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Short Title: HBV targets the cholesterol transport

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Abbreviations:
Apo: apolipoprotein
BODIPY: dipyrromethene boron difluoride
cccDNA: covalently closed circular DNA
CMFDA: chloromethylfluorescein diacetate
DNA: deoxyribonucleic acid
DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
HBeAg: HBV e antigen
HBsAg: HBV surface antigen
HBV: Hepatitis B virus
HDL: high density lipoprotein
HSPG: heparan sulfate proteoglycan
IDL: intermediate density lipoprotein
LAMP 1: lysosomal-associated membrane protein 1
LDL: low density lipoprotein
LRP: LDL-receptor related protein
LSEC: liver sinusoidal endothelial cells
L-SIGN: liver/lymph node-specific intercellular adhesion molecule-3-grabbing nonintegrin
NBD: nitrobenzoxadiazole
NPC-1: Niemann-Pick C1
NTCP: sodium taurocholate cotransporting polypeptide
Rab 11: Ras-related protein Rab-11A
TG: triglyceride
VLDL: very low density lipoprotein
VP: viral particle

Synopsis (40 words):

A single HBV virion suffices to infect an individual indicating that it hijacks a physiological pathway targeting hepatocytes. HBV associates with lipoproteins to access the perisinusoidal space or to follow the physiological cholesterol transport pathway to hepatocytes via macrophages.

Graphical Abstract: Model for HBV host cell targeting to achieve hepatocyte infection.

ApoE: apolipoprotein E; ApoA1: apolipoprotein A1; EE: early endosome; HSPG: heparan sulfate proteoglycans; KC: liver macrophages (Kupffer cell); LDL-R: LDL-receptor; LE: late endosome; LSEC: liver sinusoidal endothelial cell; Lys: lysosome; NPC1: Niemann Pick Typ C 1 protein; NTCP: sodium taurocholate cotransporting polypeptide; RE: recycling endosome; SC: Stellate cell; TRL: triglyceride-rich lipoprotein remnant

Abstract (260 words)

Background and aims: A single hepatitis B virus (HBV) particle is sufficient to establish chronic infection of the liver after intravenous injection, suggesting that the virus targets hepatocytes via a highly efficient transport pathway. We therefore investigated whether HBV utilizes a physiological liver-directed pathway that supports specific host-cell targeting *in vivo*.

Methods: We established an *ex vivo* system of perfused human liver tissue that recapitulates the liver physiology to investigate HBV liver targeting. This model allowed us to investigate virus-host cell interactions in a cellular microenvironment mimicking the *in vivo* situation.

Results: HBV was rapidly sequestered by liver macrophages within one hour after a virus pulse, but was detected in hepatocytes only after 16 hours. We found that HBV associates with lipoproteins. Electron- and immunofluorescence microscopy corroborated a co-localization in recycling endosomes within peripheral and liver macrophages. Importantly, recycling endosomes accumulated HBV and cholesterol, followed by transport of HBV back to the cell surface along the cholesterol efflux pathway. To reach hepatocytes as final target cells, HBV was able to utilise the hepatocyte-directed cholesterol transport machinery of macrophages.

Conclusions: Our results propose that HBV by binding to liver targeted lipoproteins and utilizing the reverse cholesterol transport pathway of macrophages hijacks the physiological lipid transport pathways to the liver to most efficiently reach its target organ. This may involve trans-infection of liver macrophages and result in deposition of HBV in the perisinusoidal space from where HBV can bind its receptor on hepatocytes.

Keywords

Transcytosis, trans-infection, liver targeting, lipoprotein metabolism

Introduction

Hepatitis B virus (HBV) belongs to the family hepadnaviridae and is a highly infectious and organ specific pathogen. Currently over 290 million humans worldwide are chronically infected with HBV and at high risk to develop liver cirrhosis or hepatocellular carcinoma (www.who.int/mediacentre/factsheets/fs204/en/). The successful dissemination of HBV within the human population relies mainly on vertical transmission that allows an immune escape of the virus or sexual transmission that requires an efficient transmission at a low infection dose. HBV infectivity indeed is extraordinarily high, and during experimental infection in chimpanzees, a single virion was reported to suffice to establish HBV infection[1]. Recently, the sodium taurocholate cotransporting polypeptide (NTCP) expressed on the basolateral membrane i.e. side of hepatocytes facing the liver sinusoid has been identified as a crucial receptor[2]. Following initial attachment of HBV virion to heparan sulfate proteoglycans (HSPG)[3,4], that are typically found in the space of Dissé between liver sinusoidal endothelial cells and hepatocytes, NTCP binds the myristoylated N-terminal part of the large HBV surface protein. How HBV can gain access to the liver and to its receptor on the hepatocyte so efficiently, however, is not understood.

In the liver, hepatocytes are lined and shielded from pathogens and gut-derived toxins by a layer of fenestrated liver sinusoidal endothelial cells (LSEC), on top of which Kupffer cells are located representing the largest population of organ-resident macrophages in the body. For some hepatotropic pathogens there is evidence that sinusoidal liver cells contribute to hepatocyte infection[5]. Hepatitis C virus (HCV) has been shown to be captured by the C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and a liver/lymph node-specific version (L-SIGN)[6], that both are present on LSEC[7]. Cells expressing DC-SIGN and L-SIGN can trans-infect hepatocytes with HCV[8] and LSEC can promote HCV infection in a paracrine fashion[9]. The duck analogue of HBV was reported to be taken up preferentially by LSEC and not by hepatocytes *in vivo*[10] suggesting that LSEC remove duck HBV from the circulation and subsequently deliver it to hepatocytes.

For plasmodium sporozoites, intravital microscopy of mouse liver showed sporozoite transmigration through Kupffer cells and LSEC escaping lysosomal compartments before entering hepatocytes[11,12]. Thus, there is evidence that pathogens may exploit trans-cellular transport processes to target hepatocytes in a similar fashion as molecules like transferrin or ceruloplasmin transporting iron and copper, respectively. We recently showed that HBV infection of hepatocytes is significantly reduced when lipases are inhibited, and found a reduced hepatic virus uptake *in vivo* if apolipoprotein (Apo) E is lacking[13]. These data are in line with the finding that ApoE-specific antibodies inhibit HBV infection[14]. This indicated a transport pathway employed by HBV along with lipoproteins to efficiently target hepatocytes.

The liver plays a key role in lipid metabolism. It takes up and oxidizes triglycerides (TG), converts excess carbohydrates and proteins into fatty acids and TG to provide energy and synthesizes cholesterol, phospholipids and Apo. Furthermore, it secretes cholesterol into bile or converts it to bile salts. TG and cholesterol are hydrophobic neutral lipids that travel within the water-based blood embedded within lipoprotein particles containing Apo. Lipoprotein particles are divided by their density relative to surrounding water into ultra-low density chylomicrons, very low (VLDL), intermediate (IDL), low (LDL) and high (HDL) density lipoproteins.

Ultra-low density chylomicrons transport lipids absorbed from the intestine to adipose, cardiac, and skeletal muscle tissue, where their TG components are hydrolyzed by lipoprotein lipases and released. When most of the TG have been hydrolyzed, the chylomicron remnants become enriched with ApoE and ApoC2, the coenzyme for lipoprotein lipase, and are taken up by hepatocytes where remaining neutral lipids become hydrolyzed by cellular lipases[15]. VLDL are assembled within hepatocytes and released into the blood stream to transport “endogenous” TG, phospholipids and cholesterol. VLDL release TG and become IDL and then LDL with ApoE determining their recycling to the liver. HDL particles remove cholesterol and fatty acids from cells and exchange neutral lipids with VLDL. ApoE is also present in a subfraction of HDL, HDL_E, where it serves as a major factor determining their rapid uptake into the liver[16].

There is strong evidence that in case of postprandial lipoprotein peaks or hyperlipoproteinemia Kupffer cells contribute to hepatic clearance of circulating lipoprotein, either due to higher average lipoprotein size or due to oxidative modification[17]. Once lipoproteins have passed the endothelial cell layer and reach the space of Disse, they are trapped by electrostatic interactions with HSPG. There, hepatic lipase remodels the particles, giving them an optimal size to interact with cell surface receptors and ApoE. The receptors that bind and take up ApoE-containing remnant particles include the LDL receptor, the LDL-receptor related protein (LRP)-1 and HSPG[18]. Additionally, excess cholesterol is transported back to the liver in a process known as reverse cholesterol transport mainly via high density lipoprotein (HDL). From lipoproteins initially taken up by macrophages cholesterol can be effluxed to HDL and transported to the liver. Within macrophages, cholesterol esters of endocytosed lipoproteins become hydrolysed by the endosomal acid lipase before free cholesterol is transported to the cell membrane via a Niemann-Pick C1 (NPC1)-dependent mechanism[15]. Finally, free cholesterol is released from the macrophage and can bind extracellular lipid receptors such as HDL enabling uptake into hepatocytes. Little is known about how much liver macrophages contribute to reverse cholesterol transport to hepatocytes.

Since only humans or primates are productively infected with HBV and small animal models are lacking, we developed a novel infection model system, in which human liver tissue is perfused with labelled viral particles (VP). Using this *ex vivo* liver perfusion model we unexpectedly found that HBV in association with lipoproteins was preferentially taken up by liver macrophages into recycling endosomes and only thereafter accumulated in hepatocytes. Macrophages re-secreted HBV in association with free cholesterol derived from endocytosed lipoproteins. Finally, HBV was taken up by hepatocytes along with macrophage-derived neutral lipids supporting the conclusion that HBV can exploit the macrophage cholesterol transport as one way to reach its host cell, the hepatocyte. We thus hypothesize that HBV uptake into macrophages and subsequent trans-infection of hepatocytes represents an efficient strategy for the pathogen to be sequestered in the liver, to avoid scavenger cells in the circulation or spleen and to target hepatocytes.

Results

HBV primarily associates with liver macrophages prior to infecting hepatocytes

To identify the cell populations in the liver that in a physiological fashion are rapidly associated with HBV, we established an *ex vivo* human liver tissue perfusion model with medium and human serum (**Fig 1A**). Tissue viability was confirmed after 17-hour perfusion by scavenger receptor-mediated uptake of ovalbumin into liver sinusoidal endothelial cells (**Fig 1B**). To visualise early HBV infection events, we used purified viral particles (VP) comprising a mixture of virions and filamentous subviral particles that express all three HBV envelope proteins (S, M and L), and labelled them with Alexa594 fluorochrome (VP_{594}) (**Fig 1C**). Such HBV VP_{594} were added to isolated liver macrophages and uptake of VP was detected and confirmed by HBs staining using polyclonal antibodies. This demonstrated that purification and labelling neither affected HBV envelope protein integrity nor particle uptake (**Fig 1D**). Co-localisation of VP_{594} with HBsAg-specific staining confirmed specificity of VP_{594}-fluorescence labelling. Staining of HBs after *ex vivo* perfusion of human liver tissue with HBV VP indicated uptake into sinusoidal lining cells (**Fig 1E**). Antibody staining specificity was confirmed by absence of staining of HBV-negative liver tissue (**Fig 1F**).

To study the physiological interaction of HBV with liver cells in the presence of serum components the perfusion medium was supplemented with 50% human serum collected during a postprandial phase from a HBV-naïve and non-vaccinated individual. Demonstrating a productive infection of hepatocytes by HBV in this experimental system, we detected HBV cccDNA, the nuclear transcription template of HBV, at three days after the perfusion with HBV particles, and secretion of newly synthesized HBV e antigen (HBeAg) (**Fig 2A**). Strikingly, after a 45 min perfusion of the liver tissue with VP_{594} in medium containing human serum, quantitative immunofluorescence analysis revealed that 97±2 % of CD68-positive liver macrophages but not (0.9±1.3%) CD68-negative cells such as hepatocytes had taken up VP_{594} (**Fig 2B-D**). Similar results were obtained following perfusion of human liver tissue with unlabelled HBV and detection of viral particles by immunofluorescence staining (**Fig 2E**).

Notably, HBV particles were also detected in liver macrophages in tissue sections from HBV-infected patients (**Fig 2F**). Moreover, a localization to macrophages was confirmed *in vivo* when we injected VP or HBV purified from human plasma into mice. Within 45 minutes or 1 h after intravenous injection, respectively, liver macrophages had already accumulated fluorescently labelled VP_{594} and HBV DNA (**Fig 2G-I**). Taken together, these data indicate that HBV rapidly accumulates in macrophages but not hepatocytes thus raising the question whether HBV can escape lysosomal degradation in macrophages.

HBV localizes to recycling endosomes within macrophages

Since macrophages are specialised to clear pathogens from the circulation by lysosomal degradation, we next defined the intracellular location of internalized HBV particles in liver macrophages. To proof endocytosis of infectious HBV by human liver macrophages, isolated liver macrophages were incubated with purified HBV at 4°C or 37°C, respectively, and cellular viral rcDNA content was quantified. A significant increase of HBV rcDNA was detected associated with cells incubated at 37°C illustrating that liver macrophages do not only bind but actively take up infectious HBV (**Fig 3A**).

To determine the exact intracellular localization within liver macrophages, immunostainings and confocal microscopy were performed. Already after one hour, VP_{594} accumulated in an LAMP1-negative but Rab11-positive endosome, indicative of recycling endosomes but not lysosomes (**Fig 3B,C**). Even after 17h, HBV particles remained localized in LAMP1-negative, Rab11-positive recycling endosomes (**Fig 3D,E**). The localization of VP_{594} to recycling endosomes was confirmed using macrophages differentiated from the monocyte-macrophage cell line THP (**Fig 4A,B,D,F**). In striking contrast, recombinant subviral particles containing only HBV S but lacking L and M proteins concentrated selectively in lysosomes (**Fig 4C,E,G**).

These data suggest that viral particles containing the L protein but not subviral particles without escape lysosomal trafficking and degradation within macrophages by targeting vesicles that are involved in recycling of endocytosed molecules to the cell surface.

HBV associates with lipoproteins in cell culture, in blood and in macrophages

We next addressed the question, which transport pathway within macrophages was hijacked by HBV to escape lysosomal degradation in perfused human liver tissue. Ultrastructural analysis found HBV particles accumulated in liver macrophages (**Fig 5A**). These were virions fulfilling all ultrastructural characteristics reported[19] and, to a lesser extend, filamentous subviral particles. No such particles were detected in non-perfused liver tissue. Within the macrophage, HBV was membrane-associated within intracellular, perinuclear compartments or small vesicles. Interestingly, HBV-positive vesicles also contained lipoproteins, which according to their size (70 nm) were most likely ultra-low density chylomicrons or VLDL (**Fig 5A**).

This prompted us to study whether HBV is associated with lipoproteins in sera from HBV-infected patients. Using density gradient ultracentrifugation we detected that HBV partially floated in fractions of very low density. This was corroborated by DNA dot-blot analysis and HBsAg-specific ELISA indicating an association with lipoproteins of ultra-low density (**Fig 5B,C**). Interestingly, this HBV-positive, low-density fraction was not present anymore when serum was filtrated through a 100 nm membrane. HBV-fractions of higher density, in contrast, remained constant confirming an association with lipoproteins of very low density like chylomicrons (**Fig 5D**). Furthermore, HBV started to sediment in a low-density fraction during sucrose gradient ultracentrifugation when incubated with human serum (**Fig 5E**). A mixed ELISA using Apo-B and Apo-E capture antibodies (**Fig 5F**) was able to detect HBV indicating that HBV associates to lipoproteins. Importantly, when we subjected subviral particles to mass spectrometry analysis, we detected a 28-fold enrichment of ApoE (**Fig 5G**) associated to subviral particles. Finally, an association of HBV with lipoproteins was confirmed by electron

297 microscopy after immuno-gold staining (**Fig 5H**). Immuno-gold staining of HBV produced in
298 the hepatoma cell line HepAD38 that expresses endogenously ApoE detected ApoE locating
299 close to, yet distal of the virus particle which is in agreement with HBV association with an
300 ApoE-containing lipoprotein.

301

302 **ApoE enhances HBV infection and HBV localizes to cellular compartments that**
303 **accumulate free cholesterol**

304 We took advantage of a reporter virus that allows sensitive detection and quantification of HBV
305 infection by luciferase expression to address the question whether there is a functional role for
306 the HBV-lipoprotein association during infection (**Fig 6A**). Clearly, the addition of serum to HBV
307 purified by CsCl gradient centrifugation was able to enhance HBV infection of hepatocytes (left
308 panel). This effect was diminished when the serum lacked ApoE, e.g., by using the serum of
309 ApoE-deficient mice (right panel). Moreover, the infection of differentiated HepaRG-cells with
310 HBV was significantly diminished when an LDL-receptor mediated uptake was inhibited using
311 blocking antibodies during, but not after incubation of the cells with HBV (**Fig 6B**). These data
312 support the notion of an important function of HBV binding to lipoproteins for an early step of
313 HBV infection.

314 To study the intracellular transport of HBV particles and its relation to the intracellular recycling
315 pathway of lipoprotein-derived free cholesterol, we incubated liver-derived macrophages with
316 chylomicrons containing fluorescent nitrobenzoxadiazole (NBD)-cholesterol and VP_{594}. Two
317 hours after endocytosis of the lipoproteins - when cholesterol esters were expected to be
318 hydrolyzed - perinuclear vesicles accumulating free cholesterol also contained HBV particles
319 (**Fig 6C**). Strikingly, HBV co-incubated with human serum accumulated in Rab11-positive
320 recycling endosomes (**Figs 3,4**), which is consistent with publications reporting that free
321 cholesterol derived from ApoE-enriched TRL-remnants concentrates in recycling
322 endosomes[20,21]. This indicates that HBV follows the recycling pathway of cholesterol derived
323 from chylomicrons in liver macrophages.

Recycling endosomes containing free cholesterol are intracellularly targeted by high-density lipoprotein (HDL)-derived ApoA1[20]. Since our stainings demonstrated that HBV localised in liver macrophages in compartments concentrating TRL-derived free cholesterol (**Fig 6C**), ApoA1-targeting provides a tool to track HBV particle transport in the macrophage. Indeed, serum-derived HDL, when added to the cell culture medium, localized to vesicles containing HBV particles (**Fig 6D**), and HBV, when cultured in medium containing human serum, co-localised with ApoA1 (**Fig 6E**). In summary, these results demonstrate that HBV particles accumulated in recycling endosomes together with lipoproteins enriched for cholesterol or lipoprotein-derived cholesterol in peripheral as well as liver-derived macrophages. Our data also stress the hypothesis that HBV employs the recycling pathway of lipoprotein-derived free cholesterol in macrophages.

HBV is trans-cytosed through macrophages along free cholesterol transport pathways

To track the intracellular transport of HBV particles and lipoprotein-derived cholesterol in macrophages, we used human monocyte-derived macrophages because they exhibit lower auto-fluorescence than liver macrophages and allow more sensitive detection of specific fluorescence labels. HBV particles co-localized in perinuclear and cytosolic vesicles with filipin, which binds free cholesterol (**Fig 7A**). Treating macrophages with an inhibitor of cellular cholesterol transport (U18666a) resulted in a perinuclear accumulation of cholesterol[22]. In addition, transport of HBV particles from perinuclear compartments to the cell surface was inhibited (**Fig 7B,C**) suggesting that HBV transport requires the free cholesterol transport pathway. Thus, HBV particles not only accumulated in subcellular compartments that concentrate lipoprotein-derived free cholesterol but followed the directional intracellular transport and transcytosis pathways of cholesterol.

Release of endosomal, lipoprotein-derived free cholesterol from macrophages is induced by high density lipoprotein (HDL) and HDL-derived ApoA1[20]. Treating HBV inoculated macrophages with either HDL or ApoA1 increased the secretion of HBsAg and HBV-DNA (**Fig**

7D-F). When macrophages were incubated with HBV and [^{3}H]-cholesterol-labelled chylomicrons, human serum induced their secretion and accordingly reduced their intracellular concentration (**Fig 7G**). These studies confirm that HBV is secreted from macrophages in analogy to cholesterol ready to enter the reverse cholesterol transport pathway to hepatocytes.

Redistribution of HBV particles from CD68+ macrophages to hepatocytes

Only hepatocytes support HBV replication and therefore are the final target cells of the virus as neither macrophages nor dendritic cells support HBV replication[23]. Thus, HBV particles initially taken up by liver macrophages need to remain intact and be exocytosed for subsequent infection of hepatocytes. To test this, human liver tissue was perfused for 45 min with VP_{594} followed by a chase perfusion for 16h to allow for detection of redistribution of HBV particles. While after 45 min HBV particles were confined to CD68+ liver macrophages (**Fig 2B-E**), after the 16h chase, HBV localized within hepatocytes identified by HepPar1-staining (**Fig 8A,B**) with approximately 34±9% of hepatocytes having internalized HBV. At this time point, 50±2% of CD68+ liver macrophages still stained positive for HBV, but with overall fewer particles per cell (**Fig 8C**).

Cholesterol transport directly from macrophages to hepatocytes would provide a directional pathway for HBV to target hepatocytes. To investigate whether this is an option, we visualized intercellular lipid transport by staining neutral lipids including cholesterol esters and free cholesterol with non-polar BODIPY 493/503. Hepatocytes showed a plasma membrane staining along with distinct intracellular, vesicular lipid droplets, while macrophages showed a predominant intracellular staining pattern (**Fig 8D,E**). Of note, hepatocyte plasma membranes in close proximity to macrophages showed a markedly enhanced lipid staining, suggesting membrane lipid exchange between the cells (**Fig 8F**). Additionally, macrophage membrane protein CD68 was detected in hepatocyte cell membranes closely located to liver macrophages, supporting the hypothesis of a direct intercellular membrane component exchanges (**Fig 8G**). In hepatocytes, HBV was exclusively detected at plasma membranes

with enhanced lipid staining (**Fig 8D**), or within lipid containing structures proceeding from the plasma membrane into the interior of the hepatocyte (**Fig 8E**) supporting our model of reverse transport of HBV from macrophages to hepatocytes.

HBV particles taken up by liver macrophages trans-infect hepatocytes

To characterize whether liver macrophages promote HBV-infection of hepatocytes in trans, we set up co-cultures of primary human liver macrophages and hepatocytes isolated from the same donor. Liver macrophages were loaded with VP_{594} and any unbound virus removed by intensive washing before co-culturing with chloromethylfluorescein diacetate (CMFDA) labelled hepatocytes. After an overnight co-culture, HBV was detected within CMFDA+ hepatocytes (**Fig 9A**). Of note, we observed filamentous intercellular connections (**Fig 9B, arrow**), and an exchange of DiI-labeled membrane patches between macrophages and hepatocytes (**Fig 9C**).

To evaluate whether macrophages can transfer infectious HBV to hepatocytes, liver macrophages were incubated with HBV at either 4°C or 37°C for 6h, washed, trypsinized and incubated with primary human hepatocytes at 37°C for 12 days (**Fig 9D**). Only incubation at 37°C that allowed virus uptake into macrophages resulted in HBV infection of hepatocytes, establishment of cccDNA (**Fig 9E**) and secretion of HBeAg and HBsAg (**Fig 9F**) while macrophage incubation at 4°C that did not allow for virus uptake did not result in HBV infection of hepatocytes (**Fig 9E,F**). Importantly, liver macrophages themselves were not productively infected (**Fig 9G**). Trans-infection was blocked by the entry-inhibitor Bulevirtide and neutralizing anti-HBs antibodies (**Fig 9H**) illustrating that trans-infection is also dependent on the bona fide HBV receptor. In addition, trans-infection was significantly reduced upon U18666-mediated inhibition of cholesterol efflux from macrophages (**Fig 9I**). However, trans-infection of hepatocytes with HBV via liver macrophages was not more efficient than direct infection of hepatocytes, suggesting trans-infection via liver macrophages is not necessarily a preferential but rather an alternative infection route, at least *in vitro* (**Fig 9J**). Taken together,

405 our findings show that macrophages can internalize HBV, form intercellular membrane bridges
406 that could potentially promote cell-to-cell transmission of HBV and trans-infect hepatocytes via
407 a cholesterol efflux-dependent pathway.

Discussion

HBV infection relies on very efficient targeting of permissive hepatocytes since one single virus is able to establish infection in a chimpanzee[1]. It remained unclear how HBV is able to reach the liver without being phagocytosed in the circulation, how it bypasses liver scavenger cells and how it is able to selectively target hepatocytes for infection. Here, we characterize these early steps of HBV uptake into the liver under physiological conditions using a novel liver perfusion and infection model that allowed us to track HBV in liver tissue. Interestingly, HBV was rapidly taken up into liver macrophages and only at a later time point detected in hepatocytes. We found that HBV associates with human serum lipoproteins and ApoE. Within macrophages, HBV localized to the same endosomal compartments as lipoproteins before being released along the cholesterol efflux pathway. HBV released after trans-infection of macrophages was able to infect hepatocytes. Since lipoprotein and reverse cholesterol transport to the liver is an efficient physiological process, we hypothesise that HBV exploits this pathway to reach the liver and infect hepatocytes.

Accordingly, we propose the following model for HBV host cell targeting. HBV - after association with ApoE-rich lipoproteins - attaches to liver-specific HSPG that protrude from the Space of Disse across endothelial fenestration into the liver sinus[24]. Subsequently, HBV may be taken up directly into hepatocytes or first into liver macrophages. The ratio of direct host cell targeting vs. trans-infection is likely to be determined by individual lipidemic[17] and/or microstructural liver sinus[25] conditions. HBV sequestered by liver macrophages, hijacks the intracellular lipoprotein metabolism and is re-secreted along the free cholesterol-recycling pathway. Free cholesterol and HBV are then delivered from the macrophages via intercellular membrane bridges to neighbouring hepatocyte plasma membranes where interaction with the HBV receptor NTCP can prime a productive infection. Thus, the hepatotropism of HBV as well its ability to survive within the circulation may depend on the utilization of cellular lipid transport pathways that are hepatocyte-directed.

The bile-acid transporter peptide NTCP has recently been identified as a HBV receptor that is exclusively expressed on hepatocytes[2]. How HBV gains access to hepatocellular NTCP *in vivo* is not understood since endothelial cells lining the sinusoids restrict passive diffusion of particles >12 nm in diameter[25]. Since HBV is an enveloped virus with a diameter of 42 nm, it seems unlikely that HBV can passively diffuse through this layer of sinusoidal cells with the efficiency needed to promote the high *in vivo* particle infectivity that has been reported[1]. Moreover, one has to consider the scavenging activity of LSECs and liver macrophages that could easily trap and lyse HBV. A directed transport exploiting a transcytosis pathway through macrophages as we have observed would be able to overcome these hurdles. Preferential uptake of HBV by liver macrophages - as we observed *in vivo* in mice - may result from initial binding to liver specific HSPG[3,4] extending into the liver sinus. Binding to these HSPG has been observed e.g. for plasmodium sporozoites[24].

Since HBV does not require distinct glycosaminoglycans found in the liver to confer hepatocyte specificity[3], the association with serum components like lipoprotein remnants may increase targeting to hepatic glycosaminoglycans and other lipoprotein receptors. This reasoning is supported by our and others recent findings that liver uptake is significantly reduced in ApoE-deficient mice and that HBV infection is inhibited by ApoE-specific antibodies *in vitro*, respectively[13,14], as well as by reports that HBV binds to ApoH and lipoprotein lipase[26,27], both surface components of lipoproteins. In our study we demonstrate that HBV associates with lipoproteins in patients' serum and when release from producer cells, and that blocking LDL-receptor function on hepatocytes markedly reduces productive infection. Importantly, efficient hepatocyte infection relied on the presence of ApoE supporting the crucial role of an association of HBV to ApoE-containing lipoproteins.

Electron microscopy analysis after immunogold staining strongly supported the association of HBV with ApoE-containing lipoproteins. Interestingly, ApoE was detected distal of the virus particle in accordance with an association of a lipid particle smaller than 100 nm in diameter, presumably an ApoE-containing HDL or a small VLDL/LDL. An exact quantification of the HBV-

lipoprotein association, however, is difficult since the purification of HBV requires ultracentrifugation that is known to dissociate proteins from lipoproteins[28]. As the virus-lipoprotein association rather is of lower affinity, virions may have dissociated during purification resulting in an underestimation of the association of HBV with lipoproteins of low density in our experiments.

In addition to mediating liver-tropism, lipoprotein embedded cholesterol may facilitate HBV recycling within macrophages as described for lipoprotein-associated proteins like ApoC, ApoE and lipoprotein lipase[21]. Lipoproteins bind to LDL-receptors on hepatocytes via ApoE, that has allelic variants ApoE1 to 4. ApoE3 more potently binds the LDL-receptor and induces cellular cholesterol recycling than its allelic variants[29]. Interestingly, the ApoE3 allele is overrepresented in HBV-infected patients and subjects with an ApoE3 allele have a lower rate of spontaneous HBsAg clearance[30,31]. Thus, clinical observations support an important role of lipoprotein metabolism and transport to the liver in early HBV infection. Finally, association of HBV infection with lipoprotein-metabolism is further supported by inhibition of an early infection step by lipase inhibitor Orlistat[13].

The contribution of macrophages to reverse cholesterol transport represents a physiological pathway, by which cholesterol derived from circulating lipoproteins is endocytosed into recycling endosomes in macrophages, effluxed to lipid acceptors and delivered to hepatocytes, especially under hyperlipidemic conditions and in the postprandial situation[15,17]. Lipids effluxed from liver macrophages are incorporated into hepatocytes *in vivo*[17]. The N-terminal acyl residue of the HBV L protein may mediate transport along the free cholesterol transport pathway as described for influenza hemagglutinin[32] and other acylated proteins including glycosylphosphatidylinositol anchored proteins.

Liver resident macrophages are located in close proximity to hepatocytes that are specialised in cholesterol uptake. Intercellular membrane bridges between neighbouring donor and acceptor cells have been suggested to support lipid transport[33]. We observed intercellular membrane bridges between liver resident macrophages or Kupffer cells and hepatocytes and

the transfer of lipids and membrane proteins from Kupffer cell to hepatocyte membranes suggest a direct exchange of membrane components between neighbouring cells. This principle is exploited by prions, which - as HBV - recycle along the free cholesterol pathway to the plasma membrane and are directly transferred to neighbouring cells via membrane bridges[34,35]. Thus, routing along the recycling cholesterol transport pathway in liver macrophages and transcellular delivery to hepatocytes through intercellular membrane bridges appear to act in concert to facilitate hepatocyte-targeting by HBV.

In conclusion, it seems that as for other viruses, specific host cell targeting by HBV does not only result from a particular virus-receptor interaction on the target cell. Our results propose that HBV *in vivo* associates with lipoproteins, may take advantage of their liver targeting and is able to hijack the cholesterol transport pathway through macrophages. These pathways have been evolutionary optimized to deliver their cargo from distant sites to the liver and may explain the highly efficient liver targeting by HBV. They are suited to support the deposition of HBV in the persinusoidal Space of Disse from where HBV can bind its receptor on hepatocytes or even be directly transport to hepatocytes by the establishment of membrane bridges.

Materials and Methods

All authors had access to the study data and had reviewed and approved the final manuscript. For additional details on materials, providers and procedures see Supplemental Information.

Ex vivo human liver perfusion

HBV viral particles were purified by sequential sucrose and CsCl gradient sedimentations and fluorescence labelled with Alexa Fluor 594® (VP_{594}). Human liver tissue samples and annotated data were obtained and experimental procedures were performed within the framework of the non-profit foundation HTCR, including the informed patient's consent. Surgical human liver biopsies (10 to 20 g) consisting of healthy tissue obtained during metastasis resection were perfused within 2 hours with medium containing HBV, VP_{594} and

515 human serum as indicated via portal vein branches at 37°C. To potentially increase HBV-TRL-
516 associations, postprandial but not preprandial serum was used. For longer perfusions, 12%
517 erythrocytes were added and oxygen was supplied.

518 **Virus uptake into liver cells**

519 Primary human hepatocytes and liver macrophages were isolated by two-step collagenase
520 perfusion followed by differential centrifugation and adherence steps and maintained as
521 described[36]. Human serum negative for anti-HBs antibodies and monocytes for differentiation
522 into macrophages were obtained from healthy volunteers.

523 Liver macrophages were incubated with HBV at a multiplicity of infection (MOI) of 100
524 enveloped, DNA-containing HBV particles, washed five times, trypsinized at 37°C for 15
525 minutes, centrifugated for 5 min at 300 g, adhered to the culture surface for 1h, three times
526 washed and subsequently hepatocytes were seeded on top. Re-secretion and (trans-)infection
527 were monitored by determining HBsAg, HBeAg and HBV-DNA in cell culture supernatant. HBV
528 cccDNA was determined in infected hepatocyte lysates by quantitative RT-PCR[23]. HBV RNA
529 was isolated using the Monarch Total RNA Miniprep Kit according to the manufacturer's
530 protocol but with 1h of DNAse digestion. cDNA was synthesized with LunaScript RT SuperMix
531 Kit (with a denaturation step of the RNA for 5 min 95°C, incubation on ice, 50 min 50°C, 5 min
532 95°C) (both New England Biolabs, Ipswich, MA, USA). For qPCR, primers/probes from Life
533 Technologies (Thermo Fisher, Frankfurt, Germany) were used.

534 To analyse the functional contribution of lipoproteins, recombinant (r)HBV expressing
535 NanoLuc-Luciferase (rHBV-Luc) was produced as previously described[37] that allows for a
536 sensitive and quantitative infection. To remove supernatant- and particle-associated proteins,
537 recombinant rHBV-Luc was highly purified via heparin-affinity chromatography and
538 subsequent sucrose- and CsCl-gradient ultracentrifugation. rHBV-Luc particles were
539 concentrated and buffer exchanged in PBS, and cells were infected with the highly purified
540 rHBV in the presence or absence of 10% FCS, or serum obtained from CH57B6/N wild-type
541 or ApoE-knockout mice. To determine infection efficacy, 10 µL cell culture supernatant were

mixed with 100 µL PBS containing 0.1% Tween 20 and 1 µM Coelenterazine N as described[38]. Luminescence was detected using a Tecan Infinite 200 reader (Tecan Group Männedorf, Switzerland).

Isolation of mouse hepatocytes and liver macrophages

Mouse livers were perfused by a standard two-step collagenase perfusion through the portal vein *in situ* before the liver was collected and the tissue was homogenized and passed through a cell strainer. The cell suspension was incubated with magnetic CD11b-specific MicroBeads and loaded onto a MACS column (both Miltenyi Biotec, Bergisch Gladbach, Germany) to collect macrophages according to the manufactures protocol. The remaining cell solution was centrifuged twice for 5 minutes at 50 g without break to collect hepatocytes.

Re-secretion from macrophages

THP macrophages were differentiated with 70 nM phorbol-12-myristate-13-acetate and incubated with $1x10^8$ HBV virions / ml (final concentration) for 3 hours and subsequently without HBV, but adding 10% human serum, 100 or 200 µg/ml HDL as indicated. To determine cholesterol secretion, 1 µg/ml [3H]-cholesterol-chylomicrons were added to the HBV incubation, and heparin was added to the washing. [3H]-cholesterol was quantified in supernatant and cell lysates by liquid scintillation counting.

Electron microscopy of liver tissue

10 µm cryosections of liver tissue or PFA-fixed cells were permeabilized with PBS / 0.5% saponin and stained with appropriate primary and indicated secondary antibodies. To stain neutral lipids BODIPY493/503 (1/250), to stain free cholesterol 50 µg/ml filipin was used. All images were acquired on an Olympus FluoView 1000-confocal laser-scanning microscope. For transmission electron microscopy, liver tissue was fixed with 2.5% glutaraldehyde in 0.1 mol/l cacodylate, post-fixed with 1% OsO4 and stained with uranyl acetate followed by lead citrate. Ultra-thin section were analyzed on a Zeiss EM 10 CR electron microscope (Zeiss, Oberkochen, Germany).

568 **Immuno-gold staining and electron microscopy of HBV virions**

569 Sucrose fractions containing HepAD38 cell-derived HBV virions were diluted with TN buffer
570 (20 mM Tris-HCL pH 7.4 and 140 mM NaCl) and fixed with 2% PFA for 4 hours at 40°C.
571 Residual sucrose and PFA were removed by using Amicon Ultra (30k) centrifugal filter units
572 by diluting and washing concentrated samples with TN buffer. Freshly glow-discharged
573 carbon- and pioloform-coated 300-mesh copper grids (Science Services GmbH, Munich,
574 Germany) were floated on top of 10 µl of different dilutions of sucrose-free HBV particles for 5
575 minutes. After removal of the sample by soaking into Whatman paper, the grid was briefly
576 washed by floating on a droplet of 3% uranylacetate (UA) solution, followed by another floating
577 on a fresh 3% UA droplet for 2 minutes. The stain was removed by soaking into Whatman
578 paper and the grid was air-dried at room temperature before staining and imaging with a JEOL
579 JEM-1400 transmission electron microscope (Jeol GmbH, Freising, Germany).

580 Quality of the HBV virion-containing samples was assessed by TEM analysis of negatively
581 stained samples. Immuno-gold labeling that was performed as described elsewhere[39]. In brief,
582 samples absorbed onto glow-discharged grids were washed with H_2O, incubated for 20
583 minutes in blocking buffer (0.8% BSA, 0.1% fish skin gelatin, 50 mM glycine in PBS) and
584 incubated with primary antibodies for 20 minutes in blocking buffer, followed by 5-times
585 washing of the grids with PBS before incubation of the grids with Protein A-10 nm gold
586 conjugate (Cell Microscopy Core, NL) in blocking solution for 20 minutes. The antibodies used
587 for immuno-gold labeling were anti-ApoE (Sigma-Aldrich - AB947), anti-HBsAg (Humabs
588 Biomed – HBC34) and goat-IgG isotype control (Santa Cruz – Sc2028). Thereafter, the grids
589 were washed again with PBS and fixed with 1% glutaraldehyde in PBS for 5 minutes.

590 **Mass spectrometry of purified HBV subviral particle fractions**

591 Subviral particles were produced in HepG2 cells stably transfected with a plasmid encoding
592 HBV genome 2415-1839 expressing all three HBV surface proteins (HepG2-LMS). Particles
593 were purified as previously described via heparin-affinity chromatography and sucrose
594 gradient ultracentrifugation[43]. Fractions containing the subviral particles were collected,

595 concentrated and buffer exchanged to PBS using VivaSpin 6 filter units (100 000 MWCO,
596 Sartorius) and analyzed via liquid chromatography/mass spectrometry (LC/MS) using an
597 Orbitrap Eclipse Tribrid mass spectrometer (Thermo Fisher). Supernatant of HepG2 cells not
598 expressing HBV proteins was purified as control. A HepG2-LMS and HepG2 control sample
599 were denatured with urea lysis buffer (8 M Urea, 50 mM Tris-HCl pH 7.5) followed by reduction
600 with 10 mM DTT for 45 min at 30°C and alkylation of cysteines with 55 mM CAA for 30 min at
601 room temperature. Samples were diluted 1:5 with digestion buffer (Tris-HCl pH 8.5, 2 mM
602 CaCl2) and digested with trypsin at 1:50 enzyme-to-protein ratio at 37°C overnight. Samples
603 were acidified with 1% formic acid (FA) followed by desalting on self-packed StageTips.
604 Peptides were eluted with 50% ACN, 0.1 % FA and dried down prior to LC-MS/MS analysis.
605 LC-MS/MS measurements were performed on a Dionex Ultimate 3000 RSLCnano system
606 coupled to an Orbitrap Eclipse mass spectrometer (Thermofisher Scientific, Bremen). Peptides
607 were reconstituted in 0.1% FA and measured in data dependent acquisition mode with a 50
608 min linear LC gradient. Peptide and protein identification and quantification was performed with
609 MaxQuant software (v. 1.6.3.3) with standard settings unless otherwise described[44]. Raw files
610 were searched against the human and bovine reference proteome (UP000005640, download
611 01/2021; UP000009136, download 04/2022), a customized HBV sequence fasta file and
612 common contaminants. Carbamidomethylated cysteine was set as fixed modification and
613 oxidation of methionine, and N-terminal protein acetylation as variable modifications. Trypsin/P
614 was specified as the proteolytic enzyme with up to two missed cleavage sites allowed. Results
615 were adjusted to 1% peptide spectrum match and 1% protein false discovery rate (FDR).
616 Displayed protein abundances are based on log2 transformed median centered iBAQ
617 intensities.

618 **Statistical analysis**

619 Data are given as mean ± SD unless otherwise indicated. Statistical analysis was performed
620 using unpaired student`s t-test or by Two-Way ANOVA when more than two groups were
621 compared.

622 **Confirmatory statement**

623 All authors had access to the study data. Additionally, the final manuscript has been reviewed
624 and approved by all authors.

625

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633

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730

Figure Legends

Fig 1: Establishment of an *ex vivo* perfusion model of human liver tissue.
(**A**) Schematic depiction of the *ex vivo* perfusion system. Inside a cell culture incubator, liver tissue was kept in a sterile container filled with medium containing human serum. Using a perfusion pump, the tissue piece was continuously perfused via branches of the portal vein. (**B**) Liver tissue was perfused for 17 h with 50% human serum containing medium. For 30 min, 20 µg/ml Alexa Fluor®647-labelled ovalbumin was added to assess receptor mediated endocytosis and confirm intact liver cell function. Fixed liver tissue sections were analysed by confocal laser scanning microscopy. (**C**) Purified HBV particles were subjected to 10% SDS-PAGE before (left) or after Alexa594 fluorochrome-labeling (VP_{594}, right). Gels were either silver stained (left) or investigated for fluorescence (λex./λem. = 365/602, right). Arrows indicate large (L), middle (M) and small (S) envelop proteins at expected molecular weight (kDa). (**D**) Isolated liver macrophages were incubated with medium containing VP594 for 1h before cells were washed and fixed. Cells were stained with HBsAg-specific antibodies and Alexa488-labelled secondary antibodies. Merged pictures show VP_{594} in red and HBsAg in green with arrows pointing at co-localising VP_{594} with HBsAg-specific staining. (**E**) After 45 min perfusion with unlabelled HBV VP liver tissue sections were stained with polyclonal rabbit anti-HBs and fluorescent secondary antibodies (left panel). As control, perfused tissue was stained with rabbit control serum (right panel) to confirm specificity of HBsAg-detection. (**F**) Liver tissue of an HCV-infected, but HBV-negative patient was fixed and stained with HBsAg- and CD68-specific primary and fluorescent secondary antibodies showing no HBsAg-detection in HBV-negative tissue. Scale bar is 10 µm in (**B, D-F**).

Fig 2: Uptake of HBV particles by liver macrophages in *in vivo* perfused human livers.
(A) Human liver tissue pieces were perfused at 37°C with medium containing $5x10^9$ HBV virions/ml and 50% human serum for 17h and subsequently with medium only for 58h. Before and at the end of perfusion, tissue sections were lysed and DNA was extracted. HBV cccDNA content was determined by a selective real time PCR and is given relative to cellular PRNP gene expression, each dot represents a randomly taken liver tissue sample (left panel); perfusate was analysed for HBeAg content by ELISA (mean ± SD of OD_{450}, duplicates), a separate piece of liver tissue from the same donor perfused with HBV-negative medium served as negative control (right panel). (**B-F**) Human liver tissue pieces were perfused at 37°C for 45 min with medium containing 50% human serum and 10^{10} VP_{594}/ml (shown in red), and fixed tissue sections were stained for macrophage marker CD68 (shown in green). (**B**) Differential interference contrast (DIC) showing liver sinusoids with hepatocytes next to CD68-stained liver macrophages. (**C**) Quantification of $CD68^+$ or $CD68^-$, HBV+ cells. Analysis of ten random vision fields (200-fold magnification; mean values ± SD). (**D**) XY- and Z-axis projection at higher magnification to visualize intracellular localization of VP_{594} in macrophages (y-z), cell nucleus stained with DAPI. (**E**) Immunofluorescence staining for CD68 and HBsAg in fixed tissue sections perfused with non-labelled HBV. (**F**) Immunostaining of liver tissue from a chronically infected HBV patient for HBsAg (shown in red) and CD68 (shown in green). Lower row shows the boxed area enlarged. (**G-I**) CH57Bl/6 mice were intravenously injected through the tail vein with (**G,H**) $2x10^{11}$ FITC (VP_{FITC}) labelled VP or **(I)** $1x10^9$ virions purified from HBV-positive plasma after filtration through a 100 nm pore size filter. (**G**) After 45 min mouse livers were fixed, liver sections were stained with macrophage maker F4/80 and analysed by confocal

laser-scanning microscopy. Y-axis (y-z) and z-axis projections (x-z) are shown. Merged pictures show VP_{FITC} in green. (**H**) Numbers of VP_{FITC}-positive, F4/80+/– cells (ten randomly selected vision fields at 200-fold magnification). (**I**) 1h after injection, liver macrophages and hepatocytes were isolated. Cellular DNA was prepared and analysed by quantitative RT-PCR for HBV-DNA relative to NID2 as house-keeping gene. Values are mean ± SEM of two independent experiments, with triplicates analyzed per experiment. **p=0,0012. All scale bars are 10 µm.

Fig 3: HBV cellular localisation in human primary liver macrophages.
(A) Primary human liver macrophages were incubated with purified HBV for 4 h either at 37°C or 4°C, and after washing were lysed and HBV DNA was quantified by qPCR. Means±SD of one representative experiment with three independent replicates in each group are shown. *p<0.5. (**B-E**) Liver macrophages were incubated with VP_{594} in medium containing 10% human serum for 1h, thoroughly washed and either immediately fixed **(B,C)** or further cultivated for 16h in absence of HBV to follow intracellular transport of HBV (**D, E**). After fixation cells were stained for LAMP1 (**B** and **D**) or Rab11 (**C** and **E**). Merged pictures show VP_{594} in red, CD 68, LAMP1, and Rab11 staining in green, and nuclear staining with DAPI in blue. Representative images of single liver macrophages are shown, scale bars are 10 µm.

Fig 4: Differential localization of HBV and recombinant HBsAg in THP macrophages.
THP macrophages were incubated with medium containing fluorochrome-labelled HBV (VP_{594}; **A, B, D, F**) or recombinant HBsAg ($HBsAg_{594}$; **C, E, G**) for 1h. Cells were either directly fixed after washing (**A**) or further cultivated without HBV for 16h (**B, C**) and stained for Rab11. (**D-G**) Cells were either directly fixed after washing (**D, E**) or after further cultivation without HBV or HBsAg for 16 h (**F, G**) and stained for LAMP1. Merged pictures show VP_{594} and $HBsAg_{594}$ in red, Rab11 and LAMP1 in green, and nuclear staining with DAPI in blue. Scale bar 10 µm. Co-localization determined in three randomly selected vision fields of each group was analyzed using Manders' correlation method. Mred (fraction of red overlapping with green) was calculated using JACoP plugin of ImageJ. The means±SD of four random views of one representative experiment are shown as bar graphs next to the respective image. ***p<0.001, ****p<0.0001.

Fig 5: Association of HBV with lipoproteins. (**A**) Human liver tissue was perfused for 45 min at 37°C with medium containing human serum and HBV (perfused tissue) or directly fixed without perfusion with HBV (non-perfused tissue) as negative control. Transmission electron microscopy shows liver macrophages (KC) next to a hepatocyte (Hep) in HBV perfused and non-perfused liver tissue (left panels), areas marked in red are shown at higher magnification (right panels). Exemplary sections are shown, demonstrating co-localization of triglyceride-rich lipoprotein (TRL) and HBV particles in perfused livers (white arrows) and an example of lipoproteins in a non-perfused liver (dark arrows). Scale bars indicate 1µm (left panels) and 100nm (right panels) LD: lipid droplets; Ery: erythrocytes. (**B-D**) The plasma sample of an HBV infected patient was separated by sucrose density gradient ultracentrifugation either directly (**B,C**) or after filtration through a membrane of 100 nm pore size (**D**). Indicated fractions (Fr.) were analysed for HBV-DNA by dot blot-analysis **(B,D)** and for HBsAg content by ELISA **(C)**. **(B,D)** Volumes dotted are indicated above, HBV-DNA-content and density of each fraction are given below the dot blots. **(C)** HBsAg content of ultra-low density fractions 25 and 26 from non-

filtered plasma is shown. Fraction 26 proved positive for HBV-DNA and HBsAg. (**E**) HBV virions contained in high density fraction 3 were further purified by CsCl-density centrifugation. After buffer-exchange to PBS, HBV virions were incubated for 30 minutes at 37°C with or without human serum (10% v/v; anti-HBs-negative) and subjected to sucrose density gradient centrifugation. HBV-DNA was detected in fractions of low density (indicated by arrows) after incubation with serum (lower row). (**F**) Mixed ELISA of human plasma containing HBV (left panel: $3x10^5$ - $3x10^7$ virions; right panel: $3x10^5$ virions) using anti-ApoB (left panel) or anti-ApoE capture (right panel) and anti-HBs detection antibodies. (**G**) Proteins detected by mass spectrometry of supernatant-purified HBV subviral particles (HepG2-LMS) or control fractions show association of ApoE and HBsAg (red cross). (**H**) HBV particles purified by sucrose density ultracentrifugation from stable producer cells HepAD38 were analysed by immune electron microscopy with gold-labelled anti-ApoE- (upper panel) and anti-HBsAg-specific antibodies (picture below). Pictures show distant localisation of ApoE and proximal localisation of HBsAg. Scale bars indicate 50 nm.

Figure 6: Functional role of the association of HBV with lipoproteins

(**A**) HepG2-NTCP cells were infected with high pure luciferase expressing recombinant HBV in the presence or absence of FCS (left) or in the presence of serum from ApoE KO or control mice (right). On day seven cell culture supernatants were analysed for luminescence intensities. (**B)** Differentiated HepaRG-cells were incubated with an LDL-receptor blocking antibody (anti-LDL-R) either before or before and during HBV infection or four days after infection. On day 12 post HBV infection HBeAg was determined in the cell supernatant by ELISA and cccDNA was quantified in cell lysates by quantitative RT-PCR. (**C-E**) Isolated liver macrophages were incubated for 1h with VP_{594} in medium containing 10% human serum and washed. In (**C**) 5 µg/ml chylomicron NBD-cholesterol was added for 2 hours, in (**D**) 2 µg/ml HDL_{488} was added for 1h before fixation. (**E**) Cells were fixed and stained for ApoA1. Merged pictures show VP_{594} in red, NBD-cholesterol, HDL_{488}, and ApoA1 staining in green, and nuclear staining with DAPI in blue. Scale bars indicate 1 µM **p<0.01; ***p<0.001; ****p<0.0001.

Fig 7: Recycling of HBV along intracellular transport pathways of free cholesterol.

(**A-C**) Monocyte derived macrophages were loaded for 16h with cholesterol using medium containing 50 µg/ml acLDL, incubated with VP_{594} containing medium for 1h and further incubated for 4h with acLDL containing medium without VP_{594}. After fixation cells were stained with filipin to detect free cholesterol. (**A**) Total cellular (total) and perinuclear (pn) areas of a representative cell are indicated by dashed lines. The boxed area is exemplarily shown enlarged below the main panels separately for each channel. Merged pictures show VP_{594} in red and filipin staining in green. Scale bar is 10 µm. (**B, C**) 5 µM U18666A was added to the culture medium as indicated. (**B**) Representative vision fields of several macrophages. (**C**) Relative fluorescence intensity (I) of filipin- and Alexa 594® in the cell periphery were calculated by subtracting pn from total signals and multiplying these with the corresponding area to quantify the content of free cholesterol and VP_{594}. The diagram gives the ratio of pn to peripheral cholesterol (Filipin, dots) and VP_{594} (triangles) in analysed cells. Scale bar is 10 µm (A) or 100 µm (B). (**D-E**) THP macrophages were incubated with HBV in serum free medium for 3h, washed and further cultured for 16h with 0,2% BSA containing medium only or medium supplemented with 100 or 200 µg/ml HDL as indicated. (**D**) Supernatants of macrophages were collected and analysed for HBsAg or (**E**) HBV-DNA content. (**F**) THP macrophages were incubated with HBV in 0,2% BSA containing medium for 3h, washed and further cultured for

16h with 0,2% BSA containing medium only or supplemented with 25 µg/ml ApoA1. (**G**) THP cells were incubated with HBV and 1 µg/ml [3H]-cholesterol-chylomicrons in serum free medium (w/o) or medium containing 10% human serum. Cells incubated with neither HBV nor [3H]-cholesterol-chylomicrons were used as negative control (–). In cell lysates and supernatant, HBV-DNA was quantified by real time PCR, and [3H]-cholesterol content was determined by liquid scintillation counting. Mean values ± SD of four independent experiments are given.

Fig 8: HBV transport from liver macrophages into hepatocytes.
(**A-E**) Human liver tissue was perfused with medium containing VP_{594} (shown in red) and 50% human serum for 1h, followed by a 16h perfusion without HBV before fixation. DAPI staining is shown in blue. All scale bars are 10 µm. (**A**) Immunofluorescence staining for CD68+ liver macrophages. The tissue shows liver sinuses with hepatocytes neighboured by several CD68+-stained liver macrophages. The boxed area is shown as enlargement below the main panels separately for each channel and focuses on one liver macrophage located in a liver sinus next to two hepatocytes. The dotted line indicates the circumference of one hepatocyte next to the macrophage. (**B**) Localization of HBV particles within one exemplary HepPar1+ hepatocyte (green). Z-axis projections are given for each channel. (**C**) Quantification of HBV-positive CD68+ liver macrophages, CD68- cells and HepaPar1+ hepatocytes in ten random vision fields at 200-fold magnification. Mean values ± SD. (**D, E**) Staining of neutral lipids with BODIPY493/503 shown in green within (**D**) a liver sinus with hepatocytes neighboured by two liver macrophages or (**E**) focussing one hepatocyte and one liver macrophage indicated by (Hep) and (KC), respectively. Hepatocytes (Hep) and liver macrophages (KC) are indicated. (**D**) shows xy images at two different z-positions of the same vision field. (**E**) Boxed areas are shown as enlargements for different z positions on the right. (**F**) Staining for SR-B1 (shown in green). The arrow indicates VP_{594} localised to SR-B1 positive plasma membranes. The area highlighted by the white box is enlarged on the right. (**G**) Relative localization of VP_{594} and CD68-positive liver macrophages (green) after 1h (left picture) and 17h perfusion (right picture). For increased contrast, DAPI staining is shown in white. Hep: hepatocyte. All scale bars are 10 µm.

Fig 9: Trans-infection of hepatocytes by HBV taken up by liver macrophages.
Primary liver macrophages (KC) and hepatocytes (PHH) were isolated from the same donors. (**A**) KC were loaded with VP_{594} for 4h, thoroughly washed and co-cultured with hepatocytes pre-labelled with 5-chloromethyl-fluorescein diacetate (CMFDA, green). One representative hepatocyte and liver macrophage each is shown. Lower panel shows Z-axis projection at the dashed line. (**B**) Macrophage / hepatocyte mix culture was incubated with 2 µg/ml Alexa Fluor 488®-labelled acLDL (green) for 6h to stain macrophages. acLDL staining (left), phase contrast (middle) and overlay (right) are shown. The arrow indicates a membrane connection between a macrophage and a hepatocyte. (**C**) Freshly isolated hepatocytes were incubated with 5 µM DiI for 20 min, washed three times by centrifugation at 300g and seeded onto a culture of liver macrophages from the same donor for 16h. A close connection that has established between a hepatocyte and a macrophage is shown. The area highlighted by the white box is shown enlarged below. Background fluorescence was acquired in a far-red fluorescence channel. Merged pictures (right panel) show DiI in red and nuclear staining with DAPI in blue. (**D**) Isolated KC were incubated with HBV for 6h at either 4°C or 37°C as indicated and washed, trypsinized, seeded and washed again before added to PHH. Widefield microscopy of

macrophage / hepatocyte mix cultures (left and middle panel) and pure liver macrophages (right panel) at day 12 post infection. (**E-G**) Liver macrophages (KC) were incubated with HBV for 6h at 4°C (dark gray bars) or 37°C (light grey bars) and washed, trypsinized, seeded and washed again before hepatocytes (PHH) were added. (**E**) Detection of HBV cccDNA in cell lysates. (**F**) HBsAg and HBeAg were measured in co-culture media. (**G**) Negative control with KC only. Values are mean ± SEM of two independent experiments, triplicates each. (**H, J**) Primary macrophages were incubated with HBV in the presence or absence of 100 µM Bulevertide or ant-HBsAg-antibodies (H) or with 5 µM U18666a (J), washed, trypsinized, seeded and washed again before hepatocytes (PHH) were added. On day 12, HBV RNAs (H) or HBV cccDNA (J) were quantified. Values are mean ± SD (H) and ± SEM (J) of two (H) and three (J) independent experiments, triplicates each. (**I**) Primary human hepatocytes only (left panel) or primary human macrophages (right panel) were incubated with $1x10^8$/ml virions. Then, cells where either washed (hepatocytes only culture) or trypsinized and thoroughly washed before hepatocytes were added to macrophages. On day 12 post infection, HBV cccDNA was quantified in cell lysates. Values are mean ± SEM of three independent experiments, quadruplicates each. **p < 0.01, ***p < 0.001 and ****p < 0.0001. Scale bars are 10 µm (**A-C**) or 100 µm (**D**).

Supporting Information (text file): EXTENDED EXPERIMENTAL PROCEDURES