SUPPLEMENTARY INFORMATION

FOR

Analysis of host responses to hepatitis B and delta viral infections in a micro-scalable hepatic co-culture system

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Running title: Transcriptional host responses to hepatitis B mono- or hepatitis B and delta co-infections in a micro-scalable hepatic co-culture system.

Key words: Hepatitis B virus, hepatitis delta virus, viral hepatitis, primary hepatocyte culture, tissue engineering

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Extended Results section:

Infection of SACC-PHH with HBV does not require DMSO but is significantly enhanced by PEG.

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% v/v). At 1% > DMSO abnormal morphology and cell death were observed (Supporting Fig.1A). At 0.5% DMSO, no statistically significant difference was observed in the activity of CYP2C9 and CYP3A4, hepatic proteins important for drug metabolism (Supporting Fig. 1B-C). SACC-PHHs were challenged with HBV +/- PEG and DMSO. HBV infection was established when PEG was present at the time of challenge, regardless of pre-treatment with or maintenance in DMSO, as demonstrated by 0.5 Au levels higher of secreted HBsAg in culture supernatants (Supporting Fig. 2A, Supporting Fig. 3A and 3C), 10-fold higher HBV DNA levels (Supporting Fig. 2B) and 1000-fold higher HBV pgRNA levels (Supporting Fig. 2C) compared to non-infected cells. However, upon omission of PEG during HBV challenge, viral persistence was only observed in cultures maintained in DMSO (Supporting Fig. 2A-C, Supporting Fig. 3B and 3D). Therefore, although DMSO was not essential for productive HBV infection when PEG was present during challenge, removal of PEG resulted in a greater need for DMSO treatment as demonstrated by the higher percentage of infected cells in cultures maintained in DMSO (~40% versus ~20% HBcAg+, treated versus untreated) (Supporting Fig. 2D-G, Supporting Fig. 4A and 4B). Infection rates were further improved when both additives were present.

Material and Methods:

Generation of self-assembling primary hepatocyte co-cultures (SACC-PHHs)

Cryopreserved human hepatocytes were obtained from Bioreclamation IVT Inc. (Westbury, NY), ThermoFisher 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 non-parenchymal mouse embryonic fibroblast 3T3-J2 cells (CCL-92, ATCC, Manassas, VA)(1-3). Cryopreserved hepatocytes were removed from liquid nitrogen and thawed in a water bath at 37ºC. Hepatocytes were transferred to a 50 mL conical tube containing 20 mL plating medium (Hµrel PlatinumHeps plating medium™, Hµrel Corporation, New Brunswick, NJ), and centrifuged at 150xg for 10 minutes at room temperature. After removing the supernatant, the cells were re-suspended in Hµrel Platinum Heps plating medium™ and cell number as well as cell viability were assessed using trypan blue exclusion. 3T3-J2 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Inoza, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 200 U/mL penicillin/streptomycin. Cells were maintained at 37°C in a 5% CO_2: 95% air-humidified atmosphere until experimental plating. On plating day, cells were detached from the plate surface using trypsin (0.25%), suspended in 15 mL DMEM medium and centrifuged at 200xg for 5 minutes at room temperature. After removing the supernatant, the cells were re-suspended in

plating medium (Hμrel PlatinumHeps™, Hμrel Corporation, New Brunswick, NJ) and cell number and viability were determined using trypan blue exclusion.

All co-cultures were plated on collagen type-I coated, tissue culture treated plates 96 well and 24 well (Corning Inc, Corning NY). Hepatocytes were seeded at a density of 11,000, 30,000, 90,000 and188,000 cells in each well of a 384, 96, 8 chamber glass slide, and 24 well plate, respectively. 3T3-J2 cells were added the next day at 5,000, 15,000, 90,000 and 90,000 per well of a 384, 96, 8 chamber glass slide, and 24 well plate, respectively. Hμrelhuman™-24 and Hμrelhuman™-96, and Hμrelhuman™-384 SACC-PHH are distributed by the Hμrel Corporation (New Brunswick, NJ). Cells were maintained in 500 μl for 24 well plates and 150 μl for 96 well plates, and 50 μl for 384 well plates in Hμrel PlatinumHeps maintenance medium™ (Hμrel Corporation, New Brunswick, NJ). Medium was replaced every 2 days. The cells were co-cultured at 37°C in a 5% CO_2 for 10 days prior to HBV infections.

Production of cell-culture derived HBV

HepG2.2.15 cells(4) were grown in media containing tetracycline until they reached a confluency of 100%. At this time media was changed to DMEM F12 media supplemented with 10% FBS, 1% Pen/Strep. Media from the HepG2.2.15 culture was collected every two to three days for approximately 3 weeks. The collected media was sterile-filtered through a 0.22 μm filter (Millipore, Darmstadt, Germany) and was then concentrated ca. 100-fold using a stir cell concentrator (Millipore, Darmstadt, Germany). After concentration, the virus was run over a 5 mL HiTrap heparin column (GE, Fairfield, CA) to further concentrate and purify infectious virus particles from non-infectious sub-viral particles. Concentrated virus was applied to a heparin column, which was washed with 5 column volume of wash buffer (20 mM phosphate buffer, 50 mM NaCl, pH= ~7). Afterwards, the virus was eluted with elution buffer (20 mM phosphate buffer, 2 M NaCl, pH= ~7). Once all virus was eluted, the viral stock was dialyzed with sterile 1X PBS using a 30 kDa dialysis cassette (Millipore, Darmstadt, Germany). After dialysis, virus was aliquoted into cryovial tubes and cryopreserved at -80°C until use.

Production of materials for mock infections

DMEM F12 media supplemented with 10% FBS, and 1% Penn/Strep spent media that had been used on HepG2 cells was concentrated 100-fold from 4 liters to 40 mL's using stir cell concentrator (Millipore, Darmstadt, Germany). This material was passed over a 5 mL HiTrap heparin column (GE, Fairfield, CA) using the same purification conditions as for HBV virus. Once eluted the material was dialyzed with sterile 1X PBS using a 30 kDa dialysis cassette (Millipore, Darmstadt, Germany). This material was frozen down in 1 mL aliquotes into cryovial tubes and cryopreserved at -80°C until use in mock infection conditions.

Generation of HBV Envelope expressing HepG2 cells

The large (L), medium (M), and small (S) HBV envelope proteins (HBV Genotype C, Genebank # FJ518810) followed by an internal ribosome entry site (IRES) and tagBFP fluorescent reporter were cloned into the pTrip-tagBFP-DEST lentiviral backbone using Gateway cloning (Invitrogen, Carlsbad, CA), generating the construct pTrip-CMV-HBV-ENV-IRES-tagBFP-DEST. This construct was transformed into Sure2 *E. coli* cells (Invitrogen, Carlsbad, CA). Colonies were picked, grown under ampicillin selection, mini-prepped (Qiagen, Hilden, Germany) and sequenced (MacrogenUSA, New York City, NY). Lentiviral pseudoparticles were generated by co-transfecting 4x $x10^6$ 293T cells in a 10 cm plate using Xtremegene (Sigma-Aldrich, St. Louis, MO) with plasmids expressing the pTrip-CMV-HBV-ENV-IRES-tagBFP-DEST proviral DNA, HIV-1 *gag–pol*, and VSV-G in a ratio of 1/0.8/0.2, respectively, as previously described(5). Supernatants were collected at 24 and 48 hours, pooled, and filtered through a 0.45 μm filter

(Millipore, Darmstadt, Germany). Filtered lentiviral supernatants were supplemented with polybrene (1:1000 by volume) and 1M HEPES (1:50 by volume), aliquoted, and stored at -80°C until use. HepG2 cells were seeded at $2.5x10^5$ cells per well in a full 6 well collagen-coated polystyrene plate (Greiner Bio-One, Monroe, NC) and transduced with lentivirus expressing the HBV-Env-BFP-IRES-tagBFP by spin inoculation (2000 rpm, 37°C, 2 hours). The transduced cells were then single-cell sorted on a BD FACSVantage SE w/Diva (BD, San Diego, CA) for those cells with high tagBFP expression into collagen-coated 96 well plates (Greiner Bio-One, Monroe, NC). HBV Env BFP HepG2 clones were expanded, BFP expression determined by FACS using a BD LSR2 flow cytometer (BD Biosciences, San Diego, CA) and data analyzed using FlowJo (Treestar Software, Ashland, OR). Clones with high BFP expression were kept and tested for expression of HBV envelope genes and HBsAg production by RT-qPCR and HBsAg ELISA, respectively. The best clone, 4C8, was utilized for hepatitis delta virus (HDV) production.

Production of cell-culture derived hepatitis delta virus (HDV)

HBV Env BFP HepG2 (4C8) cells were seeded in a 15 cm collagen coated polystyrene tissue culture plate. Once the cells had reached confluency they were transfected with the pSVL(D3) plasmid (Addgene, Cambridge, MA, plasmid # 29335) which contains three head-to-tail copies of the HDV genome(6). After 6 hours post transfection, medium was replaced with fresh DMEM media containing 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin. Supernatants were collected over a 4 week period, filtered through a 0.45 μm filter (Millipore, Darmstadt, Germany), concentrated ca 100 fold using a stir-cell concentrator stir cell concentrator (Millipore, Darmstadt, Germany). run over a 5 mL HiTrap heparin column (GE, Fairfield, CA), and dialyzed using a 30 kDa cutoff 30 mL dialysis cassette (Millipore, Darmstadt, Germany). HDV stocks were aliquoted and cryopreserved at -80°C until use.

Human albumin ELISA

Chromatographically purified human albumin was obtained from MP Biomedicals (catalogue #2191349, Santa Ana, CA) and horseradish peroxidase-conjugated goat IgG to human albumin was obtained from Bethyl Labs (catalogue # A80-129P, Montgomery, TX). Following completion of the desired infection and treatment period, serum albumin content was quantified by competitive enzyme linked immunosorbent assay (ELISA). Absorbance was measured at 490 nm. All 384-well, 96-well and 24-well plates were assayed in an identical manner.

HBsAg ELISA

Detection and quantification of HBsAg levels was performed by ELISA according to the manufacturer's instructions (GS HbsAg EIA 3.1, Bio-Rad, Hercules, CA). Briefly, a 100 µl sample of a 1:20 dilution of supernatant was prepared in 1x PBS was used in lieu of undiluted supernatant. Absorbance was read at 450λ on the BertholdTech TriStar (Bad Wildbad, Germany).

HBV DNA isolation from supernatants

HBV DNA was isolated using the Qiamp MinElute Virus Spin Kit (50), (Qiagen, Hilden, Germany). HBV DNA was eluted in 60 µl of elution buffer, and 5 µl was used per well in the HBV DNA qPCR reaction.

HDV RNA isolation from supernatants

HDV RNA was isolated using the ZR Viral RNA KIT (200 preps) (Zymo, Irvine, CA). HDV RNA was eluted in 20 µl of DNase/RNase free water and 5 µl was used per well in the HDV RNA RT-qPCR assay.

Total HBV DNA isolation and quantification from infected cells

To isolate total HBV DNA from HBV challenged SACC-PHHs, 300 µl (24 well) or 100 µl (96 well) of lysis buffer was added respectively to the corresponding sample (50 mM Tris-Base, 50 mM EDTA, 1% SDS, 100mM NaCl pH 8.0). The sample was further digested with 20 µl of Proteinase K per sample from a QIAMP DNA mini kit (Qiagen, Hilden, Germany) for an hour at 37ºC. RNAse A (SigmaAldrich, St. Luis, MO) was added to the lysate (1 µl) and incubated at room temperature for 2 minutes. 500 µl of AL lysis buffer (Qiagen, Hilden, Germany) was subsequently added to the solution. The samples were incubated at 70ºC for 4 hours and were vortexed every 20 minutes to digest the cells completely. Following this step, 500 µl of 100% (v/v) EtOH were added and mixed thoroughly by inverting 10 times. This suspension was then applied to a Qiamp DNA mini kit column and centrifuged for 1 minute at 13,000 revolutions per minute (rpm). The samples were spun again in new tubes for 1 min at 13,000 rpm to dry. The DNA was then eluted with 50 µl of AE buffer and concentrations measured using a Nanodrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA).

A 5 µl aliquot of HBV DNA isolated from lysed cells was used per reaction well. To amplify HBV DNA the following primers and probes were used: CCGTCTGTGCCTTCTCATCTG (forward primer), AGTCCAAGAGTCCTCTTATGTAAGACCTT (reverse primer), and probe FAM-CCGTGTGCACTTCGCTTCACCTCTGC-TAMRA 22. Primers were kept at a concentration of 600 nM and probe at 300 nM final concentration in the reaction. A master mix was created containing 2X TaqMan reaction mix (AppliedBiosystems, Foster City, CA), primer/probe mix and ddH_2O. The master mix was then applied with the samples to the respective wells. 5 µl of the standards and the samples were added to the respective wells. The following PCR program was run on a Step One Plus qPCR machine (Life Technologies, Carlsbad, CA): 50ºC for 5 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec, 56ºC for 40 sec, and 72ºC for 20 sec.

HBV pgRNA isolation and quantification from infected primary hepatocytes

SACC-PHHs were lysed with 350 µl RLT (Qiagen, Hilden, Germany) buffer supplemented with 2-Mercaptoethanol for 10 minutes at room temperature (RT). The cells were then pipetted into an RNase free Eppendorf tube and passed through a 26 ½ gauge needle 5 times in order to facilitate cell lysis. Once cells were lysed the manufacturers protocol was followed Qiagen RNAeasy kit (Qiagen, Hilden, Germany) for elution where the sample was eluted twice once with 50 µl of Rnase free water and then addition 30 µl.

To quantify HBV pgRNA a modified Luna® Universal One-Step RT-qPCR Kit (NEB, Ipswitch MA) protocol was used. A primer mix with each primer at 3 µM was created with the forward primer GAGTGTGGATTCGCACTCC and the reverse primer GAGGCGAGGGAGTTCTTCT 2. A master mix was created as follows per reaction: 5 µl of SYBR mix, 0.125 µl of reverse transcriptase, 1 µl of primer mix, and 1.875 µl of ddH2O. The following cycling times was used: reverse transcription and amplification step at 50ºC for 10 min and 95ºC for 1 min; 40 cycles of 95ºC 15 sec, and 60ºC for 1 min. The melt curve was performed at 95ºC for 5 sec, 65ºC for 5 sec, 95ºC for 15 sec, and 50ºC for 5 sec.

HDV viral proximity ligation assay for RNA (vPLAYR), Tyramide signal amplification (TSA), and HBcAg co-staining of HBV, HDV, and co-infected SACC-PHHs

SACC-PHHs were seeded in a collagen-coated 8 chamber slide at a density of 90,000 PHHs and 30,000 NIH 3T3 J cells. These were then challenged with HBV (MOI= 4,000), HDV (MOI= 1,000), or both viruses as described above. After challenge, cells were washed with Hµrel maintenance media 5 times and Hµrel maintenance media supplemented with 0.5% DMSO was added and replaced every two days. At days 8 and 28 post-infection, cells were fixed with 4% PFA for 20 minutes at room temperature. Cells were then stored in RNA Later (Thermo Fischer, Waltham, MA) overnight at 4ºC.

The following day, an adapted vPLAYR/TSA was performed (7, 8). First, the cells were washed once with general wash buffer (1X sterile PBS, 0.1% Tween, with 4U/mL of RiboLock RNase Inhibitor, (ThermoFisher, Waltham MA) to remove any residual 4% PFA. Subsequently, cells were incubated for 20 minutes at 40ºC in incubation buffer (2x saline-sodium citrate stock (SSC, Sigma-Aldrich, St. Louis MO), 2.5% v/v polyvinylsulfonic acid (Sigma-Aldrich, St. Louis MO), 20 mM ribonucleoside vanadyl complex (Sigma-Aldrich, St. Louis MO), 40 U/mL RiboLock RNase Inhibitor, 0.1% Tween, 100 µg/mL salmon sperm DNA (Sigma-Aldrich, St. Louis MO), and 10% v/v formamide (Sigma-Aldrich, St. Louis MO)) without oligo probes added. After 20 minutes, oligo probes were added at a final concentration of 100 nM, and slides were put back at 40ºC for 1 hour. To reduce background, after the 1 hour incubation with probe the slides were incubated for 20 minutes at 40ºC using a 2x SSC with 10% v/v formamide solution. Cells were then washed three times with general wash buffer at room temperature for 5 minutes per wash. After washes, cells were incubated for 30 minutes at 37ºC with hybridization buffer (100 nM final backbone/insert oligos, 1X SSC, 0.1% Tween, 40 U/mL RiboLock RNase Inhibitor, brought up in 1X sterile PBS). Again, after incubation cells were washed three times with general wash buffer, 5 minutes per wash at room temperature. Cells were then incubated for 1 hour at 37ºC in ligation solution (0.125 units/ul T4 ligase (New England Biolabs, Ipswich, MA), 1X T4 ligase buffer (NEB), 250 µg/mL BSA, 0.05% Tween, 40 U/mL RiboLock RNase Inhibitor, brought up with DEPC treated ddH_2O). Afterwards, cells were washed once with general wash buffer and were incubated for 3 hours at 37ºC with rolling cycle amplification buffer (0.1 U/µL Phi29, 1X Phi29 buffer, 250 µM dNTPs, 5% glycerol, 250 µg/mL BSA, 0.05% Tween, 40 U/mL RiboLock RNase Inhibitor brought up in DEPC treated ddH_2O).

A Tyramide signal amplification (TSA) was then performed using the Invitrogen Alexaflour 488 Tyramide Superboost kit (Invitrogen, Carlsbad, CA) as follows: After the 3 hour incubation, the cells were washed three times with general wash buffer. Next, to remove any endogenous peroxidase activity, cells were treated with 3% H_2O_2 for 30 minutes at room temperature. After H_2O_2 treatment, the cells were washed 5 times with general wash buffer to remove any residual H_2O_2. To block any endogenous biotin or streptavidin SACC-PHHs were incubated with biotin first for 20 minutes, were washed once and subsequently incubated with streptavidin. Afterwards, SACC-PHHs were washed an additional three times with general wash buffer and then incubated with biotinylated probe (10 nM, 1X SSC, in 1X sterile PBS) for 45 minutes at 37ºC. Cells were then washed three times with sterile 1X PBS. Two to three drops, or about 200 µL, of poly-HRP-conjugated secondary antibody was added to cover the cells, which were then incubated at room temperature for 45 minutes. Afterwards, the cells were washed with 1X PBS five times. Approximately 200 µL of tyramide working solution were added per well following the Invitrogen TSA protocol and incubated for 10 minutes. Stopping solution was then added and cells were washed three times with sterile 1X PBS.

In order to stain for HBV-infected cells, the following procedure was followed after vPLAYR/TSA amplification of HDV RNA. First, the SACC-PHHs were blocked with goat serum (Invitrogen, Carlsbad CA) for 1.5 hours. Then the cells were incubated with the primary anti-HBcAg antibody (DAKO rabbit polyclonal, catalog #B0586, Santa Clara, CA) at a 1:400 dilution for 1 hour. After the 1-hour incubation with the primary antibody, the cells were washed 5 times with 1X PBS. The secondary was then added (Anti-rabbit Alexa 594, 1:400, Fisher Scientific, Waltham, MA) along with the nuclear Hoechst dye (1:10000, Sigma-Aldrich, St. Louis, MO) and the cells were incubated for an additional hour. After the incubation the cells were washed 5 times and then incubated with 1X PBS supplemented with DAPI (1:5000) for 30 minutes at room temperature. The cells were then washed three times with sterile 1X PBS and the plastic well container was removed and a small amount of ProLong Gold Antifade Mountant (Thermo Fischer, Waltham, MA) was used. A cover slip was added and sealed with nail polish. The cells were stored at 4ºC until imaging. Images were taken on a Nikon A1 (Nikon, Minato, Tokyo, Japan) confocal microscope in the Princeton confocal core facility using a 20X objective.

Image processing was performed using Fiji and Nikon elements software (Nikon, , Minato, Tokyo, Japan). Quantification was performed using an in-house written processing script and Nikon elements software (Nikon, Minato, Tokyo, Japan). Three panels per experimental condition were quantified for the number of HDV (488/FITC channel), HBcAg (Alexa647, Cy5 channel), dual stained, and cell nuclei (DAPI).

Oligo probes used in incubation buffer are shown below:

HDV (+) vRNA insert1_1	ATC AGA CCC CGA GAG TGA GAA AAA AAA AAA CTC AGT CGT GAC ACT CTT
HDV (+) vRNA insert1_2	GGT GCG AGG AAC CTC GGA GAA AAA AAA AAA GAC GCT AAT ATC GTG ACC
HDV (+) vRNA insert1_3	CAA TGC TCC CAA GAA GCA AAA AAA AAA AAA CTC AGT CGT GAC ACT CTT
HDV (+) vRNA insert1_4	AGA TTC GGA TGA TTC TCC CGA AAA AAA AAA GAC GCT AAT ATC GTG ACC
HDV (+) vRNA insert1_5	CAA GTT TGG AGA GCA CTT CGA AAA AAA AAA CTC AGT CGT GAC ACT CTT
HDV (+) vRNA insert1_6	TTG AGA AGC CTT CAG AGG GAA AAA AAA AAA GAC GCT AAT ATC GTG ACC
HDV (+) vRNA insert1_7	AAA TCA CCT CCA GAG GAC CCA AAA AAA AAA CTC AGT CGT GAC ACT CTT
HDV (+) vRNA insert1_8	CAG AAG AGC CCT GGC CGA GCA AAA AAA AAA GAC GCT AAT ATC GTG ACC
HDV (+) vRNA insert1_9	GAG ATG CTA GGA GTG GTA GGA AAA AAA AAA CTC AGT CGT GAC ACT CTT
HDV (+) vRNA insert1_10	GAG GGG AAA AGT AAA GAA AGC AAC AAA AAA AAA AGA CGC TAA TAT CGT GAC C

Backbone and insert probes for hybridization buffer are shown below as well as the biotinylated detection probes.

Backbone	P-ATTAGCGTCCAGTGAATGCGAGTCCGTCTAGGAGAGTAGTACAGCAGCCGTCAAGAGTGTC
Insert 1	P-ACGACTGAGTTTGGTCACGAT
Insert 2	P-CCAAGGTAGTTTAGTAGCCTG
Detection 1	Biotin-ACGACTGAGTTTGGTCACGAT
Detection 2	Biotin-CCAAGGTAGTTTAGTAGCCTG

Immunofluorescence imaging of HBcAg in HBV mono-infected primary hepatocytes

HBV infected SACC-PHHs were washed once with sterile 1X phosphate buffered saline (PBS, Life technologies, Carlsband, CA) and then fixed for 20 minutes with 4% (w/v) paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO) at RT. After fixation, the cells were washed five times with sterile 1X PBS. Cells were then incubated for 30 minutes in blocking buffer (10% bovine serum albumin in sterile 1X PBS, Sigma-Aldrich, St. Louis, MO). After 30 minutes the SACC-PHHs were incubated with a primary antibody mix of primary anti-HBcAg antibody (DAKO rabbit polyclonal, catalog #B0586, Santa Clara, CA) at a 1:400 dilution for 1 hour. After the 1-hour incubation with the primary antibody the cells were washed 5 times with 1X PBS. The secondary was then added (Anti-rabbit-Alexa594 1:1000, Fisher Scientific, Waltham, MA) along with the nuclear Hoechst dye (1:400, Sigma-Aldrich, St. Louis, MO) and

the cells were incubated for an additional hour. After the incubation the cells were washed 5 times and then incubated with 1X PBS supplemented with DAPI (1:5000) for 30 minutes at room temperature. The cells were then washed three times with sterile 1X PBS and the plastic well container was removed and a small amount of sterile 1X PBS was added, a cover slip was added and sealed with nail polish. The cells were stored at 4ºC until imaging.

Drug inhibition assays

Therapeutic drug treatment with myrcludex B (MyrB, kindly provided by Stephan Urban, University of Heidelberg or entecavir (ETV, Sigma Aldrich, St. Louis, MO) were performed in a 384 well format with SACC-PHHs. MyrB and ETV were solubilized in sterile 1X PBS. Prior to drug treatment cells were treated with 0.5% DMSO for 24 hours and were either challenged with HBV or HBV/HDV. Once HBV persistence had been established (10 days) drug treatment was started. A series of concentrations was used for each drug. For MyrB of $1x10^3$, $1x10^2$, and $1x10^1$ nM were used, while for ETV concentrations of 250, 125, 63, 32, and 16 nM were used. Drug treatment was performed over 10 days during which the inhibitors were freshly supplied every 2 days at each media change with monitoring of HBsAg and hAlb levels over this period of time. At the end of the 10 days cells were lysed with BioBasic RLT supplemented with 2-Mercaptoethanol for 10 minutes at RT. Total HBV DNA, pgRNA, and HDV genomic RNA were quantified by qPCR and RT-qPCR respectively. As controls, a set of wells were only challenged with HBV or HBV/HDV and had no drug treatment or control peptide administered over the course of the experiment. Another set of SACC-PHHs were not infected with either virus and had no drug treatment administered over the course of the experiment. Ever condition had minimum 16 biological replicates.

Prophylactic drug treatment with MyrB and ETV were performed in a 384 well plate seeded with SACC-PHHs. MyrB and ETV were solubilized as described above. One day prior to HBV or HBV/HDV co-infection SACC-PHHs were treated with the respective drug. SACC-PHHs were challenged 24 hours later with either HBV or HBV/HDV in the presence of 4% PEG, 0.5% DMSO, and the respective concentration of drug. Every two days media was collected and new media with the respective concentration of inhibitor was added in order to maintain a constant level of drug throughout the experiment. For MyrB concentrations of $1x10^3$, $1x10^2$, and $1x10^1$ nM were used. While for ETV concentrations of 250, 125, 63, 32, and 16 nM were used. Drug treatment was performed over the course of 20 days with HBsAg and hAlb levels being monitored over time. At the end of 18 days the cells were lysed with BioBasic RLT supplemented with 2-Mercaptoethanol for 10 minutes at RT. Total HBV DNA, pgRNA, and HDV genomic RNA were quantified by qPCR and RT-qPCR respectively. As controls, a set of wells were only challenged with HBV or HBV/HDV and had no drug treatment or Ctrl peptide administered over the course of the experiment. Another set of SACC-PHHs were not infected with either virus and had no drug treatment administered over the course of the experiment. Ever condition had minimum 16 biological replicates.

Image processing and quantitation of HBcAg staining

Image processing was performed in MATLAB. For the [red] images, high intensity punctate structures were picked out using three criteria: intensity, gradient, and difference from the local median. To produce the median subtracted image, a 2-dimensional median filtered (100 μm^2 neighborhood) copy of the image was subtracted from the original image to pick out punctate locally high intensity small structures. The gradient magnitude image of the [red] was produced using the Sobel gradient operator. K-means clustering was then used to pick out the highest bin in the gradient, intensity, subtracted median images, as well as the intensity in the DAPI channel. For the clustering, three bins were used for original intensity images and five were used for the filtered images. The overlap between these segmented masks in the [red] channel formed the master mask for [red] structures and the high DAPI bin was used as the nuclear

mask. Cleanup of both masks was performed by removing structures too large or small to be single nuclei (<7 μm^2 or >325 μm^2) or [red] puncta (<2.6 μm^2 or >290 μm^2). In order to, count [red] puncta which were in the cell body as well as the nucleus but not in areas where cells were not present or counted, the area around each nucleus was thickened by up to 10 µm in all directions, not overlapping with other cells. The fraction of these cell areas which overlaps with the [red] mask was then calculated.

cDNA library preparation for RNAseq transcriptomic sequencing

HBV mono-, HBV/HDV co- or mock infected SACC-PHHs were lysed with 350 µl RLT (Qiagen, Hilden, Germany) buffer supplemented with 2-Mercaptoethanol for 10 minutes at room temperature (RT). The cells were then pipetted into an RNase free Eppendorf tube and passed through a 26 ½ gauge needle 5 times in order to facilitate cell lysis. Once cells were lysed the manufacturers protocol was followed Qiagen RNAeasy kit (Qiagen, Hilden, Germany) with the omission of the DNaseI digestion step. Samples were eluted once with 50 µl of Rnase free water.

The integrity of total RNA samples was assessed on a Bioanalyzer 2100 using RNA 6000 Nano chip (Agilent Technologies, CA); only samples with RNA Integrity Number (RIN) greater than 9.0 were used for RNA-seq. The poly-A containing RNA transcripts were enriched from 1µg of total RNA for each sample using oligo-dT bead, and further converted to cDNA then Illumina sequencing library using PrepX RNA-seq library kit on the automated Apollo 324TM NGS Library Prep System (Wafergen Biosystems, CA) according to the manufacturer's protocol. Different DNA barcodes were added to each RNA-seq library. The libraries were examined on Agilent Bioanalyzer DNA High Sensitivity chips for size distribution, and quantified by Qubit fluorometer (Invitrogen, CA). The RNA-seq libraries were pooled at equal molar amount and sequenced on Illumina HiSeq 2500 Rapid flowcells as single-end 75 nucleotide reads following the standard protocol at a depth of 30 million reads per sample. Raw sequencing reads were filtered by Illumina HiSeq The following samples were sequenced as described above:

Donor	D8	D28	Infection
HU1007	X	X	HBV
HU1007	X	X	HBV
HU1007	X	X	HBV
HU1007	X	X	mock
HU1007	X	X	mock
HU1007	X	X	mock
HU1019	X	X	HBV/HDV
HU1019	X	X	HBV/HDV
HU1019	X	X	mock
HU1019	X	X	HBV
HU1019	X	X	HBV/HDV
HU1019	X	X	HBV/HDV
HU1019	X	X	HBV/HDV
HU1020	X	X	mock
HU1020	X	X	HBV

HU1020	X	X	HBV/HDV
HU1020	X	X	HBV/HDV
HU1020	X	X	HBV/HDV
HU1020	X	X	HBV/HDV
HU1016	X	X	HBV
HU1016	X	X	HBV/HDV
HU1016	X	X	mock

Analysis of RNAseq data for HBV and HBV/HDV co-infected SACC-PHHs under both acute and chronic conditions.

The samples were run on two HiSeq 2500 Rapid Run mode flowcells. Base calling was performed by Illumina RTA version 1.18.64.0. BCL files were then converted to fastq format using Illumina's bcl2fastq version 1.8.4. Reads that aligned to phiX using Bowtie 1.1.1 were removed as well as reads that failed Illumina's default chastity filter(9). The fastq files from each lane were then combined before splitting the reads from each sample using the barcode sequences allowing 1 mismatch, using the barcode_splitter program, version 0.18.2.

A reference genome was created using the NCBI GRCh38 human reference (iGenomes), the NCBI GRCm38 mouse reference (iGenomes), the NCBI U95551.1 Hepatitis B virus subtype ayw, complete genome, and the NCBI M21012.1 Hepatitis delta virus RNA, complete genome. The reference sequences (fasta) and gene annotations (gtf) were combined to form a single unified reference. The read alignment, quantification, and differential expression analysis was done using a locally installed instance of the Galaxy workflow platform(10). The reads for each sample were then aligned to the combined reference using STAR (Galaxy Version 2.5.2b-1) with default parameters(11). Quantification for each gene was then done using featurecounts (Galaxy Version 1.6.0.1) with default settings(12). Due to issues in the mouse gene annotations (GTF files), we removed lines from the featurecounts output that contained empty gene_ids prior to importing into R version 3.3.3 (2017-03-06) using scripts run in RStudio version 1.0.136(13, 14). Differential expression was then determined with DESeq2 (version 1.12.4) using either the two factors (donor + treatment) or (donor + time; **Supplementary Figure 12**) as the experimental design(15). Transcript counts were normalized using DESeq2 default options and transformed using the regularized $\log_2$ function in DESeq2 for clustering and PCA analysis. Differential gene expression was determined using the standard DESeq2 filters and nbinomWaldTest. Results were extracted from the DESeq2 analysis and annotated using Bioconductor's AnnotationDbi (version 1.34.4) and org.Hs.eg.db packages (version 3.3.0). Pathways with the most significantly altered gene expression and significant enrichment for members of gene ontology (GO) biological processes terms were identified using R package Generally Applicable Gene-set Enrichment (GAGE; version 2.22.0) and the accompanying supportive package gageData (version 2.10.0)(16). GO terms with a q.val $\leq$ 0.05 were slimmed down using the online tool REVIGO(17) – the GO term IDs and $\log_{10}$(q.val) as determined by GAGE were entered and the following settings used: "allowed similarity" = "medium"; "the numbers associated to GO categories" = " some other quantity, where larger absolute value is better"; "select a database with GO term sizes" = "whole UniProt"; and "select a semantic similarity measure to use" = "SimRel." The resultant REVIGO output were saved and the package GOplot (version 1.0.2) used with some author-made modifications to the circle_dat and GOCircle functions(18). For these modifications and additional details concerning the R

packages (including versions) used and the specific conditions for analysis, all R code can be accessed at https://github.com/aploss/SACC-PHH-RNASeq.

HBV infection conditions

Infections used tissue culture-derived HBV from HepG2.2.15 cells at an MOI of 4,000 in the presence of 4% polyethylene glycol (PEG) 8,000 (Sigma-Aldrich, St. Louis, MO) 0.5% (v/v) dimethylsulfoxid (DMSO, Sigma-Aldrich, St. Louis, MO) unless otherwise indicated. After a 24-hour incubation with HBV infection solution the HBV inoculum was removed using a sterile 1 mL filter pipet tip. The SACC-PHHs were washed five times with Hµrel PlatinumHeps maintenance medium™, (Hµrel Corporation, New Brunswick, NJ). After washes, fresh Hµrel PlatinumHeps maintenance medium™, (Hµrel Corporation, New Brunswick, NJ) supplemented with 0.5% DMSO was added unless indicated otherwise. For mock infected SACC-PHHs a corresponding amount of concentrated DMEM F12 media supplemented with 10% FBS, 1% Penn/Strep was added to mimic the concentrated nature of purification process that the HBV virus had gone through. Media was changed every two days.

HBV/HDV infection conditions

HBV/HDV infections of SACC-PHHs were performed as follows. Tissue culture derived HBV and HDV were mixed together to make a viral stock solution HBV was used at a MOI of 4,000 GE/cell, while HDV was at an MOI of 1,000 GE/cell. Infections were carried out in the presence of 4% polyethylene glycol (PEG) 8,000 (Sigma-Aldrich, St. Louis, MO) 0.5% dimethylsulfoxid (DMSO, Sigma-Aldrich, St. Louis, MO) unless otherwise indicated. After 24 hours of challenge SACC-PHHs were washed 5 times with Hµrel PlatinumHeps maintenance medium™, (Hµrel Corporation, New Brunswick, NJ). After washed fresh Hµrel PlatinumHeps maintenance medium™ (Hµrel Corporation, New Brunswick, NJ) supplemented with 0.5% DMSO was added unless indicated otherwise. For mock infected SACC-PHHs a corresponding amount of concentrated DMEM F12 media supplemented with 10% FBS, 1% Penn/Strep was added to mimic the concentrated nature of purification process that the HBV and HDV virus had gone through. Media was changed every two days.

Poly(I:C) transfection of non-infected SACC-PHHs

To test if innate immune pathways could be activated in SACC-PHHs 500 ng of Poly(I:C) was transfected into a 24 well plate of SACC-PHHs (188,000 cells per well) using a TransIT®-mRNA transfection Kit (Mirus Bio, Madison, WI). 350 µl RLT (Qiagen, Hilden, Germany) buffer supplemented with 2-Mercaptoethanol for 10 minutes at room temperature (RT). The cells were then pipetted into an RNase free Eppendorf tube and passed through a 26 ½ gauge needle 5 times in order to facilitate cell lysis. Once cells were lysed the manufacturers protocol was followed Biobasics Total RNA isolation kit (BioBasics, New York City, NY) for elution the sample were eluted with 50 µl of RNase free water.

Pre- and Post-treatment with Poly(I:C) of HBV mono- or HBV/HDV co-infected SACC-PHHs

SACC-PHHs in either a 24 or 96 well plate format were pre-treated with poly(I:C) after 24 hours of 0.5% DMSO treatment by transfecting in 500 ng (24 well plate) or 75 ng (96 well) of poly(I:C) using a TransIT®-mRNA transfection Kit (Mirus Bio, Madison, WI). After 12 hours, media was change and cells were then subsequently infected with either HBV or co-infected with HBV/HDV as described above. For SACC-PHHs that were persistently infected with HBV or co-infected HBV/HDV for 12 days poly(I:C) was then transfected 500 ng (24 well plate) or 75 ng (96 well) of poly(I:C) using a TransIT®-mRNA transfection Kit (Mirus Bio, Madison, WI). A set of wells were lysed 12 hours post poly(I:C) transfection with RLT and total RNA was isolated using a Biobasics Total RNA isolation kit (BioBasics, New York City, NY) for elution the sample were

eluted with 50 µl of RNAse free water. These cells were used to observe the effect of innate immune activation of intracellular HDV RNA levels. Four wells of cells had been lysed before treatment/infection and subsequently an additional 4 wells were also lysed on day 22 at the end of the experiment for measuring intracellular HDV RNA and relative fold expression of ISG's OAS-1, MX1, and ISG15 to housekeeping GAPDH. Also, supernatants were also collected every two days during the duration of the experiment for HBsAg measurements in order to determine the effect of poly(I:C) transfection on HBV infection/persistence.

RT-qPCR of interferon stimulated genes OAS1, MX1, and ISG15

To quantify fold changes of OAS1, MX1, and ISG15 levels in SACC-PHHs total RNA was isolated from lysed cells using an EZ-10 Spin Column Total RNA Miniprep Super Kit (BioBasics, New York City, 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. The following forward and reverse primers were used to quantify OAS-1 (fwd= CTGATGCAGGAACTGTATAGC, rev= CACAGCGTCTAGCACCTCTT), MX1 (fwd= GTGCATTGCAGAAGGTCAGA, rev= TCAGGAGCCAGCTGTAGGTGT), and ISG15 (fwd= TCCTGCTGGTGGTGGACAA, rev= TTGTTATTCCTCACCAGGATGCT). The master mix for the detection of the appropriate gene was aliquoted 8 ul per well and 2 ul of respective isolated RNA was then added. The plate was centrifuged at 3,000 rpm for 1 min. The following PCR program was run on a Step One Plus qPCR machine (Life Technologies, Carlsbad, CA): 50ºC for 10 min, 95ºC for 1 min, followed by 40 cycles of 95ºC for 15 sec, 60ºC for 1 min, followed by a melt curve of 95ºC for 5 sec, 65ºC for 5 sec, 95ºC for 5 sec and 50ºC for 5 sec.

RT-qPCR of host gene Cadherin 5

To quantify fold changes of Cadherin 5 levels in SACC-PHHs total RNA was isolated from lysed cells using a EZ-10 Spin Column Total RNA Miniprep Super Kit (BioBasics, New York City, 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. The following forward and reverse primers were used to quantify (fwd= TTGGAACCAGATGCACATTGAT, rev= TCTTGCGACTCACGCTTGAC). The master mix for the detection of the appropriate gene was aliquoted 8 ul per well and 2 ul of respective isolated RNA was then added. The plate was centrifuged at 3,000 rpm for 1 min. The following PCR program was run on a Step One Plus qPCR machine (Life Technologies, Carlsbad, CA): 50ºC for 10 min, 95ºC for 1 min, followed by 40 cycles of 95ºC for 15 sec, 60ºC for 1 min, followed by a melt curve of 95ºC for 5 sec, 65ºC for 5 sec, 95ºC for 5 sec and 50ºC for 5 sec.

Statistical analysis

Statistical analysis was performed using Graphpad Prism Software (Graphpad, La Jolla, CA). Either a one-way ANOVA analysis using Bonferroni parameters or a nonparametric t test was performed. P values less than 0.05 were considered statistically significant.

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Supporting Figures:

Table 1: Donor information of the PHH lots used for this study

Table 2: Characterization of induced enzymatic activity of plated SACC-PHHs.

Supporting Fig. 1: Effect of DMSO on SACC-PHH morphology and CYP2C9 and CYP3A4 activity.

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Supporting Fig. 3: Longitudinal characterization of contribution of polyethylene glycol (PEG) and DMSO to the HBV infection efficiency of SACC-PHHs as assessed by HBsAg ELISA.

Supporting Fig. 4: Output for quantification of HBcAg IF images.

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Supporting Fig. 6: Minimal differences in human albumin levels regardless of infection condition.

Supporting Fig. 7: Quantification of number of HDV and HBV infected SACC-PHHs in HDV mono, HBV mono, and HBV/HDV co-infected samples.

Supporting Fig. 8: Detection of HDV RNA in supernatants of co-infected SACC-PHHs provides further evidence for *bona fide* HDV replication.

Supporting Fig. 9: SACC-PHHs in 384 well format can be productively infected with HBV/HDV.

Supporting Fig. 10: Minimal variation in human albumin levels in 384 wells either therapeutic and prophylactic treated with MyrB and ETV.

Supporting Fig. 11: RNAseq samples show donor variability, not infection type, contributed more to variance between samples

Supporting Fig. 12: Heat map of sample-to-sample distances for the regularized $\log_2$-transformed counts of human genes for each sequenced sample

Supporting Fig. 13: Surface and X genes are the most highly transcribed HBV genes.

Supporting Fig. 14: GO analysis shows that members of the term “type I interferon signaling pathway” are significantly enriched amongst upregulated genes in HBV-infected day 8 samples as compared to mock controls

Supporting Fig. 15: Transcriptional differences over time in mock-, HBV mono-, and HBV/HDV co-infected

Supporting Fig. 16: Minimal transcriptional changes occur in non-parenchymal stromal cells regardless of experimental condition

Supporting Fig. 17: SACC-PHHs from donor HU1003 have increased levels of ISG15 at day 8 post infection but not OAS-1.

Supporting Fig. 18: Corroboration of RNAseq data by RT-qPCR.

Supporting Fig. 19: Longitudinal HBsAg data for poly(I:C) treated HBV mono- and HBV/HDV co-infected SACC-PHHs.

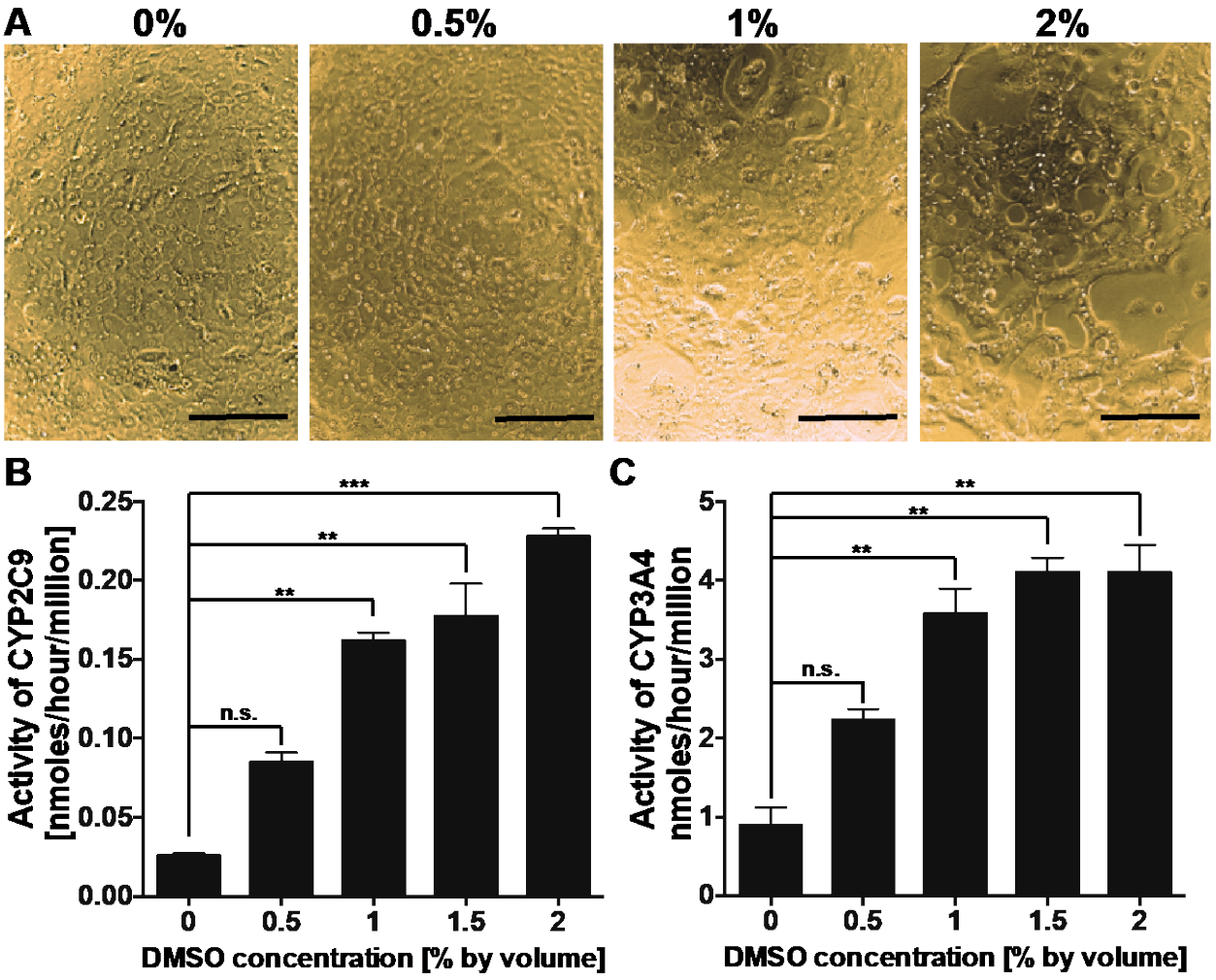
Supplemental Table 1: Donor information of the PHH lots used for this study

				Serological Testing						
Human donor #	Age (years)	Gender	Race	EBV	RPR	CMV	Hep B	Hep C	HIV	Social History
Mixed donor HU1007	Age range 23-69	Male (3), Female (2)	Cauc. (4), Arabic (1)	P (1), NR (4)	N	P(2), N(3)	N	N	N	Drug use (1), Heavy drinker (2), No alchohol use (2)
Single donor HU1003	64	Male	Caucasian	P	N	N	N	N	N	Drug use (N), Alchohol (N)
Single donor HU1010	52	N/A	Caucasian	P	N	P	N	N	N	Drug use (N), Alchohol (N)
Mixed donor HU1016	Age range 3-63	Male (6), Female (4)	Cauc (9), African American (1)	P(5), N(1), NR(4)	N	P (6), N(4)	N	N	N	N/A
Single donor HU1019	49	Female	Caucasian	P	N	P	N	N	N	N/A
Single donor HU1020	49	Male	Caucasian	P	N	P	N	N	N	N/A
Single donor HU1018	19	Male	Caucasian	P	N/R	N	N	N	N	Drug use (N), Alchohol (N)
Single donor HU1051	16 months old	Male	Caucasian	N/A	N/A	P	N	N	N	Drug use (N), Alchohol (N)
Single donor HU1052	24	Female	Caucasian	N/A	N/A	N	N	N	N	Drug use (Y), Alchohol (Y)

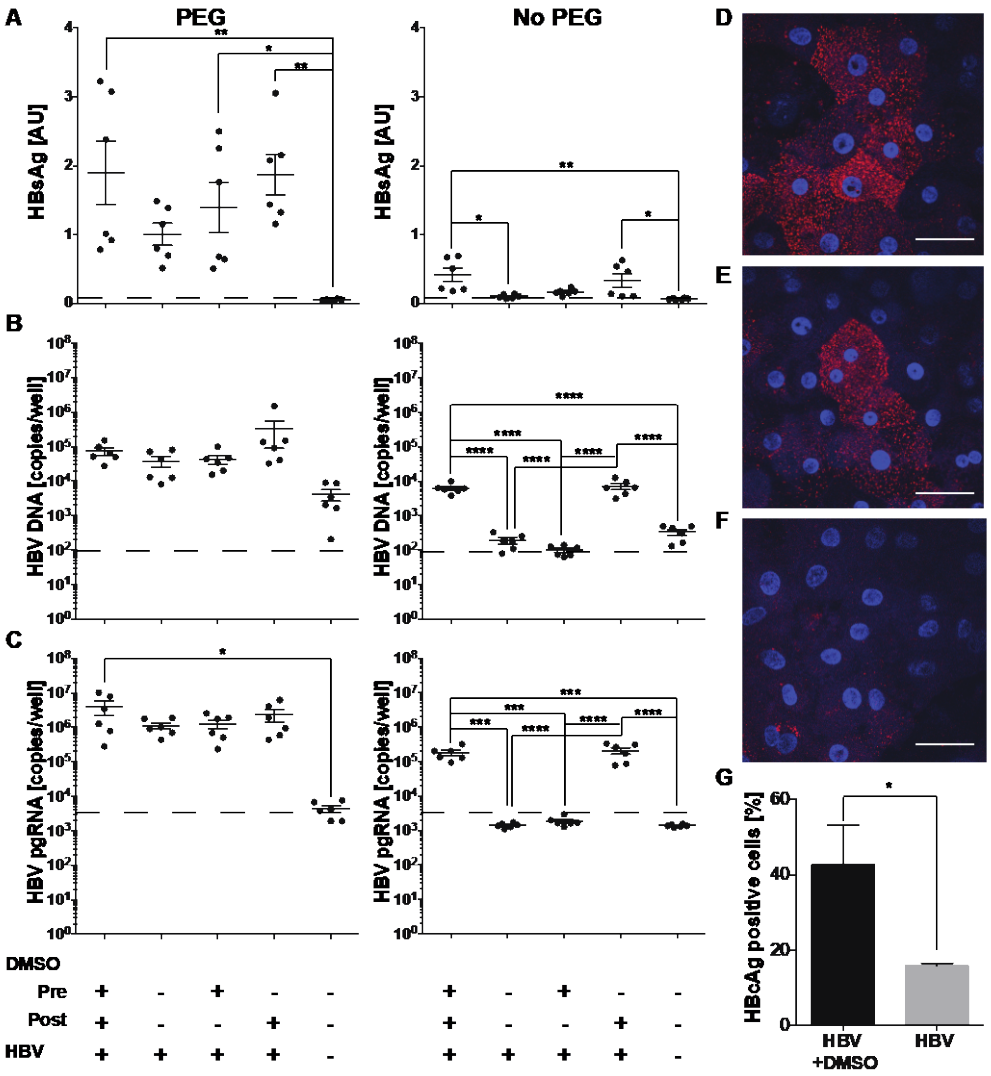
Terms used: Epstein-Barr virus (EBV), Rapid plasma regain (RPR), Cytomegalovirus (CMV), Hepatitis B virus (Hep B), Hepatitis C virus (Hep C), Human immunodeficiency virus (HIV), Not applicable (N/A), Not reported (N/R), Positive (P), and Negative (N)

Supplementary Table 2: Characterization of induced enzymatic activity of plated SACC-PHHs.

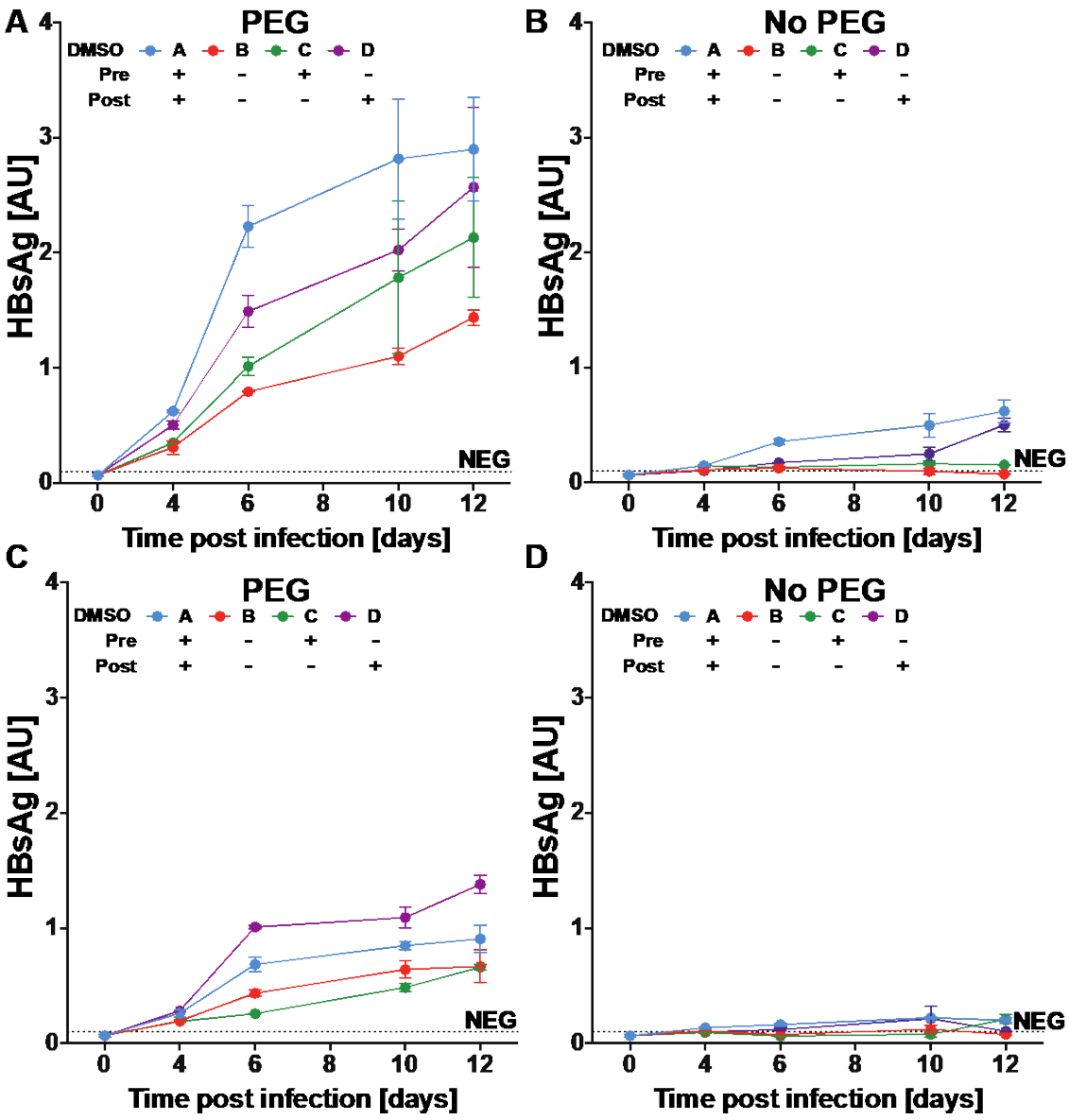
Donor	Substrate	Enzyme	Concentration (µM)	Day 1	Day 7	Day 14
Single donor HU 1003	Midazolam	CYP3A4	5	0.242	0.386	0.422
	Dextromethorphan	CYP2D6	20	0.598	0.468	0.282
	Tolbutamide	CYP2C9	20	0.018	0.013	0.004
	Substrate	Enzyme	Concentration (µM)	Day 1	Day 4	Day 8
Mixed Donor HU 1007	Midazolam	CYP3A4	5	2.49	3.99	2.52
	Dextromethorphan	CYP2D6	20	2.14	5.2	3.32
	Tolbutamide	CYP2C9	20	0.4	0.5	0.41
	Substrate	Enzyme	Concentration (µM)	Day 1	Day 3	Day 8
Single donor HU 1010	Midazolam	CYP3A4	5	1.03	1.23	0.79
	Dextromethorphan	CYP2D6	20	0.53	0.31	0.18
	Tolbutamide	CYP2C9	20	0.14	0.13	0.09
	Substrate	Enzyme	Concentration (µM)	Day 1	Day 4	Day 8
Mixed donor HU 1016	Midazolam	CYP3A4	5	1.15	1.28	0.94
	Dextromethorphan	CYP2D6	20	0.54	0.6	0.72
	Tolbutamide	CYP2C9	20	0.12	0.14	0.13
	Substrate	Enzyme	Assay (metabolite)	Intrinsic Clearance (mL/hr/10^6 cells)	Metabolite Formation Velocity (nmol/hr/10^6 cells)	
Single donor HU 1019 (BD330)	Midazolam	CYP3A4	intrinsic clearance	0.33		
	Dextromethorphan	CYP2D6	intrinsic clearance	0.23		
	Phenacetin	CYP1A2	Metabolite Formation Velocity (4-acetamidophenol)		0.76	
	Diclofenac	CYP2C9	Metabolite Formation Velocity (4-hydroxydiclofenac)		1.3	
	Estradiol	UGT1A1	Metabolite Formation Velocity (estradiol 3-glucuronide)		1.2	
Single donor HU 1018	Substrate	Enzyme	Concentration (µM)	Day 1	Day 4	Day 8
	Midazolam	CYP3A4	5	0.816	0.77	0.463
	Dextromethorphan	CYP2D6	20	0.863	0.953	0.75
	Tolbutamide	CYP2C9	20	0.12	0.12	0.04
Single donor HU 1051	Substrate	Enzyme	Concentration (µM)	Day 1	Day 4	Day 8
	Midazolam	CYP3A4	5	1.046	0.956	1.063
	Dextromethorphan	CYP2D6	20	0.031	0.018	0.026
	Tolbutamide	CYP2C9	20	0.029	0.039	0.02
Single donor HU 1052	Substrate	Enzyme	Concentration (µM)	Day 1	Day 4	Day 8
	Midazolam	CYP3A4	5	0.54	0.826	0.297
	Dextromethorphan	CYP2D6	20	0.413	0.88	0.587
	Tolbutamide	CYP2C9	20	0.009	0.018	0.01
Single donor HU 1020 (BD405A)	Substrate	Enzyme	Concentration (µM)	Day 1	Day 4	Day 8
	Midazolam	CYP3A4	5	0.414	0.362	0.28
	Dextromethorphan	CYP2D6	20	0.551	0.81	0.704
	Tolbutamide	CYP2C9	20	0.008	0.01	0.016



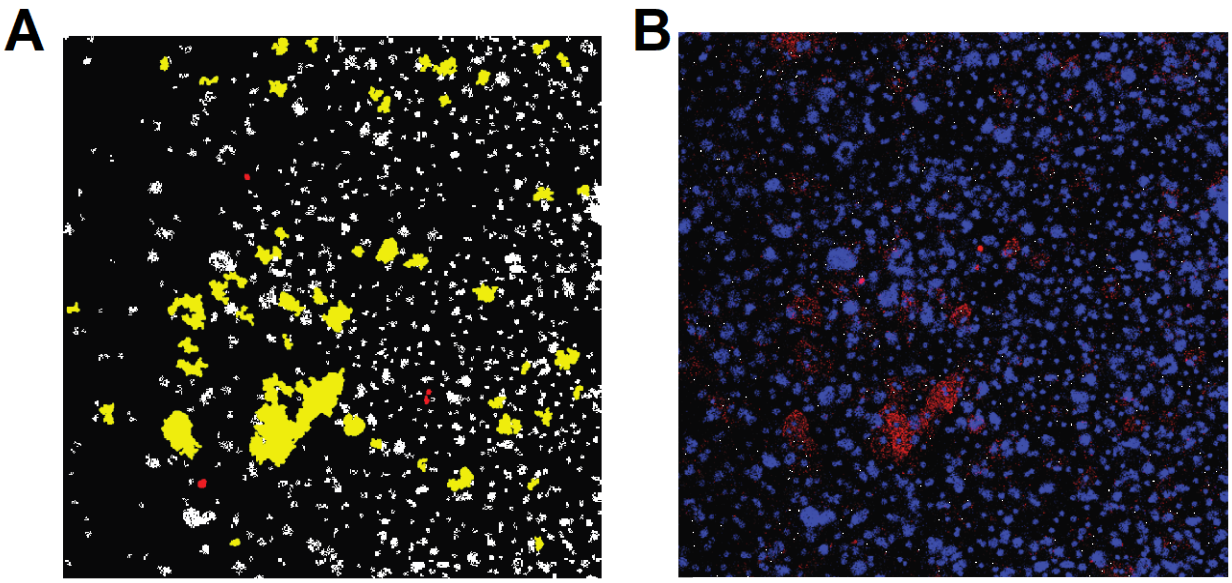
Supporting Fig. 1: Effect of DMSO on SACC-PHH morphology and CYP2C9 and CYP3A4 activity. SACC-PHHs were treated with increasing amounts of DMSO. (**A**) Light microscopy images of SACC-PHHs treated with 0, 0.5, 1, 1.5, and 2% DMSO by volume (scale bar= 200 nm). (**B**) CYP2C9 activity as assessed by 0H tolbutamide formation. (**C**) CYP3A4 activity as assessed by 1-0H midazolam formation.



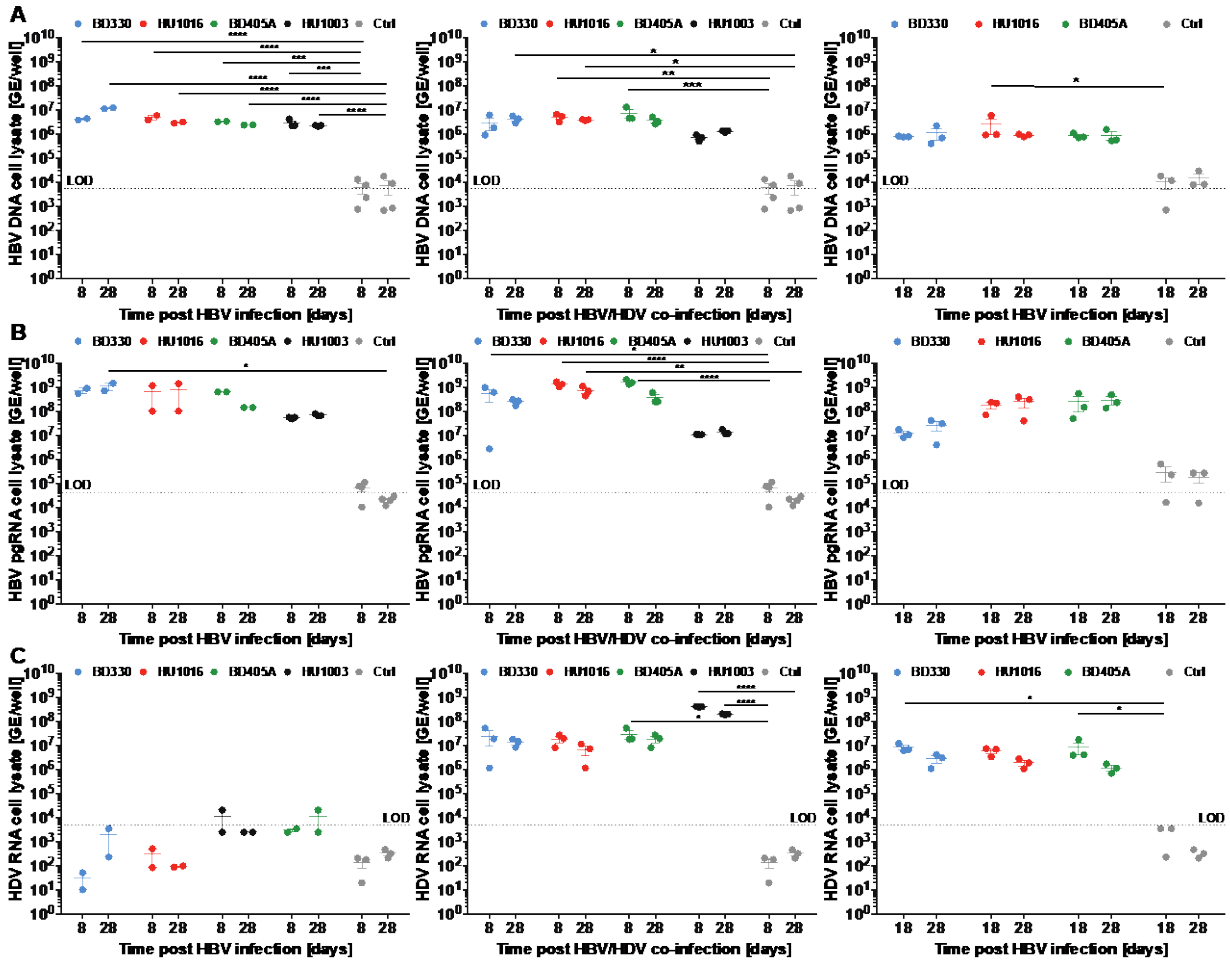
Supporting Fig. 2. Impact of polyethylene glycol (PEG) and DMSO on HBV infection of SACC-PHHs. SACC-PHHs were infected in the presence or absence of 4% PEG under conditions where the cells were treated with either 0.5% DMSO before infection or were maintained in the presence of DMSO. HBsAg ELISA (**A**) was performed on supernatants, HBV DNA qPCR (**B**) and HBV pgRNA RT-qPCR (**C**) were performed on cell lysates. High resolution HBcAg immunofluorescent images were taken of SACC-PHHs under the following conditions: (**D**) HBV challenged, 4% PEG, 0.5% DMSO present throughout experiment; (**E**) HBV challenged, 4% PEG, no DMSO at any point during the experiment; (**F**) non-infected SACC-PHHs. (**G**) Quantification of HBV infection in HBV-challenged SACC-PHHs. For all intracellular viral parameters, six biological replicates were performed. Scale bar = 200 nm. All data are presented as means ± s.e.m. Statistical significance was determined using ANOVA * <0.05. ** <0.001, *** <0.0001, and **** <0.00001.



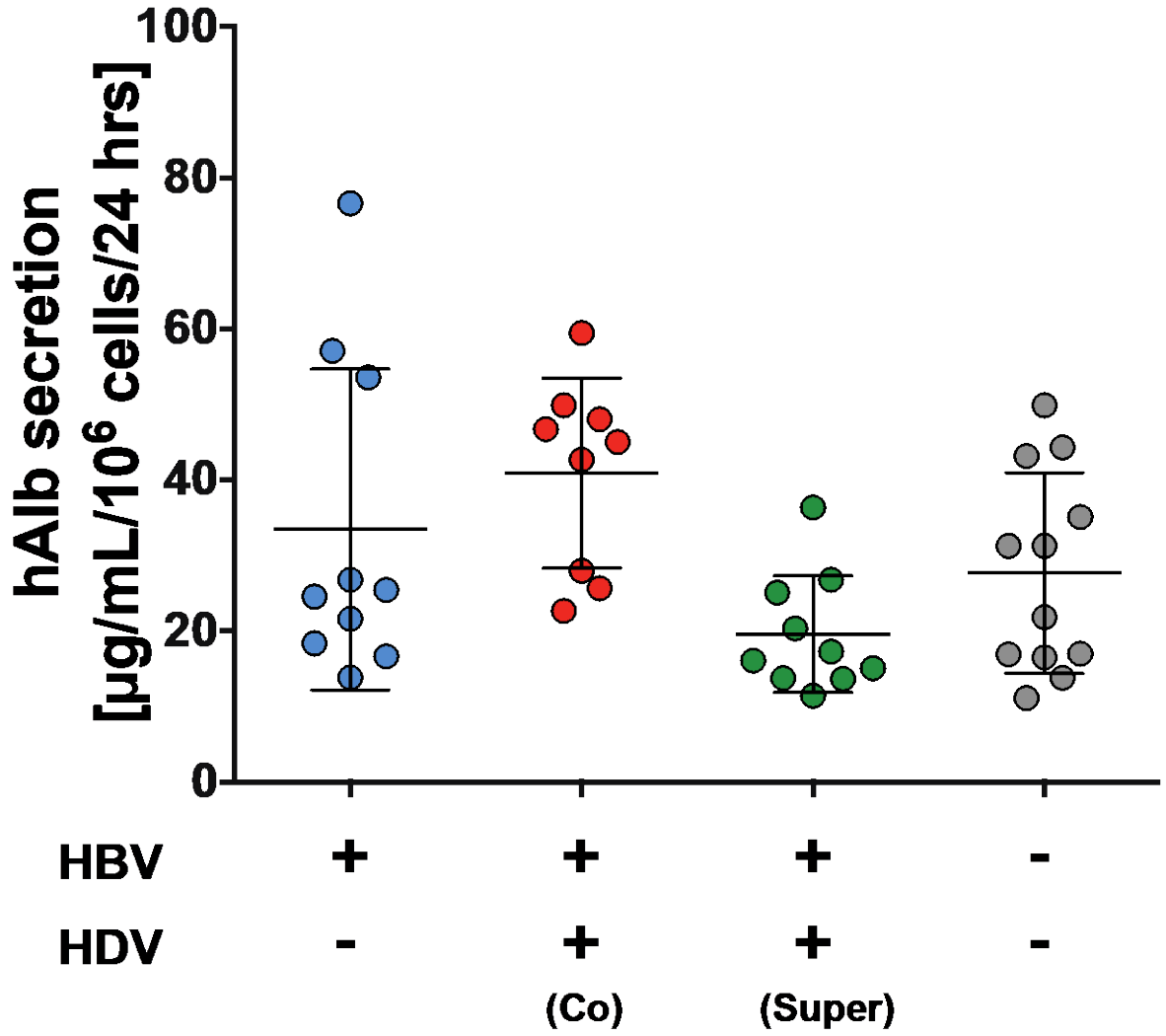
Supporting Fig. 3: Longitudinal characterization of contribution of polyethylene glycol (PEG) and DMSO to the HBV infection efficiency of SACC-PHHs as assessed by HBsAg ELISA. SACC-PHH donors HU1003 (left column) and HU1010 (right column) were challenged with HBV in the presence or absence of 4% PEG and varying the timing of the 0.5% DMSO treatment. (**A**) HBsAg ELISA data for HU1003 donor with 4% PEG present during infection. (**B**) HBsAg ELISA data for HU1010 donor with 4% PEG present during infection. (**C**) HBsAg ELISA data for HU1003 donor without PEG present during infection. (**D**) HBsAg ELISA data for HU1010 donor without PEG present during infection.



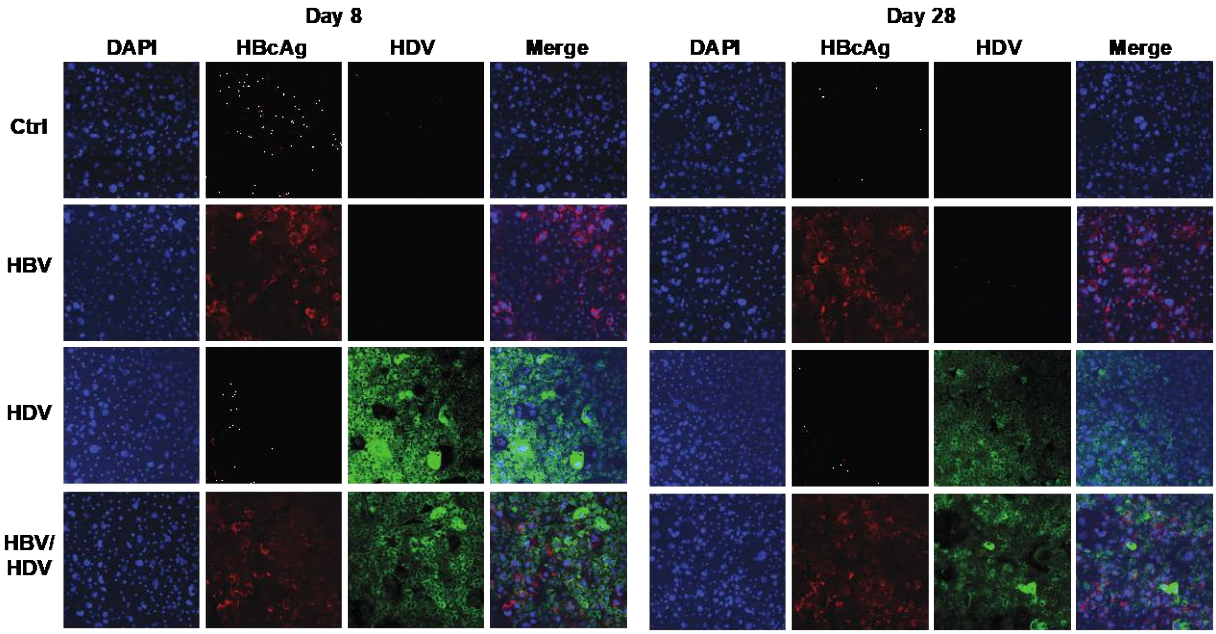
Supporting Fig. 4: Output for quantification of HBcAg IF images. Samples infected with HBV were immunofluorescence stained for HBcAg. HBcAg positive cells were quantified using the algorithm described in the materials and methods. (**A**) Graphical output file for quantification of HBcAg positive cells Yellow indicates signal from HBcAg+ cells and white indicates DAPI-stained nuclei. (**B**) Microscopy image from HBV-infected SACC-PHHs. HBcAg=red, DAPI=blue.



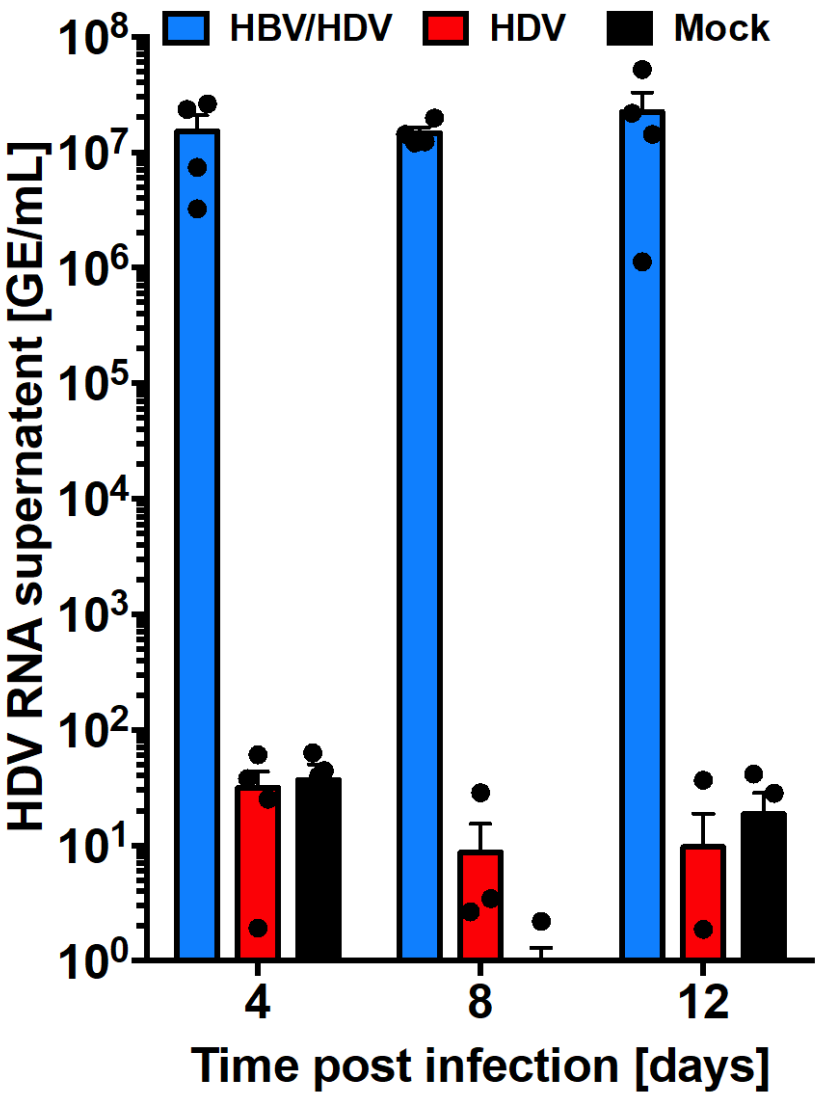
Supporting Fig. 5: Characterization for HBV mono-infected, HBV/HDV co-infected, and HBV/HDV superinfected SACC-PHHs from cell lysates of mixed and single donors. SACC-PHHs were challenged with either HBV, HBV/HDV, or first with HBV and upon establishment of persistent infection were infected with HDV. (**A**) HBV DNA qPCR data. (**B**) HBV pgRNA RT-qPCR data. (**C**) HDV RNA RT-qPCR data. All data are presented as means ± s.e.m. Statistical significance was determined using ANOVA t-test * <0.05. ** <0.001, *** <0.0001, and **** <0.00001



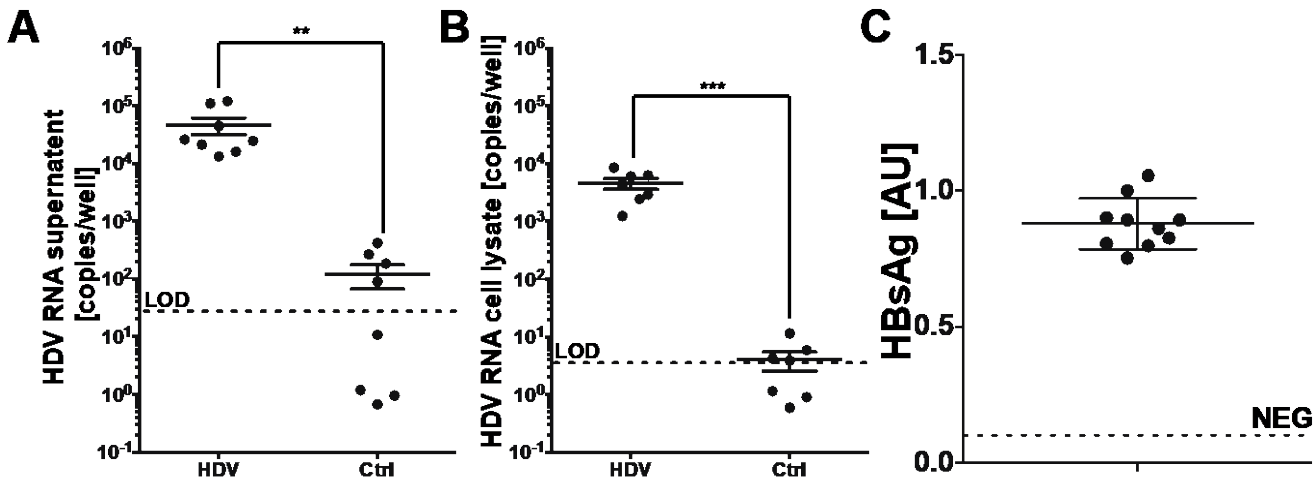
Supporting Fig. 6: Minimal differences in human albumin levels regardless of infection condition. SACC-PHHs were challenged with either HBV, co- or superinfected with HBV/HDV. Human albumin (hAlb) levels were assessed by hAlb ELISA.



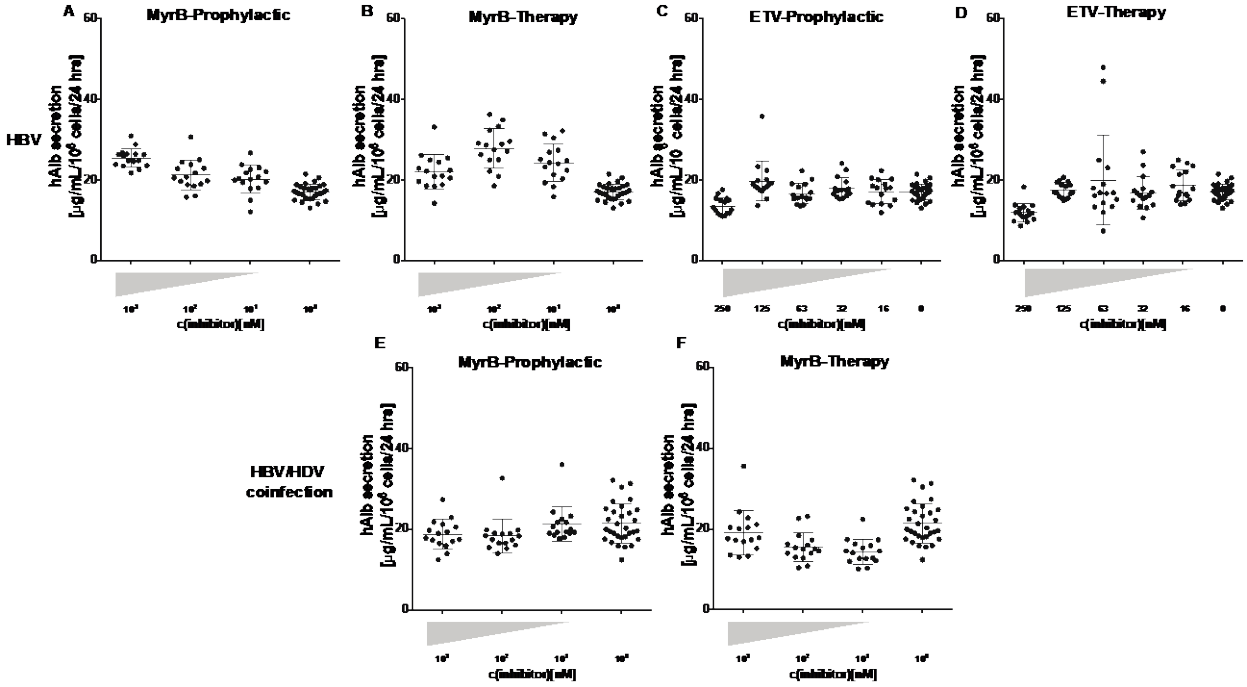
Supporting Fig. 7: Quantification of number of HDV and HBV infected SACC-PHHs in HDV mono, HBV mono, and HBV/HDV co-infected samples. SACC-PHHs were seeded on a glass slide and were subsequently infected with either nothing (Ctrl), HDV, HBV, or HBV/HDV. At day 8 (left) and day 28 (right) post infection, cells were fixed and stained for HDV genomic RNA (green) by vPLAYR/TSA amplification, HBcAg (red), and DAPI (blue).



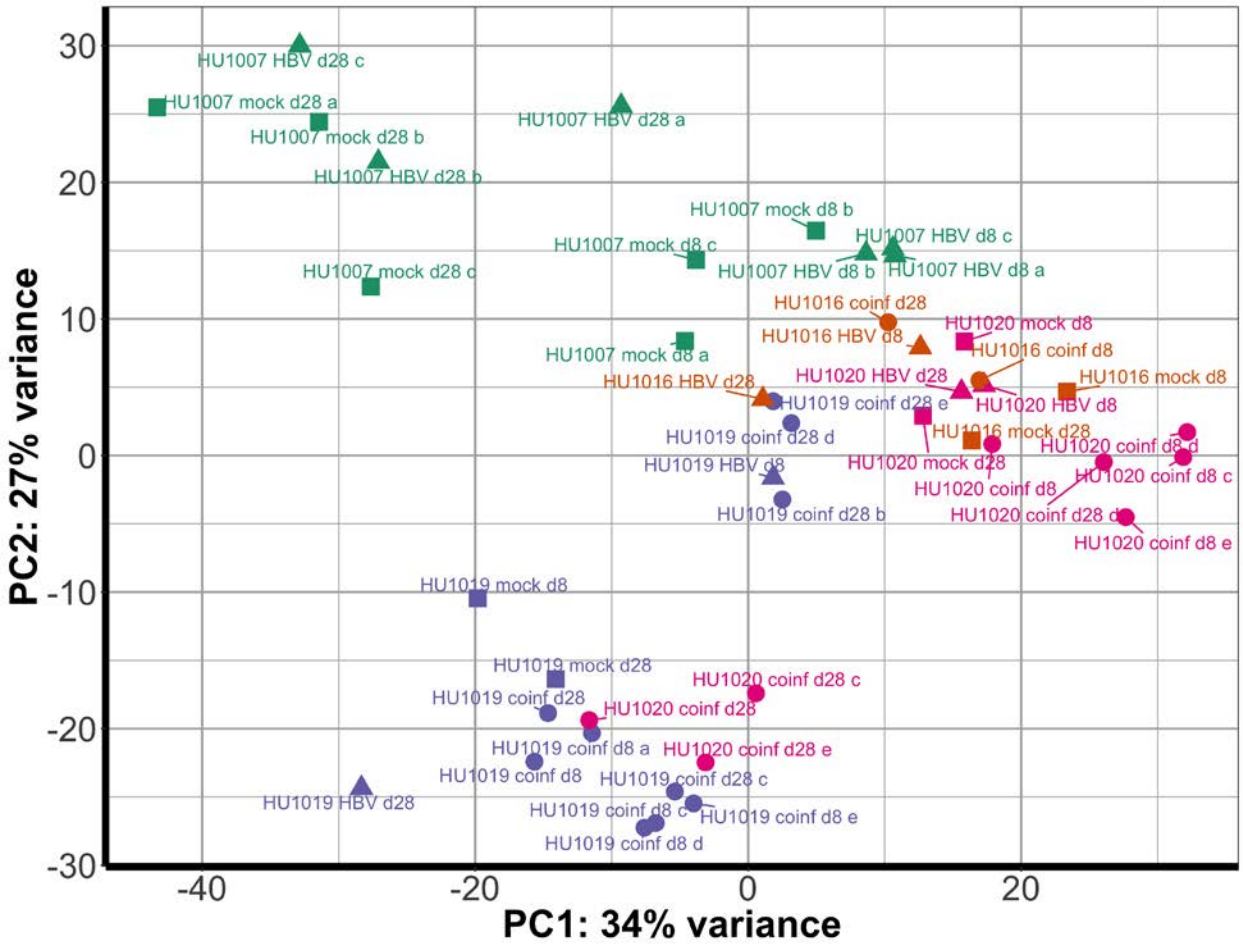
Supporting Fig. 8: Detection of HDV RNA in supernatants of co-infected SACC-PHHs provides further evidence for *bona fide* HDV replication. SACC-PHHs were co-infected with HBV and HDV, HDV mono-infected, or non-infected. HDV genomic RNA was quantified by RT-qPCR.



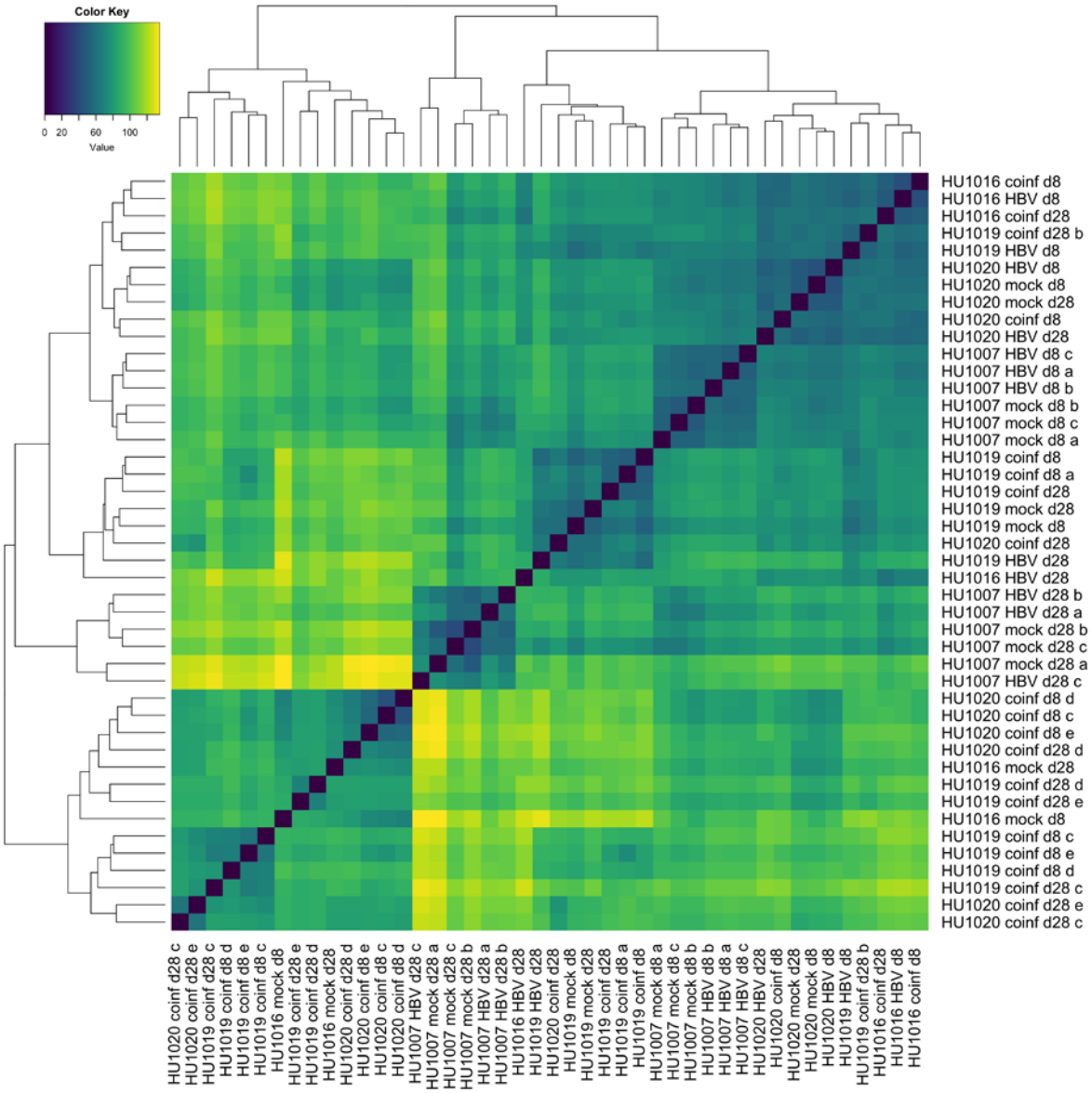
Supporting Fig. 9: SACC-PHHs in 384 well format can be productively infected with HBV/HDV. SACC-PHHs were seeded in a 384 well format and were challenged with HBV/HDV. HDV infection was ascertained by isolating HDV RNA from supernatants (**A**) or cell lysate (**B**) and quantifying the amount of genomic HDV RNA by RT-qPCR. (**C**) HBsAg levels at 12 days post HBV/HDV challenge were measured for 10 randomly chosen wells across the plate.



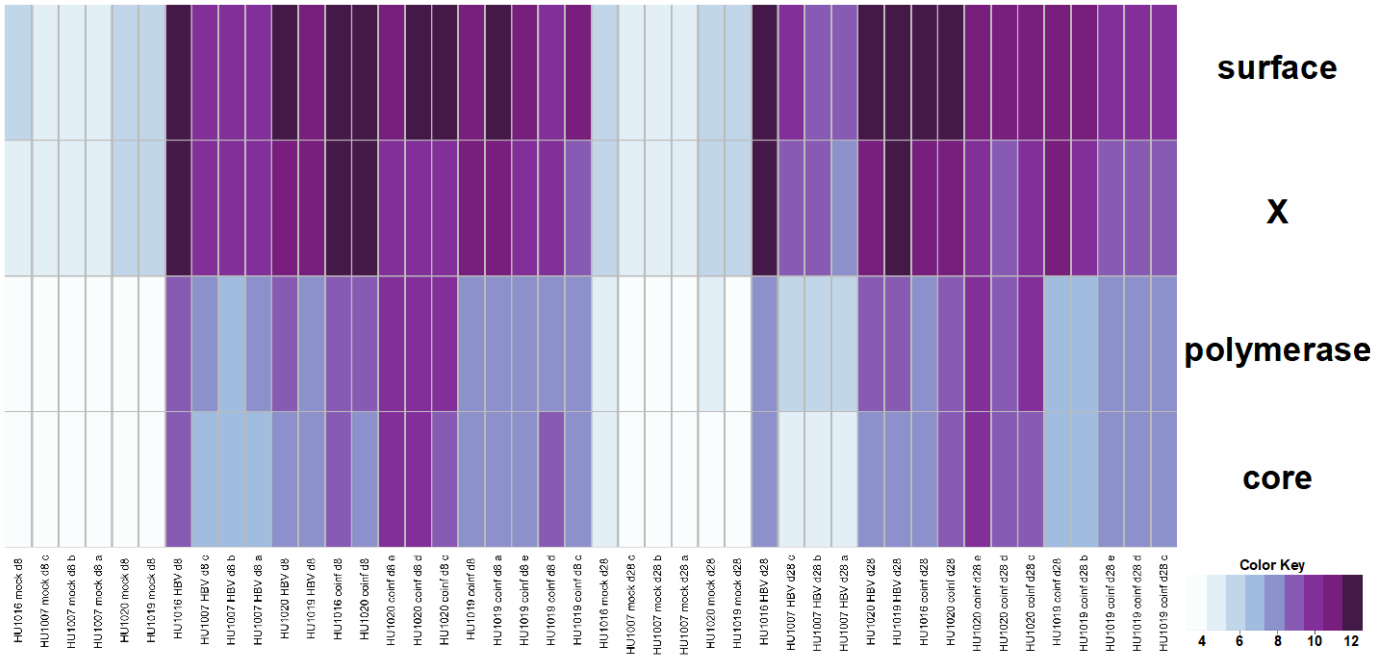
Supporting Fig. 10: Minimal variation in human albumin levels in 384 wells either therapeutic and prophylactic treated with MyrB and ETV. SACC-PHHs were challenged with either HBV (**A-D**) or HBV/HDV (**E-F)**. Infected SACC-PHHs were treated either prophylactically with MyrB (A and E) or ETV (**C**). SACC-PHHs that had already an established persistent HBV or HBV/HDV co-infection were treated therapeutically with MyrB (**B and F**) or ETV (**D**), respectively.



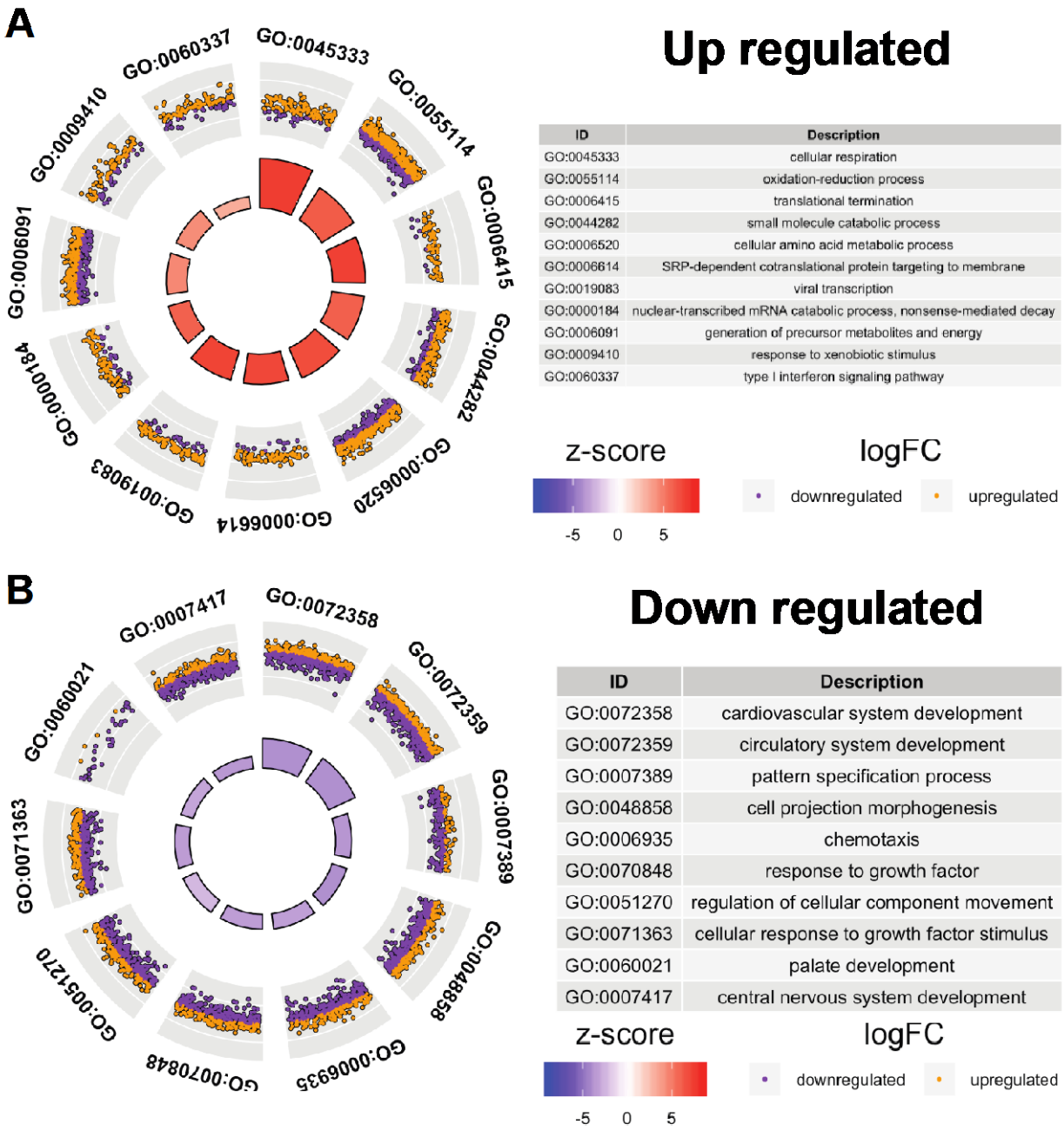
Supporting Fig. 11: RNAseq samples show donor variability, not infection type, contributed more to variance between samples. Principal component analysis based off the regularized $\log_2$-transformed counts for human and HBV genes in HBV mono-infected, HBV/HDV co-infected, and control RNAseq samples from donors HU1007, HU1016, HU1019, and HU1020.



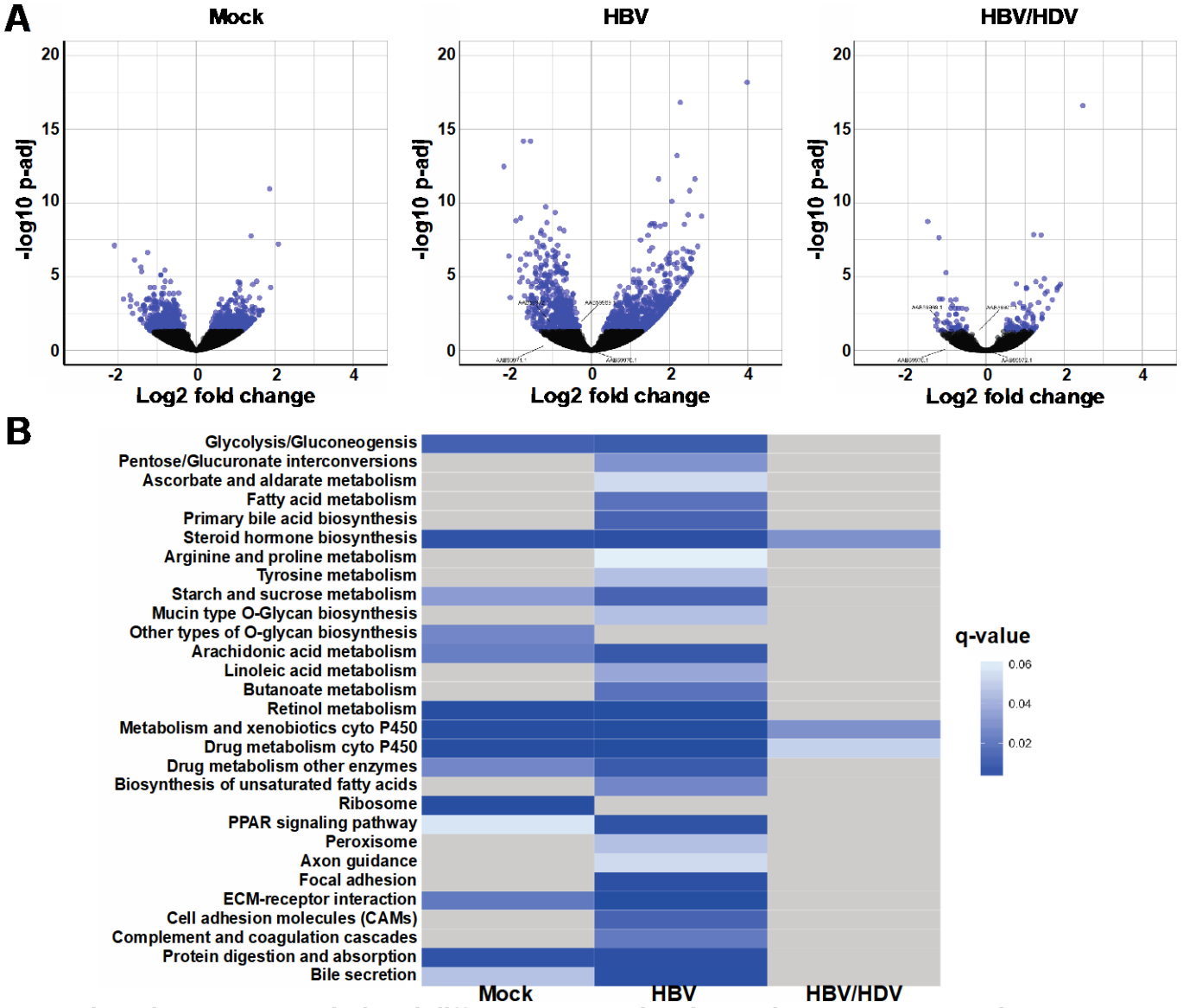
Supporting Fig. 12: Heat map of sample-to-sample distances for the regularized log_2-transformed counts of human genes for each sequenced sample. Each row/column represents a single sample. RNASeq performed on HBV mono-infected, HBV/HDV co-infected, and control samples from donors HU1007, HU1016, HU1019, and HU1020 at 8 and 28 days post infection.



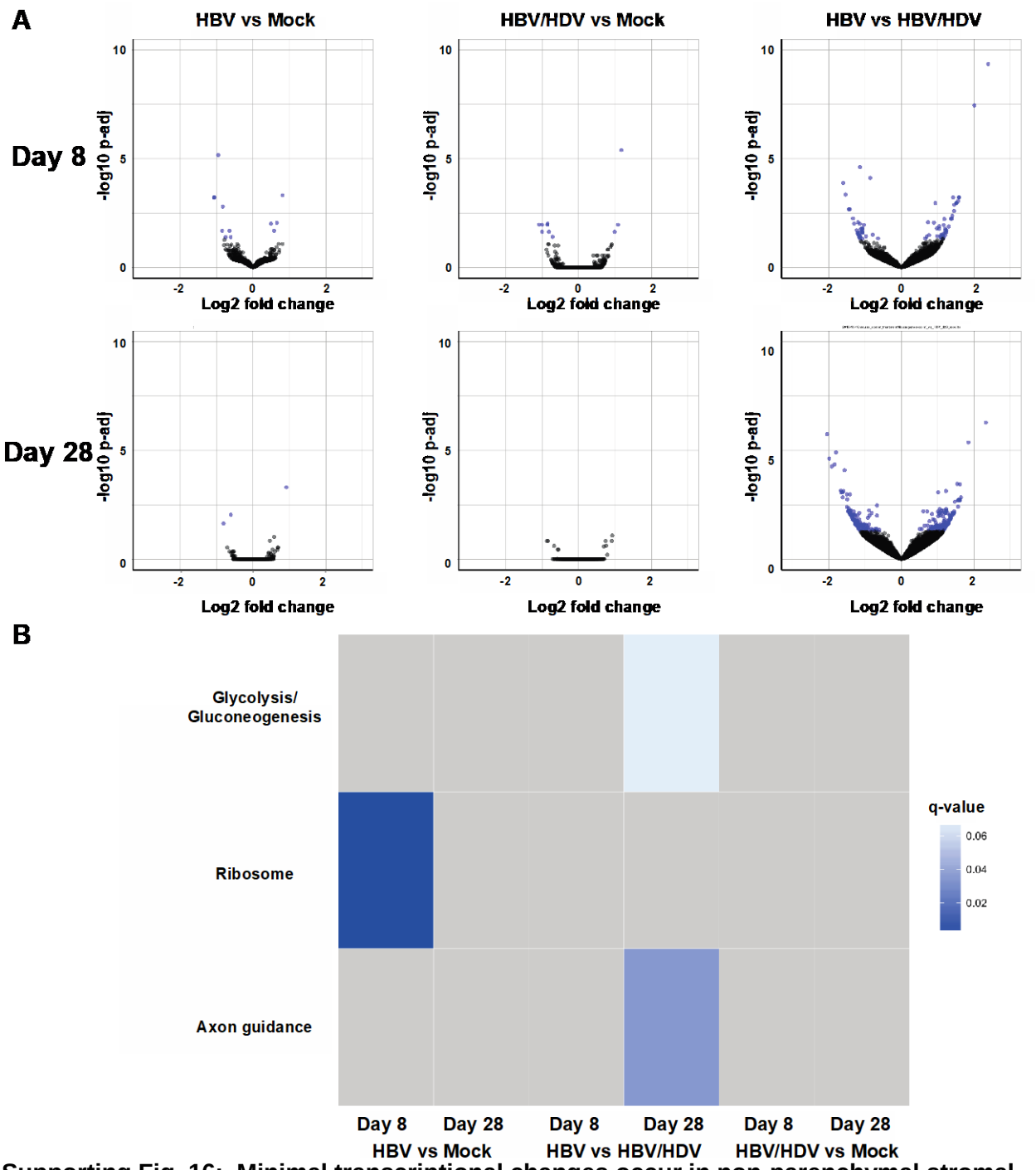
Supporting Fig. 13: Surface and X genes are the most highly transcribed HBV genes. Heat map of the regularized $\log_2$-transformed counts of HBV viral genes in HBV mono-infected, HBV/HDV co-infected, and control RNAseq samples from donors HU1007, HU1016, HU1019, and HU1020 at 8 and 28 days post infection.



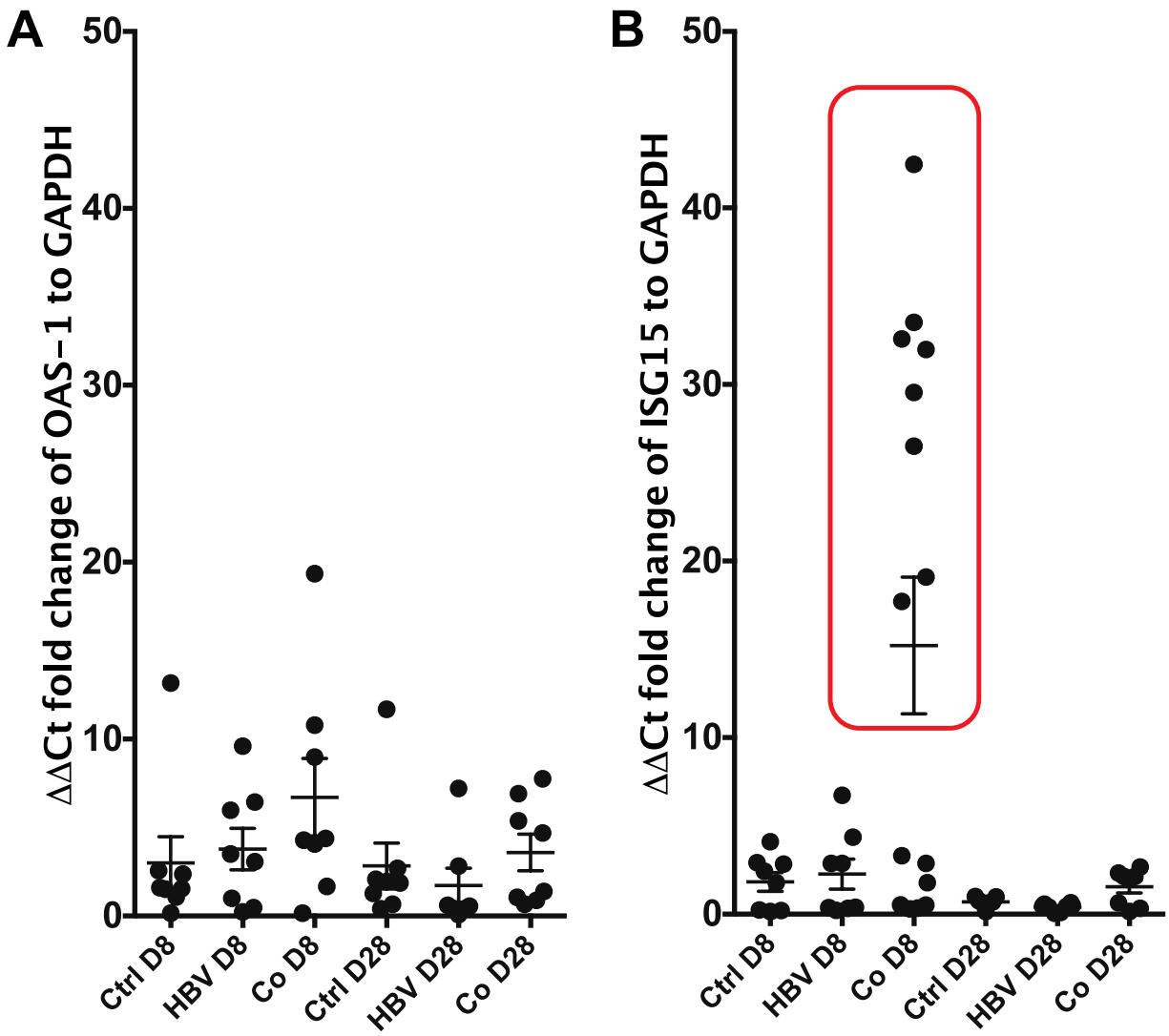
Supporting Fig. 14: GO analysis shows that members of the term "type I interferon signaling pathway" are significantly enriched amongst upregulated genes in HBV-infected day 8 samples as compared to mock controls. GO term enrichment analysis was performed on HBV mono-infected and mock control samples on day 8. Enrichment for GO biological processes terms amongst upregulated (**A**) and downregulated (**B**) genes in HBV infected versus mock-infected SACC-PHHs are shown. All terms shown are significant at a q. val $\leq$ 0.05 but due to space constraints, not all significant terms for all tested conditions are depicted here. Please go to https://github.com/aploss/SACC-PHH-RNASeq to see the complete output (tables and plots) as well as code.



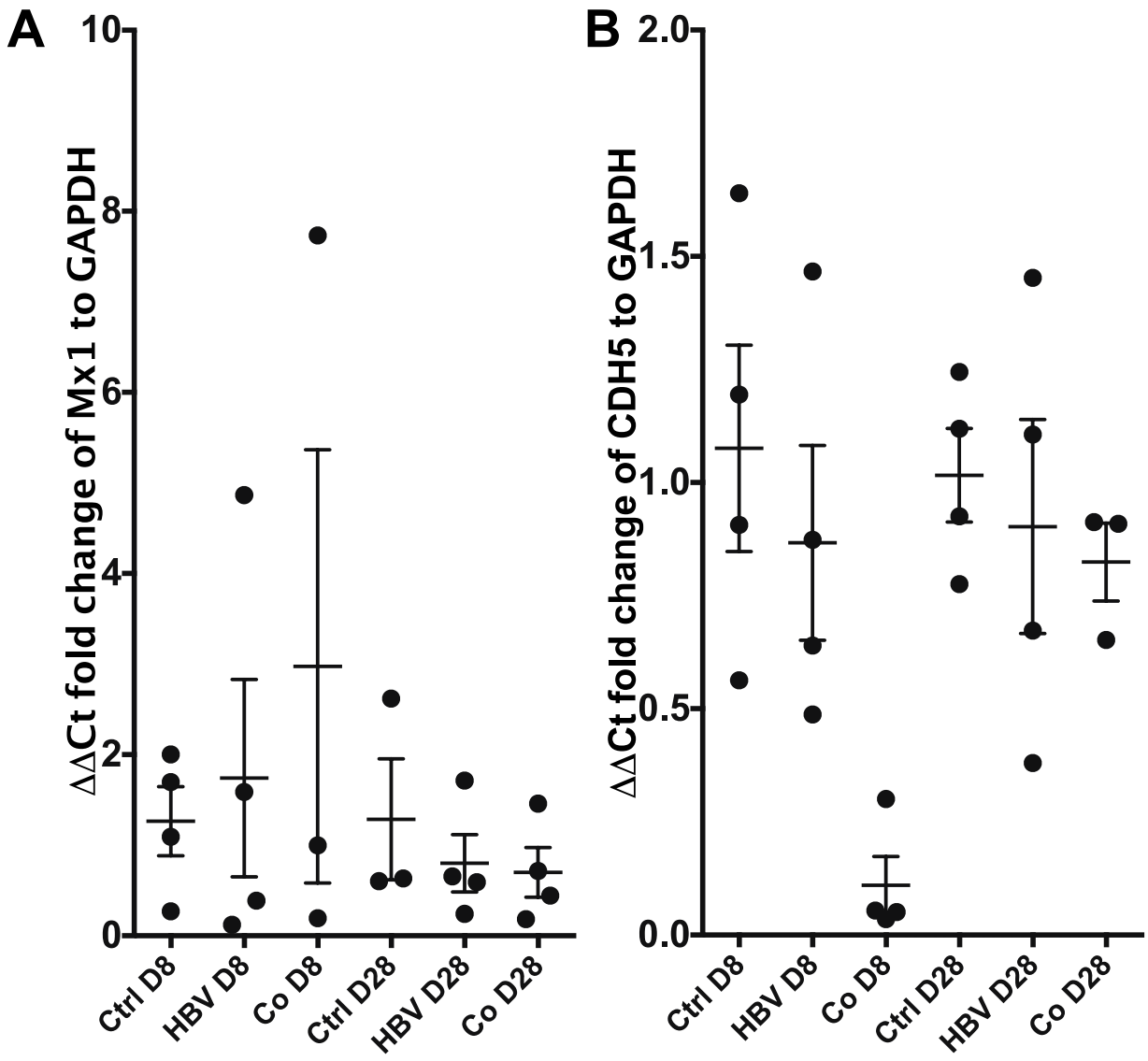
Supporting Fig. 15: Transcriptional differences over time in mock-, HBV mono-, and HBV/HDV co-infected. SACC-PHHs were challenged with either HBV, or co- or superinfected with HBV/HDV. (**A**) Volcano plots showing differential human gene expression (donor + time set as the two factors for the experimental design) from 8 to 28 dpi in control, HBV mono-infected, and HBV/HDV co-infected RNAseq samples from donors HU1007, HU1016, HU1019, and HU1020. Points in blue have a padj $\leq$ 0.05. (**B**) Longitudinal GAGE pathway analysis, probing the transcriptional differences between day 8 and day 28 in mock (column 1), HBV mono-infected (column 2), and HBV/HDV co-infected (column 3) samples.



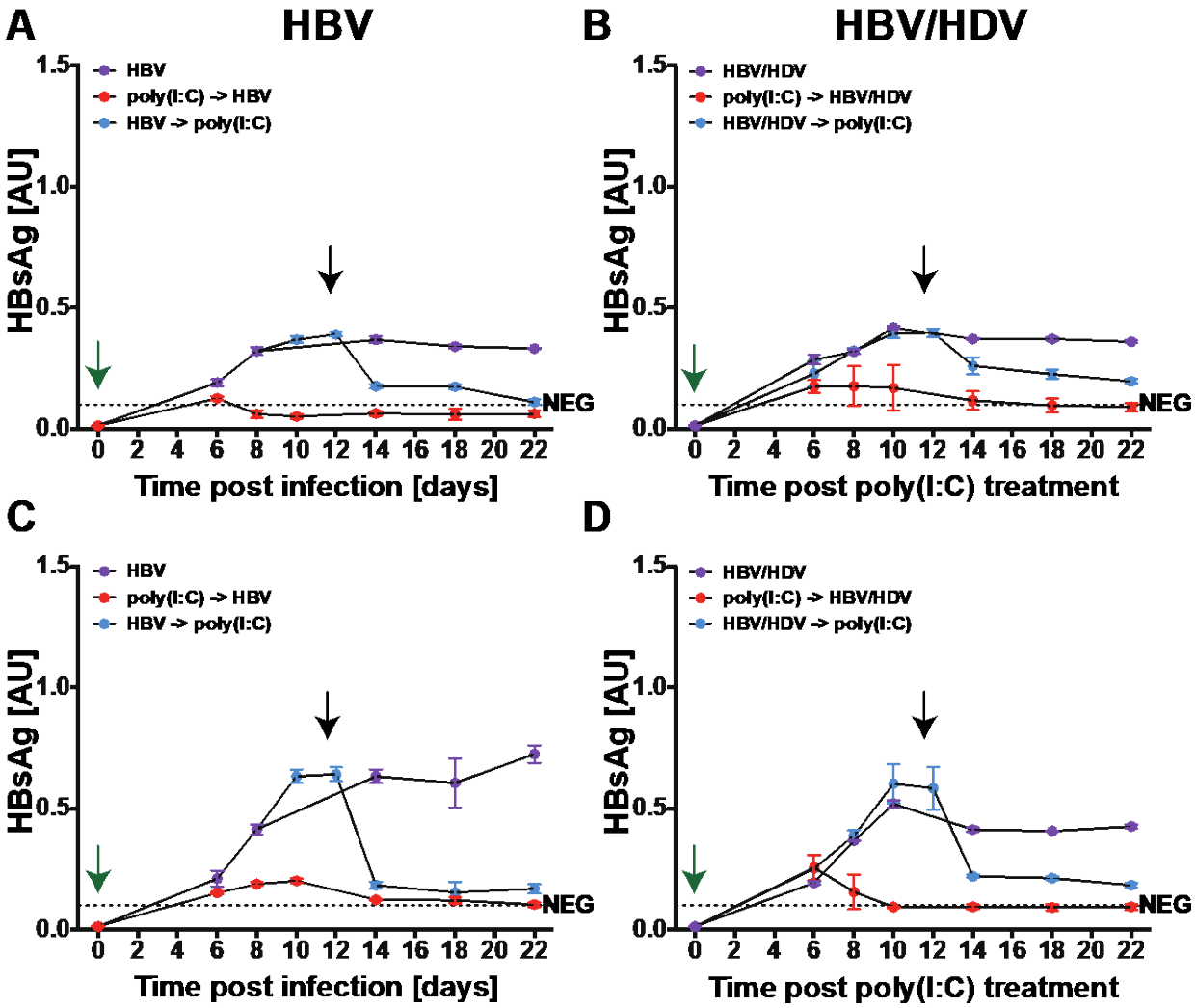
Supporting Fig. 16: Minimal transcriptional changes occur in non-parenchymal stromal cells regardless of experimental condition. SACC-PHHs were challenged with either HBV, or co- or superinfected with HBV/HDV. Non-parenchymal stromal cells are not susceptible to HBV or HDV infection. (**A**) Volcano plots of differential murine gene expression (donor + treatment set as the two factors for the experimental design) between infection conditions (HBV mono-infected vs mock, HBV/HDV co-infected vs mock, and co-infected vs mono-infected) in RNAseq samples from donors HU1007, HU1016, HU1019, and HU1020. (**B**) GAGE pathway analysis of the differential gene expression for HBV vs mock, HBV vs HBV/HDV, and HBV/HDV vs mock at 8 and 28 dpi from donors HU1007, HU1016, HU1019, and HU1020.



Supporting Fig. 17: SACC-PHHs from donor HU1003 have increased levels of ISG15 at day 8 post infection but not OAS-1. Mock, HBV mono-infected, and HBV/HDV co-infected samples from day 8 and 28 post infection from donors HU1003, HU1007, HU1016, HU1019, and HU1020 had total RNA isolated from cell lysates and OAS-1 (**A**) and ISG15 (**B)** levels were quantified by RT-qPCR. Red circle indicates 8 biological replicates from HU1003 that showed high levels of ISG15 induction upon HBV/HDV co-infection at day 8.



Supporting Fig. 18: Corroboration of RNAseq data by RT-qPCR. Mock, HBV mono-infected, and HBV/HDV co-infected samples from day 8 and 28 post infection that had been RNA sequenced had MX1 (**A**) and CDH5 (**B)** levels quantified by RT-qPCR.



Supporting Fig. 19: Longitudinal HBsAg data for poly(I:C) treated HBV mono- and HBV/HDV co-infected SACC-PHHs. Three experimental time courses were taken for two SACC-PHH donors. (1) SACC-PHHs were either pre-treated with poly(I:C) and infected with either HBV or co-infected with HBV/HDV. (2) SACC-PHHs with a persistent HBV mono- or HBV/HDV co-infection were subsequently poly(I:C) treated. (3) SACC-PHHs were either HBV mono- or HBV/HDV co-infected with no poly(I:C) treatment as a control. HBV challenged (**A**) HU1051 and (**C**) HU1052 donor. HBV/HDV challenged SACC-PHHs (**B**) HU1051 and (**D**) HU1052. Arrows indicate when poly(I:C) was added dark green (pre-treatment) and black (post 12 days of infection).