh7, susceptible to both HBV and HDV infection.[2,9] Although low-cost and easy to culture, hepatoma lines remain less than ideal due to their abnormal proliferation and aberrant gene expression profiles as compared with PHHs.

Previous studies have shown that PHHs and fetal human hepatocytes are susceptible to infection with HBV and other hepatotropic viruses.[10-12] However, monoculture PHHs rapidly de-differentiate following plating, with concomitant loss of susceptibility to HBV, limiting any analysis to a few days. This de-differentiation can be delayed by co-culturing with nonparenchymal cells.[13,14] In both self-assembling co-cultures of PHHs (SACC-PHHs) and micropatterned co-cultures (MPCCs), PHHs are stabilized, maintaining a phenotype similar to hepatocytes in the 3D context of the liver. MPCCs of PHHs are susceptible to a variety of liver-tropic pathogens.[15,16] However, HBV infections of MPCCs, which persisted only up to 16-19 days, required preselection of susceptible donors and blocking of innate immune signaling.[17] The requirement for JAK/STAT inhibition consequently reduces this platform's suitability for thoroughly investigating host–virus interactions. A recent publication on a 3D microfluidics PHH system established infection for up to 40 days in a 12-well format,[18] but this system is not easily scalable and requires advanced technical skills to operate.

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Potential conflict of interest: Dr. Ploss advises Hurel. Dr. Novik is employed, owns stock, and holds intellectual property rights with Hurel.

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Recently, we showed that SACC-PHHs can be robustly and persistently infected with HBV for up to 40 days with little donor-to-donor variability and without suppressing innate immune signaling.[19] Building on this work, we demonstrate here that PHHs in SACCs support long-term HBV-HDV co-infection in microwell plate formats. Establishing long-term HBV/HDV infection in a microscale, 384-well format is a significant improvement even over 96-well formats, as it would enable high-throughput screens in automated settings. We further used this model to probe the host responses to both viruses, and we confirm that HBV is a "stealth virus." Going beyond this, we investigated the role of innate immune activation on HBV and HBV/HDV viral persistence, giving insight into viral dynamics and the clinical significance of innate immune recognition. Our work demonstrates the versatility of the SACC-PHH platform for modeling complex liver diseases, such as HBV, HBV/HDV, and potentially other clinically relevant liver infections, in a controlled experimental setting.

Materials and Methods

For full experimental details, please see the Materials and Methods section in the Supporting Information.

GENERATION OF SELF-ASSEMBLING PRIMARY HEPATOCYTE CO-CULTURES

Cryopreserved human hepatocytes were obtained from Bioreclamation IVT Inc. (Westbury, NY), Thermo Fisher Scientific (Waltham, MA), Sekisui Xenotech LLC (Kansas City, KS), and Corning Inc. (Corning, NY). The co-culture model consists of a mixture of human hepatocytes and nonparenchymal mouse embryonic fibroblast 3T3-J2 cells (CCL-92, ATCC, Manassas, VA).[14,20] All co-cultures were plated on collagen type-I coated, tissue culture–treated 96-well and 24-well plates (Corning Inc.).

REAL-TIME QUANTITATIVE PCR OF ISGS OAS-1, MX1, AND ISG15

To quantify fold changes of OAS-1, MX1, and ISG15 levels in SACC-PHHs, the total RNA was isolated from lysed cells using an EZ-10 Spin Column Total RNA Miniprep Super Kit (BioBasics, New York, NY). A mastermix for each gene to be quantified was made following the Luna Universal One-Step RT-qPCR Kit (New England Biolabs, Ipswitch, MA) protocol with forward and reverse primers at a 3-μM final concentration (see Supporting Information for primer sequences). The plate was centrifuged at 3,000 rpm for 1 minute. The following PCR program was run on a Step One Plus qPCR machine (Life Technologies, Carlsbad, CA): 50˚C for 10 minutes, 95˚C for 1 minute, followed by 40 cycles of 95˚C for 15 seconds, 60˚C for 1 minute, followed by a melt curve of 95˚C for 5 seconds, 65˚C for 5 seconds, 95˚C for 5 seconds, and 50˚C for 5 seconds.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism Software (GraphPad, La Jolla, CA). A one-way analysis of variance (ANOVA) using Bonferroni parameters was used. *P* values less than 0.05 were considered statistically significant.

Results

INFECTION OF SACC-PHH WITH HBV DOES NOT REQUIRE DMSO BUT IS SIGNIFICANTLY ENHANCED BY POLYETHYLENE GLYCOL

To facilitate robust HBV infection in both human hepatoma cells overexpressing hNTCP as well as in PHH monocultures, polyethylene glycol (PEG) and dimethyl sulfoxide (DMSO) are routinely added during infection.[21] Optimal DMSO concentrations used for HBV infection and maintenance in hepatoma cells and PHHs vary from 1%-2.5% by volume. We titrated the DMSO concentration in our SACC-PHHs (0%-2% vol/vol). At 0.5% DMSO, no statistically significant difference was observed in the activity of CYP2C9 and CYP3A4, hepatic proteins that are important for drug metabolism (Supporting Fig. S1). SACC-PHHs were challenged with HBV +/- PEG and DMSO. HBV infection was established when PEG was present at the time of challenge, regardless of pretreatment with or

maintenance in DMSO (Supporting Figs. S2 and S3). However, after omitting PEG during the HBV challenge, viral persistence was only observed in cultures maintained in DMSO. Therefore, although DMSO was not essential for productive HBV infection when PEG was present, removal of PEG resulted in a greater need for DMSO treatment (Supporting Figs. S2-G and S4A,B). Infection rates were further improved when both additives were present (please see Supporting Information for more details).

SACC-PHHS SUPPORT HBV/HDV CO-INFECTION AND SUPER-INFECTION

Previous work demonstrated that PHHs are susceptible to HDV virions enveloped with proteins from the related hepadnavirus WHV.[22] However, there is limited information on HBV/HDV co-infection or super-infection in cultured PHHs; therefore, we sought to characterize viral dynamics during such infections in SACC-PHHs. Cultures were challenged with either HBV alone, co-infected with HBV/HDV, or super-infected with HDV once HBV persistence was established for 10 days. Regardless of the donor used (Tables 1 and 2), PHHs became robustly infected with HBV during mono-infection, reaching 2.5-3 arbitrary units (Au) of HBsAg in culture supernatants (Fig. 1A). Infection was further corroborated by 100-1,000 times higher HBV DNA levels (Fig. 1C and Supporting Fig. S5A) and about 10,000 times higher HBV pregenomic RNA (pgRNA) levels (Fig. 1D and Supporting Fig. S4B) in the cell lysates of HBV-challenged versus mock-infected cells. Regardless of co-infection or super-infection, levels of HBsAg exceeded 3 Au in the supernatants of all donor cultures (Fig. 1A). SACC-PHH cultures became HDV viremic during both co-infections and super-infections, as evidenced by about a 1,000-fold increase of HDV RNA from prechallenge levels in the culture supernatants (Fig. 1B and Supporting Fig. S4C).

Differences in HBV replication intermediates were observed across infection conditions, with about 0.5-log higher levels of HBV DNA in HBV mono-infected cultures as compared with HBV/HDV super-infected cultures (Fig. 1C). Although there was no significant difference in HBV DNA levels between mono-infected and co-infected cultures at day 8, there was a significant reduction by day 28 in super-infected versus mono-infected cultures (Fig. 1C). HBV pgRNA levels, however, were 10-fold higher in co-infected SACC-PHH lysates than those of super-infected cells (Fig. 1D). Of interest, the level of HDV genomic RNA was about 10-fold higher in the cell lysates of HBV/HDV co-infected samples compared with super-infected samples (Fig. 1E). The observed differences in viral replication intermediates were not attributable to culture deterioration, as human albumin (hAlb) levels were similar across groups (Supporting Fig. S6).

DETERMINATION OF NUMBER OF HBV, HDV, AND CO-INFECTED SACC-PHHS

To determine the infection rate, we challenged SACC-PHHs with either HBV, HDV, HBV/HDV, or no virus. At 8 days and 28 days post infection (dpi), cells were fixed and stained for nuclei, HBcAg, and HDV genomic RNA using a viral proximity ligation assay for RNA and tyramide signal amplification (vPLAYR/TSA) (Fig. 2A and Supporting Fig. S7). We have used the vPLAYR/TSA technique previously to determine the frequency of HDV infected cells in liver tissue from 1.3× HBV/hNTCP bacterial artificial chromosome transgenic NRG mice.[23] vPLAYR/TSA is a highly sensitive technique that was developed and used to directly detect small RNA fragments.[24] The percentage of infected cells across multiple frames (about 800 cells over three frames) was then quantified for each condition (Fig. 2B-D). For HBV mono-infected cells, approximately 30%-45% were HBcAg+ at either time point. However, dual-positive frequencies in HBV/HDV co-infected cells were 29%-36% at 8 dpi and 15%-28% at 28 dpi. When detecting HDV genomic RNA-positive cells, HDV mono-infected SACC-PHHs had 60%-85% of cells infected on day 8, but this was slightly reduced to 42%-52% by day 28. These data show collectively that there is a large fraction of cells infected with HBV and/or HDV. HDV RNA was readily detectable in the supernatant of HBV/HDV co-infected cultures (Supporting Fig. S8), providing further evidence for bona fide HDV replication.

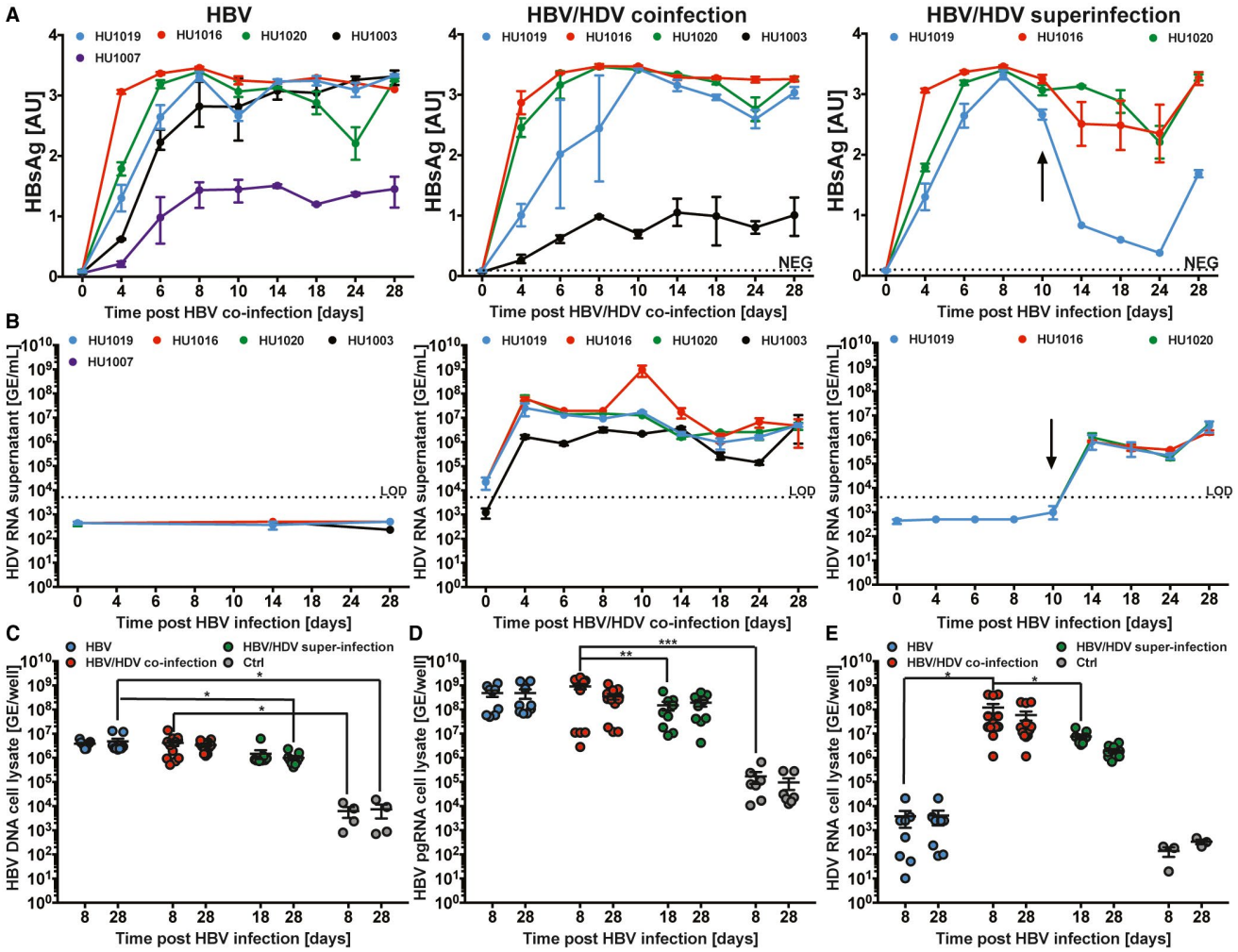

FIG. 1. HBV mono-infection, HBV/HDV co-infection, and HBV/HDV super-infection kinetics in SACC-PHHs. SACC-PHHs were infected with either HBV, HBV/HDV (co-infection), or were first persistently infected with HBV and then with HDV (super-infection). (A) Longitudinal HBsAg enzyme-linked immunosorbent assay (ELISA) data. (B) HDV RNA real-time quantitative PCR data from supernatants. HBV DNA (C), HBV pgRNA (D), or HDV RNA (E) in the cell lysates. All data are presented as mean ± SEM. Statistical significance was determined using ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

CHARACTERIZATION OF HBV INFECTION IN A MICROSCALE 384-WELL FORMAT

To further increase the utility of our platform for high-throughput screening (HTS), we scaled the SACC-PHH cultures down to a 384-well format. In this microformat, SACC-PHHs remained susceptible to HBV infection, as seen by greater than 3 Au HBsAg in the supernatants and only 3.12% variation across all 384 wells following challenge with cell culture–produced HBV (HBVcc) (Fig. 3A,B). This was corroborated by 2.5-logs higher levels of HBV DNA (Fig. 3C) and 4-logs higher HBV pgRNA (Fig. 3D) in the cell lysates of HBV-challenged versus mock-infected SACC-PHHs.

ASSESSMENT OF CLINICALLY RELEVANT DRUG CANDIDATES FOR HBV MONO-INFECTION AND HBV/HDV CO-INFECTION

In this microformat, we then tested the prophylactic and therapeutic effects of clinically relevant DAAs on either HBV mono-infection or HBV/HDV co-infection. In SACC-PHHs, prophylactic entecavir

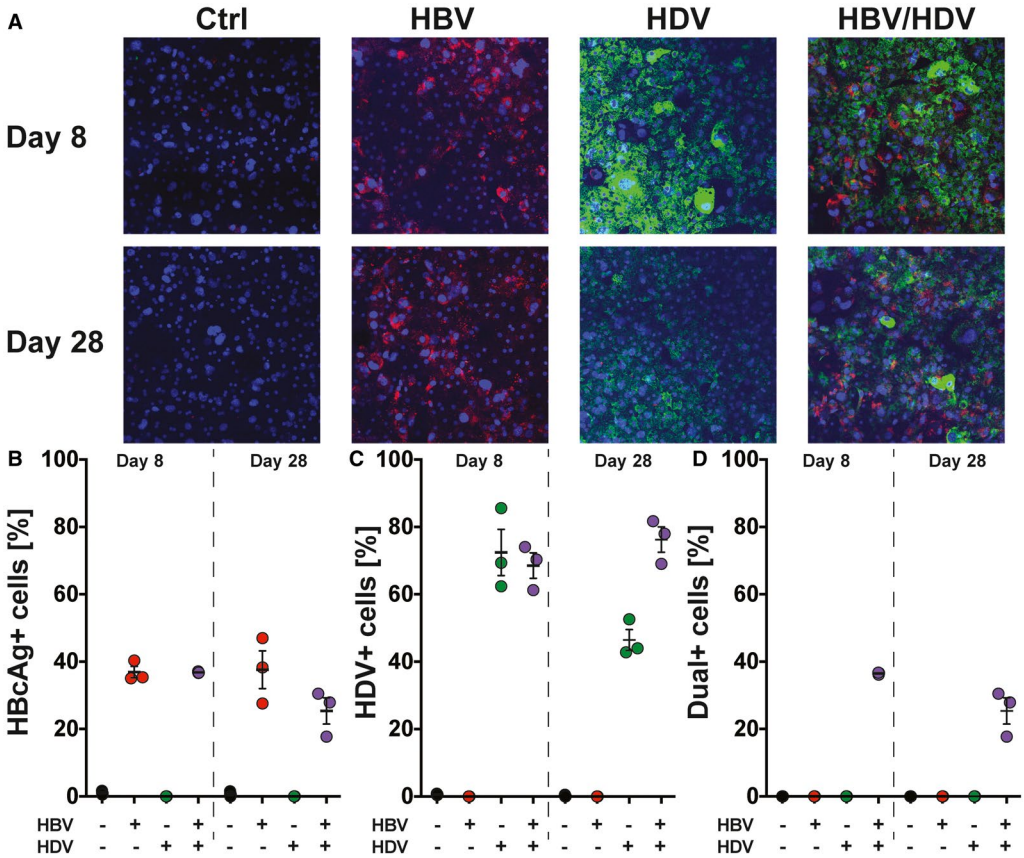

FIG. 2. Quantification of infection in HBV, HDV, and co-infected SACC-PHHs by HDV vPLAYR/TSA and anti-HBcAg immunofluorescence staining. SACC-PHHs were either infected with HBV (multiplicity of infection [MOI] = 4,000), HDV (MOI = 1,000), or both HBV/HDV (HBV MOI = 4,000, HDV MOI = 1,000). (A) At 8 dpi (top) and 28 dpi (bottom), control and infected SACC-PHHs were fixed and stained for HDV genomic RNA (green) by a vPLAYR/TSA procedure and for HBcAg (red) by an anti-HBcAg antibody as well as with 4′,6-diamidino-2-phenylindole (blue) for nuclear DNA. Quantification of three different images for each experimental condition were performed (approximately 800 cells total per condition) for HBcAg positive cells (B), HDV genomic RNA positive cells (C), and dual-positive cells (D).

(ETV) treatment reduced HBV DNA levels in a titration-dependent manner by about 99% at a concentration of 250 nM to about 40% at a concentration of 16 nM (Fig. 3E). When persistently HBV-infected SACC-PHHs were treated with ETV, a similar titration-dependent reduction in HBV DNA levels was observed, from about 95% at 250 nM to about 50% at 16 nM (Fig. 3E).

Recently, the HBV/HDV entry inhibitor Myrcludex B (MyrB) was shown to suppress both HBV DNA and HDV RNA in co-infected patients.[25] SACC-PHHs plated in the 384-well format were either pre-incubated with MyrB or a control peptide[19] before HBV infection, or MyrB was administered therapeutically. In agreement with previous studies, pre-incubation with MyrB effectively blocked about 99% of HBV at a concentration of 1,000 nM, 95% at 100 nM, and about 80%-85% at 10 nM relative to treatment with the control peptide (Fig. 3E). Moreover, when persistent HBV infections were first established and SACC-PHHs were therapeutically treated with MyrB, infection was reduced by about 97% at 1,000 nM and 50% at 10 nM (Fig. 3E).

We next extended the analysis to treatment of HBV/HDV co-infected cultures in 384-well plates. Following challenge with HBV/HDV, HDV RNA levels in the supernatant were 500-fold higher compared with the control (Supporting Fig. S9A). HDV genomic RNA levels in cell lysates were 3-3.5 logs higher than mock wells (Supporting Fig. S9B). In addition, high levels of HBsAg, between 0.8 and 1 Au, were observed for co-infected samples (Supporting

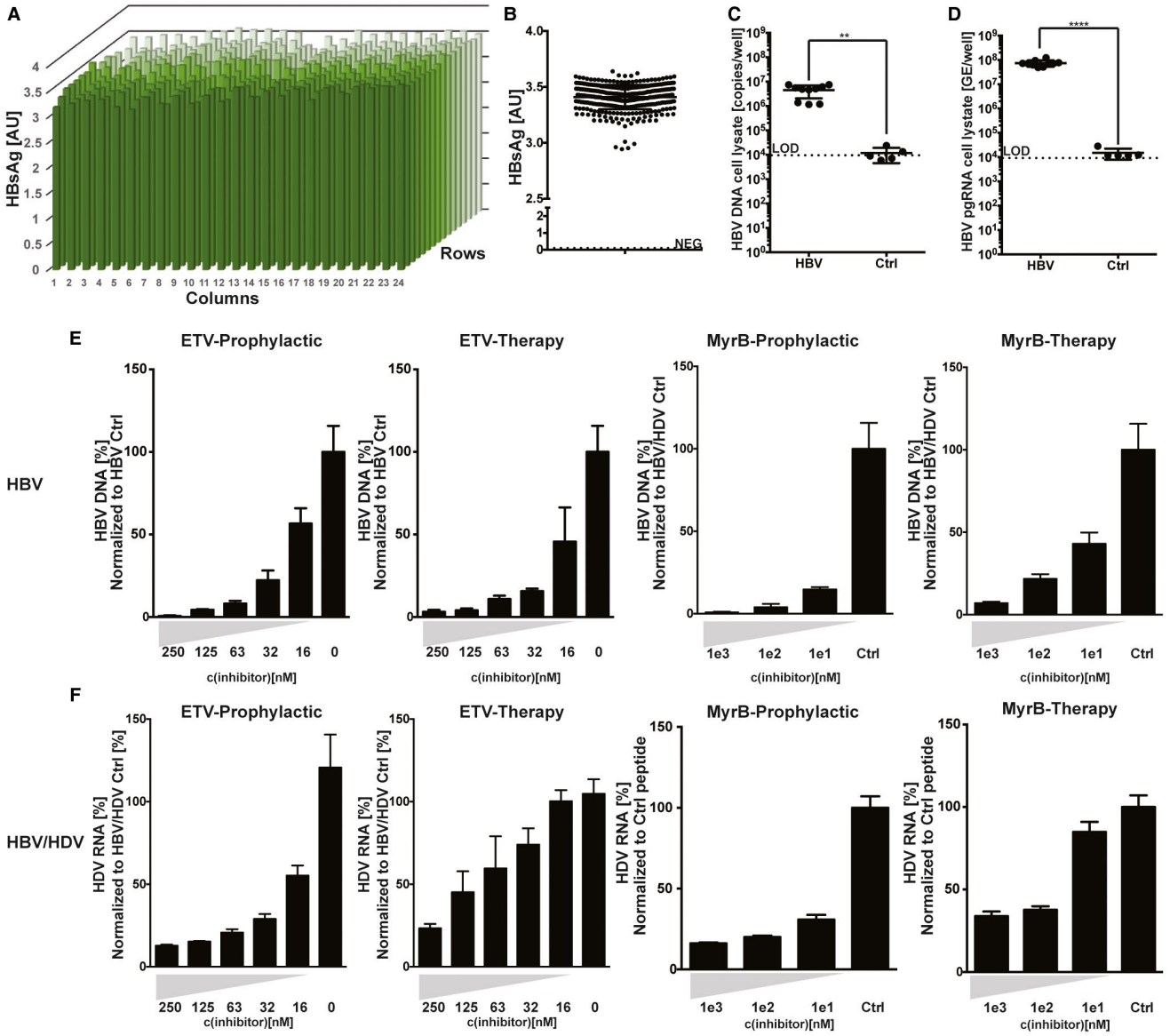

FIG. 3. Assessment of clinically relevant drug treatments for HBV mono-infection and HBV/HDV co-infection of SACC-PHHs in a 384-well microwell format. (A) SACC-PHHs in a 384-well format were infected with HBV, and viremia was assessed for each well by HBsAg ELISA. (B) Summary of HBsAg concentrations determined across the plate shown in (A). Coefficient of variance across the 384-well plate equaled 3.2%. HBV DNA (C) and HBV pgRNA (D) from cell lysates of HBV-infected versus control cultures as assessed by real-time quantitative PCR (HBV, n = 10; control, n = 5). (E) SACC-PHHs were challenged with HBV. The x-axis shows the drug concentration and the y-axis shows the amount of HBV DNA (for ETV) or HBsAg secretion (for MyrB) normalized to untreated, HBVcc-infected untreated control cells, or HBVcc-infected cells treated with a control peptide, respectively. (F) SACC-PHHs were co-infected with HBV/HDV, and HDV RNA was quantified by real-time quantitative PCR in supernatant (far left) and cell lysate (second from left). Co-infected SACC-PHHs were treated with the entry inhibitor MyrB prophylactically (second from right) or therapeutically (far right), with the x-axis showing drug concentration and the y-axis showing the amount of HDV RNA present in the supernatant normalized to that secreted by co-infected cells treated with a control peptide. All samples were run in a 384-well plate with 10 biological replicates. All data are presented as mean ± SEM. Statistical significance was determined using ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Fig. S9C). Together, these data indicate that SACC-PHHs can be successfully co-infected with HBV/HDV in this microwell format.

Next, we aimed to ascertain the effect of ETV on HDV in HBV/HDV co-infected SACC-PHHs. Prophylactic ETV treatment resulted in a

dose-dependent inhibitory effect, with an approximate 80% reduction in HDV RNA levels in the supernatant at 250 nM to a 45% reduction at 16 nM relative to control (Fig. 3F). Therapeutic ETV treatment was less effective, with about 70% inhibition at 250 nM to only 20% at 64 nM. These effects are presumably due to the inhibition of HBV replication at these doses. The effect of ETV at 32 and 16 nM was minimal. Prophylactic MyrB treatment reduced HDV RNA levels by about 87% at 1,000 nM and about 70% at 10 nM (Fig. 3F), whereas therapeutic treatment resulted in about 60% or 15% reduction in HDV RNA levels at 1,000 nM or 10 nM, respectively (Fig. 3F). Notably, no major differences in hAlb levels were observed for HBV mono-infected or HBV/HDV co-infected samples, regardless of treatment (Supporting Fig. S10).

CHARACTERIZATION OF TRANSCRIPTIONAL CHANGES OCCURRING IN HBV MONO-INFECTED AND HBV/HDV CO-INFECTED SACC-PHHS AT EARLY AND LATE STAGES OF INFECTION

The transcriptional changes that occur in PHHs following HBV mono-infection or HBV/HDV co-infection remain incompletely characterized due to rapid de-differentiation and subsequent loss of infection in traditional PHH culture systems. With our platform's improved ability to maintain persistent mono-infection or co-infection for up to 28 days, we sought to characterize the host response to viral infection at both early (8 dpi) and late (28 dpi) timepoints. We compared the cellular transcriptome by using RNA sequencing (RNASeq) of control, HBV mono-infected, and HBV/HDV co-infected SACC-PHH cells from donors HU1007, HU1016, HU1019, and HU1020, as they had similar viral kinetic profiles (Fig. 1). Principal component analyses and heat maps of sample–sample distances based on the regularized $\log_2$-transformed (rlog) counts (routinely used to decrease the variance across samples for genes with low counts) for human and HBV genes demonstrated that variability among samples was due more to variability among donors and not infection, as samples from the same donor generally clustered together regardless of infection status (Supporting Figs. S11 and S12).

We also used our RNASeq data to confirm our SACC-PHH platform's faithful recapitulation of the liver-specific transcriptional profile observed in PHHs within the 3D context of the liver. We used a previously generated list of drug-metabolizing enzymes, predominantly regulated at the transcriptional level and expressed in the human liver, that has been used to evaluate the hepatic phenotype of human ectopic artificial livers as well as fetal hepatocytes and hepatocyte-like cells.[26] We assessed rlog counts for this panel of genes in each of our samples and found that they remained largely unchanged independent of donor, time, or infection (Fig. 4).

We then assessed the differential gene expression between infection conditions after accounting for donor variation. Compared with mock-infected controls, HBV mono-infected and HBV/HDV co-infected SACC-PHHs had more differentially expressed genes ($p_{adj} \leq 0.05$ and absolute value[$\log_2$ (fold change)] ≥ 0.5) at 8 dpi (mono-infected, 90 genes; co-infected, 53 genes) versus 28 dpi (mono-infected, 0 genes; co-infected, 3 genes) (Fig. 5A,B). For both mono-infections and co-infections compared with mock, more genes were down-regulated, but all four HBV genes were consistently up-regulated at both timepoints (Fig. 5A), with normalized counts of HBsAg and viral polymerase higher than those of HBx and core across all infected samples (Supporting Fig. S13). Besides the viral genes, four other genes were down-regulated in both acutely and chronically HBV/HDV co-infected SACC-PHHs compared with mock cultures: platelet and endothelial cell adhesion molecule 1 (*PECAM1*), deoxyribonuclease 1 like 3 (*DNASE1L3*), cadherin 5 (*CDH5*), and phosphatase domain containing paladin 1 (*PALD1*). When examining the co-infected versus mono-infected samples, seven genes were down-regulated at both 8 dpi and 28 dpi: ficolin 3 (*FCN3*), *DNASE1L3*, *CDH5*, roundabout guidance receptor 4 (*ROBO4*), *PECAM1*, kinase insert domain receptor (*KDR*), and *CD93*.

Among the differentially expressed genes of HBV mono-infected versus mock SACC-PHHs, the pathways oxidative phosphorylation and extracellular matrix (ECM)-receptor interactions were enriched at 8 dpi ($p_{adj} \leq 0.07$; Fig. 5C). When comparing differentially expressed genes in the co-infected to

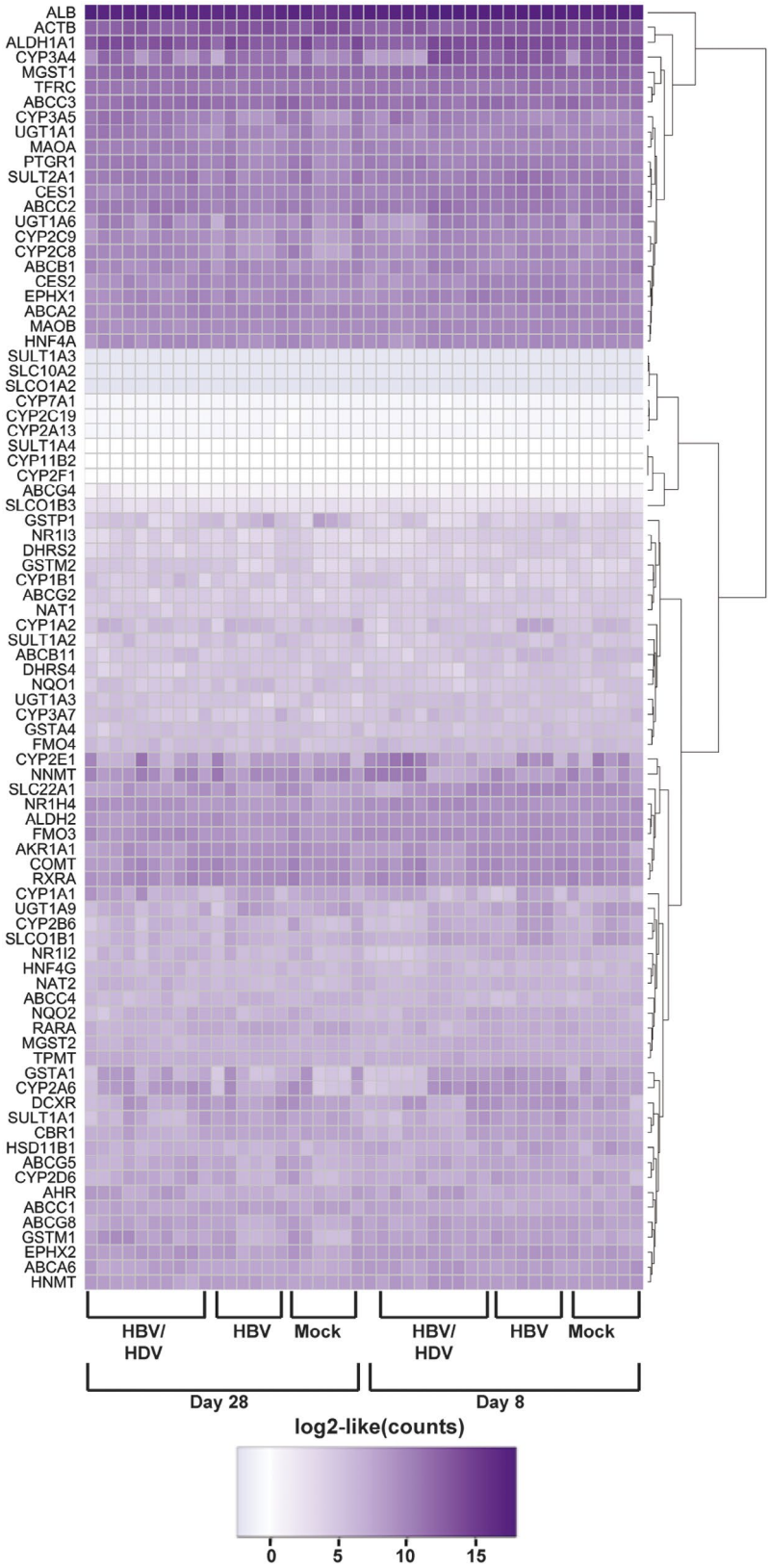
ALB
ACTB
ALDH1A1
CYP3A4
MGST1
TFRC
ABCC3
CYP3A5
UGT1A1
MAOA
PTGR1
SULT2A1
CES1
ABCC2
UGT1A6
CYP2C9
CYP2C8
ABCB1
CES2
EPHX1
ABCA2
MAOB
HNF4A
SULT1A3
SLC10A2
SLCO1A2
CYP7A1
CYP2C19
CYP2A13
SULT1A4
CYP11B2
CYP2F1
ABCG4
SLCO1B3
GSTP1
NR1I3
DHRS2
GSTM2
CYP1B1
ABCG2
NAT1
CYP1A2
SULT1A2
ABCB11
DHRS4
NQO1
UGT1A3
CYP3A7
GSTA4
FMO4
CYP2E1
NNMT
SLC22A1
NR1H4
ALDH2
FMO3
AKR1A1
COMT
RXRA
CYP1A1
UGT1A9
CYP2B6
SLCO1B1
NR1I2
HNF4G
NAT2
ABCC4
NQO2
RARA
MGST2
TPMT
GSTA1
CYP2A6
DCXR
SULT1A1
CBR1
HSD11B1
ABCG5
CYP2D6
AHR
ABCC1
ABCG8
GSTM1
EPHX2
ABCA6
HNMT
HBV/ HDV
HBV
Mock
HBV/ HDV
HBV
Mock
Day 28
Day 8
log2-like(counts)
0
5
10
15

FIG. 4. Regardless of the experimental condition, minimal variation was observed for 86 drug-metabolizing, liver-specific transcripts in SACC-PHHs. The rlog (function in DESeq2) RNASeq counts are shown from the SACC-PHHs mono-infected with HBV, co-infected with HBV/HDV, or mock-infected for 86 drug-metabolizing enzymes, predominantly regulated at the transcriptional level and expressed by the human liver.

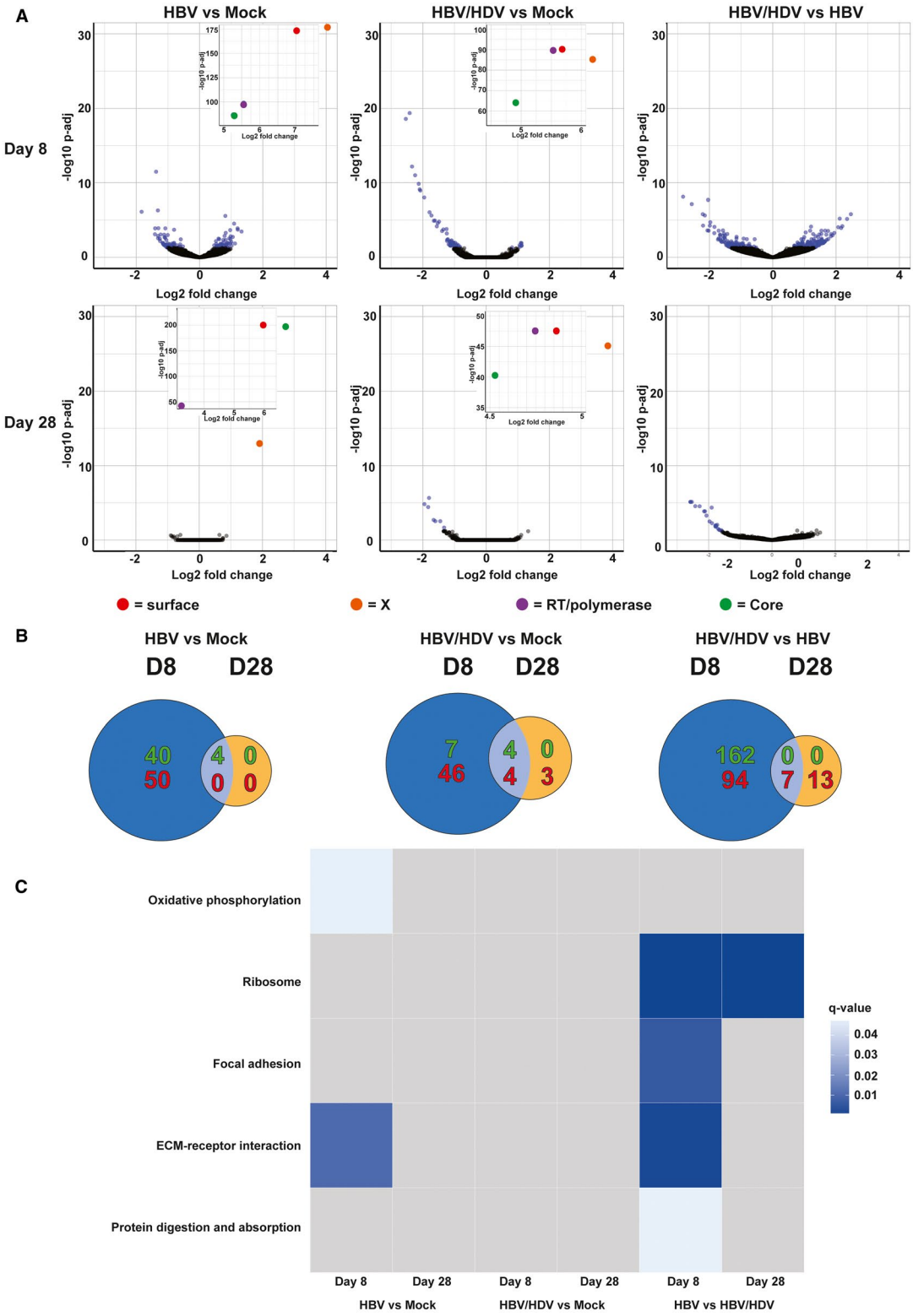

FIG. 5. Transcriptomic analysis of "early"-stage and "late"-stage HBV mono-infected and HBV/HDV co-infected SACC-PHHs. RNA was isolated at 8 dpi and 28 dpi from SACC-PHHs that were HBV mono-infected, HBV/HDV co-infected, or mock-infected. A cDNA library was then generated and sequenced. (A) Volcano plots of differentially expressed genes in mono-infected versus mock, co-infected versus mock, or co-infected versus mono-infected at 8 dpi (top row) and 28 dpi (bottom row). Each point represents a gene ($p_{adj} \leq 0.05$, blue; $p_{adj} > 0.05$, black). HBV genes are indicated in red, orange, purple, or green. (B) Venn diagram of genes up-regulated (green) or down-regulated (red) between mono-infected versus mock, co-infected versus mock, and co-infected versus mono-infected at 8 dpi and 28 dpi. Genes were considered up-regulated or down-regulated if the absolute value of $\log_2$(fold change) ≥ 0.5 and $p_{adj} \leq 0.05$. (C) Significantly enriched pathways (q-value ≤ 0.07) determined using GAGE comparing mono-infected versus control, co-infected versus control, and co-infected versus mono-infected at 8 dpi and 28 dpi.

mono-infected samples, three additional pathways (ribosome, focal adhesion, and protein digestion and absorption) were significantly enriched at 8 dpi. Only the ribosome pathway was still significantly enriched at 28 dpi. No pathways were enriched for the co-infected samples relative to mock, and significant enrichment of innate immune pathways was not observed under any condition. In contrast, by gene ontology (GO) term analysis, there was significant enrichment (q.val ≤ 0.05) among up-regulated genes for members of "type I IFN signaling pathway" at 8 dpi for HBV mono-infected samples compared with mock (Supporting Fig. S14). When comparing differential gene expression from 8 dpi to 28 dpi for each infection condition, only some metabolism pathways were uniquely enriched in the HBV mono-infected samples, along with complement and coagulation cascades and cell adhesion molecules (Supporting Fig. S15). Other metabolism and biosynthesis pathways were consistently enriched across all infection conditions. Minimal transcriptional changes occurred in the murine nonparenchymal stromal cells across all conditions tested (Supporting Fig. S16). Having observed from our transcriptional analysis that HBV/HDV co-infection did not induce strong innate immune activation, we compared OAS-1 and ISG15 induction by real-time quantitative PCR across these donors plus donor HU1003, which had shown very different viral kinetics (Fig. 1, middle). We observed that OAS-1 was consistently not induced in any donor under any condition. However, ISG15 was induced in HU1003 SACC-PHHs at 8 dpi (Supporting Fig. S17A,B). In corroboration of our RNASeq analysis, real-time quantitative PCR of our RNASeq samples showed little change in the average expression of MX1 (Supporting Fig. S18A) and a marked decrease in CDH5 at 8 dpi in the co-infected samples (Supporting Fig. S18B).

HBV/HDV CO-INFECTION DOES NOT DISRUPT INNATE IMMUNE ACTIVATION BY POLY(I:C)

As we observed no enrichment of innate immune pathways but did observe enrichment of members of the GO term "type I IFN signaling pathway" among up-regulated genes in HBV versus mock infected samples at 8 dpi, we wanted to confirm that our SACC-PHHs were competent for ISG expression. As such, poly(I:C), a double-stranded RNA mimic that is a strong inducer of cell-intrinsic innate immune responses, was transfected into SACC-PHHs (Fig. 6A), resulting in induction of OAS-1 (Fig. 6B, left), MX1 (Fig. 6B, middle), and ISG15 (Fig. 6B, right). Next, we aimed to determine whether an established co-infection with HBV/HDV would prevent innate immune activation by poly(I:C); thus, we transfected poly(I:C) into cells persistently co-infected with HBV/HDV for 12 days (Fig. 6A). Innate immunity activation following poly(I:C) transfection was not inhibited by the established HBV/HDV infection; rather, we observed increases in relative OAS-1 (Fig. 6C, left), MX1 (Fig. 6C, middle), and ISG15 (Fig. 6C, right) expression in cell lysates at 12 dpi/12 hours following poly(I:C) transfection. Thus, in contrast to other viruses, the lack of antiviral innate immune signatures during HBV/HDV co-infection cannot be attributed to a generalized suppression of innate immune pathways.

CHARACTERIZATION OF HBV VIRAL KINETICS AND INNATE IMMUNE ACTIVATION FOLLOWING POLY(I:C) TRANSFECTION

We then tested whether inducing these responses inhibited HBV mono-infection, challenging our

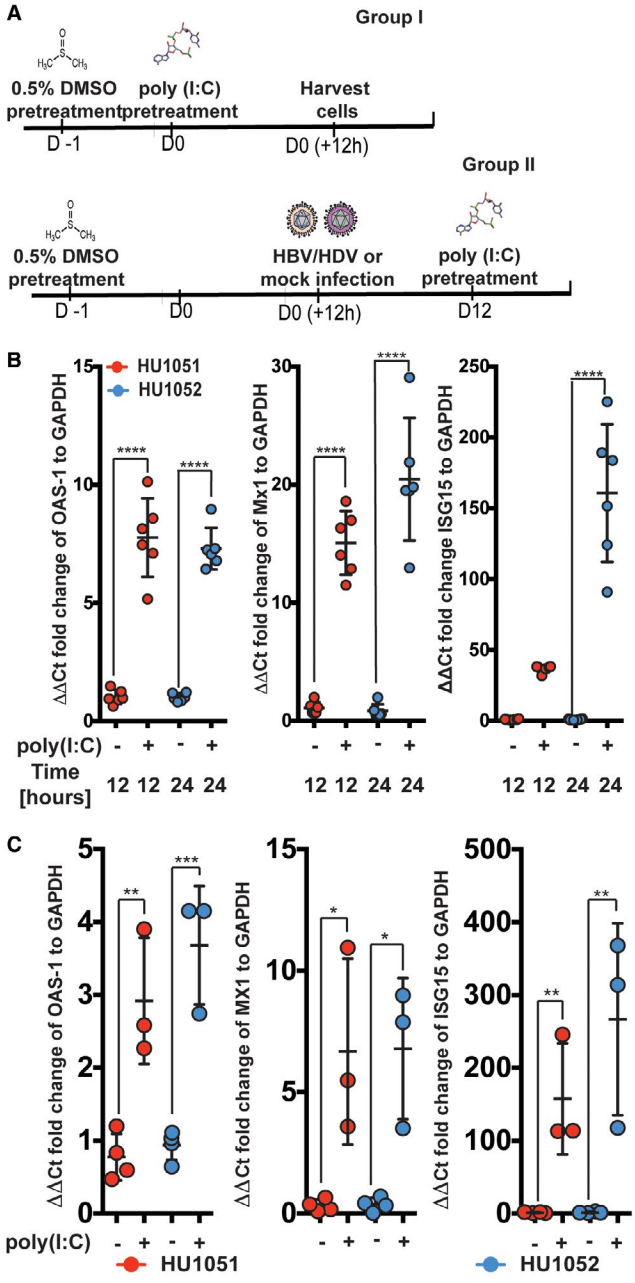

FIG. 6. HBV/HDV co-infection does not generally impair cell-intrinsic innate defenses. (A) Schematic of experimental time course. (B) Fold increase in the ISGs OAS-1 (left), MX1 (middle), and ISG15 (right) normalized to glyceraldehyde 3-phosphate dehydrogenase, 12 and 24 hours following poly(I:C) transfection, quantified by real-time quantitative PCR in the absence of infection. (C) Analysis of OAS-1 (left), MX1 (middle), and ISG15 (right) mRNA expression 12 hours following poly(I:C) exposure of SACC-PHHs co-infected 12 days beforehand. Different colors represent indicated hepatocyte donors. All data are presented as mean ± SEM. Statistical significance was determined using ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

SACC-PHHs either (1) with HBV, (2) pretreating with poly(I:C), or (3) first infecting with HBV and then transfecting in poly(I:C) at 12 dpi (Fig. 7A). Once more, untreated SACC-PHHs were readily infected with HBV, exhibiting high levels of HBsAg in the supernatants (Fig. 7B,C). However, cells treated with poly(I:C) before infection demonstrated HBsAg levels 0.5-0.7 Au lower than non-poly(I:C)-treated samples (Fig. 7B,C and Supporting Fig. S19A,C). SACC-PHHs already persistently infected with HBV for 12 days and then treated with poly(I:C) showed a decline of 0.5-0.8 Au in HBsAg levels over the subsequent 10 days (Fig. 7B,C and Supporting Fig. S19A,C). This suggests that in our SACC-PHHs, induction of cell-intrinsic innate immune responses can inhibit HBV infection.

CHARACTERIZATION OF HBV/HDV VIRAL KINETICS AND INNATE IMMUNE ACTIVATION FOLLOWING POLY(I:C) TRANSFECTION

Pegylated interferon is the only currently approved treatment for HBV/HDV co-infected patients and has debatable efficacy. We next wanted to test whether inducing innate immunity inhibited co-infections, challenging our SACC-PHHs either (1) with HBV/HDV, (2) pretreating with poly(I:C) and then co-infecting, or (3) first co-infecting and then transfecting in poly(I:C) at 12 dpi (Fig. 8A). Following co-infection, high levels of HBsAg were observed (Fig. 8B,C), but pretreatment or treatment 12 dpi with poly(I:C) dampened HBsAg levels in the supernatants (Fig. 8B,C and Supporting Fig. S19B,D). This suggests that co-infection with HDV does not antagonize the innate immune response initiated by poly(I:C). In co-infected, non-poly(I:C)-treated samples, intracellular HDV genomic RNA levels were high (1×10^5 genome equivalents/well) at both 12 dpi and 22 dpi (Fig. 8D, left). In contrast, intracellular HDV genomic RNA was about 1 log lower 12 hours following poly(I:C) transfection (Fig. 8D, middle), but by 22 dpi it was comparable to the non-poly(I:C)-treated controls. Notably, pretreatment with poly(I:C) resulted in about 1 log lower levels of intracellular HDV genomic RNA at both 12 dpi and 22 dpi (Fig. 8D, right).

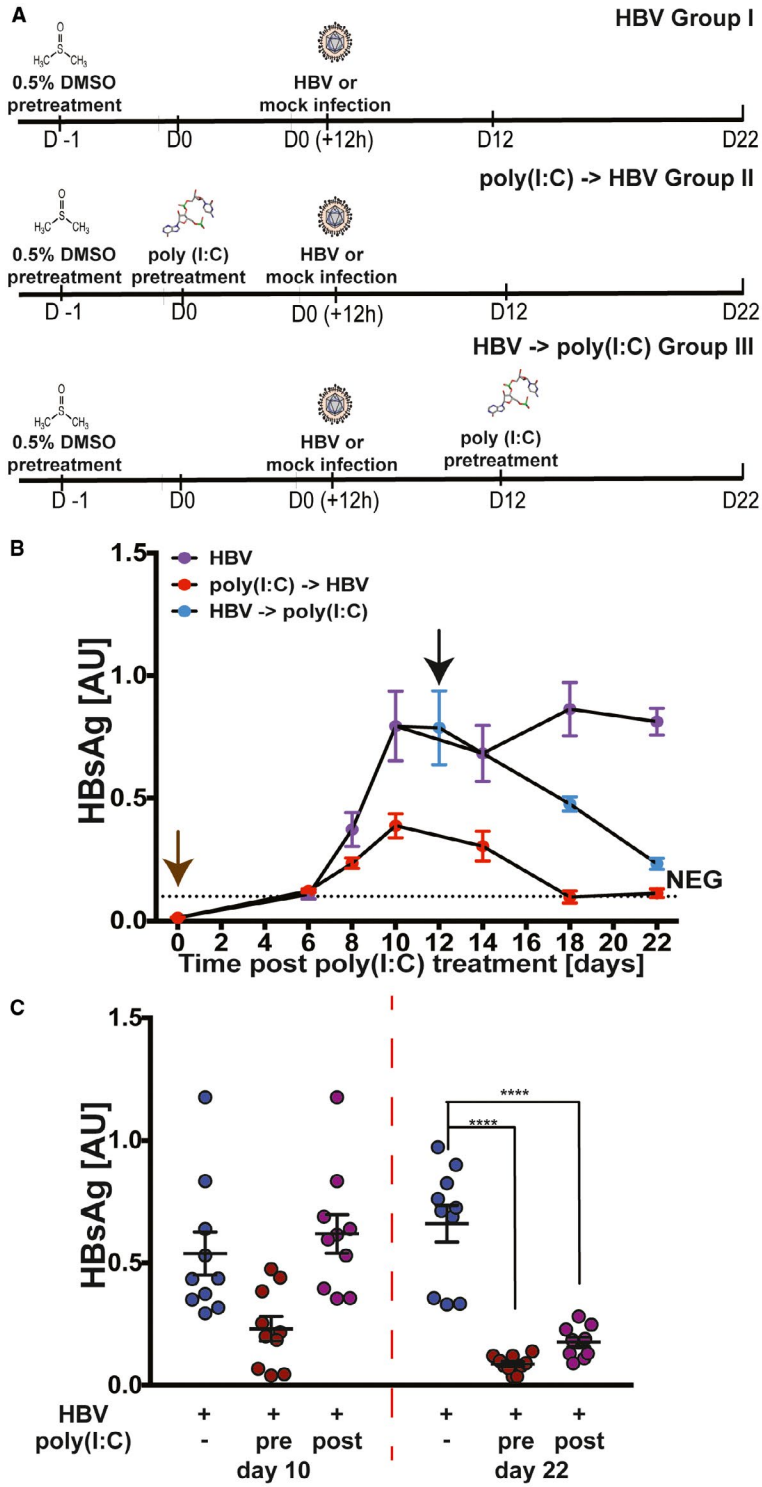

FIG. 7. Induction of innate immunity by poly(I:C) treatment leads to suppression of HBV. (A) Schematic of experimental time course for poly(I:C)-transfected SACC-PHHs infected with HBV. (B,C) Longitudinal measurements of HBsAg by ELISA in supernatants of SACC-PHHs +/- poly(I:C) as shown in (A). Brown and black arrows indicate poly(I:C) transfection either before or after establishment of persistent infection, respectively. Co-infected SACC-PHHs +/- poly(I:C) were lysed 12 dpi and 22 dpi. A separate set of pre-infected SACC-PHHs for each donor was also lysed. Statistical significance was determined using ANOVA. $^{****}P < 0.0001$.

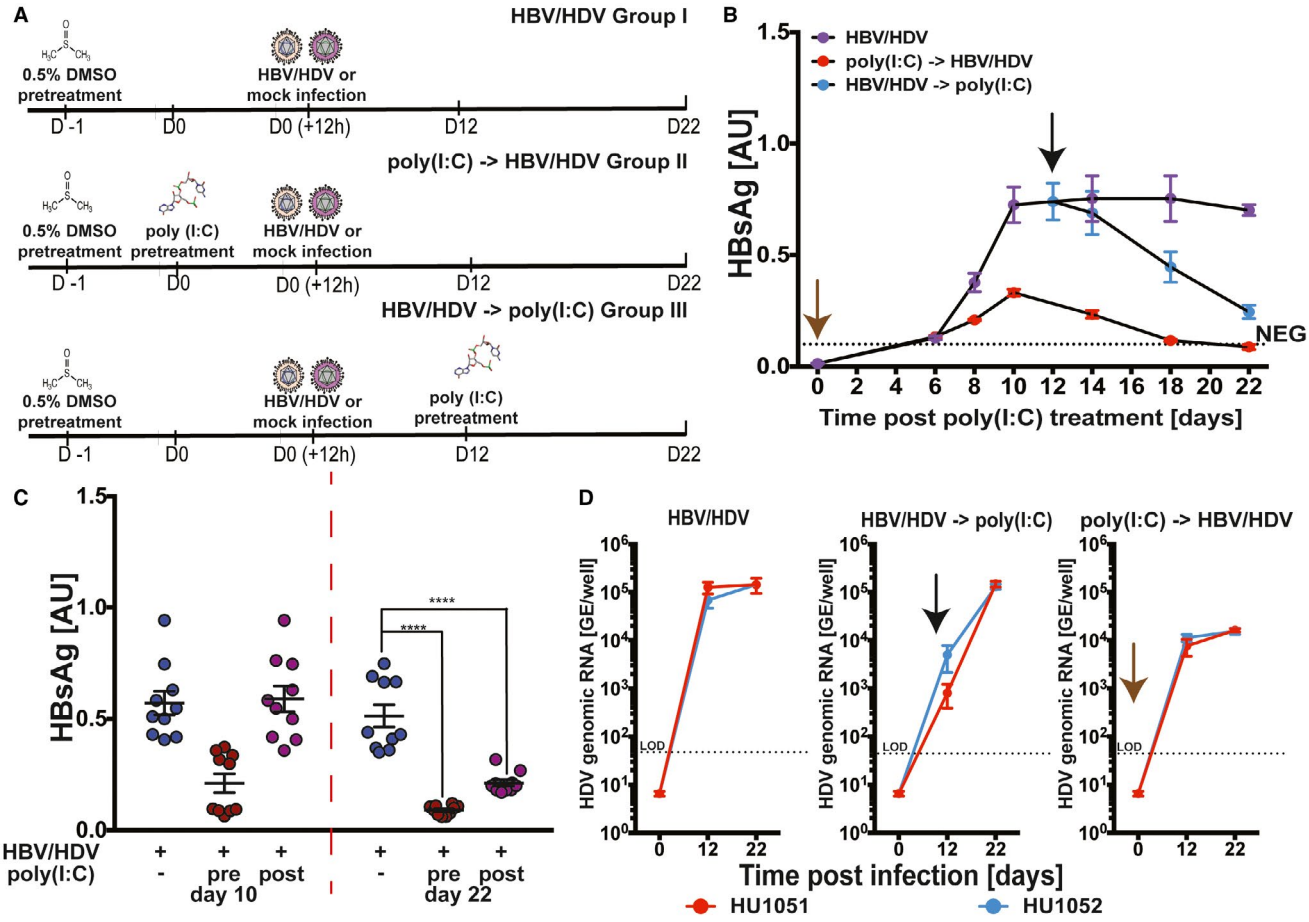

FIG. 8. Induction of innate immunity has little effect on HDV infection. (A) Schematic of experimental time course for poly(I:C)-transfected SACC-PHHs co-infected with HBV/HDV. (B,C) Longitudinal measurements of HBsAg by ELISA in supernatants of co-infected SACC-PHHs +/- poly(I:C) as shown in (A). Brown and black arrows indicate poly(I:C) transfection either before or after establishment of persistent infection, respectively. Co-infected SACC-PHHs +/- poly(I:C) were lysed 12 dpi/hpi and 22 dpi. A separate set of pre-infected SACC-PHHs for each donor was also lysed. (D) Quantification of intracellular HDV genomic RNA by real-time quantitative PCR at 12 dpi and 22 dpi in cultures mock-treated (left) or exposed to poly(I:C) before (middle) or after (right) infection. Statistical significance was determined using ANOVA. ****$P < 0.0001$.

Discussion

Historically, there have been several impediments to successfully using PHH culture systems for the investigation of HBV mono-infection and HBV/HDV co-infection. Additives such as DMSO and PEG are relied on to achieve robust infection. In SACC-PHHs, robust HBV mono-infection or HBV/HDV co-infection can be achieved with pretreatment and maintenance with 0.5% DMSO without perturbing the PHH gene-expression profile. Although PEG can also be removed from the viral challenge conditions, its presence did greatly enhance the infection in SACC-PHHs.

The rapid de-differentiation of PHHs has been a second difficulty in generating advanced PHH culturing systems that can support persistent infections. We aimed to establish HBV/HDV co-infection in our SACC-PHHs. In patients, acute HDV is mostly caused by HBV/HDV co-infection and resembles an acute HBV mono-infection in adults, with less than 5% becoming chronic.[27] However, superinfection, in which a chronic HBV patient is infected with HDV, results in more severe acute hepatitis that leads to chronicity in up to 80% of individuals.[28] During co-infection, high serum levels of both HBV DNA and HDV RNA are observed. However, during superinfection, HBV levels are

suppressed.[28] Similar to co-infected patients, the supernatants of our co-infected SACC-PHH cultures contained high levels of both HBsAg and HDV RNA over the course of infection, whereas superinfected SACC-PHHs exhibited reduced HBsAg levels that rebounded by 18 days following HDV infection and 10-fold lower HBV DNA by 28 dpi, indicating HBV suppression. These data suggest that SACC-PHHs can recapitulate the infection kinetics observed in both acute and chronic HBV and HBV/HDV-infected patients.

We observed high levels of infection in HBV-challenged cells (36%-50% HBcAg+), between 40% and 80% HDV mono-infection, and about 15%-38% dual-infected cells in HBV/HDV co-infected SACC-PHHs. HDV mono-infection rates in mono-cultured PHH's have been previously reported at the high end, with 37%-40%.[29] We used a vPLAYR/TSA procedure that allowed for sensitive detection of HDV genomic RNA. Of interest, we observed a range of signal intensities within the infected cell population, possibly indicating heterogeneity in the levels of HDV replication across the cell population.

We successfully scaled the SACC-PHH platform to 384 wells and demonstrated persistent mono-infection or co-infection for up to 28 days. Of note, susceptibility was not donor-dependent, as all donors tested supported persistent infection. We validated our platform for HTS by testing the efficacy of drugs in clinical trials for treating HBV and HDV, observing in the 384-well format the same inhibitory effects seen in patients.

Finally, it has been unclear how well cultured PHHs resemble those in the 3D context of the liver at the transcriptomic level. Here, we demonstrated that regardless of time, donor or infection, most liver-specific transcripts commonly used as markers of a hepatic phenotype remained up-regulated in our SACC-PHHs, arguing that the differential gene-expression profiles derived from these samples were a result of infection, not culture deterioration. Furthermore, the nonparenchymal cells co-cultured with our PHHs demonstrated little differential transcriptional activity in infected versus noninfected cultures, suggesting minimal impact on infection. Regardless of timepoint, HBV viral genes remained highly up-regulated in both mono-infected and co-infected samples, corroborating the persistence of infection. Of note, HBsAg was the most up-regulated HBV transcript and HBx was the least up-regulated across all experimental conditions.

The host response to HBV mono-infection and HBV/HDV co-infection has been a contentious area of research, especially regarding innate immunity. Although some studies have observed innate immune activation following infection,[30] others have reported minimal to none, leading to the characterization of HBV as a "stealth" virus.[31] For HBV to evade inducing an innate immune response, it must either never be detected or inhibit activation. Recently, it was observed that liver biopsies freshly isolated from chronic HBV patients did not have an induced interferon (IFN) or ISG response unless poly(I:C) was transfected into cells,[32] suggesting that HBV evades detection. In the SACC-PHH platform, most of the changes in gene expression relative to mock occurred at 8 dpi. However, only the HBV mono-infected samples at 8 dpi exhibited coordinated changes of genes involved with pathways such as oxidative phosphorylation and ECM-receptor interaction, confirming other studies that have suggested how HBV hijacks the host machinery to promote viral production and persistence.[33] The lack of enrichment for pathways in the co-infected versus mock samples was not surprising, given the lower level of differential gene expression. Although we tested multiple donors in multiple replicates for this study, we still cannot be certain that additional samples might not lead to a greater differential.

In this study, we did not observe significant pathway enrichment for innate immune activation in response to HBV mono-infection. Only at 8 dpi did we see among the up-regulated genes enrichment for the GO term "type I IFN signaling pathway." At best, this indicates a weak innate immune response, potentially disjointed in its manifestation, as we did not detect coordinated changes in other related pathways or GO terms. Similarly, we did not observe innate immune activation in the HBV/HDV co-infected samples. This is in contrast to data that showed HDV elicited about an 1.5-log peak induction of IFN-β and IFN-λ expression at 5 dpi and a decrease by 7 dpi in mono-cultured PHHs.[29] However, there are several factors that make the comparison of this study to the present one questionable, including cell type (HepaRG and hNTCP-expressing HepG2 cells versus PHHs), type of infection (HDV mono-infection versus our co-infections), and variable timepoints (3-7 dpi versus 8 dpi and 28 dpi in our study). Importantly, the "early" and "late" timepoints were

unattainable in previous studies, as PHH cultures could not be maintained for long periods of time, only allowing for a short window of investigation. However, more broadly the unbiased approach to investigating the transcriptome of infected PHHs shows how our platform can be used to probe virus–host interactions to gain a better understanding of the changes occurring within infected human hepatocytes that promote establishment and persistence.

We also corroborated that the lack of a robust innate immune response to mono-infection or co-infection in our transcriptomic data is biologically relevant and not merely a consequence of our co-culture platform. Our data suggest that HBV is likely undetected by host immune pathways, as triggering ISG induction by poly(I:C) transfection led to a significant reduction in HBV viral parameters, indicating that the virus remains sensitive to innate immune responses. It is feasible that HBV may not be able to overcome a strong coordinated innate immune response as induced by poly(I:C). If a weak or deficient innate immune response is triggered following HBV infection, the virus might still be able to evade or modulate the host response. Additionally, we observed that there is undeniably a genetic component to the differences in observed response, depending on the donor used. For example, one donor (HU1003), although readily susceptible to HBV and HBV/HDV co-infection, had markedly different viral kinetics compared with the donors used for RNASeq and, unlike these other donors, exhibited up-regulated levels of ISG15 at 8 dpi. It will be of interest in future work to use larger panels of donors classified into subgroups by their viral kinetics to assess the possible connections between their transcriptomic and infection profiles.

The SACC-PHH platform has several advantages over current systems, including improved infection conditions, scalability to a 384-well format, enhancing the utility for HTS or large-scale genetic screens. Both a benefit and a drawback are that the SACC-PHH system consists of only permissive PHHs and nonpermissive nonparenchymal stromal cells. Thus, innate/cell-intrinsic immune activation is decoupled from the adaptive immune response. Co-culturing SACC-PHHs with other nonparenchymal and/or immune cells, such as $CD8^+$ T cells, would be a further advance of this system that could aid in our understanding of T-cell exhaustion and HBV pathology. Lastly, the RNASeq data set described here is a resource to begin understanding the global effects, or lack thereof, that occur during the early and late stages of infection in PHHs.

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Supporting Information

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