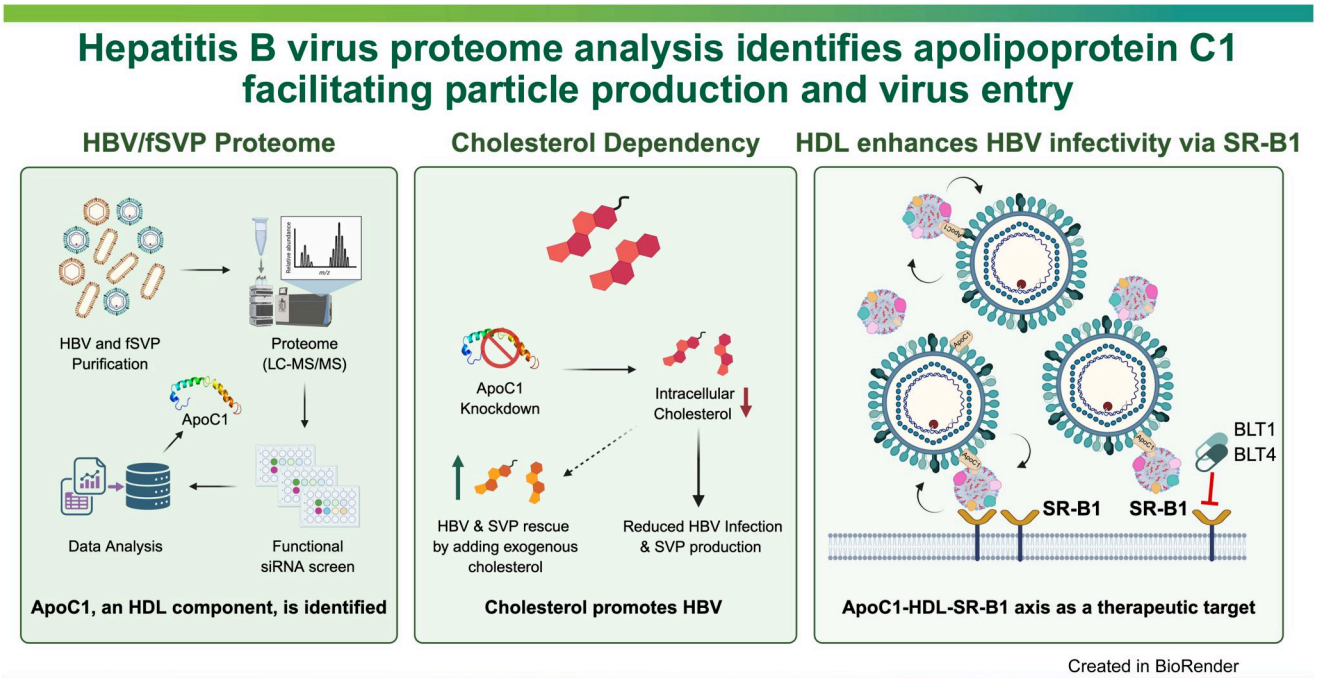
Hepatitis B virus proteome analysis identifies apolipoprotein C1 facilitating particle production and virus entry

VISUAL ABSTRACT



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ORIGINAL ARTICLE

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Hepatitis B virus proteome analysis identifies apolipoprotein C1 facilitating particle production and virus entry

Shangqing Yang[1,2] | **Firat Nebioglu**[1,2] | **Minh Tu Pham**[1] | **Yen-Chia Lin**[3] | **Andreas Pichlmair**[4,5] | **Shirin Nkongolo**[6,7] | **Pietro Scaturro**[3,4,8,9] | **Stephan Urban**[1,7] | **Stefan Seitz**[1] | **Ralf Bartenschlager**[1,2,7]

[1]Medical Faculty Heidelberg, Department of Infectious Diseases, Molecular Virology, Center for Integrative Infectious Disease Research (CIID), Heidelberg University, Heidelberg, Germany

[2]Division Virus-Associated Carcinogenesis, German Cancer Research Center (DKFZ), Heidelberg, Germany

[3]Leibniz Institute of Virology, Hamburg, Germany

[4]Institute of Virology, School of Medicine and Health, Technical University of Munich, Munich, Germany

[5]Systems Virology, Institute of Virology, Helmholtz Munich, Munich, Germany

[6]Internal Medicine IV (Gastroenterology, Hepatology, Infectious Diseases), University Hospital Heidelberg, Heidelberg University, Heidelberg, Germany

[7]German Center for Infection Research (DZIF), Heidelberg Partner Site, Heidelberg, Germany

[8]German Center for Infection Research (DZIF), Hamburg-Lübeck-Borstel-Riems Partner Site, Hamburg, Germany

[9]Department of Biology, Faculty of Mathematics, Informatics and Natural Sciences, University of Hamburg, Hamburg, Germany

Abstract

Background and Aims: Antiviral therapies targeting the hepatitis B virus (HBV) suppress viral replication, but rarely achieve a functional cure. Understanding HBV–host cell interaction is crucial for developing novel therapeutic approaches. Here, we report host cell proteins associated with HBV virions and filamentous subviral particles (fSVPs) and characterize one host cell protein, apolipoprotein C1 (ApoC1), mechanistically.

Approach and Results: Highly purified HBV virions and fSVPs were obtained by sequential use of several biophysical methods. Particles were analyzed by mass spectrometry, and associated proteins were evaluated phenotypically using an HBV infection model. The top hit, ApoC1, was characterized in detail. Alongside the established HBV-associated chaperones HSP90AB1 and HSC70, we identified several apolipoprotein-related factors associated with virions and fSVPs. RNAi-based phenotypic validation identified the strongest effects for ApoC1, likely due to two complementary effects. First, ApoC1 depletion reduced intracellular cholesterol level, impairing HBV infection and SVP production, which was compensated by exogenous cholesterol addition. Second, ApoC1, which is mainly enriched in high-density lipoprotein (HDL), associates with HBV virions and fSVPs and increases HBV infectivity. The same was found for hepatitis D

Abbreviations: ApoC1, apolipoprotein C1; BLT, block lipid transport; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; FBS, fetal bovine serum; fSVP, filamentous subviral particle; FPLC, fast protein liquid chromatography; GE, genome equivalent; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; HDV, hepatitis D virus; HSC70, heat shock cognate protein 70; HSP90AB1, heat shock protein 90 alpha family class B member 1; HSPGs, heparan sulfate proteoglycans; IP, immunoprecipitation; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MOI, multiplicity of infection; MyrB, Myrcludex B; NTCP, sodium taurocholate cotransporting polypeptide; PCA, principal component analysis; PEG, polyethylene glycol; pgRNA, pregenomic RNA; PHHs, primary human hepatocytes; PPIA, cyclophilin A; rcDNA, relaxed circular DNA; siNT, non-targeting siRNA; SR-B1, scavenger receptor class B type 1; sSVP, spherical subviral particle.

Shangqing Yang and Firat Nebioglu contributed equally to this article.

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Correspondence
Ralf Bartenschlager, Department of Infectious Diseases, Molecular Virology, Center for Integrative Infectious Disease Research (CIID), University of Heidelberg, Im Neuenheimer Feld 344, 1st Floor, 69120 Heidelberg, Germany.
Email: ralf.bartenschlager@med.uni-heidelberg.de

virus (HDV), a satellite virus utilizing HBV envelopes. Supplementation of exogenous HDL enhanced infection via scavenger receptor class B type 1 (SR-B1), the natural HDL receptor. Consistently, inhibition of SR-B1 suppressed HBV and HDV infection.

Conclusions: We established a method for obtaining highly purified HBV virions and fSVPs and identified the HDL component ApoC1 to associate with both particle types. ApoC1 promotes HBV and HDV infection most likely via SR-B1 and facilitates viral entry.

Keywords: HBV proteome, hepatitis D virus (HDV), high-density lipoprotein (HDL), scavenger receptor class B type 1 (SR-B1), subviral particles (SVPs)

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major public health burden affecting an estimated 254 million people worldwide.[1] They have a high risk of developing severe liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC), accounting for a total of 1.1 million deaths per year.[1] Antiviral therapies suppress viral replication but do not eliminate the covalently closed circular (ccc) DNA, the viral persistence reservoir residing in the nucleus of infected cells.[2] In addition, excessive amounts of non-infectious subviral particles (SVPs) are released from infected hepatocytes, causing dysfunction of the immune response targeting the major viral antigen, the hepatitis B surface antigen (HBsAg). Therefore, to achieve curative therapy, novel approaches are needed.

HBV particles are enveloped and contain 3 viral glycoproteins called S, M, and L (small, medium, and large, respectively). They share the same C-terminal S-region but have additional N-terminal extensions: the preS2 region of M and the preS1 region of L, the latter binding to the viral entry receptor NTCP.[3,4] Initial attachment of HBV to hepatocytes is facilitated by heparan sulfate proteoglycans (HSPGs) interacting with the viral surface proteins.[5] Subsequently, the preS1 region binds to NTCP, followed by virion entry. Upon transport into the nucleus, the viral genome is converted into the cccDNA that forms a stable episome from which all HBV RNAs are transcribed.[6] Transcription requires the action of the HBx protein, preventing the epigenetic silencing of the cccDNA template.[7] Infected cells release an array of HBV-derived particles, including infectious virions (Dane particles), DNA-lacking (empty) virions,[8] non-enveloped nucleocapsids with and without viral RNA, and excessive quantities of non-infectious SVPs. Depending on the L–M–S ratio, SVPs can form: highly abundant spherical SVPs (sSVPs), 20–25 nm in diameter and predominantly composed of S; less abundant filamentous SVPs (fSVPs) containing S and also L.

Several studies reported the incorporation of host cell proteins into HBV nucleocapsids or virus particles. Early work demonstrated that the interaction between HBV polymerase and pregenomic (pg) RNA requires the heat shock protein HSP90, detected in purified nucleocapsids together with the chaperone HSP70.[9] In addition, cyclophilin A (PPIA) and apolipoprotein E (ApoE) associate with secreted particles,[10,11] the latter enabling HBV to use the reverse cholesterol transport pathway through hepatic macrophages to facilitate virion uptake by hepatocytes.[12]

Despite these advances, we still do not know the host cell proteins carried along within HBV virions and delivered into the infected cell. Here, we established a particle purification pipeline enabling the proteomic analysis of HBV virions and fSVPs with high specificity and sensitivity. We identified apolipoprotein C1 (ApoC1), a component of high-density lipoprotein (HDL), as a critical host cell dependency factor and revealed its contribution to HBV infection and particle production, most likely via a dual-mode of action.

METHODS

Cell culture

Huh7, HepG2-NTCP-A3, HepG2-HB2.7, HEK293T, and HepAD38 cells were cultured as described in Supplemental Materials and Methods, https://links.lww.com/HEP/K558. To suppress HBV production, 1 μg/mL doxycycline (Sigma-Aldrich, USA) was added to the HepAD38 medium for general maintenance of the cells.

Cryopreserved primary human hepatocytes (PHHs) were purchased from Thermo Fisher Scientific and cultured according to the manufacturer's instructions.

HBV particles production and purification

HepAD38 cells containing an overlength HBV genome under the control of a tetracycline-inducible promoter were cultured as previously described.[13] HBV production was induced by omitting doxycycline from the culture medium. Culture supernatants were collected every 4 days, sterile filtered, and stored at 4 °C until further processing. HBV particles were purified as described in the Results section and Supplemental Materials and Methods, https://links.lww.com/HEP/K558.

Ethics statement

Use of human clinical samples was approved by the Ethics Committee of the Medical Faculty of Heidelberg University (approval no. S-522/2024) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients whose samples were collected prospectively. For retrospectively collected samples, the requirement for informed consent was waived by the ethics committee.

For all further details, see Supplemental Materials and Methods, https://links.lww.com/HEP/K558.

RESULTS

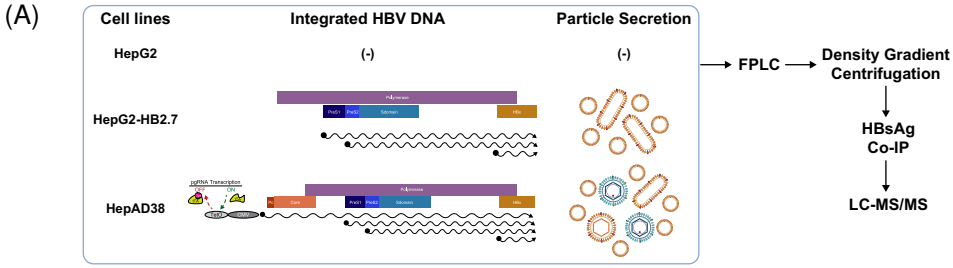
Purification of HBV virions and filamentous SVPs

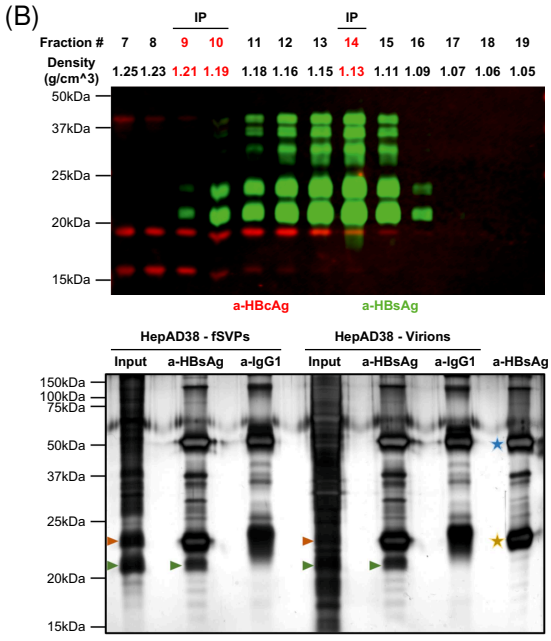
To obtain HBV virions and fSVPs, we used 2 HBV-containing HepG2-derived cell lines (Figure 1A): HepG2-HB2.7 cells containing only a partial 2.7 kb HBV genome encoding the envelope proteins and the HBx protein and supporting only SVP production; HepAD38 cells harboring a 1.1-overlength HBV genome under a tet-off inducible promoter and releasing both HBV virions and SVPs upon tetracycline removal from the culture medium.[13] The parental HepG2 cell line was used as a control to determine contaminating proteins in the proteomics analysis (Figure 1A). To reduce contamination of the preparations by fetal bovine serum (FBS) components, we generated 5 independent biological replicates: 3 from cells cultured with 10% FBS (replicates 1–3) and 2 cultured with 5% FBS (replicates 4–5).

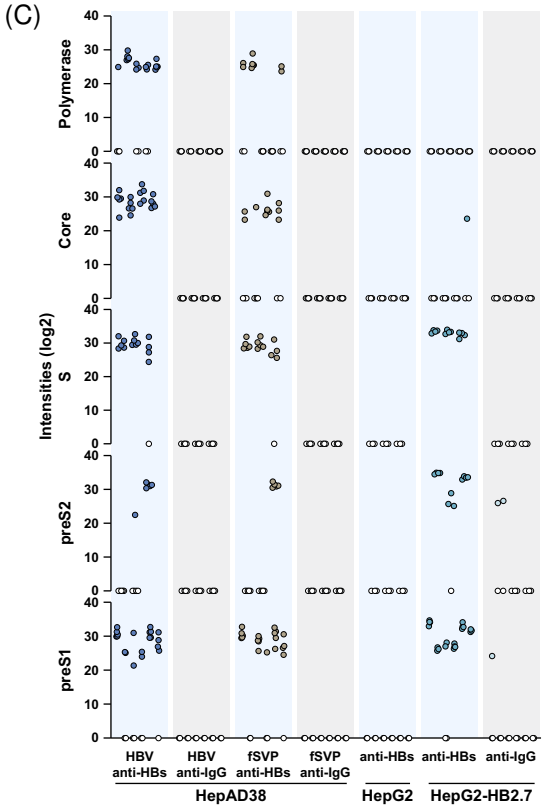
To obtain mass spectrometry-grade HBV particles, we employed our previously reported purification protocol comprising fast protein liquid chromatography (FPLC) with a heparin column and size-exclusion chromatography (Figure 1A).[14] To remove the majority of spherical SVPs, we intentionally selected only the early fractions of the size-exclusion chromatography, containing Dane particles and fSVPs. Pooled fractions were then subjected to density gradient centrifugation. Dual-fluorescence western blot analysis of the gradient fractions demonstrated distinct distribution of particles: naked capsids (density ~1.23 g/cm^3; indicated also by HBeAg that cross-reacts in the core ELISA); virions (~1.20 g/cm^3; indicated by HBsAg and HBV DNA) and fSVPs (density of ~1.13 g/cm^3; indicated by high amounts of HBV envelope proteins and low HBcAg and HBV DNA) (Figure 1B, upper panel; Supplemental Figure S1, https://links.lww.com/HEP/K558). Pooled virion fractions and the selected fSVP fraction were then analyzed for total protein by using silver-stained protein gels. We observed high amounts of protein in both samples, dampening the detection sensitivity of the HBV proteome (Figure 1B, lower panel, input lanes; gradient fractions in Supplemental Figure S2A, https://links.lww.com/HEP/K558). The same was true for fSVPs purified from the culture supernatant of HepG2-HB2.7 cells (gradient fractions in Supplemental Figure S2B, https://links.lww.com/HEP/K558 and S3A, https://links.lww.com/HEP/K558, input lane). To reduce impurities, we performed HBs-specific immunoprecipitation (IP); IP with an IgG isotype served as a specificity control. Protein amounts and complexities of all samples were greatly reduced after the IP (Figure 1B, lower panel; Supplemental Figure S3A, https://links.lww.com/HEP/K558). Notably, core protein remained detectable in the virion fractions after IP, indicating preserved particle integrity (Supplemental Figure S3B, https://links.lww.com/HEP/K558, left panel), although we cannot exclude the presence of partially damaged particles or the co-IP of empty virions secreted by HepAD38 cells. As expected, re-probing the blots with HBs-specific antibodies revealed HBV envelope proteins of different isoforms in the virion fractions (Supplemental Figure S3B, https://links.lww.com/HEP/K558, right panel).

Proteomics analysis of purified HBV virions and filamentous SVPs

Proteins contained in IP-purified virion and fSVP samples were identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS). For each experimental condition, 4 or 5 biologically independent replicates of affinity-purified complexes obtained from IgG or HBs-specific immunoprecipitations were analyzed. Although the abundance of each unique viral peptide varied between the different replicates, HBV envelope proteins, core protein, and polymerase (P) protein were consistently detected in all virion fractions, with the best peptide coverage in replicate #5 (Figure 1C; Supplemental Table S1, https://links.lww.com/HEP/K558). Notably, we detected 5 different peptides derived from the HBV P-protein that is present in only one copy per particle.[15] In addition, we detected


(A)
Cell lines
Integrated HBV DNA
Particle Secretion
HepG2
(-)
(-)
HepG2-HB2.7
HepAD38
FPLC
Density Gradient Centrifugation
HBsAg Co-IP
LC-MS/MS


(B)
IP
IP
Fraction # 7 8 9 10 11 12 13 14 15 16 17 18 19
Density (g/cm^3) 1.25 1.23 1.21 1.19 1.18 1.16 1.15 1.13 1.11 1.09 1.07 1.06 1.05
50kDa
37kDa
25kDa
20kDa
15kDa
a-HBcAg
a-HBsAg
HepAD38 - fSVPs
HepAD38 - Virions
Input a-HBsAg a-IgG1 Input a-HBsAg a-IgG1 a-HBsAg
150kDa
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa


(C)
Intensities (log2)
Polymerase
Core
preS2
S
preS1
HBV anti-HBs
HBV anti-IgG
fSVP anti-HBs
fSVP anti-IgG
anti-HBs
anti-HBs
anti-IgG
HepAD38
HepG2
HepG2-HB2.7

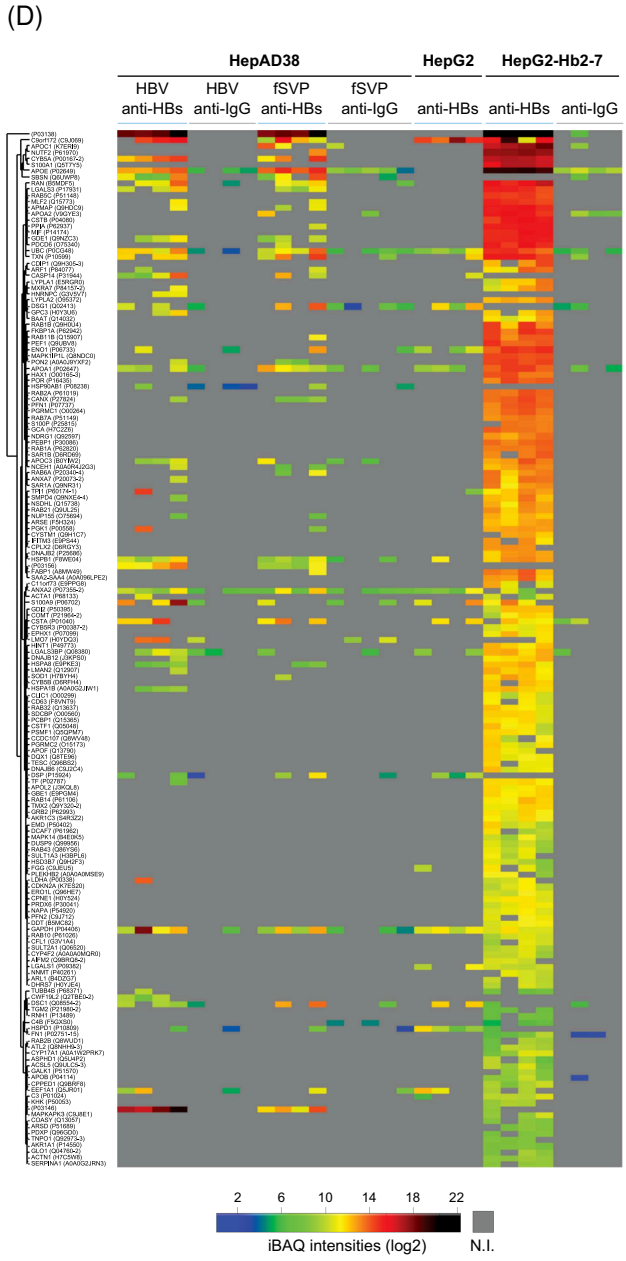

(D)
HepAD38
HepG2
HepG2-Hb2-7
HBV anti-HBs
HBV anti-IgG
fSVP anti-HBs
fSVP anti-IgG
anti-HBs
anti-HBs
anti-IgG
2 6 10 14 18 22
iBAQ intensities (log2)
N.I.

FIGURE 1 Purification of HBV virions and filamentous SVPs. (A) Purification pipeline of HBV virions and fSVPs from HepG2-HB2.7 and HepAD38 culture supernatants. Yellow particles represent subviral particles and empty virions; cyan particles represent Dane particles. (B) Upper panel: Sucrose fractions of one representative equilibrium density gradient were analyzed by dual-fluorescence western blot detecting HBV envelopes (green) and core proteins (red), with densities specified above each lane. Fractions enriched for virions (9–10) or fSVPs (14) were used for HBsAg-specific pull-down. Lower panel: Pull-down samples and corresponding inputs were analyzed by SDS-PAGE and silver staining. Arrowheads indicate glycosylated (orange) and non-glycosylated (green) small HBs proteins; stars indicate IgG heavy (blue) and light (gold) chains. (C) Viral peptides identified by AP-LC-MS/MS across all 5 biological replicates are categorized according to viral proteins. Individual values represent spectral intensities (log2) of unique peptides from pull-down samples across independent biological replicates (white circles = not identified). (D) Proteomic analysis of affinity-purified particles by AP-LC-MS/MS ($n \geq 4$). The heatmap represents intensity-based quantification (iBAQ) values of significantly enriched proteins in virion or fSVPs pull-downs (log2). Each column corresponds to one pull-down sample from one independent biological replicate. Abbreviations: AP-LC-MS/MS, affinity purification–liquid chromatography–tandem mass spectrometry; FBS, fetal bovine serum; FPLC, fast protein liquid chromatography; fSVPs, filamentous subviral particles; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; iBAQ, intensity-based absolute quantification; IgG, immunoglobulin G; N.I., not identified; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SVPs, subviral particles.

core-specific and envelope-specific peptides, whereas HBx-specific and HBe-specific peptides were not identified. In contrast, only some P-specific and much lower amounts of core-specific peptides were detected in the fSVP fraction from HepAD38 cells, demonstrating a strong depletion of virions from the fSVP fraction. Much stronger signals for HBV envelope proteins were detected in fSVP fractions derived from HepG2-HB2.7 cell supernatants, consistent with their higher SVP production rate, whereas P-specific and core-specific peptide signals were absent (Supplemental Table S1, https://links.lww.com/HEP/K558). No or only background levels of HBV-specific peptides were identified in the IgG control IP or in samples derived from HepG2 cells.

Regarding non-viral proteins co-purifying with HBV virions and fSVPs, a total of 787 host cell proteins were detected (Supplemental Table S2A, https://links.lww.com/HEP/K558). Principal component analysis (PCA) of the individual samples confirmed high correlation within biological replicates (Supplemental Figure S4A, https://links.lww.com/HEP/K558). For further analysis, only proteins consistently identified in at least 3 biological replicates of at least one experimental group were retained ($n = 266$ host proteins; Supplemental Table S2B, https://links.lww.com/HEP/K558). This list was additionally filtered to retain only proteins completely missing from all IgG controls or displaying at least a median log2 (fold-change) ≥ 3 for each HBs-specific pull-down compared with the corresponding IgG control group. In this way, 165 host cell proteins were identified, especially in the fSVP fractions of HepG2-HB2.7 cells, consistent with their higher SVP production rate compared with HepAD38 cells (Figure 1D, Supplemental Table S2B, https://links.lww.com/HEP/K558 and Table S3, https://links.lww.com/HEP/K558). Nevertheless, a substantial subset of these proteins was also detected in virion and fSVP fractions from HepAD38 cells. Among all identified host proteins, chaperones of different families were enriched in all conditions, including HSPB1 that modulates cccDNA activity and HSC70 that binds to PreS1 and regulates L protein topology (Supplemental Figure S4B, https://links.lww.com/HEP/K558).[16,17] Three proteins, CASP14, CWF19L2, and HNRNPC, were highly enriched in virion fractions with little to no signal overlap with peptides detected in the fSVP preparations (Figure 1D; Supplemental Table S2B, https://links.lww.com/HEP/K558). Moreover, several members of the apolipoprotein family were detected in HBV particles and fSVPs derived from both cell lines (Figure 1D; Supplemental Figure S4B, https://links.lww.com/HEP/K558).

Taken together, we identified a set of previously published host proteins, along with several hitherto unknown cellular proteins selectively enriched with highly purified HBV virions and/or fSVPs.

Functional validation of virion-associated and fSVP-associated host proteins

To determine whether these proteins contribute to the HBV replication cycle, 48 candidates were prioritized based on their consistent identification across HBV virions or fSVPs samples and validated by RNAi-based screening in an HBV infection assay (Figure 2A). Two days after reverse transfection of siRNAs, HepG2-NTCP-A3 cells were infected with HBV, and the amounts of secreted HBeAg, HBsAg, intracellular core protein (HBcAg), and viral RNAs were measured 5 days after infection (Figures 2B–E; Supplemental Figures S5A, B, https://links.lww.com/HEP/K558). Cytotoxicity assays identified only one knockdown as cytotoxic (PCBP1; Supplemental Figure S5C, https://links.lww.com/HEP/K558). For each parameter, values were normalized to a non-targeting siRNA pool (siNT). Cells treated with a siRNA targeting viral transcripts (siHBV) or the peptidic entry inhibitor bulevirtide (Myrcludex B; MyrB) were used as positive controls; both reduced HBV parameters without cytotoxic effects (Figures 2B–E; Supplemental Figure S5, https://links.lww.com/HEP/K558). Considering the dynamic range of the various HBV markers in this setup, phenotypes were ranked based on secreted HBeAg levels, a sensitive HBV replication marker. Overall, the results of the different

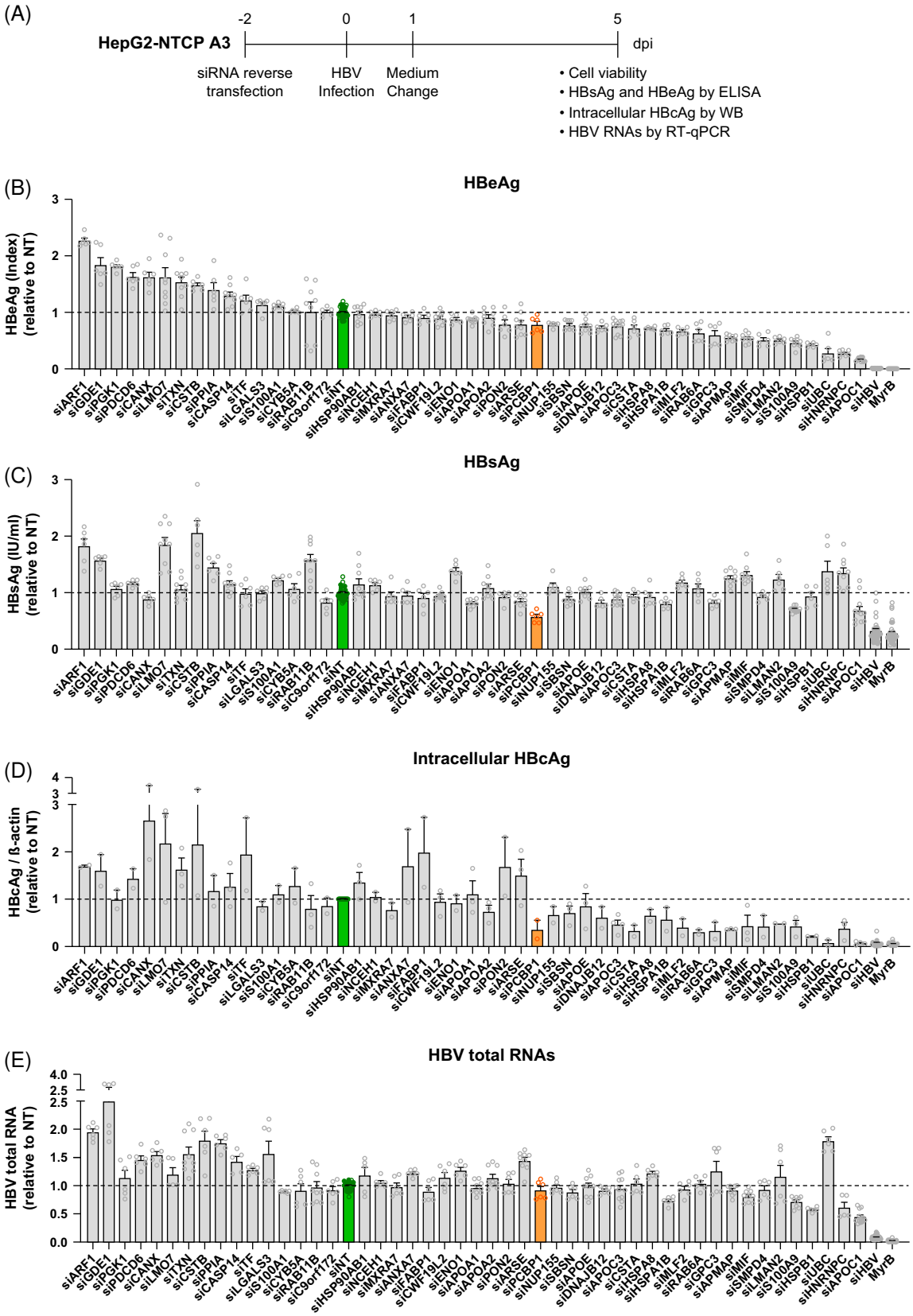


FIGURE 2 siRNA-based screen identifies novel HBV host-dependency factors. (A) Schematic of the experimental approach. HepG2-NTCP-A3 cells were reverse-transfected with a gene-specific siRNA pool for 48 hours and subsequently infected with HBV [500 genome equivalents (GEs)/cell with 4% PEG8000]. After 24 hours, the inoculum was removed, the cells were washed, and cultured for an additional 5 days. Cells treated with the entry inhibitor bulevirtide (MyrB) or transfected with siRNA targeting HBV transcripts (siHBV) served as positive controls. (B, C)

HBV infection was evaluated by measuring secreted HBeAg and HBsAg at 5 days post-infection (dpi) by ELISA. (D) Intracellular HBcAg was detected by western blot and quantified using Image Lab. (E) HBV total RNAs were quantified by RT-qPCR. Values were normalized to cells transfected with non-targeting siRNA (siNT), set to 1 (green bar). Data are presented as mean ± SEM from at least 2 independent experiments performed in triplicate. Orange bar, flawed result because of cytotoxicity (see Supplemental Figure S5C, https://links.lww.com/HEP/K558).
Abbreviations: dpi, days post-infection; ELISA, enzyme-linked immunosorbent assay; GEs, genome equivalents; HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MyrB, Myrcludex B; NTCP, sodium taurocholate cotransporting polypeptide; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SEM, standard error of the mean; siHBV, small interfering RNA targeting hepatitis B virus transcripts; siNT, non-targeting small interfering RNA; siRNA, small interfering RNA.

HBV replication parameters were consistent, although changes in HBsAg levels were less pronounced, while intracellular core protein measurements had higher variability than HBeAg ELISA. Focusing on host cell factors whose knockdown reduced viral parameters, ApoC1 knockdown gave the most consistent and strongest effect, decreasing all viral parameters in a very reproducible manner (Figures 2B–E).

As noted above, 5 human apolipoproteins were identified in our proteomic analysis. Among these, ApoE, ApoA1, and ApoC3 were detected in virions and fSVPs, whereas ApoA2 and ApoC1 were only detected in highly concentrated fSVPs derived from the high-producer HepG2-HB2.7 cells (Supplemental Figure S4B, https://links.lww.com/HEP/K558). While ApoE was previously reported to enhance HBV infection and production[10] ApoC1 has not yet been studied. Therefore, we compared the roles of the apolipoproteins identified in our proteome for their impact on HBV infection/replication and SVP production. Knockdown efficiency measured 7 days after siRNA transfection was high (Figure 3A). Zooming into the RNAi validation results (Figure 2), among the 5 apolipoproteins, ApoC1 knockdown caused the strongest reduction of secreted HBsAg and HBeAg (Figure 3B), intracellular HBV transcripts and pgRNA (Figure 3C), as well as intracellular core protein (Figure 3D), all determined 5 days after infection. Notably, also in the context of the HepG2-HB2.7 cell line, extracellular HBsAg was reduced most by ApoC1 knockdown (Figure 3E).

ApoC1 is a proviral host protein promoting the HBV replication cycle

To assess whether the 5 apolipoproteins affect HBV replication at a post-entry stage, we employed HepG2.2.15 cells containing 2 head-to-tail HBV genome dimers.[18] In line with our finding in HepG2-HB2.7 cells, ApoC1 knockdown reduced HBsAg levels (SVP production), while HBeAg and HBV DNA levels did not decrease, but rather increased (Supplemental Figure S6A, https://links.lww.com/HEP/K558). Complementing these data, we depleted apolipoproteins in the HBV infection system just several days after infection to avoid any effect on HBV entry (Supplemental Figure S6B, https://links.lww.com/HEP/K558). Although knockdown efficiency was limited compared with reverse siRNA transfection used for all other experiments, siHBV treatment significantly decreased measured HBV parameters, demonstrating that we could detect antiviral effects even under these conditions. No changes of HBV parameters were observed in ApoC1 knockdown cells, arguing against a post-entry role of ApoC1 in HBV infection.

ApoC1 is the smallest, exchangeable, and most positively charged apolipoprotein. It regulates the activity of several lipid metabolizing enzymes and the function of lipoprotein receptors, including SR-B1.[19] Since ApoC1 was identified as the top hit from the phenotypic screen, we further validated its relevance for the HBV replication cycle by time-course experiment. ApoC1-depleted HepG2-NTCP cells were infected with HBV, and the kinetics of secreted HBsAg, HBeAg, and HBV DNA were evaluated 5, 7, and 9 days post-infection. ApoC1 knockdown significantly reduced HBsAg and HBeAg, comparable to NTCP control knockdown at all time points (Figure 4A). Extracellular HBV DNA, a marker for virus particle production, detected 5 days post-infection, was derived from the inoculum, as levels were equivalent among all conditions, including cells treated with the entry inhibitor MyrB (Figure 4A, lower panel). However, at later time points when most of the input had decayed, HBV DNA was significantly reduced upon ApoC1 knockdown relative to siNT-transfected control cells, with the reduction being comparable or even stronger to NTCP depletion. Quantification of the number of infected cells and cccDNA levels confirmed the ApoC1 knockdown phenotype (Figures 4B, C, respectively). The analogous result was found when using HBV infection in the absence of polyethylene glycol (PEG), commonly used to enhance virion binding and internalization (Supplemental Figure S7, https://links.lww.com/HEP/K558).

Since previous studies reported functional redundancy of ApoC1 and ApoE,[20] we investigated possible additive or even synergistic effects of combined ApoE/ApoC1 depletion. However, secreted HBeAg levels remained comparable between HBV-infected ApoC1/ApoE double and ApoC1 single knockdown cells (Supplemental Figure S8, https://links.lww.com/HEP/K558).

These results suggest that ApoC1 plays a role at an early step of the HBV life cycle, and, at least to some extent, in the production or release of SVPs from integrated viral sequences.

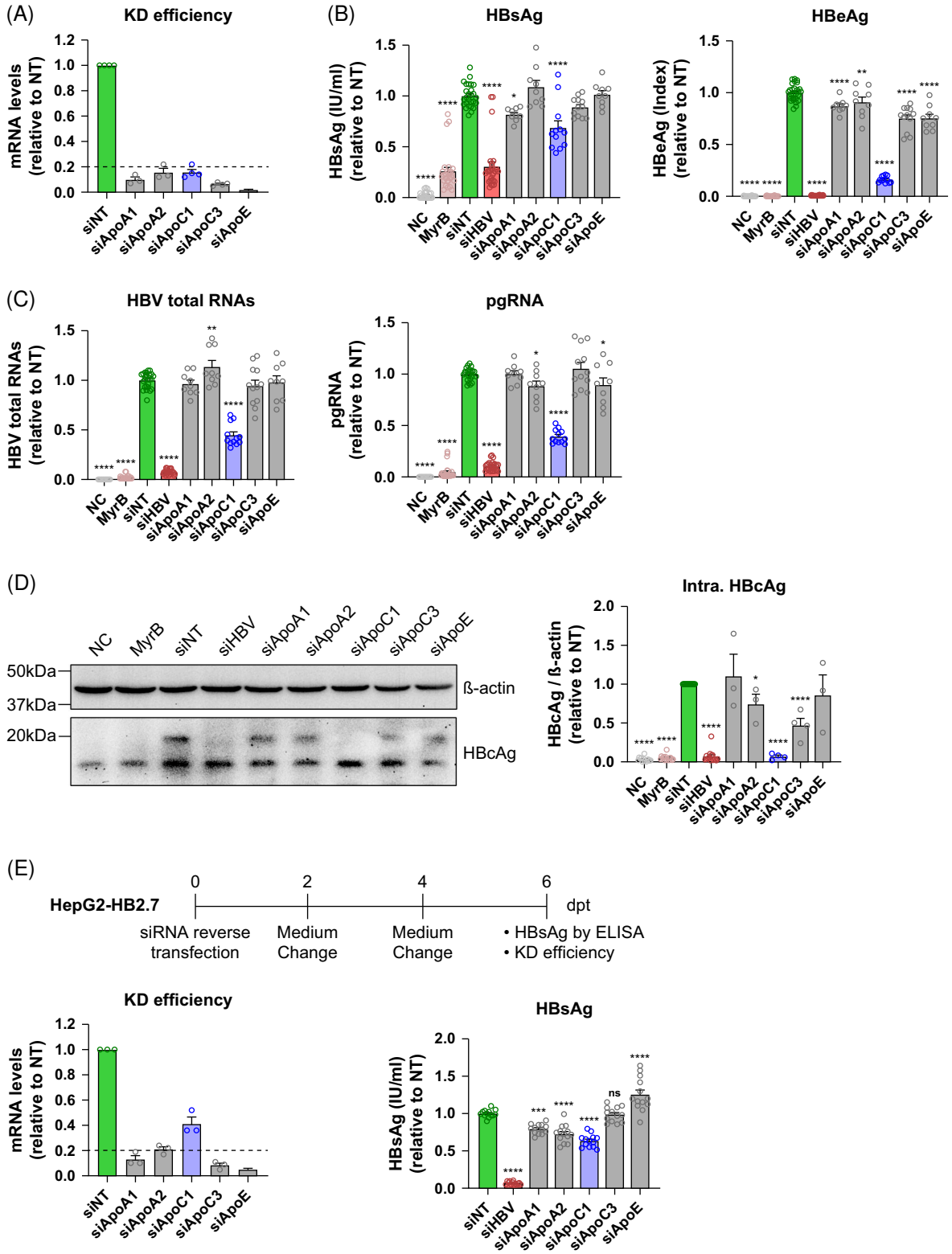


FIGURE 3 Comparative analysis of the impact of apolipoprotein depletion on HBV infection and HBsAg production. (A–D) Detailed view of apolipoprotein knockdown results from Figure 2. (A) Knockdown efficiency was assessed by RT-qPCR. (B) Secreted HBsAg and HBeAg were measured by ELISA. (C) HBV total RNAs and pgRNA were quantified by RT-qPCR. (D) Intracellular HBcAg was detected by western blot and quantified using Image Lab. One out of at least 3 independent experiments is shown. (E) HepG2-HB2.7 cells were reverse-transfected with siRNAs; medium was refreshed after 48 hours. Cells were harvested 6 days post-transfection. Knockdown efficiency and secreted HBsAg were measured by RT-qPCR and ELISA, respectively. Values were normalized to siNT, set to 1 (green bar). Data represent mean ± SEM from at least 3 independent experiments. B, C, D, E, one-way ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, and ns—not significant

($p > 0.05$). Abbreviations: ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; HBV, hepatitis B virus; KD, knockdown; MyrB, Bulevirtide-treated cells; NC, no infection control; ns, not significant; pgRNA, pregenomic RNA; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SEM, standard error of the mean; siNT, non-targeting small interfering RNA; siRNAs, small interfering RNAs.

ApoC1 regulates intracellular cholesterol levels, affecting HBV and HDV entry

Cholesterol plays an important role in the HBV replication cycle.[21] Moreover, ApoC1 has been reported to regulate intracellular cholesterol levels.[22] Therefore, we quantified cholesterol amounts in HepG2-NTCP cells depleted for ApoC1 or any of the other 4 different apolipoproteins identified in our proteome (Figure 4D, left panel). Only ApoC1 knockdown caused a small, yet significant, reduction of cholesterol level. To determine whether intracellular cholesterol amounts affect the HBV replication cycle, we conducted rescue experiments by replenishing ApoC1 knockdown cells with different concentrations of water-soluble cholesterol added to the cell culture medium during HBV infection in the absence of PEG (Figure 4D, right panel). Although we observed an increase in the HBV replication marker HBeAg in ApoC1 knockdown cells by exogenously added cholesterol (around 2-fold at high cholesterol concentration), an analogous increase was found in cells transfected with siNT. This result indicated that cholesterol is a more generally limiting factor in the HBV replication cycle and not specific to ApoC1 depletion. Nevertheless, the reduction of cholesterol we observed in ApoC1 knockdown cells could explain, at least in part, the impairment of the HBV replication cycle observed in these cells. Notably, cholesterol addition restored HBV replication close to the level detected in siNT-transfected control cells (Figure 4D, right panel). Moreover, we found that cholesterol substitution also boosted SVP production in the context of HepG2-HB2.7 cells (Figure 4E), regardless of whether FBS was absent or present (Supplemental Figure S9, https://links.lww.com/HEP/K558). These results suggest that cholesterol supports several steps of the HBV replication cycle, including SVP production.

Association of ApoC1 with HBV virions and filamentous SVPs

In addition to regulating intracellular cholesterol levels, ApoC1 appeared to promote an early HBV replication cycle step (Supplemental Figure S6, https://links.lww.com/HEP/K558). Therefore, we analyzed 2 independent heparin-purified cell culture-derived HBV stocks by HBs-specific or ApoC1-specific IP and quantified captured particles (Figure 5A). We observed co-IP of ApoC1, HBc protein, and HBV DNA when capturing the particles with an HBs-specific antibody; vice versa, co-IP of HBc protein and viral DNA was found when we captured the particles with an ApoC1-specific antibody (Figure 5A). The analogous result was found with particles derived from Huh7 cells, excluding confounders due to HepG2 cells or heparin purification (Figure 5B; Supplemental Figure S10A, https://links.lww.com/HEP/K558). Notably, analysis of HBV particles contained in sera of 3 patients with CHB corroborated the association of ApoC1 with HBV particles (Figure 5C; Supplemental Figure S10B, https://links.lww.com/HEP/K558).

To confirm this association, HepAD38-derived and purified HBV virions or fSVPs were analyzed by immuno-EM using ApoC1-specific or HBs-specific primary antibodies that were detected with Protein A-coupled to 10 nm gold particles. HBs proteins were present on the surface of virions and fSVPs (Figure 5D; Supplemental Figure S10C, https://links.lww.com/HEP/K558). Notably, ApoC1 was also detected in association with virions and fSVPs. Quantification of gold-labeled particles revealed ~10% of HBV particles being ApoC1 positive (Figure 5D). Interestingly, ApoC1 gold labeling was observed both in direct proximity to HBV virions and fSVPs, and adjacent to HDL-like structures residing next to these particles. Thus, ApoC1 may associate with HBV particles both directly and indirectly via HDL-containing lipoprotein particles (Figure 5D; Supplemental Figure S10C, https://links.lww.com/HEP/K558).

These results confirm ApoC1 association with HBV virions and fSVPs, and they suggest that at least a fraction of ApoC1 associates with these particles in the form of HDL.

ApoC1 regulates HBV and HDV entry

To study the functional role of ApoC1 in HBV uptake into liver cells, we performed ApoC1 antibody-mediated neutralization and ApoC1 protein competition assays in the context of HBV infection. An ApoC1-specific antibody dose-dependently reduced all HBV parameters (Figure 6A). The analogous result was obtained using recombinant ApoC1 protein (Supplemental Figure S11, https://links.lww.com/HEP/K558), suggesting that viral entry is facilitated by HBV-associated ApoC1.

HBV and its satellite virus HDV share the same envelope and thus, the early steps of hepatocyte infection. Consistently, we found that ApoC1 knockdown impaired HDV infection (Supplemental Figure

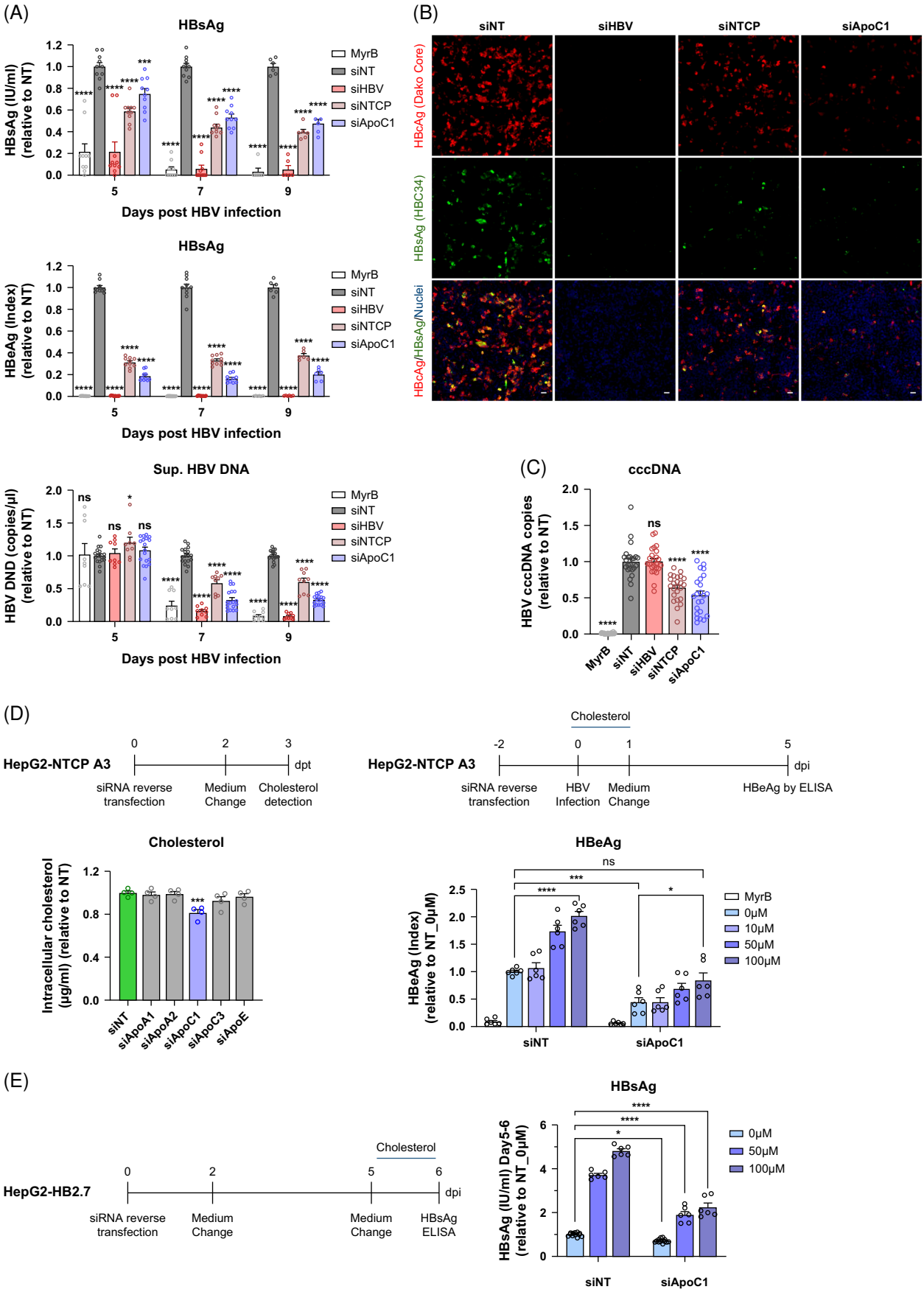

(A)
HBsAg
HBsAg (IU/ml) (relative to NT)
Days post HBV infection
MyrB
siNT
siHBV
siNTCP
siApoC1
HBeAg (Index) (relative to NT)
Sup. HBV DNA
HBV DND (copies/µl) (relative to NT)
(B)
siNT
siHBV
siNTCP
siApoC1
HBcAg (Dako Core)
HBsAg (HBC34)
HBcAg/HBsAg/Nuclei
(C)
cccDNA
HBV cccDNA copies (relative to NT)
(D)
HepG2-NTCP A3
dpt
siRNA reverse transfection
Medium Change
Cholesterol detection
Cholesterol
HepG2-NTCP A3
dpi
siRNA reverse transfection
HBV Infection
Medium Change
HBeAg by ELISA
Cholesterol
Intracellular cholesterol (µg/ml) (relative to NT)
siNT
siApoA1
siApoA2
siApoC1
siApoC3
siApoE
HBeAg
HBeAg (Index) (relative to NT_0µM)
0µM
10µM
50µM
100µM
(E)
HepG2-HB2.7
dpi
siRNA reverse transfection
Medium Change
Medium Change
HBsAg ELISA
Cholesterol
HBsAg
HBsAg (IU/ml) Day5-6 (relative to NT_0µM)

FIGURE 4 Role of ApoC1 for HBV infection and intracellular cholesterol level. (A–C) HepG2-NTCP-A3 cells were reverse-transfected with siRNAs for 48 hours and then infected with HBV (500 GE/cell; 4% PEG8000). After 24 hours, the inoculum was removed, the cells were washed, and harvested at different time points. (A) Secreted HBsAg, HBeAg, and HBV DNA were quantified by ELISA and qPCR at 5, 7, and 9 dpi, respectively. (B) Intracellular HBc and HBs proteins were assessed by immunofluorescence at 5 dpi. Red, green, and blue signals depict HBc protein, HBs protein, and nuclei, respectively. Scale bar: 100 μm. (C) HBV cccDNA was detected by qPCR at 5 dpi. (D) HepG2-NTCP-A3 cells were reverse-transfected with siRNAs, and the medium was replaced with FBS-free medium after 48 hours. Left: Intracellular cholesterol levels, measured 1 day later. Right: Cholesterol supplementation during HBV infection in the absence of PEG8000 and FBS; secreted HBeAg was measured at 5 dpi. (E) HepG2-HB2.7 cells were reverse-transfected with siRNAs, and 48 hours later, the medium was refreshed. Cells were treated with water-soluble cholesterol between days 5 and 6, and secreted HBsAg was quantified. Data represent mean ± SEM from at least 3 independent experiments. A, D (right panel), E, two-way ANOVA; C, D (left panel), one-way ANOVA; *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, and ns—not significant ($p > 0.05$). Abbreviations: ApoC1, apolipoprotein C1; cccDNA, covalently closed circular DNA; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HBc, hepatitis B core; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NTCP, sodium taurocholate cotransporting polypeptide; PEG, polyethylene glycol; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean; siRNA, small interfering RNA.

S12, https://links.lww.com/HEP/K558). Therefore, we used HDV as a tractable surrogate to determine how ApoC1 depletion affects virion infectivity. We employed Huh7-END cells[23] stably releasing HDV and depleted these cells for ApoC1 (Figure 6B). HDV released from these cells and from siNT-transfected Huh7-END cells was quantified for viral genomes, and equal amounts of genome equivalents were used to infect permissive Huh7-NTCP cells. ApoC1-depleted HDV had significantly lower specific infectivity as compared with wild-type HDV virions (Figure 6B). Moreover, infection by ApoC1-free HDV produced in HEK293T cells that do not express endogenous ApoC1,[24] could be enhanced by adding low amounts of exogenous ApoC1. High ApoC1 amounts diminished HDV infection in this setting (Figure 6C), most likely due to competition, similar to the competition we observed for HBV (Supplemental Figure S11, https://links.lww.com/HEP/K558). These results suggest that ApoC1 associated with HBV or HDV particles increases their infectivity.

Both HBV and HDV use NTCP as an entry receptor. To assess whether ApoC1 knockdown affects cell surface availability of NTCP, we employed a fluorophore-conjugated preS1 peptide.[3] Using HepG2 or Huh7 cells stably expressing NTCP, ApoC1 depletion had no effect on NTCP cell surface abundance (Figures 6D, E).

Impairment of HBV infection by pharmacological inhibition of SR-B1

Since ApoC1 is mainly associated with HDL (Figure 7A), the role of HDL in HBV infection was investigated. Pretreatment of purified HBV stocks with exogenous HDL increased HBsAg and HBeAg secreted from infected cells, indicating enhanced viral infectivity by HDL (Figure 7B). Since SR-B1 is a natural receptor of HDL, we next studied the possible contribution of SR-B1 to HBV infection. Cells depleted of either SR-B1 or ApoC1 were infected in the presence of HDL. Depletion of either protein substantially attenuated the HDL-induced increase of HBV infection (Figure 7B), indicating HDL facilitated HBV infection through an SR-B1 and ApoC1-dependent mechanism.

Earlier studies reported facilitated HBV infection by ApoE, which binds to LDL receptors.[25] We therefore compared the impact of combined SR-B1–LDL or SR-B1–VLDL depletion on HBV infection. Single knockdown of SR-B1 or LDL-receptor significantly reduced HBV infection, whereas knockdown of the VLDL-receptor had no effect (Supplemental Figure S13A, https://links.lww.com/HEP/K558). A combination of SR-B1 and LDL-receptor knockdown had no additive effect. Analogous results were obtained with HDV, consistent with the same entry route used by both viruses (Supplemental Figure S13B, https://links.lww.com/HEP/K558).

SR-B1 mediates bidirectional lipid transport between HDL and cells by facilitating the selective uptake of cholesterol esters from HDL into liver cells for bile acid synthesis and the efflux of free cholesterol to HDL.[26] We explored this SR-B1 function for HBV infection using the pharmacological inhibitors BLT1 and BLT4. They bind to SR-B1 in an irreversible or reversible manner, respectively, and inhibit SR-B1–mediated lipid transfer activity between HDL and cells.[27,28] HepG2-NTCP-A3 cells were infected with HBV for 4 hours in the presence of BLT compounds, and viral replication was measured 5 days later. Both BLT compounds significantly reduced HBV replication, indicating that SR-B1 is a potential cofactor for HBV entry (Figure 7C). To investigate whether SR-B1 inhibition exerts effects beyond the viral entry step, we treated the cells with BLT1 at later stages of infection. BLT1 treatment 3 days after inoculum removal no longer reduced HBeAg levels, whereas treatment during the infection period reduced HBeAg levels by ~30% (Supplemental Figure S14, https://links.lww.com/HEP/K558), consistent with the SR-B1 knockdown phenotype. These findings suggest that SR-B1 does not affect HBV at a post-entry step. To corroborate this conclusion in a more relevant cell system, the effect of BLT1, the more potent and specific inhibitor,[27,29] was evaluated in HBV-infected primary human hepatocytes (PHHs) derived from 2 different donors (Figure 7D). The

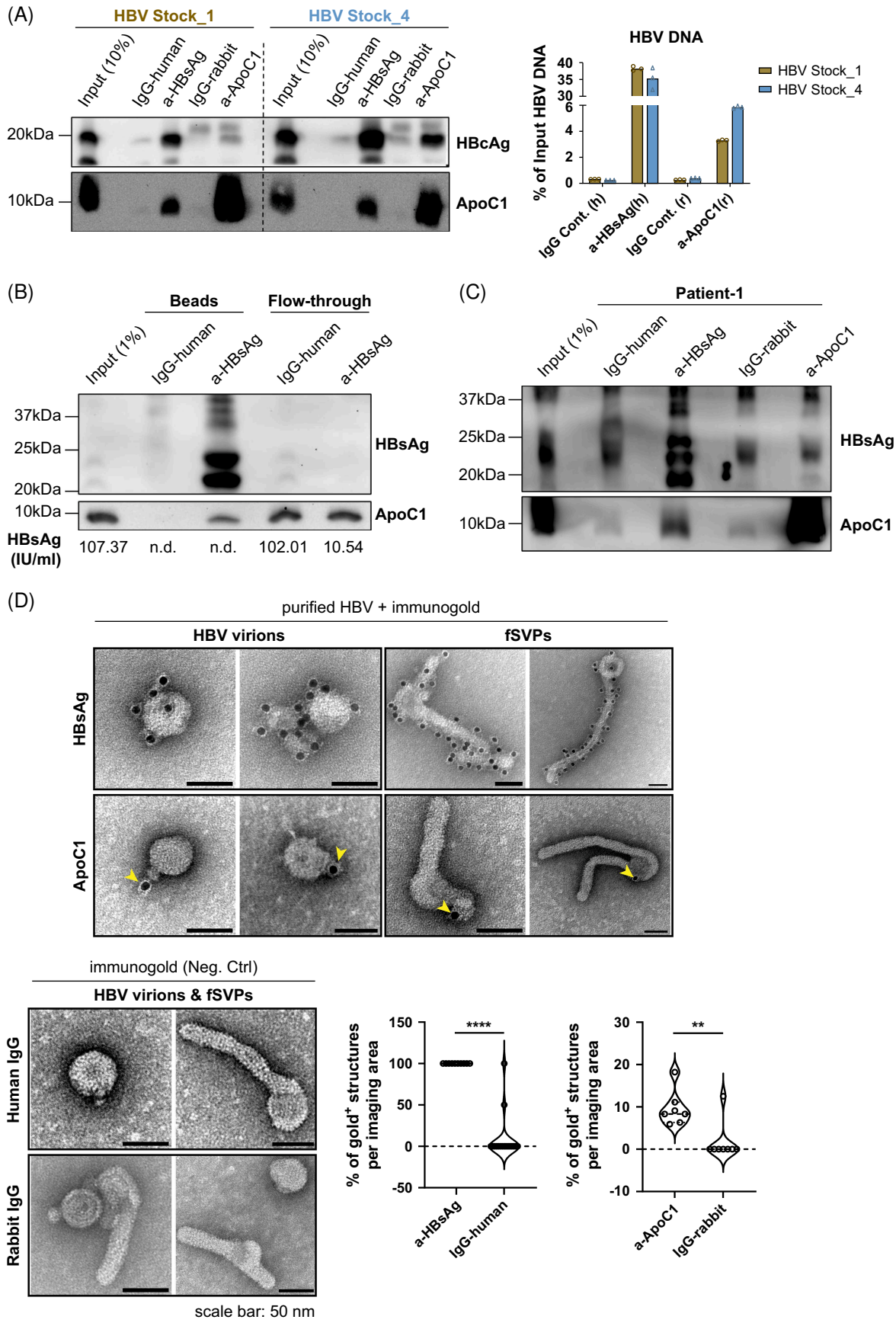


FIGURE 5 ApoC1 associates with HBV virions and filamentous SVPs. (A) Two different heparin-purified HBV stocks were subjected to

immunoprecipitation using HBsAg-specific, ApoC1-specific, or IgG antibodies. Eluates were analyzed for ApoC1 and HBcAg by western blot or HBV DNA by qPCR (normalized to input). (B) Huh7 cells were transfected with a plasmid containing a 1.1-overlength HBV genome. HBV accumulating in culture supernatant between day 4 and 7 post-transfection was subjected to immunoprecipitation with HBsAg-specific or IgG antibodies. HBsAg ELISA values of input and flow-through fractions are indicated at the bottom. (C) HBV particles in the serum of a patient with CHB were analyzed by immunoprecipitation and western blot. (D) HBV virions from HepAD38 cells purified by heparin affinity chromatography, size-exclusion chromatography, and sucrose gradient centrifugation were immunogold-labeled for HBsAg and ApoC1 and visualized by transmission electron microscopy. IgG antibodies (Neg. Ctrl) served as specificity controls. Quantification of gold-positive structures is shown below. Scale bar: 50 nm. Unpaired *t* test; $^{**}p < 0.01$ and $^{****}p < 0.0001$. Abbreviations: ApoC1, apolipoprotein C1; CHB, chronic hepatitis B; Ctrl, control; ELISA, enzyme-linked immunosorbent assay; fSVPs, filamentous subviral particles; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IgG, immunoglobulin G; N.d., not detectable; Neg., negative; qPCR, quantitative polymerase chain reaction; SVPs, subviral particles.

reduction of extracellular HBsAg and HBeAg observed at non-cytotoxic concentrations of BLT1 (Supplemental Figure S15, https://links.lww.com/HEP/K558) supported our conclusion on the important role of SR-B1 in HBV entry. Consistently, HDV infection was also reduced by both SR-B1 inhibitors (Figure 7E).

These results suggest that HDL enhanced HBV and HDV infection in an ApoC1-dependent and SR-B1-dependent manner. ApoC1, probably in part via HDL, targets virions to SR-B1 that might act as a cofactor to facilitate infection. This would explain why supplementation of exogenous HDL enhanced HBV infection while pharmacological inhibition of SR-B1 suppressed the infection.

DISCUSSION

Here, we comprehensively determined the first proteome composition of HBV particles, allowing the detection of very low abundance proteins such as the HBV P-protein that is present at approximately one copy per DNA-containing virion.[15] Consistent with earlier studies, the HBx protein was not detected.[30] Regarding host cell proteins, a recent cryo-EM study of pgRNA-containing nucleocapsids revealed an electron-dense region within the capsid interior, proposed to contain entrapped host factors.[31] Consistently, we identified 3 cellular proteins (CASP14, CWF19L2, and HNRNPC) highly enriched in the virion/fSVP preparation derived from HepAD38 cells, but not in fSVPs from HepG2-HB2.7 cells. Whether these proteins play a role in HBV biology remains to be determined. We also detected several chaperones in the virion and fSVP fractions, including HSP90AB1, earlier shown to facilitate HBV polymerase–RNA interactions and assembly of the reverse transcription initiation complex.[32]

Additional chaperones we identified associated with viral envelope proteins included HSC70 and its interaction partner, the cytosolic anchorage determinant binding protein. These proteins inhibit co-translational translocation of the PreS1 domain through the viral envelope, enabling the dual topology of the L protein.[17] Similarly, PPIA has been reported to interact with HBsAg.[11] In our fSVP proteome, we also detected the HSP40 chaperone DNAJB12, an interaction partner of the nucleic acid polymer REP-2139, interfering with assembly and release of HBV SVPs, and currently in clinical trials for HBV and HDV infections.[33] These findings underscore the superior properties of our particle purification method.

We also identified HDL-enriched apolipoproteins, 5 co-purifying with fSVPs and 3 also with virions. HDL mediates cholesterol transport from peripheral tissues to the liver by carrying free cholesterol and cholesteryl esters as part of the reverse cholesterol transport pathway. Recently, macrophages were shown to internalize lipoprotein-associated HBV particles into recycling endosomes enriched in cholesterol, which are subsequently trafficked back to the cell surface to facilitate hepatocyte infection.[12] The co-accumulation of HDL apolipoproteins with cholesterol in the HBV lipid envelope and host endosomal membranes might confer favorable biophysical properties promoting virus entry. Cholesterol influences membrane fusion in several enveloped viruses, including Ebola virus, HIV, Zika virus, and SARS-CoV-2.[34,35] Consistently, cholesterol-depleted HBV virions have reduced particle diameter and lower infectivity, both parameters being restored upon cholesterol replenishment.[21] In our study, HDL supplementation during infection enhanced viral entry, supporting a role for lipoproteins during HBV infection (Figure 7B). Moreover, pharmacological inhibition of cholesterol and cholesteryl ester transport via SR-B1 reduced HBV and HDV infection (Figures 7C–E). While these results suggest that SR-B1 might act as auxiliary HBV receptor, consistent with an earlier study,[36] we note that SR-B1 splice variants localize to distinct subcellular compartments, including plasma membrane and endosomes.[37] This localization may extend the role of SR-B1 beyond its interaction with HBV particles at the cell surface. Moreover, SR-B1 association with lipoproteins is not limited to HDL, as interactions with LDL and VLDL have also been reported.[37,38] Therefore, we cannot exclude the involvement of other lipoproteins in HBV entry, which might explain the earlier report of LDLR being an important host cell factor for HBV infection.[25]

Comparing the 5 different apolipoproteins identified in our proteome, ApoC1 depletion had the most

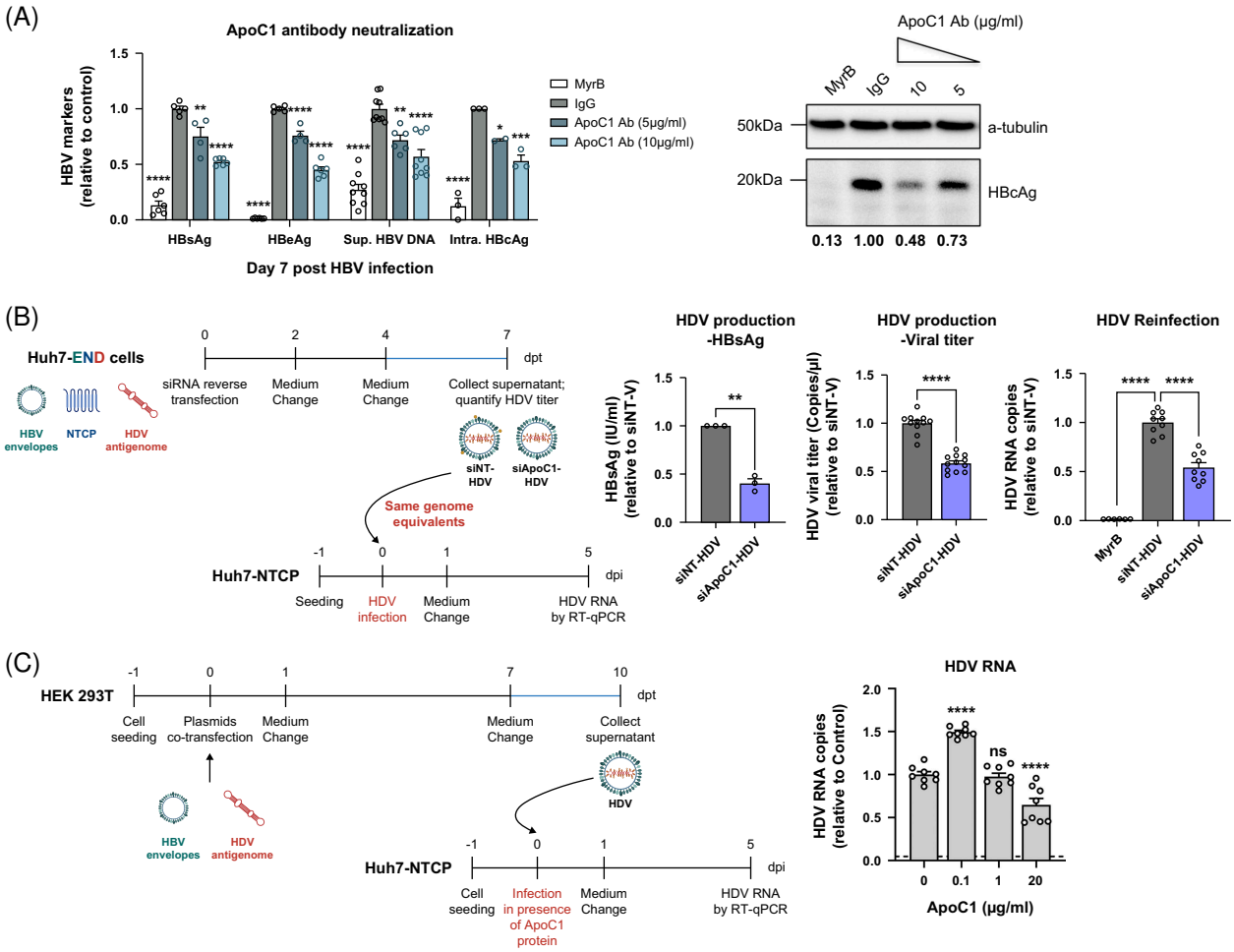

(A)
ApoC1 antibody neutralization
HBV markers (relative to control)
MyrB
IgG
ApoC1 Ab (5µg/ml)
ApoC1 Ab (10µg/ml)
HBsAg
HBeAg
Sup. HBV DNA
Intra. HBcAg
Day 7 post HBV infection
ApoC1 Ab (µg/ml)
MyrB
IgG
10
5
50kDa
a-tubulin
20kDa
HBcAg
0.13 1.00 0.48 0.73
(B)
Huh7-END cells
dpt
siRNA reverse transfection
Medium Change
Medium Change
Collect supernatant; quantify HDV titer
HBV envelopes
NTCP
HDV antigenome
siNT-HDV
siApoC1-HDV
Same genome equivalents
Huh7-NTCP
dpi
Seeding
HDV infection
Medium Change
HDV RNA by RT-qPCR
HDV production -HBsAg
HBsAg (IU/ml) (relative to siNT-V)
HDV production -Viral titer
HDV viral titer (Copies/µl) (relative to siNT-V)
HDV Reinfection
HDV RNA copies (relative to siNT-V)
(C)
HEK 293T
dpt
Cell seeding
Plasmids co-transfection
Medium Change
Medium Change
Collect supernatant
HDV
HBV envelopes
HDV antigenome
Huh7-NTCP
dpi
Cell seeding
Infection in presence of ApoC1 protein
Medium Change
HDV RNA by RT-qPCR
HDV RNA
HDV RNA copies (relative to Control)
ApoC1 (µg/ml)
(D)
MyrB
siNT
siNTCP
siApoC1
MyrBatto
MyrBatto/ Nuclei
Huh7-NTCP
Fluorescent area / cell (relative to NT)
(E)
MyrB
siNT
siNTCP
siApoC1
MyrBatto
MyrBatto/ Nuclei
HepG2-NTCP
Fluorescent area / cell (relative to NT)

FIGURE 6 ApoC1 enhances HBV and HDV infectivity. (A) HBV stocks were pre-incubated with ApoC1 antibody for 1 hour at room temperature before infecting HepG2-NTCP-A3 cells (1500 GE/cell) in the absence of PEG8000 and FBS for 24 hours. Secreted HBsAg, HBeAg, and HBV DNA were quantified by ELISA and qPCR at 7 dpi, respectively. Intracellular HBcAg (right panel) was detected by western blot at 7 dpi and quantified using Image Lab. One out of 3 representative experiments is shown. (B) To obtain HDV depleted for ApoC1, Huh7-END cells (schematic on the left) were transfected with siApoC1 or siNT. HBsAg levels and HDV titers were determined by ELISA and RT-qPCR, respectively. Owing to lower HDV production in ApoC1-depleted cells (upper right panels), Huh7-NTCP target cells were inoculated with the same HDV genome equivalents to evaluate the impact of ApoC1 depletion on HDV infectivity. At 5 dpi, intracellular HDV RNAs were quantified by RT-qPCR (lower right panel). Data represent mean ± SEM from 3 independent experiments. (C) HDV was produced in 293T cells by co-transfection of vectors encoding HBV surface proteins and a vector harboring a 1.1-fold cDNA copy of the HDV antigenome. HDV particles contained in the supernatant were pre-incubated with different concentrations of ApoC1 protein for 1 hour at room temperature before infecting Huh7-NTCP cells. HDV infection of cells was assessed by quantification of intracellular HDV RNAs at 5 dpi, and is displayed relative to untreated control cells. The dashed line indicates HDV RNA amounts in cells treated with MyrB. Data are presented as mean ± SEM from 2 independent experiments. (D) Huh7-NTCP or (E) HepG2-NTCP-A3 cells were reverse-transfected with the given siRNAs for 48 hours and subsequently exposed to 200 nM atto488-labeled MyrB peptide ($MyrB^{atto}$) for 30 minutes at 37 °C. Green and blue signals in the left panels depict $MyrB^{atto}$ and nuclei, respectively. Cells incubated with an excess of unconjugated MyrB peptide served as a nonspecific binding control. Scale bar: 100 μm. Data represent mean ± SEM from 2 independent experiments performed in duplicate. A, B (right panel), C, D, E, one-way ANOVA; B (left and middle panels), unpaired *t* test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and ns—not significant ($p > 0.05$). Abbreviations: ApoC1, apolipoprotein C1; ANOVA, analysis of variance; FBS, fetal bovine serum; GE, genome equivalents; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDV, hepatitis D virus; MyrB, myristoylated preS1 peptide; ns, not significant; NT, non-targeting; NTCP, sodium taurocholate cotransporting polypeptide; qPCR, quantitative polymerase chain reaction; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SEM, standard error of the mean; siApoC1, small interfering RNA targeting apolipoprotein C1; siNT, non-targeting small interfering RNA.

prominent effect on HBV. The impact was even stronger than that of ApoE previously reported to enhance HBV cell surface attachment by binding to the HSPG syndecan-2 and the LDLR.[25,39] Since the bovine genome lacks an ApoC1 gene,[40] we can exclude the possibility that bovine ApoC1 contributes to any of the observed effects. However, ApoC1 released from HepG2-hNTCP cells could potentially associate with virus particles and facilitate infection. This might explain why depletion of ApoC1 in infected cells reduces HBV and HDV infection by limiting ApoC1 association with viral particles, in addition to ApoC1's role in maintaining cellular cholesterol homeostasis.

Attempts to produce ApoC1-depleted HBV particles encountered technical limitations, especially the presence of naked capsids and background signals from plasmid DNA in transfection-based systems, both of which confounded viral DNA quantification. Therefore, we employed HDV, which has the same envelope glycoproteins as HBV, but is a more tractable model for infectivity analyses. The lower infectivity of ApoC1-depleted HDV suggests that virion-associated ApoC1 enhances viral entry (Figure 6B), although we cannot exclude that ApoC1 depletion alters other lipid-related properties of the virus particles, such as their cholesterol content or impaired association with lipoproteins. In contrast, excess ApoC1 may interfere with particle integrity or compete with virion-associated ApoC1 and/or HDL for cell-surface binding, possibly accounting for reduced infection (Figure 6C). Interestingly, a role for ApoC1 in viral membrane fusion was demonstrated for HCV,[41] and a similar mechanism might operate during HBV and HDV infection. However, the lack of a robust HBV fusion assay and the limited efficiency of current in vitro infection systems preclude studying this hypothesis experimentally.

By using immuno-EM, we observed ApoC1 positivity in only ~10% of cell culture-derived highly purified HBV particles. This rather low percentage may underestimate the real ApoC1 association with HBV particles for several reasons. First, neutralization with an ApoC1-specific antibody reduced HBV infection by 30%–50% (Figure 6A), which is more than can be expected if only 10% of the particles were associated with ApoC1. Second, ApoC1 concentrations are substantially higher in human serum than in standard cell culture supernatants, indicating that in vivo, more HBV particles might be associated with ApoC1 and/or HDL (Supplemental Table S4, https://links.lww.com/HEP/K558). Whether this difference accounts for the observed difference in HBV infection efficiency of cell lines in vitro versus animals in vivo,[42,43] remains to be determined. Third, ApoC1 association with HBV particles might be highly dynamic and influenced by the harsh sample preparation and fixation conditions we had to use for the EM-based analysis. Fourth, the concentration of FBS used for virus production may influence the composition of cell culture-derived HBV particles. SVPs isolated from patient serum contain ~15% cholesterol and ~14% cholesteryl esters,[44] whereas HBsAg particles produced under serum-free or low-serum (1% FCS) cell culture conditions have only ~3% cholesterol and ~1.5% cholesteryl ester.[45,46] Since cholesteryl esters are predominantly transported within circulating lipoproteins, the low cholesteryl ester content of cell culture-derived HBsAg particles is consistent with reduced lipoprotein association under in vitro conditions. Consequently, differences in lipoprotein association, including ApoC1, may contribute to the altered composition and reduced infectivity of cell culture-derived HBV particles. Overall, the proteome and lipid composition of cell culture-derived HBV

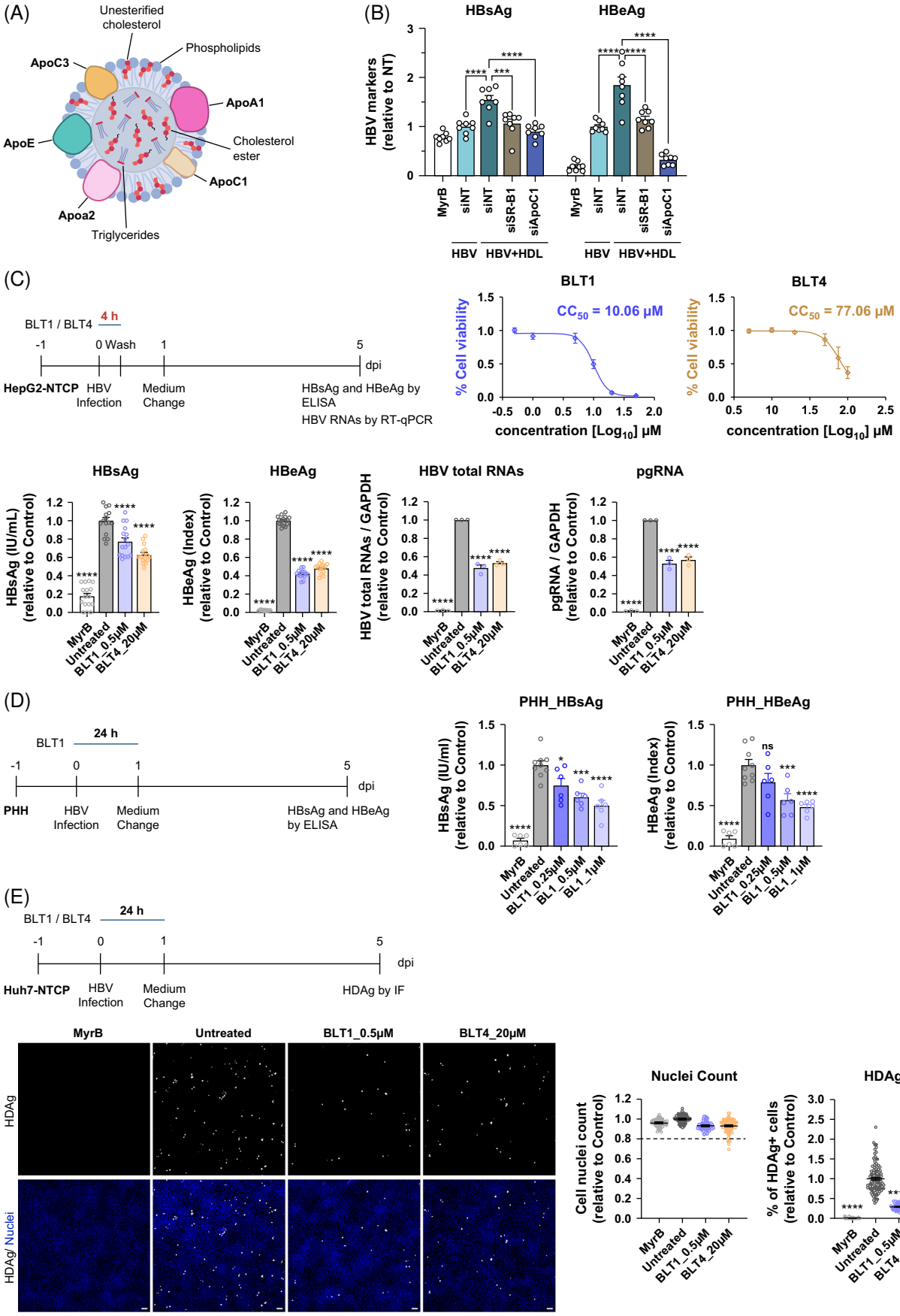

(A)
Unesterified cholesterol
Phospholipids
ApoC3
ApoA1
ApoE
Cholesterol ester
ApoC1
Apoa2
Triglycerides
(B)
HBsAg
HBeAg
HBV markers (relative to NT)
MyrB
siNT
siNT
siSR-B1
siApoC1
HBV
HBV+HDL
(C)
BLT1 / BLT4
4 h
-1
0 Wash
1
5
dpi
HepG2-NTCP
HBV Infection
Medium Change
HBsAg and HBeAg by ELISA
HBV RNAs by RT-qPCR
BLT1
BLT4
% Cell viability
CC50 = 10.06 μM
CC50 = 77.06 μM
concentration [Log10] μM
HBsAg
HBeAg
HBV total RNAs
pgRNA
HBsAg (IU/mL) (relative to Control)
HBeAg (Index) (relative to Control)
HBV total RNAs / GAPDH (relative to Control)
pgRNA / GAPDH (relative to Control)
MyrB
Untreated
BLT1_0.5μM
BLT4_20μM
(D)
BLT1
24 h
PHH
HBV Infection
Medium Change
HBsAg and HBeAg by ELISA
PHH_HBsAg
PHH_HBeAg
HBsAg (IU/ml) (relative to Control)
HBeAg (Index) (relative to Control)
MyrB
Untreated
BLT1_0.25μM
BL1_0.5μM
BL1_1μM
ns
(E)
BLT1 / BLT4
24 h
Huh7-NTCP
HBV Infection
Medium Change
HDAg by IF
MyrB
Untreated
BLT1_0.5μM
BLT4_20μM
HDAg
HDAg/ Nuclei
Nuclei Count
Cell nuclei count (relative to Control)
HDAg+
% of HDAg+ cells (relative to Control)

FIGURE 7 Inhibition of HBV and HDV infection by pharmacological block of SR-B1. (A) Schematic diagram of high-density lipoprotein (HDL). Created in BioRender. (B) HepG2-NTCP-A3 cells were reverse-transfected with siRNAs specified on the bottom for 48 hours and subsequently infected with HBV or with HBV that had been pre-incubated with HDL for 30 minutes at room temperature. The experiments were performed in the absence of 4% PEG8000, and cells were inoculated with 1500 HBV GE/cell. After 24 hours in FBS-free medium, cells were washed, and complete medium was added. Secreted HBsAg and HBeAg were quantified at 7 dpi. (C) HepG2-NTCP-A3 cells or (D) PHHs were treated with SR-B1 inhibitors (BLT1 or BLT4) throughout infection with HBV (1000 GE/cell with 4% PEG8000). Secreted HBsAg, HBeAg, HBV total RNAs, and pgRNA were quantified by ELISA and RT-qPCR at 5 dpi, respectively. (E) Huh7-NTCP cells were treated with BLT1 or BLT4, starting at 4 hours before and throughout infection with HDV (5 GE/cell with 4% PEG8000). After 24 hours, cells were washed and cultured in medium without SR-B1 inhibitors. Intracellular HDAg was assessed by immunofluorescence at 5 dpi. White and blue signals depict HDAg and nuclei, respectively. Quantification of the number of nuclei and HDAg-positive cells is shown. Scale bar: 100 μm. Data are presented as mean ± SEM from 3 independent experiments. B, C, D, E, one-way ANOVA; *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, and ns—not significant ($p > 0.05$). Abbreviations: ANOVA, analysis of variance; FBS, fetal bovine serum; GE, genome equivalents; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HDAg, hepatitis D antigen; HDV, hepatitis D virus; HBV, hepatitis B virus; HDL, high-density lipoprotein; IF, immunofluorescence; ns, not significant; NTCP, sodium taurocholate cotransporting polypeptide; pgRNA, pregenomic RNA; PHHs, primary human hepatocytes; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SEM, standard error of the mean; siRNAs, small interfering RNAs; SR-B1, scavenger receptor class B type 1.

particles might not fully recapitulate those of virions produced in vivo, which is an inherent limitation of cell culture-based HBV proteomic studies. Nevertheless, we also detected association between ApoC1 and HBV virions or SVPs in sera from patients with CHB, indicating that HBV can hijack circulating lipoproteins in vivo possibly to facilitate its transport to the liver.[23] Given these findings, combining current HBV therapies with FDA-approved serum lipid-modulating drugs may represent a novel antiviral strategy.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available within this paper and its Supplemental Information, https://links.lww.com/HEP/K558. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[47] partner repository with the dataset identifier PXD076462. Source data are provided with this paper (Supplemental Tables S1 and S2, https://links.lww.com/HEP/K558).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Shangqing Yang, Firat Nebioglu, Stefan Seitz, and Ralf Bartenschlager. Performed the experiments: Shangqing Yang, Firat Nebioglu, Minh Tu Pham, and Pietro Scaturro. Analyzed and interpreted the data: Shangqing Yang, Firat Nebioglu, Pietro Scaturro, and Ralf Bartenschlager. Contributed reagents/materials/analysis tools: Yen-Chia Lin, Andreas Pichlmair, Pietro Scaturro, Shirin Nkongolo, Stephan Urban, Stefan Seitz, and Ralf Bartenschlager. Drafted the manuscript: Shangqing Yang, Firat Nebioglu, and Ralf Bartenschlager.

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CONFLICTS OF INTEREST

Stephan Urban consults for, owns stock in, and owns intellectual property rights with Gilead. He is a co-inventor and an applicant on patents protecting HBV preS1-derived lipopeptides (Myrcludex B/bulevirtide/Hepcludex). The remaining authors have no conflicts to report.

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