# Host-Virus Interactions in Chronic Hepatitis B

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# 1. SUMMARY

Hepatitis B virus (HBV) is a blood-borne human pathogen of worldwide importance. It replicates in the hepatocytes in the liver and causes a disease known as Hepatitis B. Approximately 3% of the world population are chronically infected with HBV. Chronic Hepatitis B (CHB) is the number one cause of hepatocellular carcinoma in the world leading to close to 1 million deaths every year. Treatment options are limited and none of them are curative due to persistence of the viral covalently closed circular (ccc)DNA, a plasmid-like molecule which serves as the template for viral transcription and replication. New therapies are urgently needed that would decrease the global burden of HBV. A better understanding of the HBV immunobiology and host-virus interactions is critical in this regard. Due to the narrow species and tissue tropism, research in this area has been hindered by a lack of suitable experimental *in vivo* models of HBV infection leaving a lot of gaps in our understanding of the viral immunobiology. For example, HBV interaction with the host innate immune system has been a matter of debates for years. Although most viruses trigger various pathogen recognition receptors (PRRs) in the cells they infect leading to the induction of interferons and an antiviral state, HBV does not seem to do that. It remained controversial however, whether this is because HBV is invisible to PRRs (i.e. acting as a “stealth virus”) or because it efficiently suppresses innate immune responses very early after infection. Another example is HBV immune control. In the natural history of CHB most of the patients reach the so called HBeAg-negative chronic infections stage, when the virus is under control characterized by no or a very low viral load in the absence of noticeable immune activity that is otherwise known to control the HBV replication.

In this thesis we used human liver biopsy material from a large biobank of the University Hospital Basel in order to shed light on host-virus interactions in chronic hepatitis B. We established a novel short-term *ex vivo* liver biopsy culture system, allowing to study innate immune activation *in situ* in the human liver. We successfully used this system to demonstrate that HBV does not induce innate immune responses in the human liver in CHB. Importantly, HBV did also not interfere with the experimental induction of innate responses, suggesting that it behaves like a “stealth virus” staying under the radar of the cell’s defense systems. As a follow-up to this study, we discuss

the implication of these findings on the potential use of modulators of innate immunity as novel therapeutics for the treatment of chronic hepatitis B.

In a separate study, we sought to get an insight on how the host controls the virus during the HBeAg-negative chronic infection (ENCI) stage. By carefully analyzing HBV replication intermediates in the liver biopsies of patients of different stages of CHB, we have discovered that HBV replication is specifically inhibited downstream of pre-genomic (pg)RNA production during the ENCI stage of CHB. Our findings provide a starting point for further studies in this direction that eventually should identify the mechanism behind this inhibition and harness it for therapeutic use.

# 2. ABBREVIATIONS

| | |
|---|---|
| ALT | alanine aminotransferase |
| APOBEC | apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like |
| AVT | antiviral therapy |
| cccDNA | covalently closed circular DNA |
| cGAS | cyclic GMP-AMP Synthase |
| CHB | chronic hepatitis B |
| EDTA | ethylenediaminetetraacetic acid |
| EPCHB | HBeAg-positive chronic hepatitis B |
| EPCI | HBeAg-positive chronic infection |
| ENCHB | HBeAg-negative chronic hepatitis B |
| ENCI | HBeAg-negative chronic infection |
| (F)ISH | (fluorescent) *in situ* hybridization |
| HAV | Hepatitis A virus |
| HBV | Hepatitis B virus |
| HBcAg | Hepatitis B virus “core” antigen |
| HBeAg | Hepatitis B virus “e” antigen |
| HBsAg | Hepatitis B virus “S” antigen |
| HIV | Human immunodeficiency virus |
| IF | immunofluorescence |
| IRF | Interferon-regulatory factor |
| ISG | Interferon-stimulated gene |
| IFN | Interferon |
| IL6 | Interleukin-6 |
| NDC | nuclear DNA contamination |
| NDV | Newcastle disease virus |
| NF-κB | nuclear factor kappa B |
| NUC | nucleos(t)ide inhibitor |
| (p)STAT1 | (phospho) signal transducer and activator of transcription 1 |
| PAMP | pathogen-associated molecular pattern |
| PKR | protein kinase R |
| PRR | pattern recognition receptor |

| | |
|---|---|
| RIG-I | Retinoic acid-inducible gene I |
| repDNA | DNA replicative intermediates |
| RLR | RIG-I-like receptor |
| RT | reverse transcription |
| SeV | Sendai virus |
| TLR | Toll-like receptor |
| TNF$\alpha$ | Tumor necrosis factor alpha |

# 3. INTRODUCTION

## 3.1 Hepatitis B virus research

Hepatitis B virus (HBV) is a small DNA virus that belongs to the *hepadnaviridae* family [1]. This family consists of 2 genera: mammalian orthohepadnaviruses (with human HBV being a prototype virus) and avian avihepadnaviruses (prototype – Duck Hepatitis B Virus (DHBV)) [1]. HBV is a blood-borne pathogen that naturally infects humans and can also infect chimpanzees causing a disease known as Hepatitis B [2]. Although a possible existence of an infectious agent causing post-transfusion chronic hepatitis was evident since at least the 1940s, the first connection to the virus was made only in the 1960s, with the identification of a hepatitis-specific serum antigen in serum of Hepatitis B patients, who coincidentally happened to be predominantly Australian aborigines [3, 4]. This antigen, named therefore “Australia Antigen” (AuAg) later turned out to be a component of the infectious agent, HBV [5, 6]. These discoveries led to establishment of screening technologies and clinical diagnostic tools. Subsequent identification of similar viruses in other species, such as Woodchuck Hepatitis Virus (WHV) in woodchucks and DHBV in Peking ducks significantly accelerated HBV research [7, 8]. Because of their similarity to HBV, WHV and DHBV served as indispensable models for studying the molecular biology of hepadnaviruses and for drug screening [9]. Chimpanzees have been the most physiologically relevant and informative model of HBV infection over many years [10], however recent ethical considerations effectively shut down academic research in these animals [11], emphasizing the need for alternative models and bringing forward human liver biopsies as the only (and very rare) source of information about chronic HBV infection in the liver. In the absence of suitable immunocompetent animal model systems, human liver biopsies are currently a gold standard for validation of the results obtained in cell culture systems or in other animal models.

## 3.2 Molecular Biology of HBV

### 3.2.1 HBV particle structure and composition

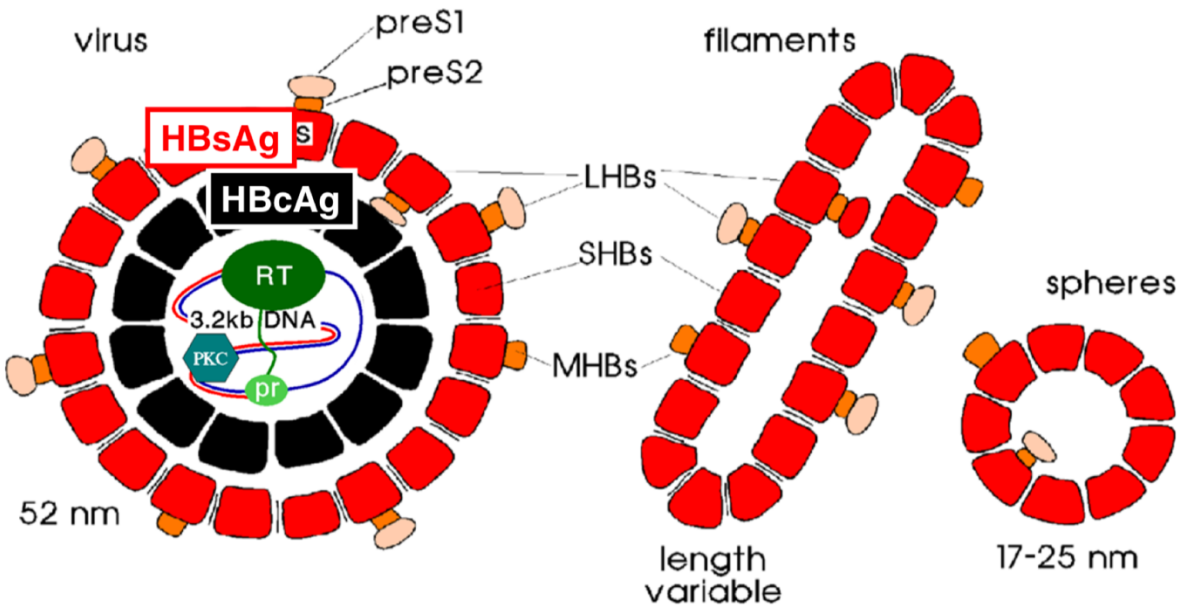


**Figure 1.** A schematic representation of HBV viral and subviral particles. Infectious virion (Dane particle) is shown on the left. Subviral particles are on the right (filaments and spheres). Red – envelope, black – capsid. HBV genomic DNA with covalently attached HBV polymerase (green) is schematically depicted inside the capsid. RT, reverse transcriptase domain of HBV polymerase; pr, primase domain. HBsAg, HBV surface antigen; HBcAg, HBV core antigen. LHBs, MHBs and SHBs are Large, Middle and Small HBs antigens, respectively. Figure modified from Gehrlich et al., (2013) [2].

Mature infectious HBV virions are called “Dane particles” after the scientist who first described them (Figure 1) [5]. A particle is ~42 nm in diameter and it carries 1 copy of the HBV genome, a ~3.2 kb relaxed circular partially double-stranded DNA molecule (rcDNA) (Figure 1 and 2) [5, 12, 13]. Both strands of the genomic DNA have gaps and the circular conformation is maintained due to overlapping complementary regions in the 5’-ends of each strand [14]. The minus(-)-strand is slightly overlength and carries a P (“polymerase”) protein covalently attached to its 5’-end [14]. The plus-strand is incomplete and its 5’-end is constituted by an RNA primer. HBV genome is encapsulated in the virus-encoded capsid which consists of the C (“core”) protein (or “HBV core antigen” (HBcAg)) homodimers [14]. Capsid is enveloped in a host derived lipid membrane containing glycosylated HBV L (“Large”), M (“Middle”) and S (“Small”) envelope proteins, collectively known as “HBV surface antigens” (HBsAg) [15]. The N-

terminal domain of the L protein is myristoylated [15]. In addition to Dane particles, serum of HBV infected individuals also contains smaller non-infectious "subviral" particles devoid of nucleic acids but containing viral envelope proteins that occur as filaments and spheres depending on their composition (Figure 1) [16] [15]. The subviral particles are present in large excess over infectious Dane particles [16].

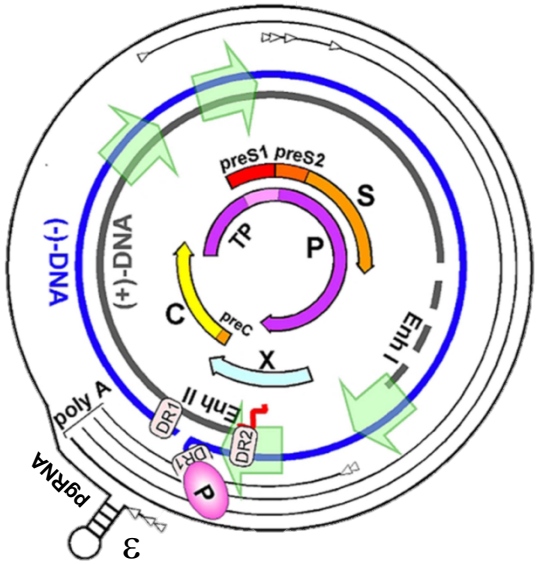


**Figure 2.** HBV genome organization. Blue and grey lines depict HBV genomic DNA in the form of relaxed circular (rc)DNA, as it exists in the virion. P, HBV polymerase covalently attached to 5'-end of the minus-strand DNA. Red wiggly line is the 5'-capped RNA primer on the plus-strand. Open reading frames (ORF) are shown in the center. Green arrows indicate four internal promoters. Enh I and Enh II are transcriptional enhancers. DR1 and DR2 – direct repeats. Outer lines are HBV transcripts, produced from covalently closed circular (ccc)DNA. Arrowheads denote transcription start sites. ε - RNA encapsidation signal on pregenomic (pg)RNA. Pre-core transcript is slightly longer than pgRNA and is not separately shown. Figure modified from Nassal et al., (2016) [14].

## 3.2.2 HBV life cycle

### *3.2.2.1 HBV entry and nuclear translocation*

Initial attachment of the virus to cells seems to happen through weak non-specific interactions with negatively charged heparan sulfate proteoglycans (HPSG) (Figure 3) [17]. This interaction is essential, but not sufficient for infection [18]. The initial attachment is followed by a specific interaction of the N-terminal part of the L-HBsAg with the cognate HBV receptor hepatocyte-specific bile acid transporter sodium-taurocholate cotransporter polypeptide (NTCP), that determines cell-type and species specificity of HBV [19, 20]. Upon receptor-mediated entry, the genome-containing capsid is released into the cytoplasm and actively transported into the nuclear basket with the help of the transport factors importin alpha and beta [21]. What happens next is not well understood, however it seems that capsid interaction with nuclear pore

proteins results in capsid disassembly and release of HBV genome into the nucleus [22].

#### *3.2.2.2 cccDNA*

In the nucleus, the HBV genome undergoes a series of modifications by nuclear enzymes. The molecular mechanisms of these modifications still remains obscure, but in general, the following events must take place: 1) synthesis of the plus-strand is completed, 2) terminal redundancy is eliminated from the minus-strand 3) HBV polymerase is cleaved off the minus-strand 5'-end, 4) RNA primer is eliminated from the 5'-end of the plus-strand, 5) the DNA ends are ligated together on both strands [14]. It is conceivable that the infected cell mistakes viral rcDNA for damaged cellular DNA and activates the DNA repair machinery, which then performs all the steps listed above. The resulting molecule is a double-stranded, covalently closed circular DNA (cccDNA) molecule, which is central for the HBV life cycle and for persistence (Figure 3) [14].

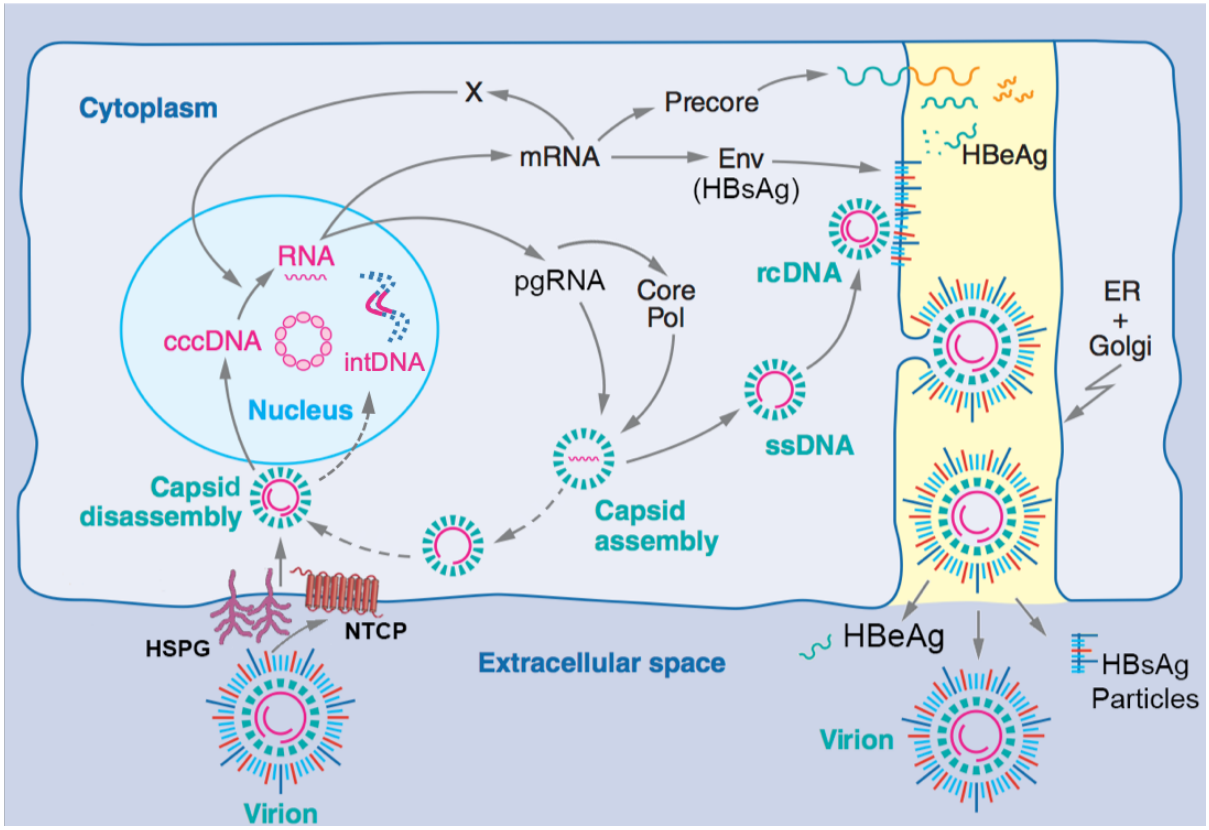


**Figure 3.** HBV life cycle (modified from Guidotti and Chisari, 2006 [23]. See text for details. NTCP, sodium taurocholate co-transporting polypeptide; HSPG, heparan sulfate proteoglycans. intDNA, integrated HBV DNA

CccDNA exists in the nucleus as an episome (or “minichromosome”) and is indistinguishable from cellular DNA by virtue of its association with histones that even carry epigenetic markers (e.g. histone modifications) [24]. CccDNA appears to be extremely stable as it can persist for years in the liver, including during antiviral therapy and even after resolution of infection [25, 26]. Elimination of cccDNA from the hepatocytes is a “holy grail” of anti-HBV drug development, however none of the current therapies specifically targets this molecule [27].

#### *3.2.2.3 HBV transcription and proteins*

HBV cccDNA serves as the template for transcription which is mediated by the cellular RNA polymerase II machinery and is regulated by liver-specific transcription factors (Figure 2) [14, 28]. The dependency on the liver-specific transcription factors is another parameter determining the hepatotropism of HBV. The viral transcripts contain a 5’-cap structure and 3'-poly-A tail and thus are indistinguishable from cellular RNA [29]. Unspliced viral RNAs are exported from the nucleus and translated by the cellular protein synthesis machinery.

Information in the HBV genome is very densely packed using all three reading frames (Figure 2, in the center) [30]. The HBV genome encodes for 7 different proteins which are produced from 5 different but partially overlapping transcripts (Figure 2, outer lines). While all HBV transcripts are terminated at the same poly-A site, they each are initiated at unique transcriptional start sites defined by 4 RNA pol II promoters and two enhancer elements (Figure 2, green arrowheads, Enh I and Enh II). The shortest transcript is 0.7 kb long and codes for the X protein (HBx). X is expressed early in infection and facilitates transcription of all other HBV RNAs [31]. It does so via binding to and targeting for degradation the “structural maintenance of chromosomes” (Smc) protein complex Smc5/6, which inhibits episomal DNA transcription [32]. The 2.1 kb PreS2/S mRNA codes for Middle and Small envelope proteins, while the large envelope (LHBs) protein is produced from a longer 2.4 kb PreS1 transcript. The 3.5 kb larger-than-genome pre-genomic RNA (pgRNA) transcript has two functions: 1) it encodes C (core) and P (polymerase) proteins and 2) serves as the template for HBV replication (discussed below). HBV core makes up the viral capsid and the polymerase replicates the viral genome. The precursor of pre-core protein, also known as “HBV e antigen” (HBeAg), is produced from a pre-core mRNA that, at the 5’-end is just a few nucleotides longer than the pgRNA [29]. This precursor is proteolytically processed to

form mature HBeAg that is efficiently secreted into the serum [33]. HBeAg is not essential for viral replication and its function is rather unclear [34]. It is believed to act as an immune modulator, ensuring “immune tolerance” to HBV infected cells [35, 36].

#### *3.2.2.4 Replication*

Hepadnaviruses are “pararetroviruses”, meaning that similar to retroviruses their replication involves a reverse transcription step. However, contrary to retroviruses, that have an RNA genome and replicate via a DNA intermediate, hepadnaviruses are DNA viruses that replicate via an RNA intermediate [29]. The HBV polymerase possesses both RNA- and DNA-dependent DNA polymerase and RNase H activities and therefore can perform all steps required for complete HBV replication [30]. The complete mechanism of HBV replication has recently been reviewed in detail by Hu and Seeger (2015) [30]. Here, I will briefly summarize the main steps of this complex process as outlined in Figure 4. HBV replication starts with a tripartite interaction between HBV polymerase, pgRNA and core. Specifically, polymerase first binds to a 5’-proximal stem-loop structure on the pgRNA called epsilon (ε) for “encapsidation”. This binding triggers packaging of the pgRNA-polymerase complex into a newly forming nucleocapsid and initiates the reverse transcription reaction which then takes place inside the viral capsid. A Tyr residue in the N-terminal domain of the polymerase serves as a primer for minus-strand DNA synthesis by providing an -OH group to which the first nucleotide will be attached. As a result of this protein-mediated priming, polymerase becomes permanently covalently attached to the newly synthesized negative DNA strand. First ~3 nucleotides are added to the growing (-)-strand using the ε element as a template, then the synthesis is transferred to a matching acceptor motif in the 3’-proximal direct repeat 1 (DR1) (first template switch; see Figure 4A). The polymerase then continues (-)-strand DNA synthesis until it reaches the 5’-end of the pgRNA thus producing a unit-length minus-strand DNA with a short redundancy (‘r’) on its 3’-end. pgRNA is directly degraded during the process by the RNase H activity of the polymerase, except for the last ~10 nucleotides which then serve as the primer for plus(+)-strand DNA synthesis (Figure 4B). To that end, the RNA primer is transferred to DR2 at the 5'-end of the (-)-strand (2-nd template switch, Figure 4C) and (+)-strand DNA synthesis is initiated and continues until it reaches the 5’-end of the (-)-strand (Figure 4D). The presence of short identical DNA sequences (‘r’) at the 5’- and 3’-ends of the (-)-strand allows the polymerase to “jump” from the 5’-end to the 3’-

end of the (-)-strand (3-rd template switch, Figure 4E) and continue (+)-strand DNA synthesis from there (Figure 4F). The (+)-strand synthesis does not proceed until the end, leaving a large (up to ~1000 nt) gap, supposedly because at this point the virions are exported out of the cell where no more nucleotides are available to continue DNA synthesis. The resulting HBV genome molecule is therefore a partially double-stranded relaxed circular DNA that has a P protein covalently attached to the 5'-end of the slightly overlength (~10 nt) (-)-strand, and an incomplete (+)-strand whose 5'-end is composed of an RNA primer. There is about a 10% chance that the 2-nd template switch does not occur resulting in *in situ* priming with the polymerase simply following all the way back along the minus-strand (Figure 4G-H). This generates a non-functional double-stranded linear (dsl) DNA genome.

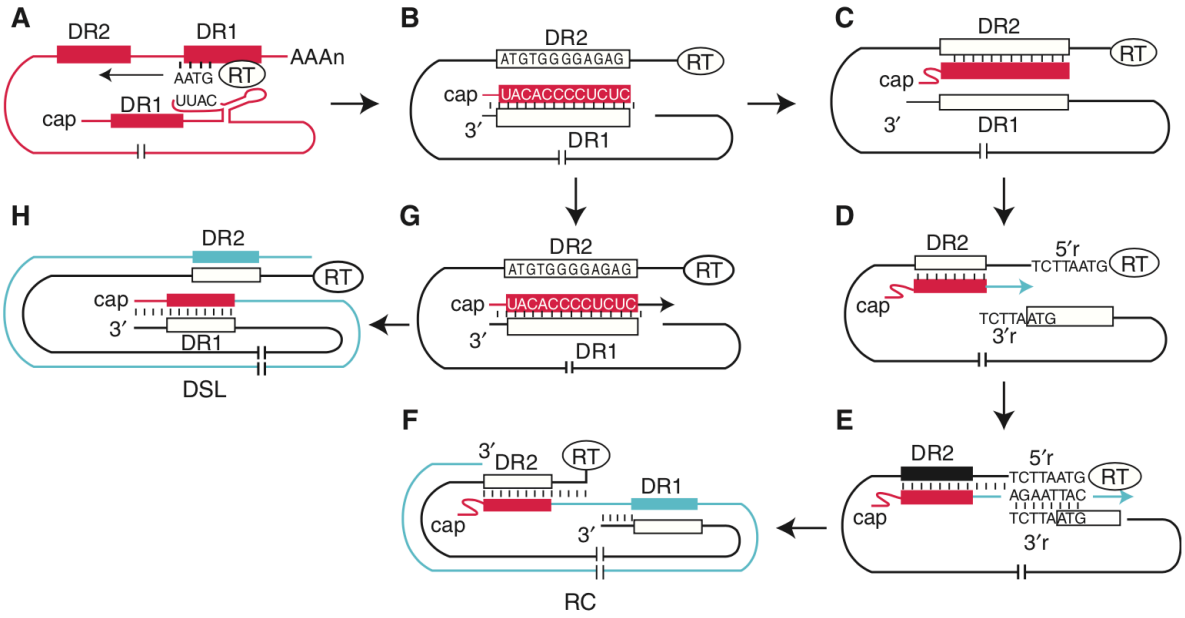


**Figure 4.** HBV replication scheme (Hu and Seeger, 2015 [30]). See text for details. Steps A, B, C, ... → F result in the production of correct rcDNA HBV genomes. Steps A, B → G, H result in replication-deficient double-stranded linear (dsl)DNA. The core protein which is required for initiation of the replication is not shown.

Capsids containing mature genomes (rcDNA or dslDNA), but not immature capsids containing RNA or ssDNA, translocate into multivesicular bodies (MVB) where they associate with the envelope proteins and get exported out of the cells [37]. This step is limited by the availability of the HBV surface proteins, especially the L-HBsAg. In case of insufficient amount of envelope proteins, the mature capsids can be transported into the nucleus and recycled, resulting in genomic DNA being released

into the nucleus and converted to a cccDNA, thus amplifying the cccDNA pool [38]. The evidence for this process is coming from experiments in the duck DHBV system where this mechanism has been shown to contribute significantly to the cccDNA pool formation. The data for human HBV suggest that this mechanism either does not exist, or at least does not play a significant role in HBV infection in humans [39, 40].

#### *3.2.2.5 HBV integration*

In contrast to retroviruses, the HBV viral life cycle does not include obligatory integration of its genome into the host genome [29]. Nevertheless, integrated HBV DNA is often found in the late stages of chronic hepatitis B (CHB) and in hepatocellular carcinomas (HCC) [41, 42]. The preferred source for HBV integration is dslDNA [43]. When a cell is infected with a virion containing dslDNA several outcomes are possible: i) dslDNA is degraded ii) dslDNA is converted into cccDNA by non-homologous end joining DNA repair pathway, often resulting in functionally defective cccDNA molecules, or iii) it can integrate into the host genome (Figure 3) [43]. Since integration is not obligatory for the HBV life cycle, it is not clear whether it plays any role at all in HBV infection [44]. Integrated HBV DNA cannot generate new virions, because it cannot produce full-length pgRNA, however the HBsAg coding ORFs are intact and can be actively expressed from their own promoters [45, 46]. In addition, truncated forms of HBx might be produced [47]. Expression of core and polymerase genes is only possible if HBV DNA integrates in the proximity of an active promoter in the host DNA, because the endogenous promoters are typically separated from the transcriptional start site in the linear copy of the integrated HBV DNA. Integration appears to happen randomly, without any preferred location in the human genome [48]. The extent of integration in chronically infected human liver is unknown, but is estimated to be ~0.01-1% in the late stages [42, 48]. There is no direct link between HBV integration and the incidence of HCC, however it is reasonable to assume that given the random nature of HBV integration, some of these integrations can be pro-oncogenic [42, 43, 48, 49].

## 3.3 Hepatitis B pathogenesis

### 3.3.1 Global importance of HBV

Hepatitis B virus infection in humans causes a disease known as Hepatitis B. The majority of adult infections result in self-limited acute Hepatitis B (AHB). However, most vertical transmissions from mother to child and ~5% of adult infections become chronic. Chronic Hepatitis B (CHB) progresses gradually over time, resulting in continuous liver damage and eventually leading to cirrhosis and/or to liver cancer. CHB is the underlying cause of more than half of liver cancers worldwide and leads to close to 1 million liver-related deaths every year [50, 51]. HBV is highly endemic in Sub-Saharan Africa and South-East Asia (Figure 5). It is estimated that ~3% of the world population is chronically infected with HBV, whereas in some highly endemic areas endemicity can reach close to 25% [51, 52]. Due to an efficient vaccination program, HBV spread has been contained in many regions of the world, however it still has a significant impact globally [51].

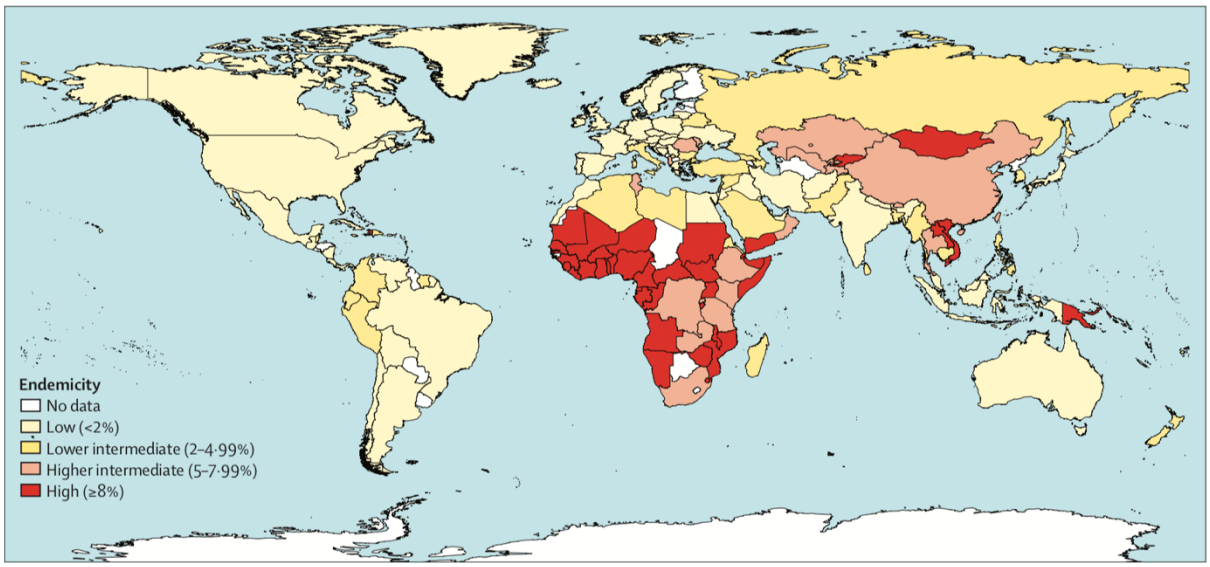


Figure 5. HBV endemicity (1957-2013). Figure modified from Schweitzer et al. (2015) [52]

### 3.3.2 Acute Hepatitis B

Acute Hepatitis B has been extensively studied in chimpanzees – another natural host for HBV infection and the only non-human primate *in vivo* model [10]. Upon initial infection HBV replicates to very high titers for several weeks before the immune

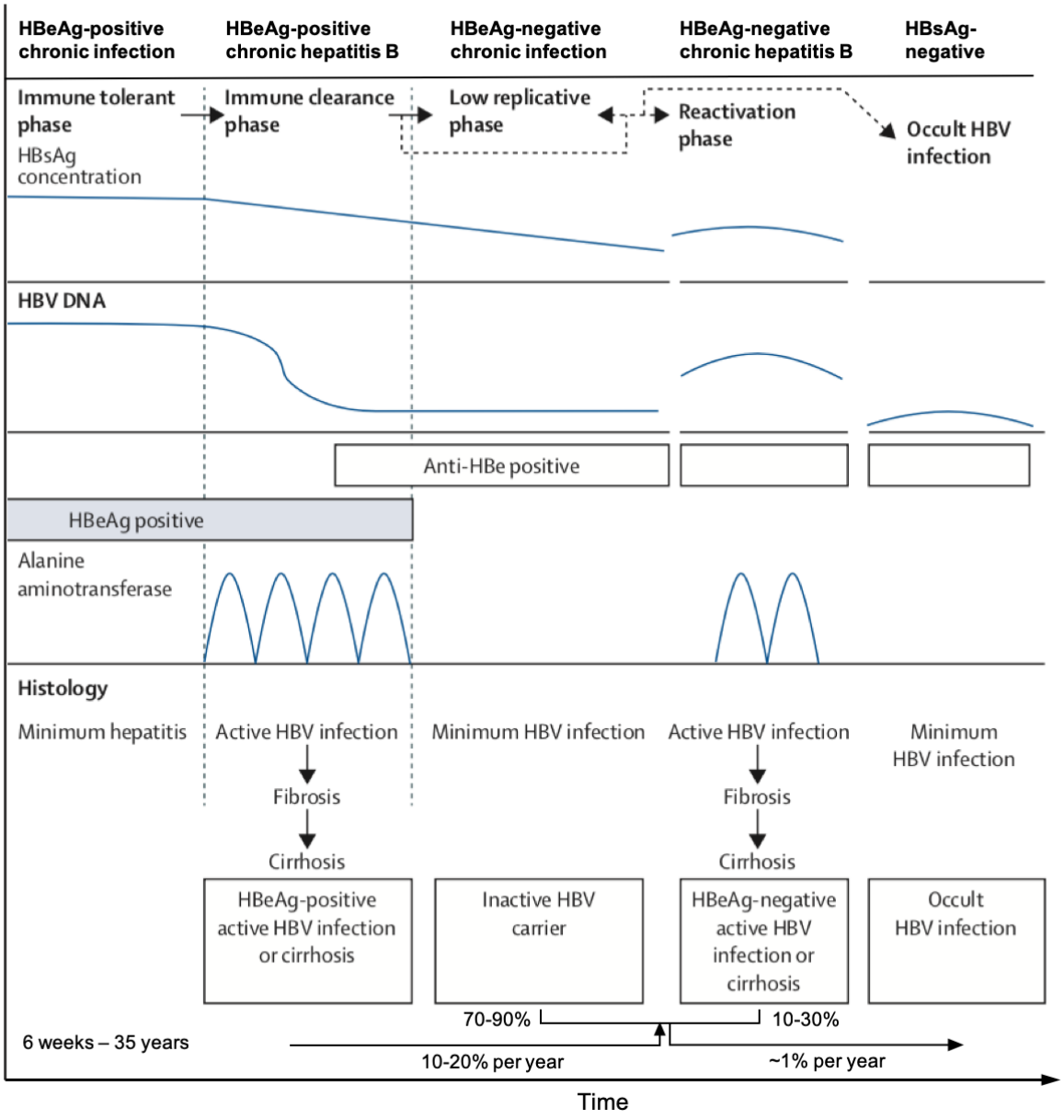


**Figure 6.** Natural history of chronic Hepatitis B. See text for details. The names of the stages according to the latest nomenclature [57] are given on top. Note that the progression of the infection following HBeAg loss is not linear (dashed arrows in the upper part of the scheme). HBeAg, HBV E antigen; HBsAg, HBV S antigen. Figure modified from Trépo, Chan and Lok (2014) [53]

system finally starts to fight the virus [53]. Strong T cell responses suppress viral replication via cytolytic and non-cytolytic mechanisms and eliminate most of the infected cells causing acute liver disease [53]. This typically leads to complete resolution of the infection with the loss of HBsAg and appearance of anti-HBs antibodies as a hallmark of successful clearance [53]. Despite clinical resolution, some HBV genomes can persist in the hepatocytes as cccDNA, without any HBV markers

being detectable in the serum. This state is sometimes referred to as “occult” infection (Figure 6) [54]. Typically, that would not lead to a recurrent hepatitis, because of the immune memory and circulating anti-HBV antibodies, however a reactivation can occur if the host’s immune system is impaired (e.g. due to an immunosuppressive therapy) [55, 56].

### 3.3.3 Chronic Hepatitis B

Chronic Hepatitis B is defined by the presence of HBsAg in the serum for longer than 6 months [53]. CHB occurs mostly after vertical transmission, infection of infants under 1 year of age, but also in ~5% of adult infections [53]. The reasons for HBV chronicity are not well understood. In case of vertical transmission neonatal tolerance is believed to be responsible. It could be caused by HBeAg that is able to cross the placenta and has been shown to induce tolerance in HBV transgenic mice [35, 36]. In the rare cases of adult HBV infections becoming chronic, weak CD4+ and CD8+ T cell responses are considered to be the main reason for not clearing the infection [58]. Viral escape mutants, inhibition of adaptive immune responses by viral proteins and the size of viral inoculum can potentially contribute to this weak immune response and persistence [23, 59, 60]. The natural history of CHB is complex and can be divided into 5 different stages according to the clinical practice guidelines of the European Association for Study of the Liver (EASL) (Figure 6) [57]. The classification is based on the viral load, presence/absence of liver disease, inflammation and HBV antigens. The first stage is called “HBeAg-positive chronic infection” (EPCI). During this phase the virus replicates to very high titers (up to $10^{10}$ IU/ml), however, and for unknown reasons, there is no apparent immune response active and therefore this phase is normally asymptomatic. Besides high viral load this stage is characterized by the presence of HBeAg in the serum, high serum HBsAg levels and no detectable liver disease/inflammation. This stage can last for several decades before the onset of an active immune response resulting in suppression of viral replication and cell death. The latter is clinically known as hepatitis and measured as an increase of serum alanine aminotransferase (ALT) as a result of hepatocyte destruction. Furthermore, the necroinflammatory activity is clearly visible at the histological level. Accordingly, this stage is called “HBeAg-positive chronic hepatitis B” (EPCHB) and it lasts until antibodies against pre-core/core (anti-HBc) appear and HBeAg disappears from the serum (HBeAg seroconversion), followed by an HBeAg-negative phase. If at this time the virus suppressed to very low

or undetectable levels (<2000 IU/ml) in the absence of an apparent immune activity (i.e. no liver inflammation), but HBsAg is still secreted, then the disease is considered to be in the “HBeAg-negative chronic infection” (ENCI) stage. Alternatively, the fight between the immune system and the virus can continue in a “HBeAg-negative chronic hepatitis B” (ENCHB) stage, with fluctuating viral loads (typically >2000 IU/ml) and persistent liver disease (elevated ALT) being the main markers of this stage. The two stages of the HBeAg-negative phase can transit into one another and back over time. The constant liver damage caused by the immune activity in the “chronic hepatitis” stage accumulates over time leading to liver fibrosis, cirrhosis and potentially liver cancer [53]. Spontaneous resolution of chronic infection, (transition into the last phase of CHB, HBsAg-negative stage) defined as the loss of HBsAg and often appearance of anti-HBs antibodies (HBsAg seroconversion), can happen with a frequency of ~1% per year in HBeAg-negative patients [61]. As in the case of AHB, virus is never completely cleared from the liver, as cccDNA persists in some hepatocytes even after clinical resolution of CHB [53]. An important question in CHB is what (and how) is controlling the virus during the ENCI (and maybe also the HBsAg-negative) stage? By definition, there is no apparent immune activity or inflammation detectable in these patients, that would otherwise suppress the virus. However, immunosuppression can lead to a reactivation of the virus, arguing that the immune system plays an active role in keeping the virus under control in this stage [62].

### 3.3.4 HBV vaccine

The first commercial HBV vaccine has been introduced in 1971 and was based on inactivated HBsAg positive patient serum [63]. Recombinantly produced HBsAg soon replaced the inactivated serum as a vaccine and is still in use today [64]. Vaccination has greatly reduced HBV morbidity worldwide, especially in the Western world [51]. The current vaccination however, only protects from infection, but has no effect on chronic HBV. Vaccination mediated eradication of HBV is still elusive however, because the vaccination coverage remains low in some highly endemic regions of the world [51].

### 3.3.5 Treatment of CHB

While most of the current antiviral therapies are very efficient in controlling viral replication, they are, despite many years of research, not curative as they cannot

actively eliminate the viral cccDNA from the liver [27]. Available options include interferon alpha (IFNα) and nucleos(t)ide analogues (NUCs) and will be described in the next paragraphs.

#### *3.3.5.1 Interferon*

IFNα is an innate immunity cytokine that acts through induction of hundreds of antiviral genes in the cells and through promoting the activation and/or differentiation of immune cells [65]. Interferon could be an exception compared to the other anti-HBV therapies, because it can induce cellular APOBEC3-family nucleic acid-editing enzymes, which could potentially modify or degrade cccDNA and/or pgRNA [66, 67]. However, the evidence for this mechanism is not compelling and also no correlation was found between APOBEC3A (A3A), A3B and A3G expression and cccDNA levels in the livers of CHB patients [68]. In 1992, interferon-alpha (IFNα) was approved as the first drug for treatment of CHB. It was soon replaced by its pegylated (conjugated with polyethylene glycol (PEG) molecule) form, which has better pharmacokinetics and a longer half-life. Peg-IFNα can suppress viral replication and even cure some patients and therefore it is still used in clinical practice. However, it is only effective in 10-40% of patients, with many patients not responding to the treatment or having a relapse after cessation of therapy [27, 57]. Patients receiving IFNα as therapy should be carefully selected and monitored because of the many side effects of interferon. Taken together, the combination of poor efficiency and side effects greatly limits the use of IFNα.

#### *3.3.5.2 Replication/RT inhibitors*

Drugs inhibiting reverse transcription were borrowed into HBV clinical practice mostly from human immunodeficiency virus (HIV) research (e.g. nucleos(t)ide analogues (NUCs) such as lamivudine, adefovir, tenofovir). That was possible because the HBV polymerase is structurally similar to the HIV reverse transcriptase (RT), so that the RT inhibitors that were already approved for treatment of HIV were found to be active against HBV. Another drug, entecavir, was originally developed against herpes simplex virus. These small molecules very efficiently inhibit HBV replication at the level of reverse transcription, but do not prevent viral antigen expression [27]. Nevertheless, and for unknown reasons, NUC therapy leads to an amelioration of the HBV associated inflammation in the liver [2, 27]. Lamivudine therapy is rarely used today

because it is frequently associated with the emergence of drug-resistant HBV mutants [69]. Adefovir has mostly been replaced by the structurally very similar tenofovir, which is superior in terms of resistance rate and side effects and is currently the drug of choice for treatment of CHB [70]. Entecavir is efficient in treatment-naïve patients and sometimes also used in combination with tenofovir [69]. Tenofovir and entecavir have little side effects, a low resistance rate and can be given lifelong [27]. The latter is crucial, because cccDNA is very stable, has a long half-life in the liver and therefore forms a persistent reservoir that can most likely only be depleted by loss during hepatocyte division or by cell killing or [26, 39]. Thus, replication inhibitors have to be administered to CHB patients lifelong or at least until functional cure occurs (HBs seroconversion), because the virus rebounds when the therapy is stopped [57].

#### *3.3.5.3 Entry inhibitors*

Myrcludex B is a synthetic peptide derived from the N-terminus of HBV large envelope protein. It functions as a competitive inhibitor of HBV attachment to its cellular receptor NTCP [71]. It is currently undergoing clinical trials, but limited available data from Phase 2 studies suggests it could be efficient against hepatitis B [72]. It should be noted however, that entry inhibitors have an inherent limitation because they can only prevent reinfection and infection of new cells, but would not affect the cells which are already infected.

#### *3.3.5.4 Novel therapies*

Future attempts to find a cure therefore focus on targeted degradation or silencing of cccDNA [73]. Cristoph Seeger pioneered this approach by implementing CRISPR-Cas9 technology, however it is still a long way until such an approach could possibly be used in humans [74]. There are many other anti-HBV therapeutic approaches currently under development, including, for example, core inhibitors [75], modulators of innate immunity [76], antisense oligonucleotides [77], immunotherapy [78], etc. Whether any of these new therapies might be efficient against cccDNA however, remains to be determined.

## 3.4 Host-Virus Interactions

### 3.4.1 Detection of viral infection by the host cell

All vertebrate cells are equipped with an ancient system of recognizing viral infections [79]. This system represents a network of pattern recognition receptors (PRRs) that detect virus-specific molecular signatures typically not present in the cell, so-called pathogen-associated molecular patterns (PAMPs), and trigger a response directed at destroying the pathogen (Figure 7) [80, 81]. This pathogen detection and elimination system is known as innate immune system and it represents a cell's first line defense against incoming pathogens. In addition, the signals produced by a virus-infected cell upon pathogen recognition are central for the activation of the effector cells of the adaptive immune system [82].

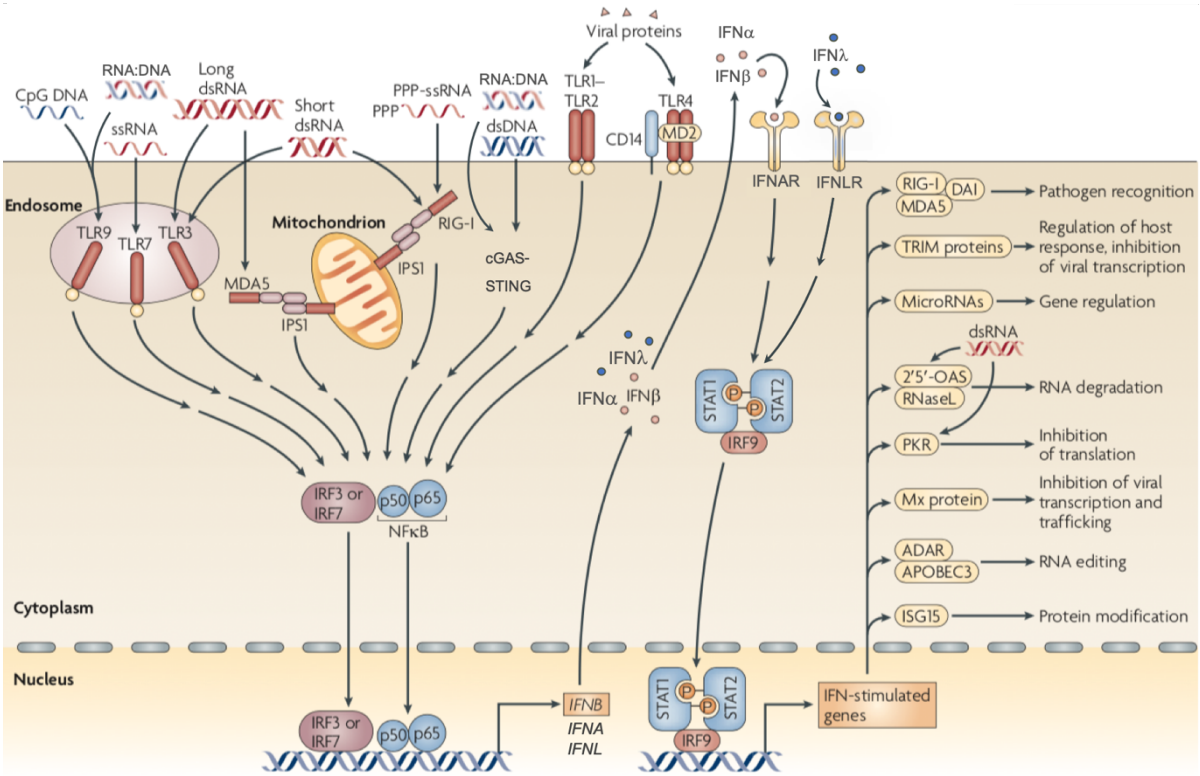


**Figure 7.** Activation of the interferon response triggered by viruses (modified from Bowie and Unterholzner (2008) [83]. See text for details. ssRNA, single-stranded RNA; dsRNA/dsDNA, double-stranded RNA/DNA; IFN, interferon; IFNAR, IFN-alpha receptor; IFNLR, IFN lambda receptor; TLR, Toll-like receptor; RIG-I, retinoic acid inducible gene I; MDA5, melanoma differentiation associated gene; IPS1, IFN-beta promoter stimulator 1; IRF, interferon regulatory factor; NF-kB, nuclear factor kappa B; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; STAT1/2, signal transducer and activator of transcription 1/2.

Pattern recognition receptors can be largely divided into two categories – cell surface or endosomal membrane bound Toll-Like Receptors (TLRs; 9 functional receptors are known in humans – TLR1-9) [80] and cytoplasmic receptors, such as RNA-sensing retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and DNA sensors, such as cyclic GMP-AMP synthase (cGAS) (Figure 7) [81]. Typical virus-specific molecules recognized by these receptors are, for example, uncapped single-stranded (ss)RNA (recognized by TLR7/8, RLRs), viral double-stranded (ds)RNA (TLR3, RLRs), viral DNA and RNA:DNA hybrids (cGAS, TLR9) and viral structural proteins (TLR1/2/4) [80, 81, 84, 85]. Recognition of PAMPs by PRRs triggers a downstream signaling cascade ultimately activating a set of transcription factors, such as interferon-regulatory factor (IRF) family and nuclear factor kappa B (NF-κB), leading to the induction and secretion of type I/III interferons (IFNs) and various pro-inflammatory cytokines (e.g. tumor necrosis factor alpha (TNFα) or interleukin 6 (IL-6)) (Figure 7) [80, 81, 86, 87]. Interferons in turn bind to their cognate cell surface receptors on the infected, as well as neighboring uninfected cells and trigger a signaling cascade leading to expression of hundreds of interferon stimulated genes (ISGs) that limit viral replication and spread (Figure 7) [88]. IFNs and pro-inflammatory cytokines also link innate immunity to adaptive immunity by activating cells of the adaptive immune system and recruiting them to the site of infection [89].

### 3.4.2 Viral evasion of immune responses

Given the cell's ability to sense and restrict viral infection at a very early stage it is not surprising that many viruses have evolved to counteract either recognition by PRRs or the effector functions of the innate immune response. For example, Hepatitis A virus (HAV) expresses a protease that can degrade mitochondrial antiviral-signaling protein (MAVS), an adaptor molecule required for induction of IFNs by RLRs, thereby limiting type I IFN response in experimentally infected chimpanzees [90, 91]. Similarly, the VP35 protein of Ebola virus and the NS1 of Influenza virus can inhibit activation of IRF3, a transcription factor required for induction of IFNs [92]. V proteins of mumps virus (SV5) and Newcastle disease virus (NDV) can degrade signal transducer and activator of transcription 1 (STAT1), a key component in type I/III IFN signaling, thus preventing the induction of an antiviral state [93, 94]. Vaccinia virus and Hepatitis C virus (HCV) can prevent synthesis of antiviral proteins in the infected cells by shutting down cap-dependent translation via inhibition of protein kinase R (PKR) [95, 96].

Finally, some viral proteins can counteract specific antiviral effectors, for example HIV Vif protein that targets host APOBEC3 family enzymes and prevents their entry into progeny virions [97].

### 3.4.3 HBV-host interactions

Hepatitis B infection, contrary to many other viruses does not seem to trigger an innate immune response (Figure 8) [98]. Experimental infection in chimpanzees did not result in the induction of a type I/III IFN response signature in the liver (Figure 8) [99]. Similar results were obtained with HBV in human liver chimeric mice and in woodchucks with WHV [100, 101]. Moreover, data available from humans with acute hepatitis B confirmed the lack of an innate immune response signature during acute infection [102, 103]. These observations have led to the hypothesis that HBV behaves as a "stealth virus" by remaining invisible to the PRRs [98].

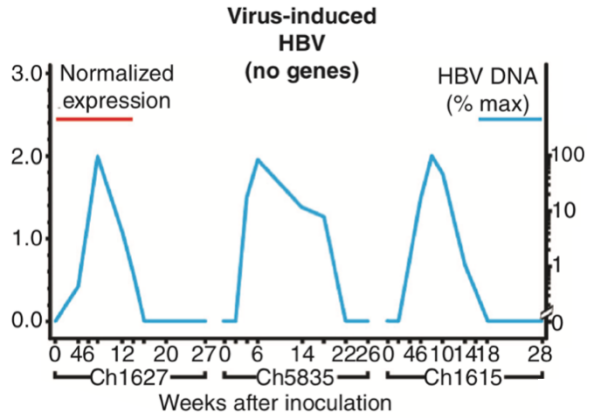


**Figure 8**. Host innate immune responses to HBV infection in chimpanzee liver (modified from Wieland (2015)). Gene expression profiling was performed in the liver of chimpanzees experimentally infected with HBV. Blue lines show viremia, red lines – gene expression. Genes correlated with viremia (innate immune response genes) are shown (no genes).

However, this concept remained controversial because it could be demonstrated that HBV indeed could trigger an innate immune response in *in vitro* HBV infection systems [104], but was able to suppress the innate immune system within 24 hours after inoculation [105]. Those findings suggested that HBV is a weak inducer, but strong suppressor of innate immune signaling and thereby appears not to trigger an innate response. Indeed, most HBV proteins have been shown to have some suppressive activity on different components of the innate immune system [106, 107]. However, many of these studies have been hampered by the lack of optimal HBV infection model systems and thus, their results remained controversial [104, 105]. Indeed, the sensitivity of HBV replication to IFN$\alpha$ or to TLR agonist induced antiviral mechanisms

in chimpanzees, HBV transgenic mice and in *in vitro* systems does not support the notion that HBV can efficiently suppress innate responses [108-111]. In an effort to resolve these contradictory results, we have for the first time investigated the interplay between HBV and the host innate immune system at the cellular level in the liver of HBV infected patients [112] (see section 5.1). As described in Suslov et al. [112] these studies unequivocally demonstrated that HBV does neither induce nor suppress the innate immune responses in the human liver and thus beaves like a stealth virus (see section 5.1) [112]. These conclusions were further supported by recent publications from other groups using state of the art cell culture systems [113, 114].

### 3.4.4 Immune control of HBV

Acute HBV infection is very efficiently controlled by the adaptive immune response [115, 116]. Cytotoxic CD8+ T lymphocytes play a central role in controlling and resolution of HBV infection by killing infected cells and IFN-gamma (IFN$\gamma$) secretion that in turn non-cytolytically inhibits viral replication [23, 115, 116]. Specifically, CD8 T cell derived IFN$\gamma$ seems to be responsible for strong suppression of HBV during the early phase of viral clearance with no or little signs of liver disease [115, 116]. Interestingly, these non-cytopathic mechanisms seem also to contribute to cccDNA elimination from hepatocytes, at least to a certain degree [116]. Final termination of acute HBV infection is associated with increased intrahepatic CD8+ T cell activity and surge of serum ALT indicating extensive killing of HBV infected cells [116].

Considering the stealth quality of HBV, it remains to be determined what exactly triggers the adaptive immune response to HBV in the absence of the activating signals from virus infected cells. Recently, Cheng et al. proposed that high titer HBV could trigger monocyte-derived macrophages to produce pro-inflammatory cytokines [113]. This hypothesis would be in line with the known capacity of Kupffer cells (i.e. liver resident macrophages) and dendritic cells to constantly sample their environment for the presence of pathogens without being productively infected [117, 118]. Thus, it is conceivable that HBV, once it reaches a high enough titer during viral spread, could activate macrophages to produce the cytokines necessary to activate the cellular and humoral immune response targeting HBV. However, further investigation will be required to validate this hypothesis in natural acute HBV infection.

As previously mentioned, in CHB, T cell responses are not capable of clearing the infection. Viral clearance still can happen in some patients, but with a very low frequency (~1% per year, [61]). In the HBeAg-negative phase of CHB, however, 70-90% of patients will have the virus under control, with viral load becoming undetectable and with no signs of inflammation (low serum ALT, minimal necroinflammation) or disease progression [53]. In this so-called “HBeAg-negative chronic infection” (ENCI) stage only presence of serum HBsAg marks the infection. At the moment it is unclear what (and how) is controlling the virus during the ENCI stage, as there is no measurable immune activity. However, as in the case of a resolved hepatitis B, immunosuppression can lead to a reactivation of the virus, arguing that immune system plays an active role in keeping virus under control in this stage [62]. From our own work, performed as a part of this thesis (see section 5.3) it appears that HBV replication is specifically inhibited in the ENCI patients compared to ENCHB patients and HBeAg-positive patients, at a step downstream of pgRNA production (see section 5.3). The mechanisms of such inhibition are currently under investigation. Understanding these mechanisms of control could lead to therapeutic strategies inducing this “natural” state of viral suppression.

# 4. AIMS OF THE THESIS

As of today, a robust and versatile immunocompetent HBV infection model is still elusive. Therefore, many aspects of HBV immunobiology are still poorly understood. Consequently, therapeutic options are currently mostly limited to replication inhibitors. Although they are very effective, they do not eliminate the viral cccDNA and thus are not curative. It is widely accepted that resolution of HBV infection will depend on effector functions of the innate and/or adaptive immune systems. However, studying intrahepatic host-virus interactions has become increasingly difficult since research involving chimpanzees, the only non-human primate HBV infection model, has been severely restricted for ethical reasons. To overcome this limitation, we sought to address these questions in surplus human liver needle biopsy tissue collected for diagnostic purposes. Access to freshly obtained liver biopsy tissue and a large human liver biopsy biobank at the University Hospital Basel provided the unique opportunity for us to study specific intrahepatic host-virus interactions as outlined below. A better understanding of these aspects will hopefully facilitate development of novel HBV therapy approaches.

1. A long-standing controversy in the HBV field is i) whether HBV can be sensed by infected hepatocytes and thereby triggers an innate immune response, and ii) whether HBV can efficiently suppress induction and/or effector functions of innate immune responses in the liver. To answer these questions, we first established a short-term *ex vivo* liver biopsy culture system using freshly obtained liver needle biopsy collected from chronically HBV infected and uninfected control patients. We then used this system to determine whether the innate immune system is activated in the liver of CHB patients and whether experimentally triggered innate immune responses are blocked the HBV positive (and/or negative) cells in the HBV infected liver. The results of this work have been published in Suslov et al. [112] and are presented in section 5.1. The potential therapeutic implications of these results for are then discussed in the section 5.2.

2. As described above, the hallmarks of the "HBeAg-negative chronic infection" (ENCI) phase of CHB are very low/no viral load in the absence of any liver disease. The mechanism(s) responsible for the efficient suppression of virus production in this

phase however are not very well understood. Likewise, it is not known what steps in the viral life cycle are inhibited in the liver of ENCI patients. In a first step to address these questions, we used selected human biopsy samples of different disease phases of CHB to identify the step(s) in the viral life cycle that are inhibited in the liver of ENCI patients. The results of these studies are presented as a manuscript draft in the section 5.3. These studies will be instrumental in identifying the host cellular mechanism(s) that control HBV in this phase and ultimately might provide the basis for the development of novel HBV therapy strategies.

# 5. METHODS, RESULTS AND DISCUSSION

## 5.1 Hepatitis B virus does not interfere with innate immune responses in the human liver

Suslov, A., Boldanova, T., Wang, X., Wieland, S. and Heim, M.H. *Hepatitis B Virus Does Not Interfere With Innate Immune Responses in the Human Liver.* Gastroenterology, 2018. **154**(6): p. 1778-1790.

For supplementary material please refer to the Appendix A of this thesis.

# Hepatitis B Virus Does Not Interfere With Innate Immune Responses in the Human Liver

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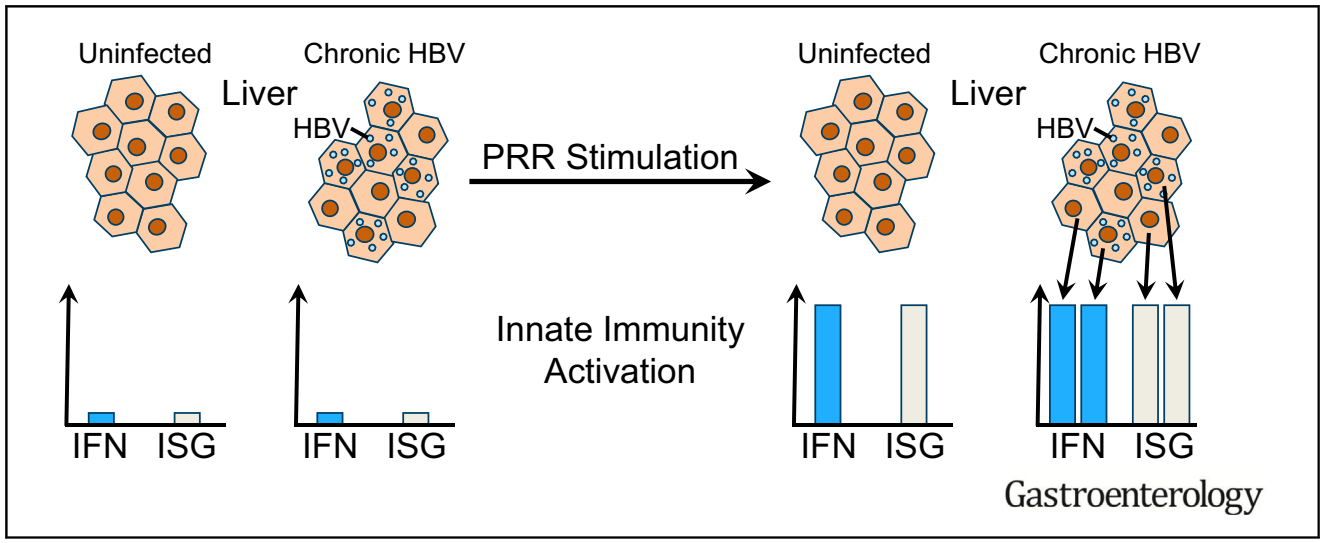


**See Covering the Cover synopsis on page 1562.**

**BACKGROUND & AIMS:** Most viruses are detected at early stages of cell infection and induce an innate immune response mediated by production of interferons (IFNs). IFNs induce expression of hundreds of IFN-stimulated genes (ISGs). Infection of chimpanzees with hepatitis C virus, but not hepatitis B virus (HBV), induces ISG expression in the liver. HBV might not induce an innate immune response because it is not detected by pattern recognition receptors (the stealth properties of HBV) or because HBV suppresses IFN production or signaling despite detection by pattern recognition receptors. We studied innate immune signaling in liver biopsies from patients with different stages of chronic HBV infection and uninfected individuals (controls). **METHODS:** We obtained liver within 10 minutes after collection from 30 patients with chronic HBV infection (hepatitis B e antigen-positive or -negative, with or without hepatitis) and 42 controls (most with fatty liver disease). The liver tissues were analyzed by histology, immunohistochemistry, quantitative reverse-transcription polymerase chain reaction, in situ hybridization, HBV RNA quantification, and HBV genotyping; some specimens were incubated with toll-like receptor (TLR) ligands (polyinosinic-polycytidylic acid) or infected with Sendai virus and then analyzed. **RESULTS:** Liver specimens from patients with HBV infection were not expressing more IFN or ISGs than those from control patients, indicating that chronic HBV infection did not activate an innate immune response. However, liver specimens from patients with HBV infection did produce IFN and induce expression of ISGs following activation of TLR3 with poly(I:C) or Sendai virus infections, so the innate immune response is not suppressed in these tissues. **CONCLUSION:** Liver tissues from patients with chronic HBV infection do not have induction of an innate immune response, but this response can be activated by other factors (TLR3 binding, Sendai virus infection) in HBV-infected liver tissue. These findings support the hypothesis that HBV is invisible to pattern recognition receptors.

*Keywords:* PRR; Virus Immune Evasion; PAMP; Ex Vivo.

[§]Authors share co-senior authorship.

***Abbreviations used in this paper:*** **CHB, chronic hepatitis B; CTRL, uninfected control; dsRNA, double-stranded RNA; HBcAg, HBV core protein; HBeAg, hepatitis B e antigen; HBsAg, HBV surface protein; HBV, hepatitis B virus; IFN, interferon; ISG, interferon-stimulated gene; ISH, in situ hybridization; JAK-STAT, Janus-associated kinase–Signal Transducer and Activator of Transcription; Mx1, Interferon-induced GTP-binding protein Mx1; OCT, optimum cutting temperature compound; poly(I:C), polyinosinic-polycytidylic acid; PRR, pattern recognition receptor; pSTAT1, phosphorylated STAT1; RIG-I, retinoic acid–inducible gene I; RLR, RIG-I-like receptor; SeV, Sendai virus; ssRNA, single-stranded RNA; TLR, Toll-like receptor.**

**Most current article**



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**EDITOR'S NOTES**

BACKGROUND AND CONTEXT

Unlike hepatitis C virus (HCV), hepatitis B virus (HBV) does not induce expression of hundreds of interferon (IFN)-stimulated genes (ISGs) in early stages of cell infection.

NEW FINDINGS

Liver tissues from patients with chronic HBV infection do not have induction of an innate immune response, but this response can be activated by other factors in HBV-infected liver tissue.

LIMITATIONS

The authors did not obtain biopsies from acutely HBV infected patients; overwhelming contribution of uninfected, normally functioning liver cells could have interfered with analysis.

IMPACT

These findings support the hypothesis that HBV is invisible to pattern recognition receptors. Further study is needed to decipher how HBV avoids detection and immune activation.

Most viruses activate the innate immune system in the cells they infect, because they bring along, or generate so-called pathogen-associated molecular patterns (PAMPs) (typically viral genomes or replication intermediates) that the host cell recognizes as foreign.[1] Cells detect those PAMPs using pattern recognition receptors (PRRs), such as cytoplasmic retinoic acid–inducible gene I (RIG-I)-like receptors (RLR) that specifically detect 5′-triphosphate-containing RNA and double-stranded RNA (dsRNA) in the cytoplasm of infected cells, and endosomal toll-like receptors (TLRs) that detect incoming dsRNA (TLR3), single-stranded RNA (ssRNA) (TLR7/8), or CpG motif-containing unmethylated DNA (TLR9).[1] The activation of these sensory pathways results in production of interferons (IFNs) and expression of interferon-stimulated genes (ISGs) that limit viral replication and spread.[2] During evolution, viruses have developed numerous strategies to escape from the host innate immune system, often involving active suppression of corresponding sensory pathways.[3]

Hepatitis B virus (HBV) is a small hepatotropic, noncytopathic DNA virus infecting humans and chimpanzees.[4] On primary infection, HBV spreads throughout the liver infecting up to 100% of hepatocytes and producing very high virus titers (up to $\sim 10^9$ – $10^{10}$ particles per mL of serum) until after 6 to 10 weeks the adaptive immune response takes control over the virus, which happens in approximately 90% of immunocompetent adults.[4,5] Approximately 5% to 10% of adult HBV infections and virtually all mother-to-infant transmissions result in chronic infection. Chronic hepatitis B (CHB) can lead to cirrhosis and liver cancer. It is estimated that HBV infections cause up to approximately 800,000 liver-related deaths per year worldwide.[6,7]

In vivo studies with experimentally infected chimpanzees showed that HBV does not induce an IFN/ISG response in the infected hepatocytes when it spreads through the liver.[8] In agreement with that, no induction of type I/III IFN was detected in the serum of human patients with acute hepatitis B infection.[9] These results suggested that the virus might not be detected by PRRs in infected cells, leading to the concept of HBV behaving like a "stealth virus."[10] Alternatively, HBV could actively interfere with downstream sensory pathways and suppress IFN induction despite being detected by PRRs. Evidence for such a transient activation followed by viral suppression of sensory pathways comes from recent work in cell culture.[11–14] Of note, one report described that early after HBV infection cells lose their ability to induce IFN-$\beta$ in response to stimulation with poly(I/C) or Sendai virus (SeV) infection.[12] Finally, HBV could also block IFN-stimulated signal transduction through the JAK-STAT (Janus-associated kinase–Signal Transducer and Activator of Transcription) pathway to inhibit ISG induction in the liver. It is well known that efficient ISG induction depends on the amplification of the initial danger signal through autocrine stimulation of the IFN receptors followed by JAK-STAT signaling.[15] Inhibition of IFN signaling by HBV infection or overexpression of viral proteins has been demonstrated in cell culture work[16–18] and more recently in a humanized mouse model.[19]

Despite this substantial evidence for an active role of HBV in suppressing innate immunity, our knowledge of the innate immune response to HBV is still hampered by technical limitations. HBV in vitro model systems do not accurately reflect the situation of in vivo HBV infection, as they are typically conducted with much higher virus and subviral particle concentrations than those achieved during natural HBV infection in humans or chimpanzees. Data from early infection states in humans are very sparse because of the difficulty in recruiting patients at the earliest presymptomatic stages of HBV infections. Experiments with chimpanzees are limited by ethical constraints and high costs.

In the present work, we developed and validated an ex vivo method using freshly obtained liver biopsies from patients with different stages of chronic HBV infection and from controls. Although we could not investigate patients with early acute HBV infection, we reasoned that inhibition of innate immunity by HBV should by detectable in ex vivo liver tissue when liver cells would be stimulated with TLR agonists or by productive viral infections.

The ex vivo analysis of liver tissue turned out to be a robust and highly informative experimental system. Freshly obtained human liver biopsies could be cultured for a least 24 hours without significant cell death or RNA degradation. The samples could be treated with TLR agonists and infected with SeV. Induction of IFNs and ISGs was readily detectable and quantifiable. Comparing liver biopsies from HBV-infected patients with uninfected controls, we could not detect any inhibition of innate responses by HBV. In situ hybridization (ISH) and immunostaining techniques allowed confirmation of this finding at the cellular level. Collectively, our data unequivocally demonstrate that the cell-autonomous innate immune system in HBV-infected human liver is intact, and support the hypothesis that HBV behaves like a "stealth" virus in vivo.

## Materials and Methods

### Patients and Liver Biopsies

Liver biopsies from patients with HBV infections and from noninfected controls were obtained in the outpatient clinic of the Division of Gastroenterology and Hepatology, University Hospital Basel, Switzerland. Liver biopsies were done using an ultrasound-guided coaxial needle technique that allowed for single-stick, multiple-pass biopsies (BioPince; Peter Pflugbeil GmbH, Zorneding, Germany). The individual biopsies are approximately 29 mm long and have a diameter of 1 mm. One cylinder was used for routine histopathological diagnostic purposes. After obtaining written informed consent, an additional biopsy cylinder was used for the ex vivo experiments described in this article. The use of biopsy material was approved by the local ethics committee (Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland). Formalin-fixed liver biopsy tissue was used for histological analysis for standard ISHAK classification (ie, determination of liver inflammation and fibrosis) and for routine immunohistochemical staining for HBV surface protein (HBsAg) and HBV core protein (HBcAg). For the HBV sample set, the histopathological analysis (ISHAK grading, HBsAg- and HBcAg-positive hepatocyte fraction), the clinical data (transaminases, HBV viral load in the serum, stage of infection, quantitative HBsAg in the serum, hepatitis B e antigen [HBeAg] status) and a summary of the ex vivo experiments performed with each biopsy are shown in Supplementary Table 1. For the control sample set, the underlying liver disease and a summary of the ex vivo experiments performed with each biopsy are shown in Supplementary Table 2.

### Ex Vivo Liver Biopsy Culture and Stimulation

Right after completion of the biopsy procedure, the liver tissue cylinders were rinsed with an excess volume of 0.9% NaCl at room temperature, followed by another wash with 1.5 mL of fresh culture medium, and cut into pieces of approximately 3 to 9 mm length for experimentation. As a baseline control, pieces were immediately snap-frozen in liquid nitrogen and embedded in optimum cutting temperature compound (OCT) for subsequent total liver RNA and histological analysis, respectively. Ex vivo liver biopsy culture was initiated within 10 minutes after the biopsy procedure by transferring the biopsy pieces into individual 2-mL tubes containing fresh culture medium (Dulbecco's modified Eagle's medium + 10% fetal bovine serum [Gibco, Waltham, MA]) followed by addition of TLR ligands or SeV. The tubes were then placed in a temperature-controlled Eppendorf tube shaker and incubated at 37°C with 5 seconds shaking at 800 to 1000 rpm every 55 seconds. Samples were collected after the appropriate time of incubation and one-third (2–3 mm) and two-thirds (4–6 mm) of each biopsy piece were snap-frozen in liquid nitrogen and embedded in OCT for subsequent total liver RNA and histological analysis, respectively. For TLR 2/4/7/8/9 stimulation, 3- to 4-mm pieces of biopsies were used and only snap-frozen samples for total RNA analysis were collected.

### TLR Ligands, SeV, and Recombinant HBsAg

The following reagents were used in this study (abbreviations and final concentrations are given in parentheses): TLR3 ligand polyinosinic-polycytidylic acid (poly(I:C); 100 $\mu$g/mL), TLR2 ligand peptidoglycan from *Staphylococcus aureus* (PGN-SA; 20 $\mu$g/mL), TLR4 ligand lipopolysaccharide from *Escherichia coli* 0111:B4 strain (LPS; 20$\mu$g/mL), TLR7 ligand imiquimod (R837; 10 $\mu$g/mL), TLR8 ligand single-stranded RNA (naked ss-poly(U); 50 $\mu$g/mL), TLR9 ligand CpG DNA (ODN 2216; 1 $\mu$M), SeV H4 strain (SeV; approximate multiplicity of infection = 10), recombinant HBsAg (3.3 $\mu$g/mL). All TLR ligands except poly(I:C) (Sigma-Aldrich, Buchs, Switzerland) were purchased from InvivoGen (San Diego, CA). SeV (H4 strain) was a gift from Prof Dominique Garcin (University of Genève, Genève, Switzerland). Chinese hamster ovary cell produced recombinant HBsAg was purchased from Jena Bioscience (Jena, Germany; cat# PR-1197).

## Results

### Ex Vivo Culture, TLR Stimulation, and SeV Infection of Human Liver Biopsies

Liver biopsies can provide valuable material to study host-virus interactions in HBV infections. They have been mainly used after immediate chemical (formalin-fixed) or thermal fixation (shock freezing in liquid nitrogen or dry ice) to avoid degradation processes that presumably start shortly after removing biopsies from the liver. To gain further insights into viral interference with innate immunity, we sought to develop a method to keep intact pieces of biopsies viable for enough time to perform TLR stimulation and SeV infection experiments. To this end, biopsy cylinders were washed with saline, cut into pieces of 3 to 9 mm length and put into fetal bovine serum–complemented Dulbecco's modified Eagle's medium at 37°C within 10 minutes of the biopsy procedure (ex vivo). Biopsy pieces were then further incubated at 37°C with occasional gentle shaking that did not affect the integrity of the biopsy tissue (Supplementary Figure 1*A*). In a dose-finding experiment, we found that stimulation of biopsy pieces with poly(I:C) at a concentration of 100 $\mu$g/mL induced readily detectable expression of IFN$\beta$, IFN$\lambda$1, and IFN$\lambda$2/3 as compared with untreated controls (Supplementary Figure 2). Using this concentration, we next performed a kinetic analysis of poly(I:C)-stimulated IFN gene induction in liver biopsy pieces over a period of 36 hours. IFN$\beta$, IFN$\lambda$1, and IFN$\lambda$2/3 expression was induced within an hour and remained elevated during the entire period, with a peak of expression between 3 and 6 hours, while it remained unchanged in untreated samples (Figure 1*A* and Supplementary Figure 3*A*). In agreement with reports from experiments with primary human hepatocytes, IFN$\alpha$2 was not induced by poly(I:C).[20,21]

The slight decline of poly(I:C)-induced IFN expression at later time points could be due to cell death. Indeed, histological analysis of biopsy pieces revealed the appearance of necrotic areas by 24 and 48 hours of ex vivo culture (Supplementary Figure 1*B*). This is consistent with the number of dead cells in the biopsies, as visualized by dead cell staining, that increased from 3.5% at time point 0 hour to 7.1%, 17.7%, and 37.0% at times points 6 hours, 24 hours, and 48 hours, respectively (Supplementary Figure 1*C*). Treatment of biopsies with poly(I:C) had no impact on the

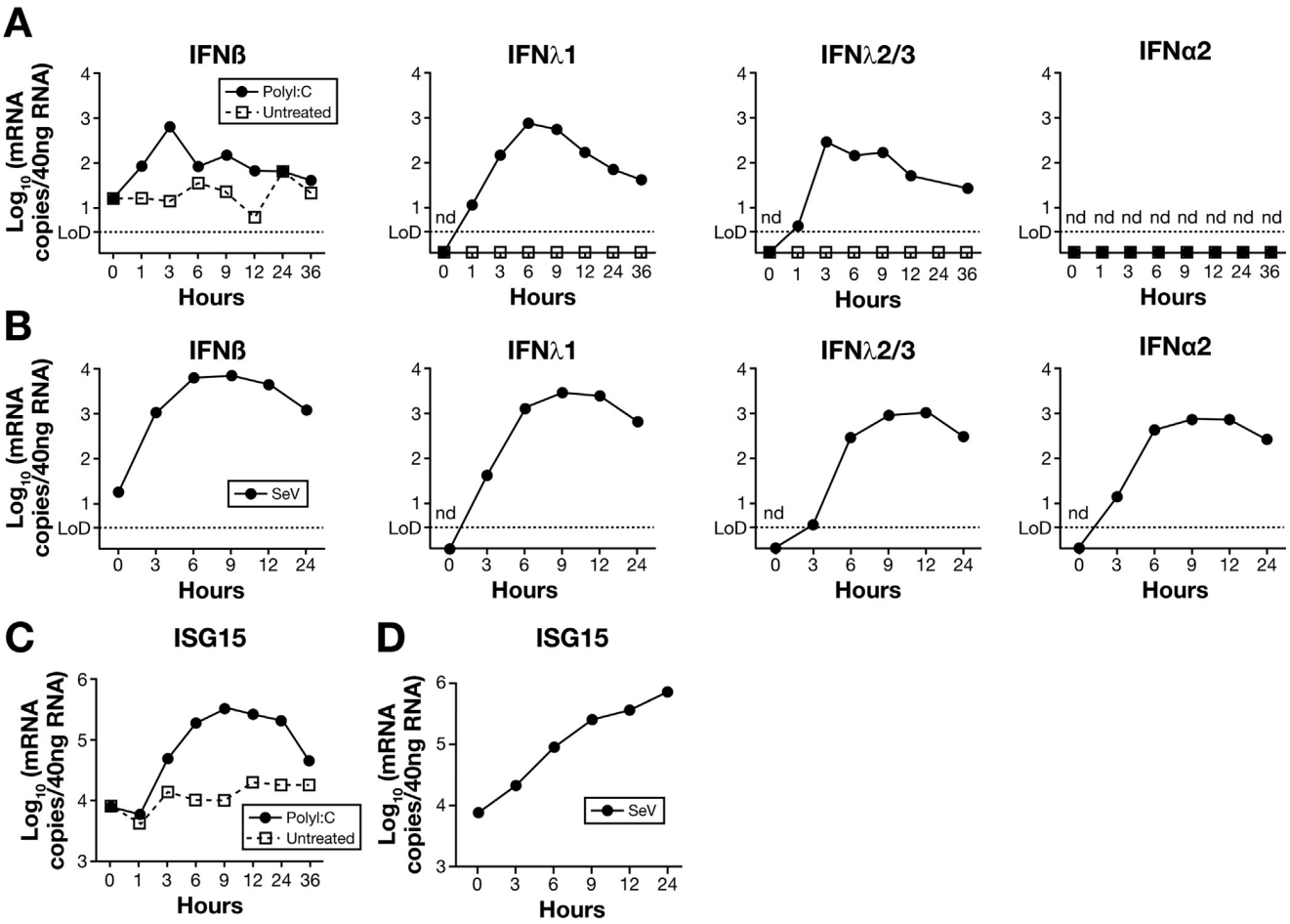


**Figure 1.** Ex vivo stimulation of human liver biopsies with poly(I:C) or SeV induces IFN and ISG expression. Fresh biopsies of patients without viral infections were cut into several pieces and immediately stimulated ex vivo with poly(I:C) (100 $\mu$g/mL) or SeV (multiplicity of infection ~10) or left untreated for up to 36 hours. Expression of IFN mRNA (*A*, *B*) and ISG15 mRNA (*C*, *D*) was analyzed by reverse-transcription–quantitative polymerase chain reaction at the indicated time points. LoD, Limit of Detection; nd, not detected. Shown are the data from representative patients (C740 in *A* and *C*, D166 in *B*). Additional samples are shown in Supplementary Figure 3.



kinetics or the extent of cell death (Supplementary Figure 1*D*). Of note, RNA integrity analysis of equal volumes of RNA samples isolated from different pieces of the same biopsy revealed no apparent RNA degradation for up to 48 hours of ex vivo culturing (Supplementary Figure 4*A*). The expression of liver-specific genes for albumin, HFN-1b and CYP3A4, was maintained during the first 12 hours, and HNF-4a during the first 6 hours in culture (Supplementary Figure 4*B*). Albumin secretion was detectable in the first 24 hours in culture (Supplementary Figure 4*C*).

We next infected ex vivo–cultured biopsy pieces with the SeV strain H4 that produces defective interfering dsRNA genomes and induces IFNs via RIG-I/MDA5.[22–24] As shown in Figure 1*B*, all IFNs, including IFN$\alpha$2, were strongly induced by SeV during ex vivo culturing of liver biopsy pieces, albeit with kinetics slightly different from poly(I:C)-induced responses, reaching maximal levels 9 to 12 hours after addition of the virus (Figure 1*A* and *B*, Supplementary Figure 3*A* and *B*). Of note, SeV stimulation induced up to 10-fold higher peak levels of IFNs compared with poly(I:C) (Figure 1*A* and *B*). To test if induction of IFNs in the ex vivo–treated liver biopsies translates into a functional response through stimulation of IFN signal transduction, we analyzed the expression of a classical interferon-stimulated gene ISG15. Both poly(I:C) and SeV strongly induced ISG15 expression (Figure 1*C* and *D*, Supplementary Figure 3). As expected for a secondary response, the kinetics of ISG15 expression was delayed compared with that of the IFN genes.

To determine whether IFN and ISG induction was triggered in the parenchymal and/or nonparenchymal compartment in the liver, we performed multiplex ISH in frozen sections of ex vivo poly(I:C)-treated or SeV-infected and -untreated control liver biopsies. We used an albumin-specific probe to mark hepatocytes, and costained the sections with probes for IFN$\beta$ or ISG15. Although IFN$\beta$ expression was detectable in SeV-infected samples (Figure 2*A*), it was undetectable in poly(I:C)-treated samples (data not shown), most likely because its expression is 10 times lower in poly(I:C)-treated samples. IFN$\beta$ was detected both in hepatocytes and in nonparenchymal cells (Figure 2*A*). Both poly(I:C) and SeV induced strong

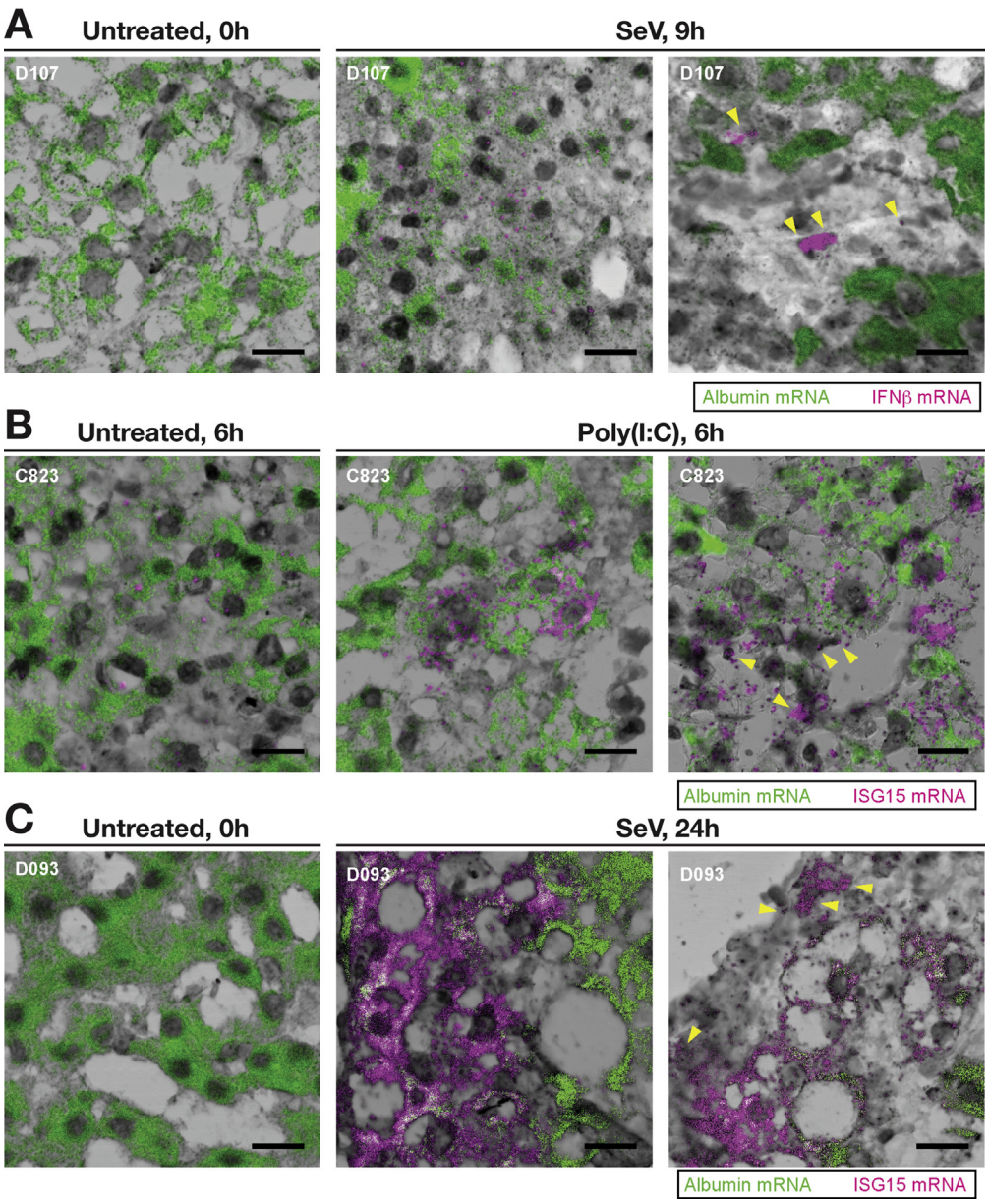


**Figure 2.** Induction of IFN$\beta$ and ISG15 expression in parenchymal and nonparenchymal cells in human liver biopsy tissue upon ex vivo stimulation with poly(I:C) or SeV. Fresh liver biopsy pieces of patients without viral infections were stimulated ex vivo with SeV (multiplicity of infection ~10), 100 $\mu$g/mL poly(I:C) or left untreated for the indicated time periods. The tissue was embedded in OCT, cryopreserved, and then subjected to ISH analysis. (*A*) Multiplex detection of IFN$\beta$ mRNA (magenta) and human albumin mRNA (green) in human liver biopsy pieces stimulated ex vivo with SeV. Nonparenchymal cells are indicated with yellow arrows. (*B, C*) Multiplex detection of ISG15 mRNA (magenta) and albumin mRNA (green) in human liver biopsy pieces stimulated ex vivo with poly(I:C) (*B*) or SeV (*C*). Nonparenchymal cells are indicated with yellow arrows. Biopsy IDs are given in the upper left corner. *Scale bar* = 20 $\mu$m.



expression of ISG15 in albumin-positive and -negative cells (Figure 2*B* and *C*, respectively), indicating that (1) IFN proteins are produced and secreted by liver cells, and (2) IFN signaling is not restricted to a specific cell type in the ex vivo–cultured liver biopsy pieces. Taken together, these results demonstrate that ex vivo–cultured liver biopsy pieces are suitable to study both IFN induction and IFN signaling.

### *IFNs Can Be Induced Normally in Ex Vivo Liver Biopsies From HBV-Infected Patients*

The ex vivo biopsy technology was then used to study innate immune responses in the liver of patients with chronic HBV infections. We selected a total number of 30 HBV-infected patients who represent all the different stages of chronic HBV infection, including HBeAg+ infection,[25] HBeAg+ hepatitis,[25] HBeAg− infection,[25] HBeAg− hepatitis,[25] and patients undergoing antiviral therapy (Supplementary Table 1). Clinical data, routine histopathology, HBV immunohistochemistry, intrahepatic HBV RNA quantification, and HBV genotyping are shown in Supplementary Table 1. Our HBV-infected cohort included samples of equally high HBsAg/HBcAg positivity (ie, >80%) in the liver, but also some patients of disproportionally high HBsAg positivity in the liver. Although the latter situation is frequently observed in CHB, the underlying molecular explanation is not known. As a control cohort, 42 patients without any viral infection were included. Most suffered from alcoholic or nonalcoholic fatty liver disease (Supplementary Table 2).

As expected from our pilot experiments described previously, there was no induction of IFNs in the untreated

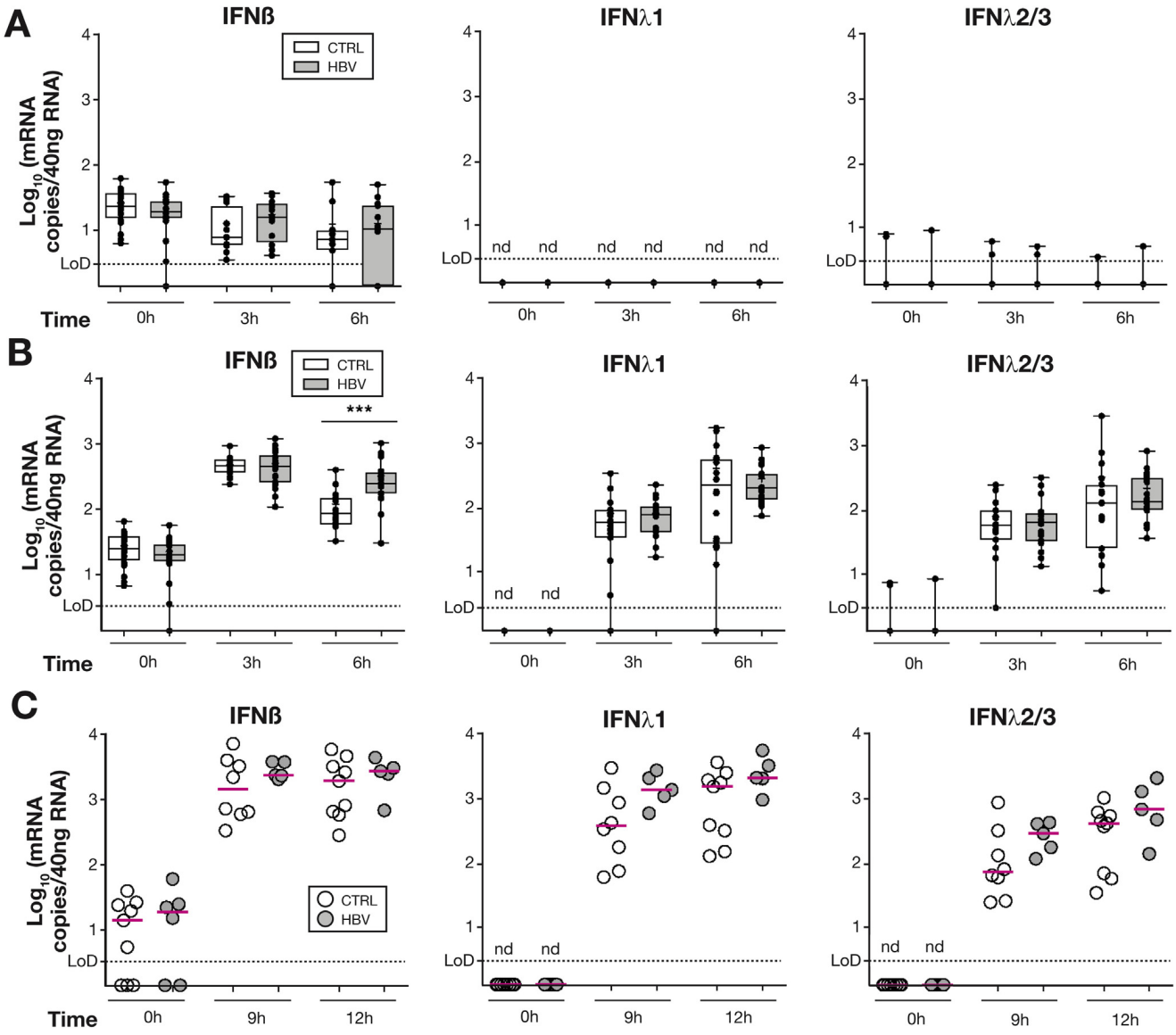


**Figure 3.** poly(I:C)- and SeV-induced IFN expression is not suppressed in the HBV-infected liver. Fresh liver biopsy pieces obtained from HBV-infected (HBV) and uninfected control (CTRL) patients were cut into several pieces and incubated for 3 to 12 hours or immediately processed as a baseline control (0 hour). (*A*) Biopsy pieces were cultured in complete medium only (untreated) or (*B*) stimulated with 100 $\mu$g/mL poly(I:C) or (*C*) infected with SeV (multiplicity of infection ~10). Total RNA was isolated from the baseline and treated samples and subjected to IFN-$\beta$, IFN-$\lambda$1, and IFN-$\lambda$2/3 specific reverse-transcription–quantitative polymerase chain reaction. Data are shown as box plots (n = 15–24) for (*A*) and (*B*), or dot plots (n = 5–9) with lines indicating the median for (*C*). Nonparametric Mann-Whitney test was used to compare IFN expression between HBV and CTRL groups (*$P < .05$, **$P < .01$, ***$P < .001$). LoD, limit of detection; nd, not detected.

samples, whereas poly(I:C) strongly induced IFN$\beta$, IFN$\lambda$1, and IFN$\lambda$2/3 in samples from patients without HBV infection (Figure 3*A* and *B*, uninfected control [CTRL]). This was not different in samples from HBV-infected patients (Figure 3*A* and *B*, HBV). The results remained the same in subsets of samples with >90% of HBsAg-positive hepatocytes or HBcAg-positive hepatocytes (Supplementary Figure 5*A* and *B*), confirming that signaling in HBV-positive cells was not suppressed. In addition, exogenous addition of HBV and viral antigens in the form of CHB patient–derived serum or recombinant HBsAg did not alter poly(I:C)-induced interferon expression in ex vivo–cultured liver biopsies (Supplementary Figure 6*A* and *B*).

Of note, levels of HBsAg and HBV DNA were increasing in the culture supernatants of HBV-positive samples over time (Supplementary Figure 7*A* and *B*), indicating that active HBV gene expression and replication continued in the ex vivo–cultured biopsy pieces. These results indicate that ongoing HBV infection does not suppress TLR3-mediated IFN induction in the liver. Furthermore, baseline IFN levels were not elevated in HBV-infected samples, suggesting that HBV does not induce IFN$\beta$ or IFN$\lambda$s in human liver.

HBV infection and HBV proteins have been shown to interfere with the expression and/or function of several other innate immune sensors and signaling pathways in cell culture or in mouse models, including various TLRs (TLR 2/4/7/8/9) and also RLRs (response to SeV infection) and IFN$\alpha$ signaling.[12,17,19,26–30] We therefore extended our analysis to other TLR family members and measured induction of type I/III IFNs in ex vivo–treated biopsies. Because for some TLRs (eg, TLR2) ligand binding mostly results in the production of proinflammatory cytokines, but not IFNs,[1] we also measured induction of the proinflammatory cytokines tumor necrosis factor alpha and interleukin-6. Stimulation of TLR8 and TLR9 with ssRNA and CpG, respectively, had no effect on the expression of these IFNs and cytokines, compared with untreated control samples (Supplementary Figure 8). This is not surprising, because TLR8 and TLR9 expression in the liver is very low, and signaling through these receptors has been so far mostly ascribed to immune cells (eg, plasmacytoid dendritic cells, which are low-abundant in human liver).[1,31] Furthermore, ssRNA (TLR8 ligand) most likely would need to be complexed with cationic lipids to produce a response.[32] Stimulation of TLR2, TLR3, TLR4, and TLR7 induced 2 or more of the tested cytokines. But importantly, there was again no difference between HBV-infected and -uninfected liver biopsy samples (Supplementary Figure 8).

We then tested whether HBV could prevent induction of innate immune responses by another virus. To that end, liver biopsy samples from HBV-infected and -uninfected patients were infected with SeV-H4. The induction of IFNs was measured by reverse-transcription–quantitative polymerase chain reaction at different time points after infection. Again, we could not detect an inhibition of SeV-induced IFN (Figure 3*C*) and other cytokine (Supplementary Figure 9) expression in HBV-infected samples or in noninfected samples incubated with recombinant HBsAg (Supplementary Figure 6*C*).

Taken together, these results demonstrate that sensory pathways downstream of PRRs are not inhibited in HBV-infected human liver biopsy samples.

## IFN Induction Is Intact in HBV-Infected Human Hepatocytes

The analysis of IFN induction using extracts from liver biopsy pieces reflects an average response from all cells in the biopsy, those that are HBV-infected as well as uninfected cells. It is therefore conceivable that we missed an inhibitory effect of HBV on sensory pathways because of the contribution of noninfected cells. The fact that >50% of our samples contained ≥90% of HBV-positive cells, and still had no impaired response to poly(I:C) (Supplementary Figure 5*A*) provides strong evidence against such a scenario. Nevertheless, we wanted to address this question directly, and analyzed IFN$\beta$ induction at the cellular level in a liver biopsy from an HBV-infected patient (80% HBsAg-positive hepatocytes by immunohistochemistry) on stimulation with SeV. We used multiplex ISH to simultaneously detect HBV RNA and IFN$\beta$ mRNA. In accordance with our previous results in noninfected biopsy (Figure 2*A*) IFN$\beta$ mRNA was detectable by ISH in the SeV-infected, but not in uninfected control biopsies, and more importantly, IFN$\beta$ mRNA was also detected in HBV RNA-positive hepatocytes (Figure 4). These results demonstrate that HBV does not interfere with IFN$\beta$ induction in infected hepatocytes.

## HBV Does Not Block IFN-induced JAK-STAT Signaling and ISG Induction in Human Hepatocytes

Having established that IFN induction is not blocked in HBV-infected human hepatocytes in vivo, we analyzed whether HBV could interfere with IFN signaling and subsequent ISG induction, as was previously reported in chimeric mice with HBV-infected human hepatocytes.[19] We chose nuclear translocation of phosphorylated STAT1 (pSTAT1) as a marker of IFN signaling. We selected 2 liver biopsies that showed positivity for HBsAg and HBcAg in 99% and 70% to 80% of hepatocytes, respectively (patients C787 and C799, Supplementary Table 1). The biopsies were treated ex vivo with 100 $\mu$g/mL poly(I:C) or left untreated for 6 hours. We then costained the biopsies with antibodies against pSTAT1 and HBcAg. Because both signals are nuclear, this approach allowed an unequivocal detection of double-positive cells. pSTAT1 was clearly detected in HBcAg-positive nuclei (Figure 5), indicating that IFN signaling is not suppressed by HBV in the infected cells. Of note, the frequency of pSTAT1-positive cells was not higher in the subset of HBcAg-negative cells compared with HBcAg-positive cells (Figure 5). A similar analysis using antibodies against HBsAg and pSTAT1 confirmed that IFN signaling was also not blocked in the HBsAg-positive cells (Supplementary Figure 10). The overall percentage of pSTAT-positive cells was not different between the HBV-positive biopsies and the control biopsy and reached approximately 32%, 45%, 55%, and 44%, respectively (Supplementary Figure 10). Again, in the HBV-infected sample C765 with 70% HBsAg-positive hepatocytes, the frequency of pSTAT1-positive cells was not higher in the subset of HBsAg-negative cells compared with HBsAg-positive cells (Supplementary Figure 10).

Next, we analyzed whether IFN induction and signaling in the ex vivo poly(I:C)-stimulated biopsies translates into induction of ISG expression. As shown in Figure 6, baseline (0 hour) ISG expression as well as expression in untreated samples was independent of HBV infection, in agreement with the lack of IFN expression in HBV-infected samples (Figure 3*A*) and indicating that ISGs are neither up- nor down-regulated during chronic HBV infection. On stimulation with poly(I:C), all 3 ISGs were induced in both groups of biopsies and with the exception of Mx1 (interferon-induced GTP-binding protein Mx1) at 6 hours of stimulation, the induction did not differ between the 2 groups (Figure 6*B*). Although there was a statistically significant difference in absolute Mx1 transcript levels between HBV and CTRL group after 6 hours of poly(I:C) stimulation, the overall fold-change induction remained similar (~9-fold change in HBV vs ~11-fold change in CTRL; data not shown). Again,

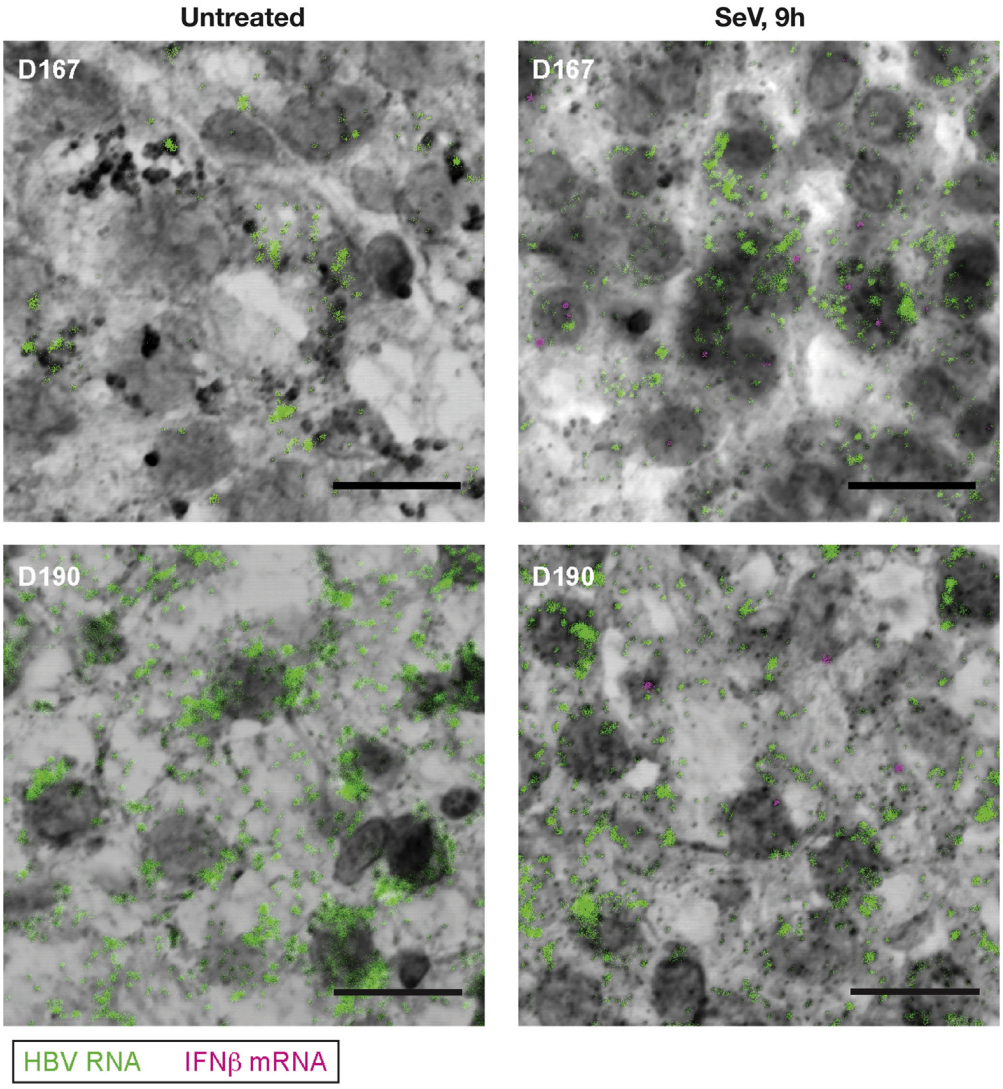


**Figure 4.** HBV-infected hepatocytes are not refractory to induction of IFN expression. Fresh liver biopsy pieces obtained from HBV-infected patients (Supplementary Table 1, D167, D190) were embedded in OCT after 9 hours of incubation in the presence of SeV (multiplicity of infection ~10) or immediately processed as a baseline control (untreated). OCT-embedded tissues were processed for multiplex ISH analysis of IFN$\beta$ mRNA (magenta) and total HBV RNA (green) as described in Material and Methods. The biopsy ID is given in the upper right corner of each image. *Scale bar* = 20 $\mu$m.

experiments with SeV-infected ex vivo biopsies confirmed that HBV infection does not inhibit ISG induction by heterologous viral stimulation of PRR sensory pathways (Figure 6*C*). Poly(I:C)- and SeV-induced ISG expression in ex vivo–cultured liver biopsies was also not affected by adding serum from patients with CHB or recombinant HBsAg to the culture medium (Supplementary Figure 6*D–F*).

Analysis of ISG induction in ex vivo–treated liver biopsies during a time period of up to 12 hours of stimulation with TLR 2/3/4/7/8/9 ligands (Supplementary Figure 11), and up to 24 hours of stimulation with SeV (Supplementary Figure 12), revealed the same pattern of ISG induction as was observed for IFNs (Supplementary Figure 8). No ISG induction could be detected on treatment with TLR 8 and 9 ligands (data not shown), correlating with the lack of significant IFN induction under the same conditions (Supplementary Figure 8). For other stimuli (TLR 2/3/4/7 ligands and SeV), induction of ISGs correlated with the induction of IFNs under the same conditions (Supplementary Figures 8, 9, 11, 12). The lack of ISG induction in the TLR2-stimulated samples (Supplementary Figure 11) suggests that the minor induction of IFN$\lambda$2/3 (Supplementary Figure 8) is not sufficient to trigger IFN signaling. Most importantly, whenever ISG induction was triggered by any given treatment, the induction was not different between HBV and CTRL biopsies (Supplementary Figure 11).

To exclude that ISG induction was restricted to HBV-negative (ie, noninfected) hepatocytes, we again performed multiplex ISH analysis of sections from liver biopsies after ex vivo treatment with poly(I:C) or infection with SeV H4. HBV-infected cells were visualized using a HBV RNA-specific probe and ISG induction was tested using an ISG15 mRNA-specific probe. ISG15 expression was strongly induced by poly(I:C) and by SeV (Figure 7). Importantly, ISG15 was readily detected in HBV-positive cells, demonstrating that HBV does not interfere with ISG induction at a cellular level (Figure 7).

Together, these results demonstrate that IFN and ISG expression is not induced during chronic HBV infection and that HBV does not interfere with induction of innate responses or with IFN signaling in the human liver.

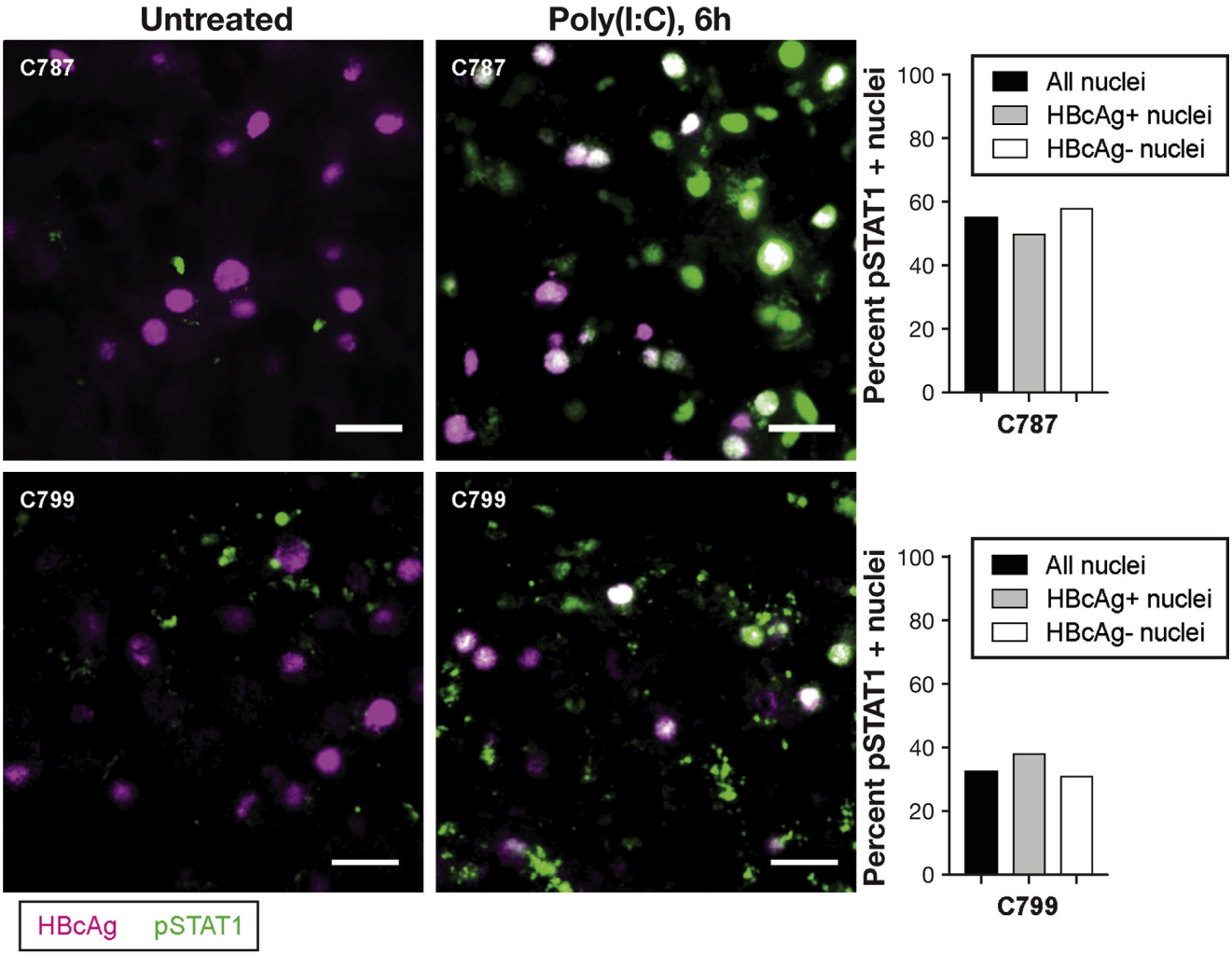


**Figure 5.** IFN signaling is not blocked in HBV-infected hepatocytes. Fresh liver biopsy pieces from 2 highly HBV-viremic patients (Supplementary Table 1, C787, C799) stimulated ex vivo with 100 $\mu$g/mL poly(I:C) or left untreated for 6 hours were embedded in OCT for cryosectioning. Cryosections were subjected to simultaneous immunofluorescence detection of nuclear HBcAg (magenta) and phospho(p)STAT1 (green). HBcAg and pSTAT1 double-positive nuclei appear white. The percentage of nuclear pSTAT1-positive cells in HBV-positive (HBcAg+) and -negative (HBcAg−) cells was quantified for the entire sections (right panels). The biopsy ID is given in the upper left corner of each image. Scale bar = 20 $\mu$m.



## Discussion

The apparent lack of an innate immune response to HBV reported in studies with experimentally infected chimpanzees[8] has led to the concept that HBV is not recognized by the sensory systems that activate the transcription of IFN genes in infected cells (the "stealth virus concept").[10] However, the lack of IFN-stimulated gene induction could also result from active viral interference with sensory pathways or with IFN signaling, and indeed, recent work in cell culture models of HBV infection provide evidence for this scenario.[11–14] In the present work, we wanted to address this question in the most relevant system, the infected human liver. For obvious ethical reasons, experimental exploration of mechanisms of viral interference with cellular processes is not possible in humans in vivo. Patient-derived cell culture systems such as primary human hepatocytes or organoid cultures have the disadvantage of disrupting the 3-dimensional architecture of the liver. We therefore developed and validated a novel ex vivo liver biopsy technique. The system is unexpectedly robust and allows keeping human liver biopsy pieces alive for at least 24 hours. The cells can be stimulated with cytokines and TLR agonists, and even be productively infected with SeV. Further work will address the usefulness of this system for addressing pharmacodynamics questions, such as mechanism of action of drugs (for example, in patients with nonalcoholic or alcoholic steatohepatitis or in viral hepatitis). In a time of growing interest in personalized health, the system might prove to be highly valuable to address mechanisms of interindividual variations of hepatic responses to insults, viruses, and drugs.

In the present study, we applied the short-term (up to 24 hours) ex vivo liver biopsy culture system to examine the impact of HBV infection on innate immune responses.

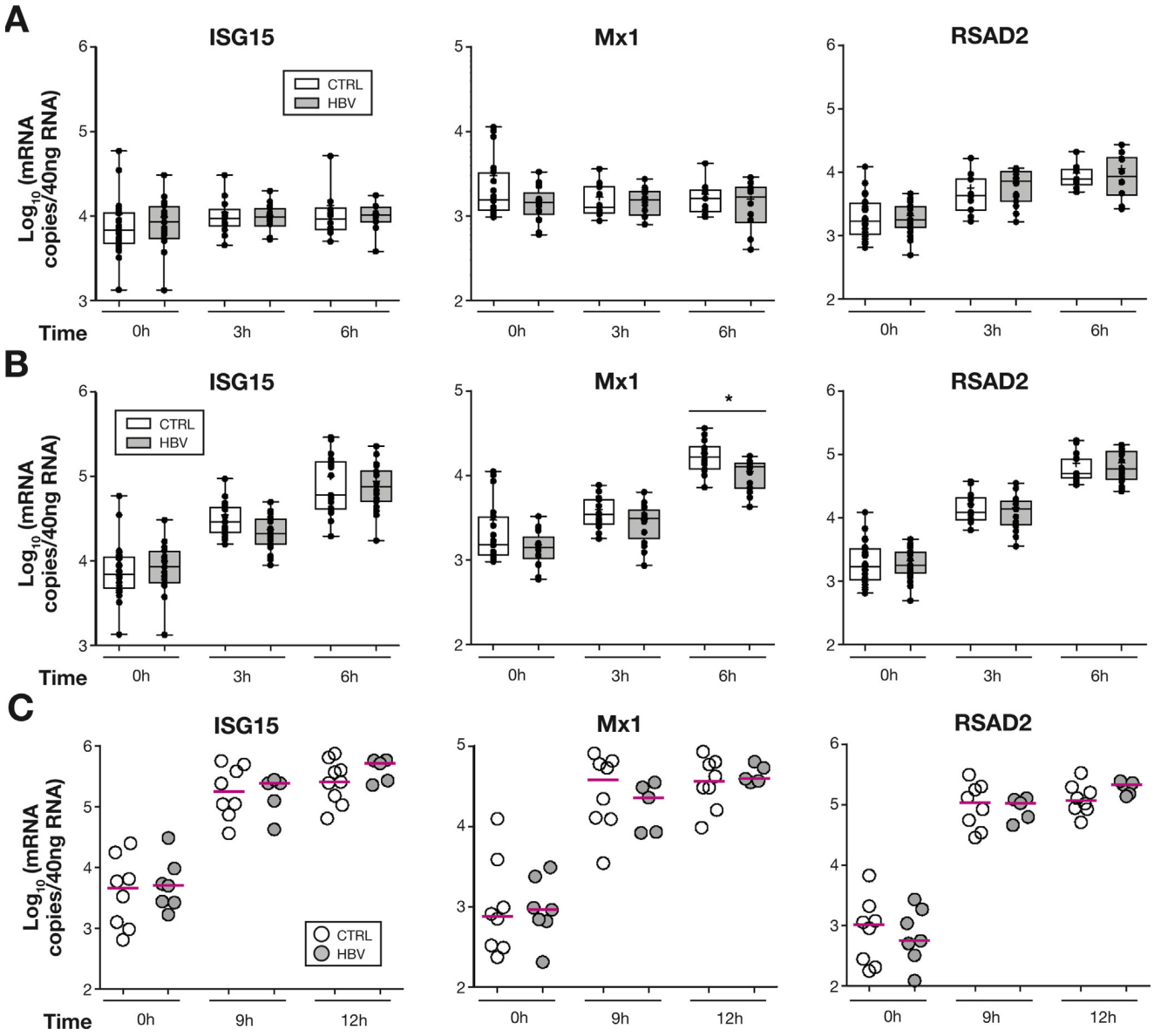


**Figure 6.** poly(I:C)- and SeV-induced ISG expression is not suppressed in the HBV-infected liver. Transcriptional induction of 3 typical ISGs (ISG15, Mx1, RSAD2 [Radical SAM domain-containing 2]) was analyzed by reverse-transcription–quantitative polymerase chain reaction in the same HBV-infected and control (CTRL) groups of untreated (*A*), poly(I:C)-treated (*B*), and SeV-treated (*C*) biopsies of HBV-infected (HBV) and uninfected (CTRL) patients used for IFN mRNA analysis in Figure 3. Reverse-transcription–quantitative polymerase chain reaction data display and statistical analysis was performed as described in the legend to Figure 3.

Consistent with previous reports in humans[9] and chimpanzees,[8] we did not observe any IFN and/or ISG induction in the liver of chronically HBV-infected patients irrespective of disease status, viral load, or HBV genotype. By stimulating ex vivo–cultured liver biopsies with different TLR ligands and SeV, we demonstrated that induction of innate immune responses as measured by IFN and ISG expression did not differ between HBV-infected and noninfected samples, suggesting that HBV infection neither induces nor interferes with innate immune responses. Of note, inductions of innate responses were very similar in all CHB samples despite them containing samples of both very high and low viremia and antegenimia and also of varying levels of liver disease. These results suggest that innate response induction was not only independent of the presence of HBV, but also the magnitude of infection and liver disease.

Although our study tested the impact of HBV on innate responses triggered by most TLRs and RLRs, we did not specifically interrogate effects on cytoplasmic DNA sensing by cGAS/STING because hepatocytes have been reported to be virtually deficient in DNA sensing.[33,34]

Incidentally, a study by Mutz and colleagues[35] also demonstrates a lack of IFN induction and suppression of innate responses in fully HBV permissive cell culture models. Together, these results differ from the observed HBV-mediated induction of innate immune responses in cell

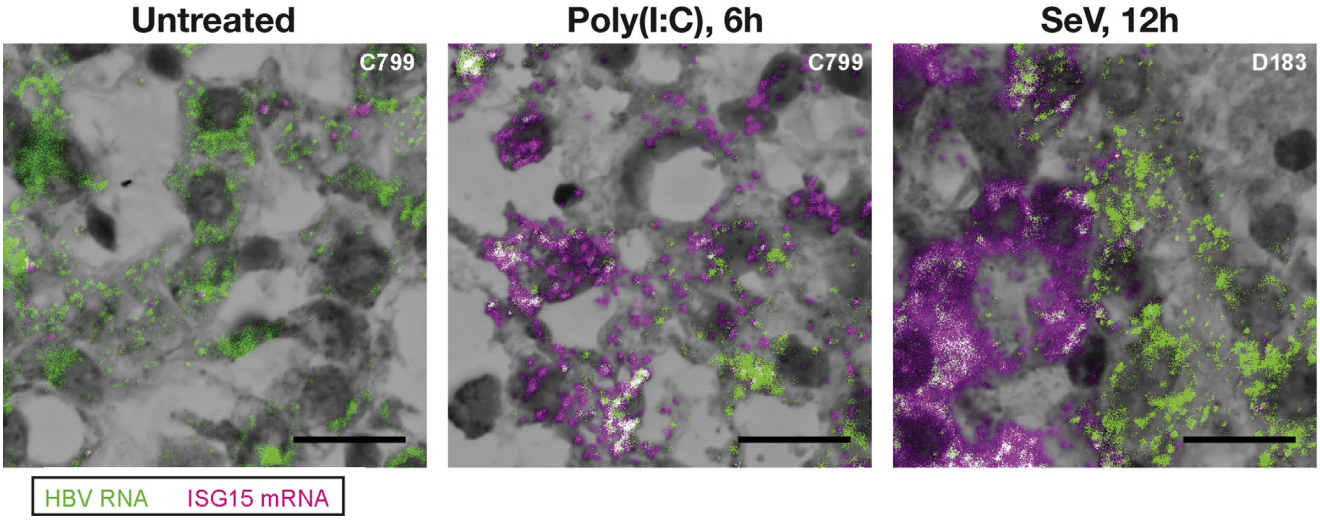


**Figure 7.** HBV-infected hepatocytes are not refractory to induction of ISG expression. Fresh liver biopsy pieces obtained from HBV-infected patients (Supplementary Table 1, C799 and D183) were embedded in OCT after 6 or 12 hours of incubation in the absence (untreated) or presence of 100 $\mu$g/mL poly(I:C) or SeV (multiplicity of infection ~10) (SeV), respectively. OCT-embedded tissues were processed for multiplex ISH analysis of ISG15 mRNA (magenta) and total HBV RNA (green) as described in Material and Methods. The biopsy ID is given in the upper right corner of each image. *Scale bar* = 20 $\mu$m.

culture models[11–13] and the apparent suppression of innate responses by HBV that were previously reported.[12,17,19,29] The discrepancies to our data could likely be a reflection of specific limitations of the different in vitro culture and murine models, including lack of proper human parenchymal and nonparenchymal liver tissue organization and artificially induced HBV gene expression and replication, that would not apply to studies in the human liver biopsy ex vivo culture system. Our results also differ from publications that reported a down-regulation of a number of genes belonging to multiple innate immunity pathways[36] or a down-regulation of TLR2 on hepatocytes and Kupffer cells[26] in liver biopsies of patients with CHB. Again, methodological differences between these studies and our work might underlie the discrepancies. On the other hand, our results are consistent with several other studies in patients,[9,37] chimpanzees,[8] and other HBV model systems[33,38,39] that did not detect a stimulatory or inhibitory effect of HBV on innate immunity.

Our ex vivo experimental approach has some shortcomings. First, we could not obtain biopsies from acutely HBV-infected patients, and, therefore, we cannot exclude that in the early phase of HBV infection the virus is actively suppressing IFN-inducing sensory pathways or IFN signaling. However, even in the chronic phase of HBV infection there is ongoing de novo infection of hepatocytes, and we should therefore have detected HBV interference with cellular innate immune pathways even in our chronically infected patients.

The second potential shortcoming concerns the spatial resolution of our analysis. In other words, it could be possible that HBV indeed interferes with innate immune pathways in infected hepatocytes, but our analysis missed this interference because of the overwhelming contribution of uninfected, normally functioning liver cells. We think that this is highly unlikely because we included several samples with very high hepatic infection rates documented by HBsAg and/or HBcAg positivity of hepatocytes. Indeed, we observed no statistically significant difference in IFN induction or ISG induction according to the extent of HBsAg and/or HBcAg staining in our entire study cohort. Finally, we addressed this concern directly in costaining experiments that showed nuclear phospho-STAT1 in hepatocytes that were positive for HBsAg or HBcAg (Figure 5 and Supplementary Figure 10) and by multiplex ISH studies that showed ISG mRNA expression in cells with detectable HBV RNA (Figure 7). In both analyses, we did indeed observe HBV-infected cells without signs of IFN effects (nuclear pSTAT1 or ISG mRNA expression). However, IFN effects were stochastically distributed in uninfected cells as well; that is, not all uninfected cells were positive for pSTAT1 or ISG mRNA expression, and indeed, we did not observe an overall difference in the percentage of nuclear pSTAT1-positive cells between uninfected and HBV-infected samples (Figure 5, Supplementary Figure 10).

In summary, we demonstrate that ex vivo liver biopsy culturing represents a valid tool for experimentation. We applied this system to the study of HBV host interactions in the liver of HBV-infected patients. We show that, in contrast to reports from cell culture and murine systems, there is no difference in ex vivo induced innate immune responses in HBV-infected livers at the whole tissue level as well as at the cellular level. Furthermore, baseline IFN and ISG expression levels did not differ between HBV-infected and noninfected control patients. Together, these results suggest that HBV does not induce or interfere with innate immune responses in vivo in the liver of HBV-infected patients, which is consistent with the observed lack of innate responses during acute HBV infection in experimentally infected chimpanzees[8] and the apparent sensitivity of HBV to

TLR-mediated induction of innate responses.[40–43] As such, and contrary to many other viruses including hepatitis C virus[44] and hepatitis A virus,[45] these results support the hypothesis that HBV behaves like a stealth virus[10] by staying under the radar of the pathogen detection system.[12]

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.01.034.

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## 5.2 Modulators of innate immunity as novel therapeutics for treatment of chronic hepatitis B

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# Modulators of innate immunity as novel therapeutics for treatment of chronic hepatitis B

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The first line defense mechanisms against viral infection are mediated by the innate immune system. Viral components are detected by infected cells and/or innate immune cells that express different sensory receptors. They in turn mediate induction of direct antiviral mechanisms and further modulation of innate and adaptive immune responses. For evading the innate system, most viruses have evolved efficient mechanisms to block sensing and/or antiviral functions of the innate response. Interestingly, hepatitis B virus (HBV) seems to act like a stealth virus that escapes cell intrinsic antiviral mechanisms through avoiding recognition by the innate system rather than blocking its effector functions. In line with this concept, agonistic activation of innate immunity has emerged as a promising novel anti-HBV therapy approach with several compounds having advanced to the clinical stage.

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## Introduction

The innate immune system represents the first line defense of a host cell to viral infection. It consists of cell surface and intracellular pattern recognition receptors (PRR) such as cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and DNA sensors, or membrane-bound toll-like receptors (TLRs) that recognize pathogen-specific structures called pathogen-associated molecular patterns (PAMPs). Incoming viruses can be sensed at the cell surface by TLRs typically recognizing PAMPs exposed on virions (TLR2, TLR4) [1,2]. Intracellularly, endosomal TLRs recognize double stranded (ds)RNA (TLR3), single stranded (ss)RNA (TLR7/8), or CpG motif-containing unmethylated DNA (TLR9) derived from incoming or replicating viruses [2]. Cytoplasmic RLRs (e.g. RIG-I, MDA5) detect virus derived dsRNA or ssRNA, while DNA sensors (e.g. cGAS, IFI16) recognize viral DNA [1]. PAMP recognition by PRRs typically results in rapid induction of type I/III interferons (IFNs) and/or other inflammatory cytokines in the infected cell [1–4]. Secreted IFNs signal to surrounding cells and induce expression of a large number of interferon-stimulated genes (ISGs) that inhibit viral replication and protect naïve cells from *de novo* infection [5]. Besides infected cells, also innate immune cells can respond to viral infection upon either directly sensing virus-infected cells (e.g. natural killer (NK) cells [6]), or engulfing viral components (e.g. dendritic cells (especially plasmacytoid (p)DCs) [7] and macrophages [8]). In the liver, also liver sinusoidal endothelial cells (LSEC) and hepatic stellate cells (HSC) contribute to innate immune responses [9]. Not surprisingly, most viruses evolved to counteract cellular innate immune responses by often actively suppressing PAMP recognition, IFN induction, IFN signaling and/or ISG activity [10,11•]. Here we briefly summarize the current understanding of the interplay between HBV and the different aspects of the innate immune system and how this knowledge is exploited for the development of novel therapies to combat chronic HBV infection.

## Lack of innate response signature during HBV infection

Consistent with observations for many different viruses, innate immune induction is also evident for infections with hepatotropic viruses including hepatitis A virus [12], hepatitis C virus (HCV) [13], hepatitis D virus (HDV) [14] and hepatitis E virus [15] all of which are associated with an intrahepatic IFN/ISG response [12,13–15]. Surprisingly however, HBV infection does not induce an intrahepatic IFN/ISG response in chimpanzees [16]. Likewise, no intrahepatic IFN/ISG response was associated with early-acute infection in the woodchuck model [17] and only minimally or not at all evident in HBV-infected human hepatocyte chimeric mice [14,18••,19]. Similarly, virtually no cytokine induction was detected in the serum of human patients with acute HBV infection [20,21]. These results suggest that HBV acts like a stealth virus efficiently spreading throughout the liver without alerting the innate immune system [16,22].

Although early studies in *in vitro* model systems suggested that HBV could transiently induce an innate

response in the infected cell, these results might have been hampered by the lack of optimal model systems at the time [23,24]. Indeed, recent studies by Cheng *et al.* [18••] and Mutz *et al.* [25••] using newly developed efficient *in vitro* HBV infection systems revealed that also *in vitro* HBV infection does not trigger a cell intrinsic innate immune response. Taken together, the stealth characteristic of HBV seems to enable the avoidance of innate recognition in its host organ and cell.

## Innate response modulation by HBV

Many viruses have evolved to actively counteract PAMP recognition, IFN induction/signaling and/or ISG function in the infected cells. Thus, it is possible that the lack of innate responses to HBV infection is due to a very robust and effective suppression of those responses by HBV. Indeed, it has recently been suggested that intrahepatic innate immune response pathways could be downregulated in chronic hepatitis B (CHB) [26]. This however, is contradictory to similar studies in patients [20,21], chimpanzees [16], and other HBV model systems [14,18••,27] where no suppression of these pathways was observed. Furthermore, innate responses can not only be easily induced in the presence of HBV but in turn also efficiently inhibit HBV replication. For example, it has been recognized early on that poly(I/C) mediated IFN-α/β induction in HBV-infected chimpanzees resulted in a reduction in many viral parameters [28]. Likewise, HBV replication in a transgenic mouse model is strongly inhibited by TLR activation [29,30]. Furthermore, and as will be discussed below, agonistic activation of TLR7 induces prolonged suppression of HBV in different CHB models [31••,32]. Also, HBV superinfection of chronically HCV-infected chimpanzees is strongly attenuated [33], suggesting that HBV is also sensitive to the HCV-triggered innate responses. Finally, HDV superinfection triggers an innate response inhibiting preexisting HBV replication *in vitro* [34•].

Nevertheless, several studies suggested that HBV proteins and replicative intermediates can interfere with innate immune responses in different *in vitro* HBV systems (reviewed in [35]). It has to be noted however, that most of these studies involved over-expression of viral components which might not reflect the situation during natural infection. Indeed, new studies utilizing recently developed efficient *in vitro* HBV infection models unequivocally demonstrate that HBV does not interfere with PRR mediated induction of innate responses in the HBV infected cell [18••,25••]. Furthermore, using *ex vivo* cultured human liver biopsies, we could show that PRR-mediated IFN and ISG induction is not suppressed in the HBV-infected hepatocytes in the liver of CHB patients [36]. In summary, the data from *in vivo* studies and state of the art *in vitro* models would suggest that HBV, besides not inducing innate responses, does also not interfere with innate immunity mechanisms in the infected cell.

## HBV and innate immune cells

Apart from cell intrinsic innate immune responses, viruses can also trigger innate responses in cells they do not infect such as innate immune cells. In the liver, these include a number of non-parenchymal cells (NPCs) such as pDCs, NK cells and liver resident macrophages (Kupffer cells). Indeed, *in vitro* exposure of human liver derived NPCs [37] or primary monocyte-derived macrophages [18••] to high doses of HBV induces cytokine production, suggesting that Kupffer cells might sense HBV in the liver. While Kupffer cell activation might therefore contribute to HBV clearance [37], studies in mice suggest that macrophage activation by HBV might also promote HBV persistence through negative modulation of HBV-specific adaptive immune responses [38•,39,40]. In addition, HBV proteins have also been implicated in downregulation of TLRs on Kupffer cells (and other cells) in CHB, possibly contributing to a proviral environment [41,42].

*NK cells.* Although NK cells are highly enriched in the human liver [43], data on the interplay of HBV with NK cells in patients is mostly derived from analysis of blood-derived NK cells [20,44–46]. Although early activation of NK cells was observed in acute HBV infection [20,45], in some cases their non-cytolytic antiviral functions seem to be suppressed at the peak of viremia [20] and also during chronic infection [44,46]. Since NK cells can kill HBV-specific T-cells [47] and exacerbate HBV-related liver damage [48,49], it appears that antiviral and host damaging effects of NK cells need to be carefully balanced to achieve HBV clearance.

*pDCs.* pDCs are specialized in IFN production upon recognition of captured viruses or viral-derived nucleic acids and therefore are indispensable for mounting a robust immune response during many viral infections (reviewed in [7]). Interestingly however, exposure of pDCs to HBV does not trigger IFN-α production, although it interferes with their capacity to respond to TLR9 stimulation [50,51]. Considering that pDC frequencies and functions otherwise are not altered in HBV-infected patients (reviewed in [52••]), it remains to be determined what role pDCs might play during HBV infection.

## Vertical HBV transmission

It is noteworthy that most studies on the interaction of HBV with different aspects of the innate immune system are focused on chronic or acute HBV infection in adults. In contrast, comparatively little is known about these interactions in newborns during vertical HBV transmission even though this is the more common route of HBV infection today. Nonetheless, experiments in animal models have suggested that HBV might induce an immunotolerant state in newborns and thereby establish persistent HBV infection [38•,53,54]. While these studies

provided an attractive explanation for the high rate of chronicity of vertically transmitted HBV infection, they are at odds with the observation that infants born to HBV-positive mothers respond well to HBV vaccination [53]. Furthermore, a recent study showed that HBV exposure *in utero* can even induce a state of trained immunity both for innate and adaptive immune functions [55••]. Taken together, these results show that the virus host interactions during vertical transmission of HBV are complex and warrant further investigation.

## Innate immune activation in HBV therapy

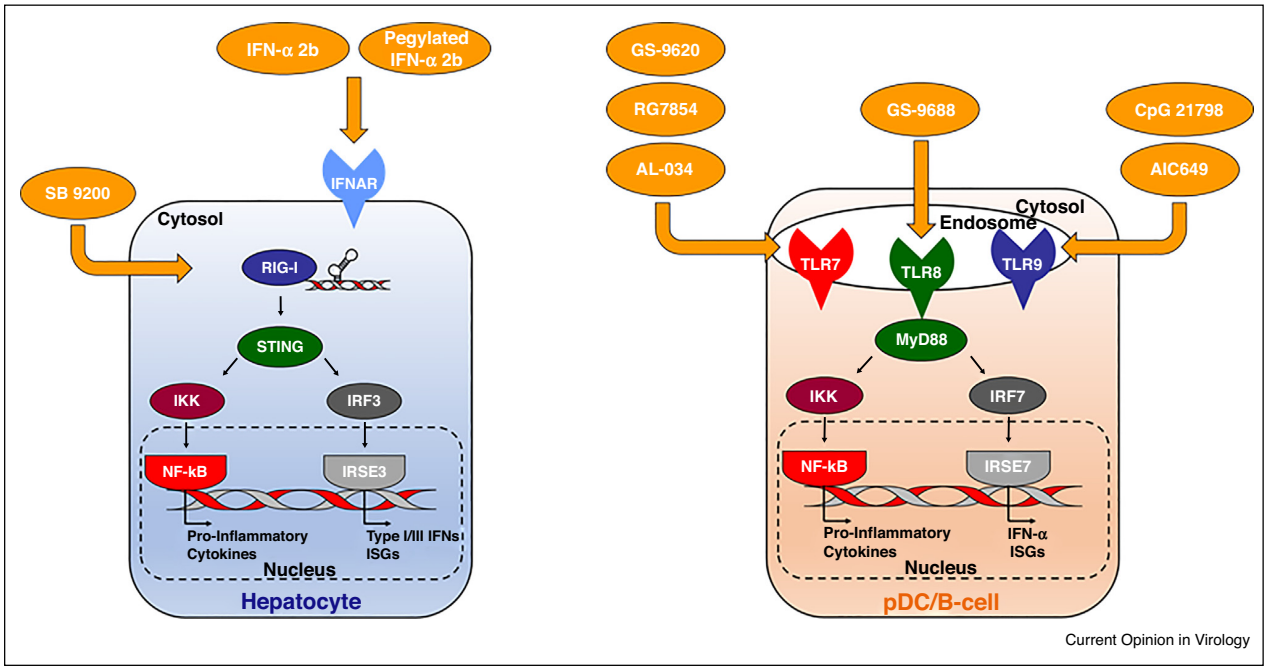
Since chronic HBV infection does not trigger a cell intrinsic innate immunity within liver, compounds capable of activating an intrahepatic, anti-HBV specific innate response are evaluated as novel therapeutic interventions of CHB [35,56–59,60••,61]. Agonistic acting immunomodulators that have already advanced to the clinical stage are discussed (Figure 1).

### IFN-α

Conventional and pegylated IFNs are the only immunomodulators currently approved for treatment of CHB. Compared to standard treatment with nucelos(t)ide analogs (NAs), a higher, although still modest, rate of functional cure is attained with IFN-α in patients during a finite treatment course [61,62]. Unlike the oral availability and safety of NAs [63,64], IFN-α treatment includes parenteral delivery and substantial systemic effects, making it impractical to administer this cytokine to all patients.

Despite clinical use for over two decades, and due to its pleiotropic effects, the mechanistic understanding of IFN-α interference with HBV is still limited [65]. IFN-α displays immunomodulatory [66–69] and direct antiviral effects [70–73,74••,75]. A study in woodchucks addressed the mechanisms of IFN-α activity after subcutaneous administration for 15 weeks [76]. Comparable to patients [77], treatment reduced replication of the

**Figure 1**




Activation of antiviral innate immunity in CHB. Activators can be separated into modulators that systemically stimulate innate immunity (e.g. IFNs and other recombinant cytokines) and modulators that act specifically and locally (e.g. agonists of cytoplasmic PRRs, IFN signaling pathway molecules, and endosomal TLRs). Available IFN-α preparations include Intron A (IFN-α 2b; Merck) and Pegasys (pegylated IFN-α 2a; Genentech). SB 9200 (Spring Bank Pharmaceuticals) is an agonist of RIG-I. GS-9620 (Gilead Sciences), RG7854 or RO7020531 (Hoffmann-La Roche), and AL-034 (Janssen) are agonists of TLR7. GS-9688 (Gilead Sciences) is an agonist of TLR8. CpG 21798 (Pfizer) and AIC649 (AiCuris) are agonists of TLR9. Upon stimulation of PRRs or TLRs by their respective ligands, downstream signaling pathways are activated, including key molecules such as STING or MyD88, leading to the nuclear translocation of the transcription factors NF-κB, IRF3 and IRF7, and subsequent transcription of IFNs, pro-inflammatory cytokines, and ISGs. Note that the figure only indicates the main cell subsets and cellular compartments that are targeted by IFN-α and agonists but does not depict any systemically induced innate immunity by these modulators as it is known for IFN-α due to the pleiotropic nature of this cytokine. IFNAR, interferon-α/β receptor; STING; stimulator of interferon genes; IKK, Kinase involved in the upstream NF-κB signal transduction cascade; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; IRF; interferon regulatory factor; IRSE, interferon-responsive sequence element; and MyD88, myeloid differentiation primary response gene 88.

HBV-like woodchuck hepatitis virus (WHV) at varying degree in responder, partial responder, and non-responder animals, with maximum mean declines in WHV DNA and WHsAg of 3.2 and 2.0 logs, respectively. Most woodchucks experienced viral rebound after the end of treatment, although viremia and antigenemia stayed suppressed in responder animals, with one achieving a functional cure. The differential treatment response to IFN-α in individual woodchucks, however, did not correlate with the intrahepatic induction of most ISGs. Instead, induction of NK and T-cell markers, elevation in liver inflammation, consistent with migration and/or proliferation of these cells, and increase in IFN-γ expression were observed, indicating that cytolytic and non-cytolytic mechanisms play a pivotal role in the IFN-α treatment response. As also described for patients [66,67], the antiviral response mediated by IFN-α in woodchucks closely correlates with its immunomodulatory effects. Altogether, these results suggest that immunomodulators, which activate antiviral immune cells in the liver like IFN-α, but are without its limitations, are promising compounds for the treatment of CHB.

## TLR agonists

Selective activation of TLRs within pDCs by agonists for the local induction of IFNs is increasingly investigated. The oral TLR7 agonist GS-9620 reduced HBV DNA in chimpanzees by 2.2 logs on average after two rounds of 4-week treatment [31••]. Durable suppression of viremia persisted at least for 2–4 months and was associated with a 50% reduction in HBsAg and HBeAg levels. In woodchucks, one of the GS-9620 treatment regimens produced a functional cure in all woodchucks [32]. GS-9620 was initially administered for 2 weeks, halted due to thrombocytopenia in some animals, and then reinitiated with half of the original dose for an additional 2 weeks. Short-term treatment resulted in a rapid decline in WHV DNA of 6.2 logs on average. Viremia stayed suppressed for more than 7 months and was accompanied by undetectable WHsAg in all woodchucks, and by anti-WHs seroconversion in a subset of animals. The antiviral effect mediated by GS-9620 in liver of chimpanzees and woodchucks following induction of IFN-α and antiviral ISGs was facilitated, at least in part, by the cytolytic activity of CD8+ T-cells and/or NK cells. In the subsequent phase Ib clinical trial, GS-9620 administered to treatment-naïve and NA-suppressed patients once or twice was well tolerated, but did not result in significant changes in HBV markers [78]. Since induction of hepatic flares or autoimmunity are main concerns in any immunomodulatory therapy, the maximum dose applied to patients was approximately 15–45-fold lower than those tested in animals. Phase II clinical trials of GS-9620, testing longer treatment duration and higher doses, alone or in combination with tenofovir, are ongoing (NCT02166047 and NCT02579382).

RG7854 [79] is another oral TLR7 agonist, which induced functional cure in woodchucks (Menne *et al.*, unpublished). The compound is currently evaluated in a phase I clinical trial (NCT02956850). The oral TLR7 agonist AL-034 showed efficacy against HBV in a mouse model [80], and is also in phase I clinical development (NCT03285620).

Agonists of TLR8 are considered important activators of innate immunity since they induce the secretion of IL-12 and IL-18 from monocytes within human liver, which in turn leads to the production of IFN-γ by intrahepatic innate immune cells such as mucosal-associated invariant T cells (MAIT) and NK cells [81]. IFN-γ has been shown to mediate HBV clearance in chimpanzees [82] and to suppress viral replication in HBV transgenic mice, woodchucks and CHB patients following treatment with IL-12 or IL-18 [83–86]. IL-12 can further rescue the antiviral function of exhausted HBV-specific CD8+ T cells *in vitro* [87].

GS-9688, an oral TLR8 agonist, was tested in woodchucks [88]. Treatment for 8 weeks reduced WHV DNA by 5 logs in most animals. Three woodchucks with sustained suppression in viremia had undetectable WHsAg, and developed anti-WHs antibody and WHV-specific T-cell responses. Comparable to patients [89], the latter suggested that the T-cell dysfunction present in CHB is reversible. A phase Ib clinical trial of GS-9688 is initiated (ACTRN12617000235303).

CpG oligodeoxynucleotides are TLR9 agonists that induce IFN-α following stimulation of pDCs and B-cells [90]. CpGs have been tested during HBV vaccine development [91,92]. Subcutaneous treatment of woodchucks with CpG 21798 in combination with entecavir (ETV) reduced WHV DNA and especially WHsAg to undetectable levels much earlier than ETV monotherapy, and delayed viral relapse [93•]. CpG monotherapy, however, was only modestly efficacious.

AIC649, an inactivated parapoxvirus ovis particle preparation, targets antigen presenting cells via TLR9 and leads to cytokine release, including IFN-α [94,95]. The antiviral efficacy of AIC649 was evaluated in woodchucks during 36 weeks of intravenous/intramuscular administration, alone or in combination with ETV during the initial 12 weeks [96]. The AIC649 dose was equal to the high dose administered to patients in an ongoing phase I clinical trial (AiCuris website). The previously observed bi-phasic response pattern [97] was confirmed during AIC649 monotherapy. Combination treatment with AIC649 and ETV produced maximum mean reductions in WHV DNA and WHsAg of 7.6 and 4.1 logs, respectively, and WHsAg became undetectable in a subset of woodchucks. WHV suppression was quite durable, but

viral rebound eventually occurred, although viremia and antigenemia stayed below pretreatment level at the end of the study. The treatment response was further associated with WHV-specific T-cell responses and anti-WHs antibodies in most woodchucks. The overall results from these studies indicate that TLR9 agonists can exhibit additive effects, when administered in combination with NAs.

## Intracellular PRR agonists

Since the above TLRs are mainly found within pDCs, but are barely expressed in other cells, PRRs that are located within the cytoplasm of HBV-replicating hepatocytes are another attractive target for stimulating antiviral innate immunity. Activation of RIG-I induces not only IFNs but has also been suggested to block HBV through sensing of the epsilon-structure within pre-genomic RNA [19]. SB 9200, a selective, oral prodrug of the dinucleotide SB 9000 has been shown in woodchucks to activate RIG-I expression in liver and to increase its protein level in hepatocytes [98]. RLR upregulation further induced the intrahepatic expression of key innate immune response molecules, including STING and IRF3, and was consistent with the induction of IFN-α/β and antiviral ISGs. Induction of innate immunity, likely in addition to the direct antiviral effect mediated by activated RIG-I, then contributed to the dose-dependent antiviral response observed with SB 9200 in woodchucks. In this context, it is noteworthy that the murine STING agonist DMXAA induced an IFN-α/β mediated cytokine release in macrophages, which suppressed HBV replication in mouse hepatocytes and liver [99]. In another woodchuck study, short-term ETV treatment was sequentially applied before or after SB 9200 treatment for 12 weeks [100]. Activation of innate immunity by SB 9200 followed by ETV resulted in maximum mean reductions in WHV DNA and WHsAg of 6.4 and 3.3 logs, respectively. The antiviral effect was transient, but WHV markers stayed suppressed until the end of the study. These results suggested that activation of intrahepatic innate immunity in CHB patients prior to initiation of standard treatment may be beneficial for augmenting the antiviral effect. This concept is investigated in a phase IIa clinical trial of SB 9200 (NCT027519968). Treatment-naïve CHB patients were pretreated with SB 9200 for 12 weeks, then switched to tenofovir, and followed for additional 12 weeks [101]. SB 9200 treatment resulted in a mean reduction in HBV DNA of 0.6 logs, and 5 of 16 patients experienced a greater than 0.5 log decline in HBsAg. The antiviral efficacy observed in patients is noteworthy considering that the applied SB 9200 dose is approximately 80-fold lower than the woodchuck dose. Following the switch to tenofovir, an enhancement of the antiviral effect was noted in HBeAg-positive patients, suggesting an added benefit of immunomodulation followed by standard treatment.

## Challenges for therapeutic use of agonistic acting immunomodulators

Although the preclinical results overall are promising, targeting innate immunity by agonists comprises challenges that need to be addressed before application in CHB patients. While clinical trials with the above agonists have not concluded, it has been suggested that the utilized animal models, in contrast to patients, may be more sensitive to this kind of therapy or may tolerate doses exceeding those considered to be physiological and safe in patients. This proposition is based on the rather disappointing outcome of monotherapy with GS-9620 in patients so far [78] using doses and dosing frequencies significantly different to the treatment applied to chimpanzees and woodchucks [31••,32]. Since monotherapy with agonistic activators of innate immunity sometimes failed to induce pronounced antiviral effects in animal models as well as patients and, unlike standard drugs, cannot be administered indefinitely, combination treatment with NAs will be required for achieving sustained suppression of HBV. Such treatment regimens (sequential or concomitant) may further allow reducing the dose of an agonist and shortening the duration of immunomodulation in patients, whereby limiting the risk that the activated adaptive immune response could target most or all HBV-infected hepatocytes and result in fatal liver damage.

## Conclusions

Considering the different models used for studying the interplay between HBV and the innate immune system, it emerges that HBV is neither sensed nor interferes with the innate immune response of the cell it infects, thus acting like a stealth virus in this regard. On the other hand, the consequences of the interplay between HBV and different cells of the innate immune system seem to substantially influence whether an HBV infection will be resolved or become persistent. The encouraging results from the above preclinical and clinical studies indicate that agonistic activation of innate immunity, most likely in combination with NAs, holds great promise for treatment of CHB.

## Conflict of interest statement

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## 5.3 Hepatitis B virus replication is inhibited downstream of pre-genomic RNA in HBeAg-negative chronic infection

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For supplementary material please refer to the Appendix B of this thesis.

# Hepatitis B Virus Replication Is Inhibited Downstream of Pre-genomic RNA in HBeAg-Negative Chronic Infection

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**Keywords:** replication, liver biopsies, immune control, integration

**Abbreviations:**

ALT, alanine aminotransferase; AVT, antiviral therapy; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; EDTA, Ethylenediaminetetraacetic acid; EPCHB, HBeAg-positive chronic hepatitis B; EPCI, HBeAg-positive chronic infection; ENCHB, HBeAg-negative chronic hepatitis B; ENCI, HBeAg-negative chronic infection; HBV, Hepatitis B virus; IFN, interferon; NDC, nuclear DNA contamination; NUC, nucleos(t)ide inhibitor; (q)PCR, (quantitative) polymerase chain reaction; repDNA, DNA replicative intermediates; RT, reverse transcription; TNF$\alpha$, Tumor necrosis factor alpha

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# ABSTRACT

A hallmark of the transition to the HBeAg-negative state during the natural history of chronic hepatitis B (CHB) is a strong reduction in serum HBV DNA levels and to lesser extent of the intrahepatic cccDNA content. However, there is no correlation between cccDNA levels and viremia, which could be attributed to partial inhibition of viral transcription. While the apparent immune activity in the HBeAg-negative chronic hepatitis (ENCHB) phase could explain this antiviral effect, it is not clear whether HBV is similarly inhibited in the HBeAg-negative chronic infection (ENCI) phase. This is of particular interest as the ENCI phase is characterized by even lower viremia, absence of liver disease and only minimal hepatic necroinflammatory activity compared to the ENCHB phase.

We now compared the relative HBV transcriptional activity and replication efficiency in the liver of CHB patients of different disease stages to identify what step(s) in the viral life cycle might be controlled in the ENCI phase. To this end, we used human liver biopsy tissue obtained from CHB patients to quantify intrahepatic HBV DNA and RNA replicative intermediates in different phases of CHB.

Our results demonstrate that viremia and cccDNA levels are, respectively, ~2.1 log and ~1.3 log lower in the ENCI compared to the ENCHB phase. But, there is no difference in the HBV pgRNA production rate between the two groups. However, we found that the intrahepatic HBV replication efficiency in the ENCHB phase was not different from that in HBeAg-positive patients, while it was ~ 16-fold lower in the ENCI phase.

Taken together our results reveal an HBV replication inhibitory activity that affects some step(s) downstream of pgRNA production in the viral life cycle in the ENCI phase. Importantly, this inhibitory activity is specific to the ENCI phase of CHB and thus, might be the key event discriminating the “immune control” phase (ENCI) from the “immune active” ENCHB phase.

# INTRODUCTION

Chronic Hepatitis B virus (HBV) infection is a global health burden affecting ~3% of the world population causing ~800 000 deaths per year due to cirrhosis and hepatocellular carcinoma [1, 2]. The causative agent, HBV is a small hepatotropic DNA virus belonging to the *Hepadnaviridae* family [3]. Upon infection of a hepatocyte, the viral genome is converted into a covalently closed circular (ccc)DNA molecule that persists in the hepatocyte nucleus as a stable episome [4]. CccDNA serves as a template for transcription of several RNAs that all encode viral proteins, and one of which, the so called pre-genomic (pg)RNA, serves as a template for HBV reverse transcription and HBV progeny production [4]. HBV encodes its own DNA polymerase which also functions as a reverse transcriptase that first converts viral pgRNA into complementary genomic minus(-)-strand DNA and then synthesizes the second, genomic plus(+)-strand DNA using the (-)-strand as template [5]. HBV polymerase is the main target of most currently available anti-HBV therapies [6].

The natural history of chronic hepatitis B (CHB) is divided into 5 stages which are defined by clinical and virological parameters and have been recently given a new nomenclature by the European Association for the Study of the Liver (EASL) [2]. The first stage, HBeAg-positive chronic infection (EPCI), is characterized by very high viremia (>$10^7$ IU/ml) and antigenemia in the absence of any disease. This phase can last for a few weeks or decades. It is typically followed by the HBeAg-positive chronic hepatitis B (EPCHB) phase characterized by elevated alanine aminotransferase (ALT) values (>40 IU/ml) indicating the presence of an antiviral immune response and is associated with a 3-5 log reduction in viremia. This disease phase is normally followed by a loss of HBeAg in the serum leading to a HBeAg-negative phase which is divided into HBeAg-negative chronic infection (ENCI) and HBeAg-negative chronic hepatitis B (ENCHB). During ENCI, the virus remains undetectable or contained (<2000 IU/ml) and there is no evidence of liver disease (ALT <40 IU/ml). ENCHB on the other hand is associated with persistent liver disease (ALT >40 IU/ml) and fluctuating viral loads >2000 IU/ml. Lastly, the HBsAg-negative stage (also known as “occult HBV infection”, or resolved hepatitis B) is defined by the disappearance of serum HBsAg and absence of liver disease, with or without the appearance of anti-HBs antibodies. While spontaneous resolution in CHB patients only occurs at a rate of ~1% per year, 95% of infections in adults will resolve within 6 months [2, 7]. But, despite the apparent clinical

resolution (“functional cure”), a hidden reservoir of viral cccDNA persists in the nuclei of some hepatocytes [8].

The ENCI patients can maintain viral control for many years without clear signs of immune system activity [2]. Nevertheless, exposure to an immunosuppressive therapy, cancer chemotherapy, or certain biologic therapies for managing inflammatory conditions can lead to HBV reactivation, indicating that the mechanisms of viral control are tightly connected to the activity of the immune system [9]. However, what step(s) in the viral life cycle are targeted by these activities and the cellular mechanism(s) involved are not well understood. Based on the mode of action of therapies that lead to HBV reactivation, such mechanism might include B-cell and T-cell-mediated control, cytokine signaling, lymphocyte proliferation and activation, and epigenetic regulation [9].

Precisely defining the key steps/events in the HBV life cycle that are controlled by the immune system during CHB are a prerequisite for determining the corresponding antiviral mechanism(s). Indeed, previous reports demonstrated a reduction in transcription and cccDNA in HBeAg-negative patients [10, 11]. However, it is not clear how ENCI compares to ENCHB. Also, previous studied did not take into account the possible contribution of integrated HBV DNA in measuring intrahepatic HBV replicative intermediates [10, 11]. Accordingly, in this study we compared the infection rate, cccDNA transcriptional activity and HBV replication efficiency between different disease stages using human liver biopsy material. Particularly, we focused on ENCI and high-viremia ENCHB stages, since the difference between them could highlight the key events required for full control over virus in ENCI compared to no control in ENCHB. To do so, we optimized extraction and analysis of HBV DNA replicative intermediates in order to avoid contributions of integrated HBV DNA.

Using this optimized procedure, we quantified the levels of cccDNA, replicative intermediates, total HBV DNA and pre-genomic RNA in liver biopsies of CHB patients from different stages of infection including HBeAg-positive (HBe+), ENCI, ENCHB, and patients undergoing antiviral therapy (AVT). We demonstrate that cccDNA transcriptional activity is similarly inhibited in the liver of ENCI and ENCHB compared to HBe+ patients. However, the HBV replication efficiency is specifically inhibited in ENCI, but not in ENCHB. Together, these results suggest the existence of antiviral mechanism(s) that are only active during ENCI.

# MATERIALS AND METHODS

## Patients and liver biopsies

Liver biopsies from patients with HBV infections and from non-infected controls were obtained in the outpatient clinic of the Division of Gastroenterology and Hepatology, University Hospital Basel, Switzerland. Liver biopsies were done using an ultrasound guided coaxial needle technique that allowed for single-stick, multiple pass biopsies (BioPince®, Peter Pflugbeil GmbH, Zorneding, Germany). The individual biopsies are 29mm long and have a diameter of 1mm. One cylinder was used for routine histopathological diagnostic purposes. After obtaining written informed consent, an additional biopsy cylinder was used for cryopreservation and subsequent extraction of nucleic acids. The use of biopsy material was approved by the local ethics committee (Ethikkommission Nordwest- und Zentralschweiz, Switzerland). Formalin fixed liver biopsy tissue was used for histological analysis for standard ISHAK classification (i.e. determination of liver inflammation and fibrosis) and for routine immunohistochemical stainings for HBV surface protein (HBsAg) and HBV core protein (HBcAg). For the HBV sample set, the histopathological analysis (ISHAK grading, HBsAg- and HBcAg-positive hepatocyte fraction) and the clinical data (transaminases, HBV viral load in the serum, stage of infection, serum HBsAg and HBeAg status) are shown in supplementary Table S1.

## RNA isolation and quantification

Total RNA was extracted from fresh-frozen biopsy pieces using the Trizol reagent (Invitrogen), or using ZR-Duet$^{TM}$ DNA/RNA MiniPrep Plus kit (cat. No. D7003, Zymo Research Corp., Irvine, CA, USA), according to manufacturer's instructions. Total RNA extracted with TRIzol was subjected to DNase I treatment using the DNA-*free*™ DNA Removal Kit (Ambion) according to manufacturer's instructions. Total RNA extracted with ZR-Duet$^{TM}$ DNA/RNA MiniPrep Plus kit was subjected to on-column DNase I treatment (provided in the kit). The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

## DNA extraction

Total DNA, nuclear DNA, cytoplasmic DNA and cccDNA were extracted from the same piece of snap-frozen liver biopsy. Tissue was homogenized in NP-40 lysis buffer (50

mM Tris-HCl (pH 8), 1 mM EDTA, 0.2% Nonidet P-40, 0.15 M NaCl) using a Dounce homogenizer (loose pestle). 60% and 20% of the homogenate were directly used to extract cccDNA and total DNA, respectively, as previously described [12]. The remaining homogenate (20% of the total lysate) was centrifuged for 5 min at 500xg and +4°C to pellet the nuclei. The supernatant containing predominantly replicative forms of HBV DNA was centrifuged again to remove any residual insoluble material yielding the pure cytoplasmic fraction. Pelleted nuclei were washed three times by resuspending them in the NP-40 lysis buffer and re-pelleting. After the last wash, the nuclei were resuspended in 100 µl of NP-40 lysis buffer (nuclear fraction). Both, nuclear and cytoplasmic fractions were further processed exactly in the same way as the samples for total DNA extraction. Extracted DNA was resuspended in 0.1x TE. CccDNA preparations were treated with 10 units of Exonuclease V (ExoV) (M0345S, New England BioLabs, Ipswich, MA, USA) for 120 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of ~11 mM and heating to 70°C for 30 min. The reactions were then directly used for qPCR.

## Reverse transcription and quantitative PCR

cDNA was synthesized from 300 ng of total RNA using MultiScribe™ Reverse Transcriptase (Applied Biosystems™) and random hexamer primers in a 25µl reaction. RT reactions omitting the reverse transcriptase served as no RT (-RT) controls. 1/10 of each RT reaction was used for subsequent SYBR-green based quantitative real-time PCR (qPCR) using the primers listed in Table S2 on an ABI 7500 Real Time PCR System (Applied Biosystems™). QPCR data were analyzed with the 7500 Software v2.0.6 (Applied Biosystems™). All reactions were performed in triplicate. Besides “-RT” controls, RT reactions without any template, qPCR reactions without any template and qPCR reactions with water as template served as negative controls on every qPCR plate.

Intrahepatic cellular and HBV DNA was analyzed by qPCR using 2.5 µl of the different DNA preparations. Genomic DNA was quantified by qPCR of the single-copy gene IMAP using serial dilutions of human genomic DNA with known concentration as a reference. Cell numbers were estimated based on the assumption that one cell contains a single diploid genome with the mass of ~7.18 pg without considering possible hepatocyte ploidy. Cell numbers estimated in the total DNA extracts were

used as a reference for calculating the amount of DNA per cell in the cytoplasmic fractions. The percentage of nuclear DNA contamination (%NDC) in the cytoplasmic fractions was calculated by dividing the IMAP signal in the cytoplasmic fraction by that in the total DNA fraction multiplied by 100. HBV DNA and pgRNA were quantified with the corresponding primers (Table S2) and using a standard curve prepared from a serial dilution of an HBV containing plasmid. The cytoplasmic HBV DNA level devoid of any signal from integrated HBV DNA was calculated by subtracting the HBV signal corresponding to the %NDC of the total DNA fraction from the HBV signal in the cytoplasmic fraction.

# RESULTS

### *Patient cohort*

The study included total of 16 HBe+ patients, 56 patients with ENCHB, 23 patients with ENCI and 7 patients undergoing antiviral treatment with NUCs (Table S1). The HBe+ group was defined only by the HBeAg-positivity without differentiating between the “chronic hepatitis B” and “chronic infection” stage. Median viral load in this group was ~$1.7x10^8$ IU/ml and the median ALT level was 63.5 IU/ml. The viral load is approximated because in many patients it was above the upper limit of quantification. In a similar way, the ongoing antiviral treatment was the only inclusion criterion for the AVT group, in which 6 out of 7 patients was HBeAg-negative. Viral load was undetectable in all 7 AVT patients and the median ALT level was 28 IU/ml. ENCHB and ENCI groups were defined based on the EASL guidelines: ENCHB included patients with a viral load >2,000 IU/ml (median ~19,274 IU/ml) with or without elevated ALT values (median 51 IU/ml), whereas the ENCI group included patients with a viral load <2,000 IU/ml (median 192 IU/ml) and normal ALT (median 25 IU/ml).

According to the EASL definitions, patients with a viral load between 2,000 and 20,000 IU/ml can be considered as ENCI if their ALT levels are persistently normal. Because ALT can be elevated due to other, non-HBV related comorbidities, the HBeAg-negative patients with viral load <20,000 IU/ml are in a “gray zone” of classification. To avoid possible result bias due to misclassification of ENCI as ENCHB patients, for specific experiments we analyzed an ENCHB sub-group with a viral load >20,000 IU/ml (median of $2.71x10^5$ IU/ml) which we refer to as ENCHB-high.

### *CccDNA level reduction alone cannot explain low viremia in ENCI*

It has been previously shown that a pronounced 3-5 log reduction in viral load upon HBeAg seroconversion is accompanied by only a modest 1-2 log decrease in cccDNA levels [10, 11]. Those studies however, did not discriminate between the patients with active high-level replication (ENCHB) and the ENCI group whose viral load is very low or absent. We now analyzed cccDNA levels separately in the ENCHB and ENCI groups and also in HBe+ and AVT patients, and correlated them with the changes in viral load among the same patients. As expected, the viral load (in IU/ml) was decreasing from a median of $1.7x10^8$ in the HBe+ group to ~$2.5x10^4$ in the ENCHB group (-3.84 log) and to ~$2x10^2$ in the ENCI group (-2.09 log to ENCHB) (Figure 1A).

There was no detectable viral load in all the patients undergoing antiviral therapy with NUCs (Figure 1A). The corresponding cccDNA levels were 3.35 copies/cell in HBe+, ~0.13 copies/cell in ENCHB and only ~0.006 copies/cell in the ENCI group (Figure 1B). With ~0.016 copies/cell, the AVT group showed slightly higher cccDNA levels than the ENCI group (Figure 1B), supporting previous observations that viral suppression with NUCs has only a limited effect on cccDNA levels [13]. The correlation between median cccDNA levels and viremia among the different stages of CHB suggests that the amount of cccDNA in the liver is indeed a limiting factor for virus production. However, in both the ENCHB and ENCI stages the viral load decreased much more than cccDNA levels compared to HBe+ patients (Figure 1A, B) confirming previous reports comparing HBe+ with HBe- patients ([10, 11]). Moreover, there was no correlation between viral load and cccDNA in the ENCHB group (Fig. 1C), suggesting that viral transcription and/or replication could also be reduced in the ENCHB group. Similarly, there was no correlation between viremia and cccDNA in the ENCI group (Figure 1D). Taken together, our results are in agreement with previous studies showing a non-linear decrease in viral load and cccDNA in HBeAg-negative patients [10, 11].

***HBV transcriptional activity in ENCI is similar to that of high-viremia HBeAg-negative patients***

It has been previously shown that HBeAg loss is associated with reduced transcriptional activity of cccDNA [10, 11]. To address whether lower cccDNA transcriptional activity could be the reason for the low viremia in ENCI patients we compared the transcription efficiency in ENCI patients with that in ENCHB patients with high viral load (ENCHB-high). To do so, we monitored intrahepatic pgRNA in the different patient groups. We focused on pgRNA as this is the template for virus production and is typically not produced from integrated HBV DNA [14].

As expected, HBV transcriptional activity measured as amount of pgRNA per cccDNA molecule was the highest in the HBeAg-positive samples with a median of 195 copies/cccDNA (Figure 2A). Again, as previously reported, the pgRNA production activity was reduced ~8-11 fold in the HBe-negative patients [10, 11]. Interestingly however, there was no difference in transcriptional activity between the ENCHB-high and ENCI group (Figure 2A), suggesting that transcriptional suppression is not a major factor driving the ENCI state. Transcriptional activity in AVT patients (most of which

are HBeAg-negative) was similar to that of both HBeAg-negative groups (Figure 2A), in agreement with the mechanism of action of NUCs, that inhibit HBV DNA synthesis, but not transcription. Taken together, these results suggest, that there is a general downregulation of cccDNA transcription upon HBeAg seroconversion, but this does not appear to be the factor that distinguishes patients with active hepatitis (ENCHB) from patients that have HBV replication under control (ENCI). Total intrahepatic pgRNA production correlated with viremia in ENCHB patients (Figure 2B), suggesting that the combination of cccDNA and transcriptional reduction can largely explain the reduced viral load at this stage. These results also suggest that viral replication steps downstream of pgRNA production are not critically affected in ENCHB. In contrast, pgRNA production in ENCI patients did not correlate with viremia (Figure 2C), further suggesting that additional steps of the HBV life cycle are suppressed in these patients.

***HBV replication inhibition in ENCI***

To determine, whether the HBV replication efficiency is indeed lower in ENCI compared to high-viremia ENCHB (ENCHB-high) patients, we analyzed production of replicative intermediates (repDNA) between the different patient groups. To avoid signals from integrated HBV DNA to impact the data, we performed subcellular fractionation of liver biopsy material and extracted DNA specifically from the cytoplasmic fraction as described in “Materials and Methods”. DNA from nuclear fraction as well as from the total biopsy lysate were extracted as control. Using this approach, we were able to keep genomic DNA contamination in the cytoplasmic fraction between 0.19% – 8.4% (Figure S1A), which enabled separation of replicative intermediates from nuclear HBV DNA in samples with very low intrahepatic levels of replication intermediates. As shown in Figure S1B, integrated DNA apparently affected quantification of HBV DNA replicative intermediates in cases with very low levels of <1 copy per cell. As expected however, analysis of higher levels of HBV DNA replicative intermediates was no longer affected by signals from integrated DNA (Figure S1B, triangles) and thus, was done using total DNA.

The total levels of repDNA per cell clearly separated by disease stage, with almost non-overlapping distribution between groups (Figure 3A). Interestingly, the repDNA levels in patients under treatment (AVT) were similar to the patients who control the virus (ENCI) (Figure 3A), suggesting that suppression of the viral replication in ENCI is similar to the NUC therapy induced suppression. Moreover, replication per cccDNA

was reduced ~11-fold more than the transcriptional activity in the liver of ENCI patients, while there was a similar reduction of replication per cccDNA and transcriptional activity in the ENCHB patients (Figure 2A and 3B). These results suggest that while the difference in replication between HBe+ and ENCHB-high might be due to differences in cccDNA transcriptional activity, the difference between ENCHB-high and ENCI requires specific inhibition of replication downstream of pgRNA. To test this hypothesis, we calculated the amount of replicative intermediates generated per pgRNA in the different CHB stages (Figure 3C). Indeed, the replication efficiency per pgRNA in the liver of ENCI patients is ~11-fold lower than that in ENCHB-high patients Figure 3C) with the latter not differing from that seen in HBeAg-positive patients. Taken together, these results clearly demonstrate that HBV replication in patients from the ENCI group is specifically suppressed downstream of pgRNA production.

# DISCUSSION

The HBeAg-negative chronic infection (ENCI) stage in the natural history of chronic HBV infection is characterized by low or non-detectable viral load the absence of liver disease and low to non-existent immune activity [2]. This can be potentially achieved in many different ways by controlling various steps/events in HBV life cycle, for example, through elimination of the majority of infected hepatocytes, transcriptional suppression of cccDNA (e.g. via epigenetic modifications), inhibition of HBV replication, modified stability of replicative intermediates, inhibition of capsid assembly, or decreased virion formation and release. In the current study we compared different replication steps and their activity in the ENCI stage with those in high-viremia stages of CHB in order to find out which of these steps are inhibited and thus might account for the low viremia in the ENCI phase of CHB. Given the very low HBV replication levels in ENCI, measuring intrahepatic HBV replicative intermediates, however, might be influenced by signals derived from integrated HBV DNA. Indeed, recent reports estimated that hepatocytes containing integrated HBV DNA can account for at least 0.01-1% of all hepatocytes in the liver [15, 16]. In low replicating HBeAg-negative patients, the frequency of infected cells can be lower than 1% (based on the cccDNA levels) [12], which might lead to a situation where integrated HBV DNA

and replicating HBV DNA would be indistinguishable. To avoid this problem, that might have influenced the results of previous studies [10, 11, 17], we prepared and analyzed cytoplasmic DNA that contains replicative intermediates, but is devoid of integrated HBV DNA.

In agreement with a central role of cccDNA in the HBV life cycle we found that CHB stages with lower viremia had on average also less cccDNA (Figure 1A, B), suggesting that cccDNA availability is indeed rate-limiting for HBV replication. However, cccDNA levels alone could not explain the viremia within the HBeAg-negative stages (Figure 1C, D). Furthermore, our results confirm that regulation of cccDNA transcriptional activity contributes to the reduced viral load in HBeAg-negative patients [10, 11]. Importantly however, our study revealed that in ENCI patients in addition to transcriptional control, also HBV replication efficiency is specifically inhibited.

We could not exclude in this study that some factors downstream of HBV genome replication, such as modified stability of replicative intermediates, inhibition of capsid assembly, or decreased virion formation and release could further contribute to establishing the ENCI state. Patients undergoing NUC treatment, however achieve a state of viral control similar to ENCI due to the inhibition of replication alone. Interestingly, the ENCI and AVT patients in our study showed similar levels of total intrahepatic replication (Figure 3A), suggesting that the combination of low cccDNA levels, transcriptional suppression and inhibition of replication in ENCI is sufficient to achieve viral control.

Besides inhibition of transcription and replication, anti-HBV antibodies in the serum, or accumulation of deficient cccDNA forms as a result of dslDNA to cccDNA conversion could contribute to the discrepancy between cccDNA and viremia [4]. Our observation that pgRNA production per cccDNA is similar in ENCHB and ENCI patients would however, suggest that deficient forms of cccDNA are not significantly affecting virus production in CHB. Likewise, the positive correlation of pgRNA and viremia in the ENCHB patients suggests that antibodies do not play a major role in controlling viremia in this phase of CHB. Rather, it appears that the combination of lower cccDNA levels and reduced transcriptional activity determine the viral load in ENCHB patients. A contribution of antibodies to the low viremia in ENCI patients however, cannot be ruled out.

Given the apparent lack of immune activity in ENCI, the mechanisms by which host achieves and maintains inhibition of HBV replication remain to be determined. HBV DNA synthesis requires 3 factors – pgRNA, core and polymerase, with both, core and pol being produced from the pgRNA [5]. Since pgRNA production is not suppressed in the ENCI patients compared to high-viremia ENCHB patients (Figure 2A), pgRNA is most likely not the limiting factor. Mutations in core and polymerase however could result in inhibition of capsid assembly and inhibition of HBV DNA synthesis by the polymerase, respectively. Therefore, it would be interesting to analyze whether such mutations are overrepresented in cccDNA of ENCI patients.

Studies in HBV transgenic mice and chimpanzees have shown that in addition to direct killing of the infected cells, T-cells also control HBV infection by non-cytolytic mechanisms, through the activity of cytokines such as interferon-gamma (IFN$\gamma$) and tumor necrosis factor alpha (TNF$\alpha$) [18-20]. Suppression of replication downstream of pgRNA is compatible with the mechanism of action of IFN$\gamma$, that is produced by T cells and was shown to interfere with pgRNA packaging and capsid formation [21]. Importantly, it is well documented that both suppression of immune cell proliferation and/or functions as well as specific inhibition of TNF$\alpha$ can lead to HBV reactivation, suggesting that TNF$\alpha$ and other cytokines produced by immune cells (e.g. IFN$\gamma$ produced by T cells) play an important role in controlling the virus in the chronic infection stage and in resolved hepatitis B [9].

B-cell depleting therapies (e.g. Rituximab) also often lead to HBV reactivation, indicating that anti-HBV antibodies (most likely anti-HBsAg antibodies) are also crucial for controlling the virus probably acting via preventing the infection of new cells [9].

The mechanism of transcriptional downregulation of cccDNA is also not well understood. Although many HBeAg-negative patients accumulate mutations in HBV pre-core / basal core promoter, a study by Volz et al. [10] has shown that such mutations are actually mostly beneficial for viral replication and that ~50% of patients did not even have such mutations. On the other hand, cccDNA is amenable to epigenetic modulation (for example by cytokines) that could change its transcriptional activity [22].

In conclusion, we identified a specific suppression of HBV replication efficiency downstream of pgRNA which might be the key event that discriminates between viral

control and lack thereof in HBeAg-negative patients. Future studies will be aimed at revealing the mechanism(s) that control HBV replication in the ENCI phase of CHB and might open new opportunities for developing novel and effective HBV therapies.

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# FIGURE LEGENDS

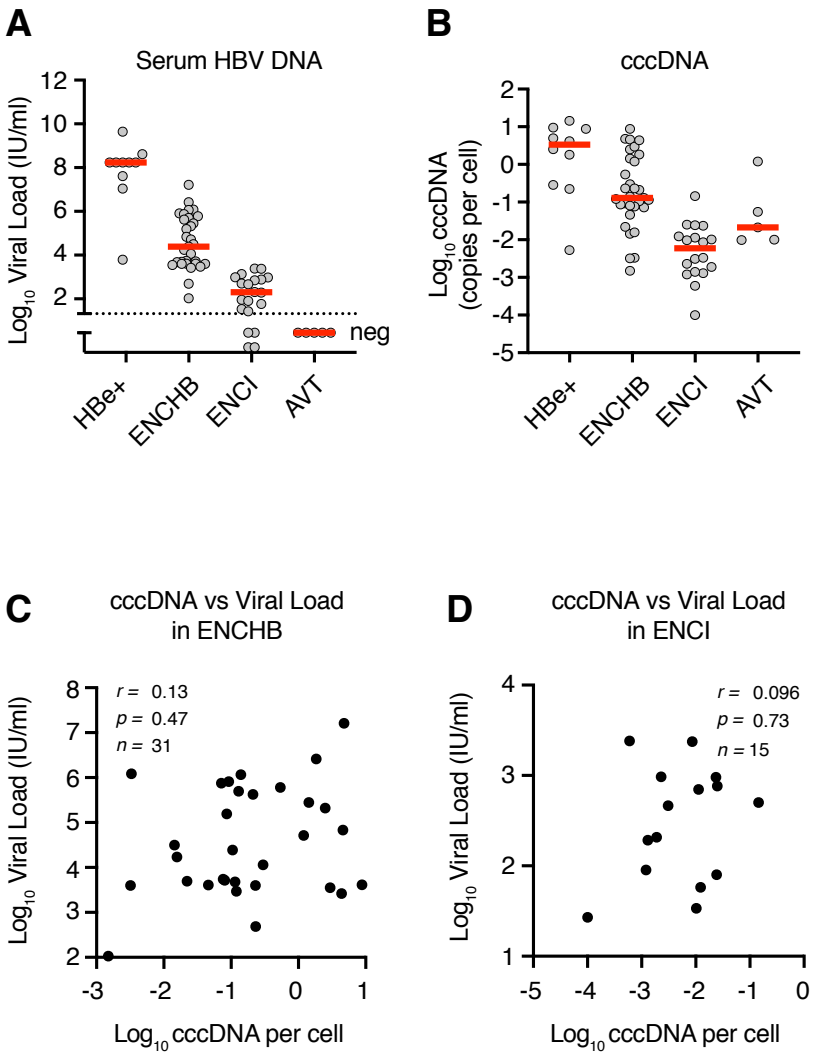
**Figure 1. Relationship between HBV viral load and cccDNA levels in different groups of CHB patients.** Serum HBV DNA levels (A) and intrahepatic cccDNA levels (B) in four groups of CHB patients (HBe+ (n=10), HBeAg-positive; ENCHB, HBeAg-negative chronic hepatitis B (n=31); ENCI, HBeAg-negative chronic infection (n=18); AVT, antiviral therapy (n=5)). Correlation between HBV viral load and intrahepatic cccDNA levels among ENCHB (C) and among ENCI (D) patients. Samples with viral load below detection limit were excluded from correlation analyses. Dotted line in (A) represents the lower limit of quantification; neg, negative for serum HBV DNA. Red lines show the median. HBV cccDNA levels are shown as copies per cell, based on the total DNA quantification and cell number estimation (see Materials and Methods). Statistical analysis of the correlations was performed using a nonparametric Spearman correlation. r, Spearman rank coefficient; n, number of samples.

**Figure 2. Production of pgRNA is inhibited in the HBeAg-negative patients.** A) CccDNA transcriptional activity in four groups of CHB patients: HBe+ (n=9), ENCHB-high (viral load >20,000 IU/ml) (n=13), ENCI (n=14), AVT (n=3). Red lines denote the median. B, C) Correlation of intrahepatic pgRNA production with viral load in ENCHB patients (B) and in ENCI patients (C). RNA values are expressed as copy numbers per cell, assuming that one hepatocyte contains ~30pg of total RNA. Statistical analysis of the correlations was performed using a nonparametric Spearman correlation. Non-parametrical Mann-Whitney test was used for group comparison. *, $p<0.05$; **, $p<0.01$; ns, not significant ($p\geq0.05$); r, Spearman rank coefficient; n, number of samples.

**Figure 3. HBV DNA replication is inhibited in ENCI patients.** HBV replicative intermediates (repDNA) were extracted from the cytoplasmic fraction of liver biopsy tissue as described in “Materials and methods” and quantified by qPCR. Shown are the amounts of replicative intermediates per cell (A) per cccDNA (B) and per pgRNA (C). The number of patients was HBe+: n=12, ENCHB-high: n=22, ENCI: n=8, AVT:

n=3 in (A), HBe+: n=10, ENCHB-high: n=16, ENCI: n=7, AVT: n=3 in (B) and HBe+: n=10, ENCHB-high: n=17, ENCI: n=7, AVT: n=2 in (C). HBV DNA measurements are expressed as copies per cell, based on the total DNA quantification and cell number estimation (see Materials and Methods). Non-parametrical Mann-Whitney test was used for group comparison. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant ($p\geq0.05$).

**Figure 1**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Log10 cccDNA (copies per cell)
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cccDNA vs Viral Load in ENCHB
r = 0.13
p = 0.47
n = 31
Log10 Viral Load (IU/ml)
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cccDNA vs Viral Load in ENCI
r = 0.096
p = 0.73
n = 15
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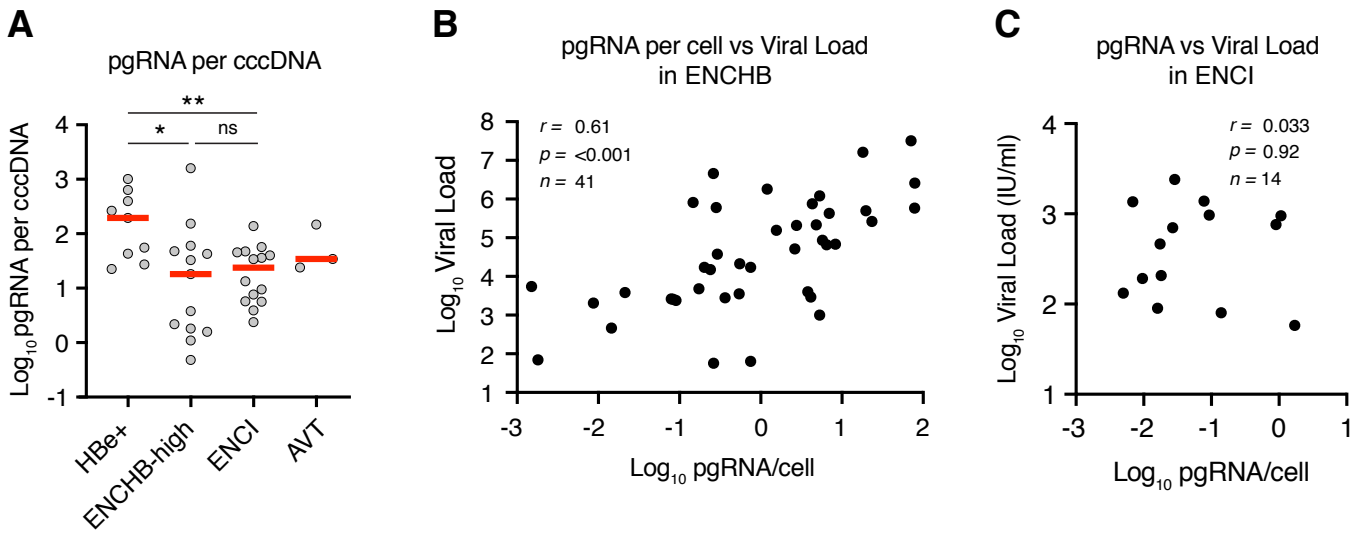
## Figure 2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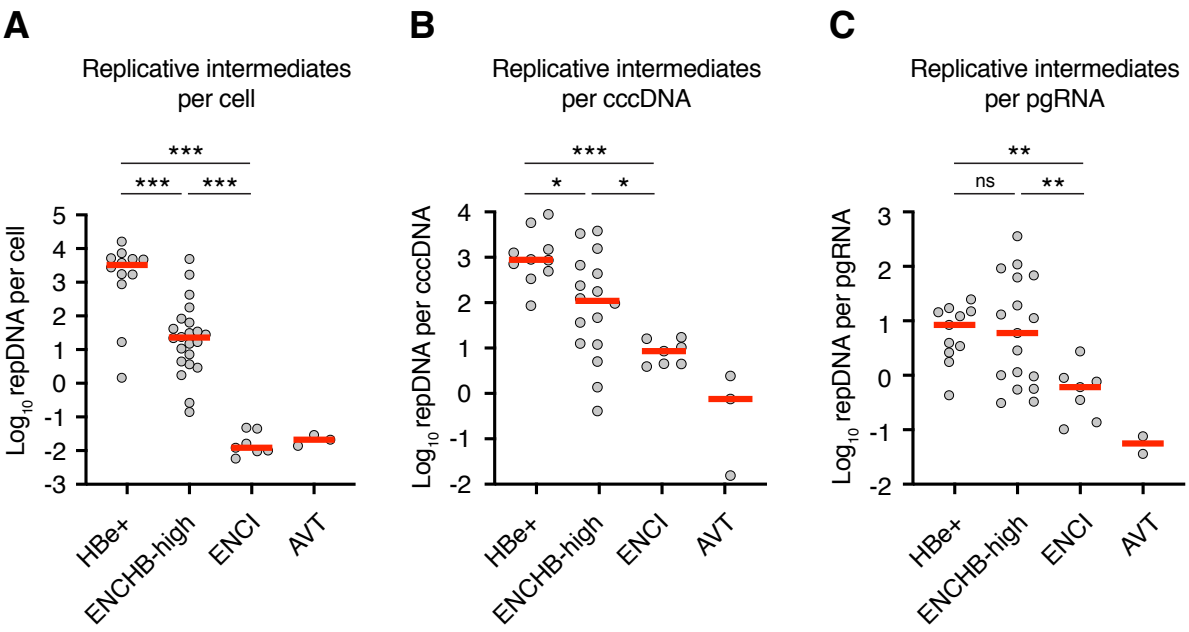
4
3
2
1
0
-1
HBe+
ENCHB-high
ENCI
AVT
B
pgRNA per cell vs Viral Load
in ENCHB
r = 0.61
p = <0.001
n = 41
Log10 Viral Load
8
7
6
5
4
3
2
1
-3
-2
-1
0
1
2
Log10 pgRNA/cell
C
pgRNA vs Viral Load
in ENCI
r = 0.033
p = 0.92
n = 14
Log10 Viral Load (IU/ml)
4
3
2
1
-3
-2
-1
0
1
Log10 pgRNA/cell

## Figure 3


A
Replicative intermediates per cell
Log10 repDNA per cell
***
***
***
HBe+
ENCHB-high
ENCI
AVT
B
Replicative intermediates per cccDNA
Log10 repDNA per cccDNA
***
*
*
HBe+
ENCHB-high
ENCI
AVT
C
Replicative intermediates per pgRNA
Log10 repDNA per pgRNA
**
ns
**
HBe+
ENCHB-high
ENCI
AVT

# 6. CONCLUDING REMARKS

Despite decades of research, hepatitis B virus still remains a global health burden and development of new curative treatments capable of eliminating viral DNA from the liver are urgently needed. This is however hampered by our limited understanding of the host-virus interactions in the human liver. With the experimental work described in this thesis we attempted to shed some light onto some of these interactions, that so far have been difficult to study because of a lack of a fully permissive immunocompetent HBV infection model. Using human liver biopsy material, we were able to overcome some of these limitations.

Liver biopsies are typically used in research as fixed tissue (formalin-fixed or cryopreserved) which limits the range of experiments that can be performed. We now developed a method to keep liver biopsies viable long enough to perform short-term (up to 24 hours) experiments. This allowed us to study, at the cellular level, the impact of HBV infection on the induction of innate immune responses by viral infection or TLR ligands. These experiments demonstrated that the presence of HBV in the liver does not affect the ability of the liver to respond to such stimuli, indicating that the virus does not interfere with the pathways leading to the induction of innate immune responses in the human liver. Lack of any HBV-associated innate immune response signature in the liver of CHB patients at a steady-state finally confirmed the hypothesis that HBV is a “stealth” virus which in contrast to other known viruses is invisible to the host cell’s innate immune system. The inability of HBV to suppress the innate immune responses explains the apparent sensitivity of the virus to some innate immune stimuli and cytokines (see section 5.2) and warrants the development of innate immune modulators as potential therapeutic agents for treatment of CHB. Indeed, several innate immune modulatory therapeutics are currently undergoing clinical trials and show efficiency against HBV (see section 5.2). Importantly, activation of the innate immune system by such therapeutics might even have the potential to eliminate

cccDNA by the IFNα or TNFα mechanisms proposed to degrade cccDNA (see section 5.2).

In a separate effort we wanted to identify the step(s) in the HBV viral life cycle whose control determines the very low viral load in the HBe-negative chronic infection phase of CHB. Those studies showed that the host can exert viral control at the level of cccDNA amounts, transcriptional control and also suppression of viral replication. Interestingly, all the antiviral activities are active even though there is no indication for any immune activity. Future studies defining the mechanism(s) responsible for these antiviral activities will certainly be instrumental in designing novel and effective HBV therapies that hopefully will be curative.

Aside from HBV, our method of short-term *ex vivo* liver biopsy culture might prove useful also in other areas of liver-related research. With the growing interest in precision medicine, this experimental system could be valuable for investigating interindividual differences in liver function and responses to various stimuli or drugs in other chronic liver diseases.

Taken together, during the work performed as a part of this thesis we developed and/or optimized methods and tools for analysis of host-virus interactions and the HBV life cycle in the human liver. Although we specifically used these tools to study HBV, their use is not limited to HBV research. Using these tools, we addressed several unanswered questions in the HBV field and gained new insights into HBV-host interactions in the human liver. We hope that our techniques and findings will prove useful for developing novel therapeutics against HBV and, finally, a long-sought, but still missing cure.

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# 9. APPENDIX

## 9.1 Appendix A. Supplementary information from section 5.1

**Supporting Information**

**Hepatitis B Virus Does Not Interfere with Innate Immune Responses in the Human Liver.**

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## Materials and Methods

### RNA isolation and quantification

Total RNA was extracted from fresh-frozen biopsy pieces using the Trizol reagent (Invitrogen) according to the manufacturer's instructions, with minor modifications. Specifically, after addition of chlorophorm and initial phase separation the aqueous phase was re-extracted once with phenol/chlorophorm mixture (5:1) (Fisher Scientific) and then once again with chlorophorm (Merck) alone. Total RNA was subjected to DNase treatment using the DNA-*free*™ DNA Removal Kit (Ambion) according to manufacturer's instructions. The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and RNA quality/integrity was assessed with an Agilent 2100 BioAnalyzer using RNA 6000 Nano Kit (Agilent Technologies).

### Reverse transcription and quantitative PCR (RT-QPCR)

cDNA was synthesized from 400ng of total RNA using MultiScribe™ Reverse Transcriptase (Applied Biosystems™) and random hexamer primers in a 25μl reaction volume. For all samples "-RT" controls (reactions omitting the reverse transcriptase) were performed. Either 1/10 (IFN$\alpha$2, IFN$\beta$, IFN$\lambda$1, IFN$\lambda$2/3) or 1/20 (TNF$\alpha$, IL6, ISG15, Mx1, RSAD2, GAPDH, HBV RNA) of each RT reaction was used in subsequent SYBR-green based qPCR reactions, that were performed using an ABI 7500 or 7500 Fast Real Time PCR System (Applied Biosystems™) and analyzed with the 7500 Software v2.0.6 (Applied Biosystems™). All reactions were performed at least in duplicates. Besides "-RT" controls, RT reactions without any template and qPCR reactions without any template served as negative controls on every qPCR plate. Target gene expression levels were quantified based on standard curves (comprised of serial dilutions of plasmids containing either cloned cDNA of the corresponding gene or a cloned PCR product) and expressed as copy numbers per 40ng of total RNA. Cutoff for transcript detection was set at 3 copies per reaction (designated as LoD (limit of detection) on the corresponding graphs). Differences in gene expression levels were tested for significance by non-parametrical Mann-Whitney test using GraphPad Prism 7 software (GraphPad Prism, Inc.). Differences with p-values $<0.05$ were considered significant. Sequences of all the primers used

for qPCR are listed in Table S1. Please note, that due to the high sequence similarity of IFNλ2 and IFNλ3 mRNA (97%), they were quantified together using one primer pair that binds to both IFNλ2 and IFNλ3 (designated as IFNλ2/3).

## H&E staining and dead cell staining and quantification

Biopsy pieces were incubated *ex vivo* for the indicated periods of time without any stimulation and then either directly processed for H&E staining or incubated with NUCLEAR-ID® Red/Green cell viability reagent (Enzo Life Sciences, Inc.,) for another 30 minutes for staining of dead cells. At the end of the incubation, biopsy pieces were immediately embedded in OCT, cryosectioned (10µm thickness) in a cryostat and mounted onto Superfrost Plus Gold glass slides (Thermo Fischer Scientific). Consecutive sections were used to visualize total number of cells (mounting with DAPI) and dead cells (mounting without DAPI), respectively. Images, corresponding to the same biopsy area were acquired from the two consecutive sections using an Olympus BX63 upright microscope (Olympus Corporation) and 10x objective. Total and dead cells were counted manually on the corresponding images of the consecutive sections. Percentage of dead cells was calculated as (dead cells/total cells)x100. For H&E staining, biopsy sections were fixed for 30 min in 4% formaldehyde at room temperature, rinsed twice with 1x PBS and then subjected to standard H&E staining. Images were acquired on an Olympus BX63 upright microscope (Olympus Corporation).

## *In Situ* Hybridization

*In situ* hybridization was performed using the ViewRNA system (Thermo Fisher Scientific) and commercially available probe sets (Thermo Fisher Scientific) as previously described [20]. Commercial type 6 probe sets were used to detect human albumin mRNA (cat# VA6-13354) and HBV RNA (cat# VF6-11745) and type 1 probe sets for detection of human IFNβ (cat# VA1-11281) and human ISG15 mRNA (cat# VA1-11634). A negative control (section of an untreated biopsy piece) was mounted together with experimental sections onto the same slide and hybridized under the same conditions. Before microscopy, slides were counterstained with Meyer's hematoxylin and embedded with DAPI-containing aqueous mounting medium (Roti-Mount FluorCare DAPI, Roth, Arlesheim, Switzerland). The images were acquired

using a laser scanning confocal microscope (LSM710, Carl Zeiss Microscopy) and Zen2 software (Carl Zeiss Microscopy). The high-power images (212.3 x 212.3µm) were acquired using the 40x objective. Colors of each fluorescent dye were assigned during acquisition (red for Fast Red substrate, green for Fast Blue substrate, and blue for DAPI). The pictures were saved in the Zeiss confocal file format (.lsm), including multicolor layers, and further processed using ImageJ software.

### Immunostaining

Frozen liver biopsy sections were fixed in freshly prepared 4% formaldehyde for 15 min at room temperature. After washing with PBS, they were permeabilized in cold methanol (-20°C) for 10 minutes. Samples were blocked with 5% normal goat serum/0.05% Tween-20 for 1h. Phosphorylated STAT1 (pSTAT1) was detected using an anti-pSTAT1 rabbit antibody 58D6 (Cell Signaling), HBsAg – using a monoclonal mouse anti-HBs antibody (Abnova) and HBcAg using a chicken anti-HBc antibody (gift from Prof. Ralf Bartenschlager, Heidelberg, Germany) in blocking buffer. For signal visualization, the following secondary antibodies conjugated to Alexa Fluor dyes (Invitrogen) were used: anti-mouse Alexa-488 or Alexa-647, anti-rabbit Alexa-568 or Alexa-647 and anti-chicken Alexa-555 in blocking buffer. Sections were mounted with Mount FluorCare DAPI (Carl Roth GmbH). Images of entire tissue sections were acquired on a Nikon Ti microscope using either a 10x or 20x objective, saved in the Nikon file format (.nd2) and further processed using ImageJ software.

### Albumin, HBsAg and HBV DNA measurement in the biopsy culture supernatant

Biopsy culture supernatant was collected at the time of tissue harvest and stored at -80°C. HBsAg levels were measured in undiluted biopsy culture supernatant using the Architect HBsAg assay (Abbott, Chicago, IL). For HBV DNA quantification, 400 µl of biopsy culture supernatant was mixed with 5x DNA lysis buffer (5% SDS, 100 mM EDTA, 200 mM Tris, pH 8.0) and treated with proteinase K (0.5 mg/ml) for >4h at 45°C. Total DNA was then extracted with phenol/chlorophorm (5:1) (Fisher Scientific), then once again with chlorophorm (Merck) alone and precipitated with isopropanol in the presence of 0.3M sodium acetate. Precipitated DNA was washed twice with 70% ethanol and dissolved in 0.5x TE buffer. HBV DNA was quantified by QPCR using a standard curve based on a plasmid containing a full-length HBV genome. For albumin

measurement in biopsy culture supernatants, liver biopsies were washed for 2-3 min with an excess volume of 0.9% NaCl at room temperature and then washed with 1.5 ml of fresh culture medium for another 10 min, cut into pieces and cultured ex vivo. Human albumin levels were measured in 50 µl undiluted cell culture supernatant using a Human Albumin ELISA Kit (AssayPro, St. Charles, USA; Cat# EA3201-1) according to manufacturer's instructions

## HBV genotyping

The same starting material that was used for HBV DNA quantification was also used for HBV genotyping. Genotyping was performed as described by Lebosse et al., J Hepatol 2017;66:897-909.

**Supplementary Table 1.** Primer sequences

| Target Gene | Primer direction | Sequence 5' --> 3' |
|---|---|---|
| GAPDH | Forward | GCTCCTCCTGTTCGACAGTCA |
| | Reverse | ACCTTCCCCATGGTGTCTGA |
| IFNα2 | Forward | TCGTATGCCAGCTCACCTTTT |
| | Reverse | TCAGTCAGCATGGTCCTCTGTA |
| IFNβ | Forward | AGTAGGCGACACTGTTCGTG |
| | Reverse | AGCCTCCCATTCAATTGCCA |
| IFNλ1 | Forward | CACAGGAGCTAGCGAGCTTCA |
| | Reverse | TTTTCAGCTTGAGTGACTCTTCCA |
| IFNλ2/3 | Forward | GCCAAAGATGCCTTAGAAGAG |
| | Reverse | CAGAACCTTCAGCGTCAGG |
| TNFα | Forward | CTCTCTAATCAGCCCTCTGGC |
| | Reverse | GCTTGAGGGTTTGCTACAACA |
| IL-6 | Forward | AATTCGGTACATCCTCGACGG |
| | Reverse | GGTTGTTTTCTGCCAGTGCC |
| ISG15 | Forward | TCCTGCTGGTGGTGGACAA |
| | Reverse | TTGTTATTCCTCACCAGGATGCT |
| Mx1 | Forward | GTGCATTGCAGAAGGTCAGA |
| | Reverse | TCAGGAGCCAGCTGTAGGTGT |
| RSAD2 | Forward | CTTTGTGCTGCCCCTTGAG |
| | Reverse | TCCATACCAGCTTCCTTAAGCAA |
| HBV RNA | Forward | TGGCCAAAATTCGCAGTCCC |
| | Reverse | GATGAGGCATAGCAGCAGGATG |
| Albumin | Forward | TGCCAAAGTGTTCGATGAAT |
| | Reverse | AGCGCATTCTGGAATTTGTA |
| HNF-1β | Forward | GGATGCTCAGTGAGGACCCT |
| | Reverse | GTTGAGATGCTGGGAGAGGT |
| HNF-4α | Forward | TCAACCCGAGAAAACAAACC |
| | Reverse | ACCTGCTCTACCAGCCAGAA |
| CYP3A4 | Forward | TGTGCCTGAGAACACCAGAG |
| | Reverse | GTGGTGGAAATAGTCCCGTG |

**Supplementary Table 2.** HBV patient characteristics and performed experiments

| ID | Geno -type | Log Viral Load (IU/ml) | Log HBV RNA Copies / 40 ng | qHBs (IU/ml) | HBs IHC (%) | HBc IHC (%) | HBeAg | ALT (U/L) | ISHAK stage | ISHAK grade | Stimulated Receptor | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | TLR | | | | | | RLR |
| | | | | | | | | | | | 3 | 2 | 4 | 7 | 8 | 9 | (SeV) |
| **HBeAg+ infection; (Immune tolerant (IT))[a]** | | | | | | | | | | | | | | | | | |
| C799 | B | >8.23[b] | 6.38 | 1477 | 99 | 80 | pos | 25 | 0 | 2 | + | | | | | | |
| D190 | D | 6.34 | 5.26 | -[c] | 100 | 5 | pos | 31 | 1 | 1 | | | | | | | + |
| **HBeAg+ hepatitis; (HBeAg+ Immune Active (IA))** | | | | | | | | | | | | | | | | | |
| C787 | D | >8.23 | 6.01 | - | 99 | 70 | pos | 46 | 1 | 5 | + | | | | | | |
| C921 | B | 3.83 | 5.80 | - | 95 | 0 | pos | 66 | 0 | 2 | + | | | | | | |
| **HBeAg- infection; (Inactive carrier (IC))** | | | | | | | | | | | | | | | | | |
| C968* | A | 1.90 | 4.60 | 6060 | 90 | 0 | neg | 8 | 1 | 3 | | | + | | | | |
| C812 | A | 1.76 | 5.24 | 3127 | 99 | 0 | neg | 17 | 0 | 3 | + | | | | | | |
| C889 | A | 3.00 | 4.80 | - | 90 | 0 | neg | 43 | 1 | 3 | + | | | | | | |
| C912* | C | 2.70 | 5.53 | 2099 | 99 | 0 | neg | 21 | 0 | 2 | + | | | | | | |
| D012* | D | 3.14 | 3.80 | 2371 | 0 | 0 | neg | 25 | 1 | 3 | + | + | | | + | | |
| D055* | D | 2.67 | 4.48 | 6626 | 0 | 0 | neg | 22 | - | - | + | | | | | | |
| D061 | B | 3.38 | 4.36 | - | 30 | 0 | - | 44 | 4 | 1 | + | + | + | + | | | |
| D459 | D | 1.46 | 4.72 | 5891 | 5 | 0 | neg | 28 | 1 | 2 | + | | | | | | + |
| **HBeAg- hepatitis; (HBeAg- Immune Active (IA))** | | | | | | | | | | | | | | | | | |
| C982** | B | 4.17 | 5.81 | - | 90 | - | neg | 45 | 0 | 2 | | + | | + | | + | |
| D021* | A | 3.46 | 5.38 | 4572 | 90 | 0 | neg | 30 | 1 | 4 | | + | + | + | + | + | |
| D054* | D | 3.62 | 4.55 | - | 10 | 0 | neg | 22 | 2 | 1 | + | | + | | | | |
| C765 | D | 5.78 | 4.90 | - | 70 | 1 | neg | 344 | 1 | 3 | + | | | | | | |
| C786 | D | 1.76 | 5.29 | 2116 | 99 | 1 | neg | 72 | 0 | 3 | + | | | | | | |
| C887 | D | 3.59 | 4.78 | - | 5 | 0 | neg | 40 | 1 | 3 | + | | | | | | |
| C911* | E | 4.23 | 4.70 | - | 30 | 0 | neg | 31 | 0 | 3 | + | | | | | | |
| C920 | A | 2.58 | 5.47 | - | 90 | 0 | neg | 45 | 0 | 3 | + | | | | | | |
| C926 | D | 3.41 | 4.23 | - | 10 | 0 | neg | 57 | 2 | 3 | + | | | | | | |
| C930* | E | 3.59 | - | - | 80 | 0 | neg | 30 | 2 | 3 | | | | | | | |
| D010* | D | 5.77 | 5.45 | 4931 | 0 | 40 | neg | 124 | 3 | 6 | + | | | | + | | |
| D139** | C | 6.08 | 5.36 | 5276 | 95 | 5 | neg | 35 | 1 | 3 | | | | | | | + |
| D167** | D | 4.33 | 5.05 | - | 80 | 0 | neg | 19 | 0 | 2 | | | | | | | + |
| D183** | D | 6.66 | 5.36 | 4926 | 100 | 0 | neg | 40 | 2 | 4 | | | | | | | + |
| **Under therapy** | | | | | | | | | | | | | | | | | |
| D445[d,e] | D | 1.83 | 5.28 | 2156 | 90 | 0 | neg | 157 | 4 | 17 | + | | | | | | + |
| C706[f] | D | <1.30[g] | 4.40 | 6125 | 20 | 0 | neg | 32 | 3 | 4 | + | | | | | | |
| C809[h] | D | <1.30 | 4.08 | 297 | 10 | 1 | pos | 26 | 3 | 1 | + | | | | | | |
| D187[i, **] | D | <1.30 | 3.79 | 7408 | - | - | neg | 24 | 3 | 5 | | | | | | | + |

[a] Nomenclature according to old EASL guidelines (in parentheses)
[b] Outside maximal assay range
[c] No information
[d] interferon therapy for three months, but stopped due to adverse effects and biopsied 1 month later.
[e] co-infection with Hepatitis D virus (HDV), but immunohistologically negative for HDV Ag
[f] Lamivudine treatment for 12 years. Biopsy obtained 1 year after stop of therapy.
[g] Below detection limit
[h] Initially under Interferon therapy followed by different combinations of Lamivudin, Adefovir and Tenofovir for 4 years and 4 years on Tenofovir at the time of biopsy.
[i] Interferon therapy followed by 11 years of Lamivudin including at the time of biopsy
* HBsAg and HBV DNA measured in biopsy culture supernatant (at 6 and 12 hours)
** HBsAg and HBV DNA measured in biopsy culture supernatant (at 6, 12 and 24 hours)

**Supplementary Table 3.** Control patient characteristics and performed experiments

| ID | Diagnosis | Stimulated Receptor | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | TLR | | | | | | RLR |
| | | 3 | 2 | 4 | 7 | 8 | 9 | (SeV) |
| D477 | AIH | | | | | | | |
| C824 | ALD | + | | | | | | |
| D438 | ALD | + | | | | | | |
| D478 | ASH | | | | | | | |
| C778 | ASH, Cirrhosis | + | | | | | | |
| NN2 | ASH, Cirrhosis | + | | | | | | |
| C927 | ASH, Cirrhosis | + | | | | | | |
| D191 | ASH, Cirrhosis | | | | | | | + |
| D052 | ASH/NASH | | + | + | | | | |
| D116 | ASH/NASH | | | | | | | + |
| C917 | ASH/NASH | + | | | | | | |
| D486 | ASH/NASH | | | | | | | |
| D093 | ASH/NASH, Cirrhosis | | | | | | | + |
| D107 | Cirrhosis | | | | | | | + |
| C888 | DILI | + | | | | | | |
| C981 | DILI | | | | | + | + | |
| D198 | DILI | | | | | | | + |
| D364 | DILI (40% steatosis) | | | | | | | |
| D439 | DILI | + | | | | | | |
| D450 | DILI | + | | | | | | + |
| D149 | GvHD | | | | | | | + |
| D480 | GvHD | | | | | | | |
| C817 | Med-Tox | + | | | | | | |
| C780 | Minimal unspecific hepatitis | + | | | | | | |
| C823 | NAFLD | + | | | | | | |
| C826 | NAFLD | + | | | | | | |
| C890 | NAFLD | + | | | | | | |
| C980 | NAFLD | | | | | + | | |
| D057 | NAFLD | + | | | | | | |
| C740 | NASH | + | | | | | | |
| C956 | NASH | + | | | | | | |
| D005 | NASH | | + | + | + | + | + | |
| D042 | NASH | + | + | + | + | | | |
| D137 | NASH, Cirrhosis | | | | | | | + |
| C928 | NASH, high grade fibrosis | + | | | | | | |
| NN1 | Non-tumour part of HCC liver | + | | | | | | |
| D006 | Normal | | | | + | | + | |
| C814 | Steatosis | + | | | | | | |
| D166 | Toxic hepatopathy | | | | | | | + |
| D060 | Transplant rejection | | | | | | | + |
| D437 | Unspecific steatosis | + | | | | | | |
| D461 | Cholestatic liver disease | + | | | | | | + |

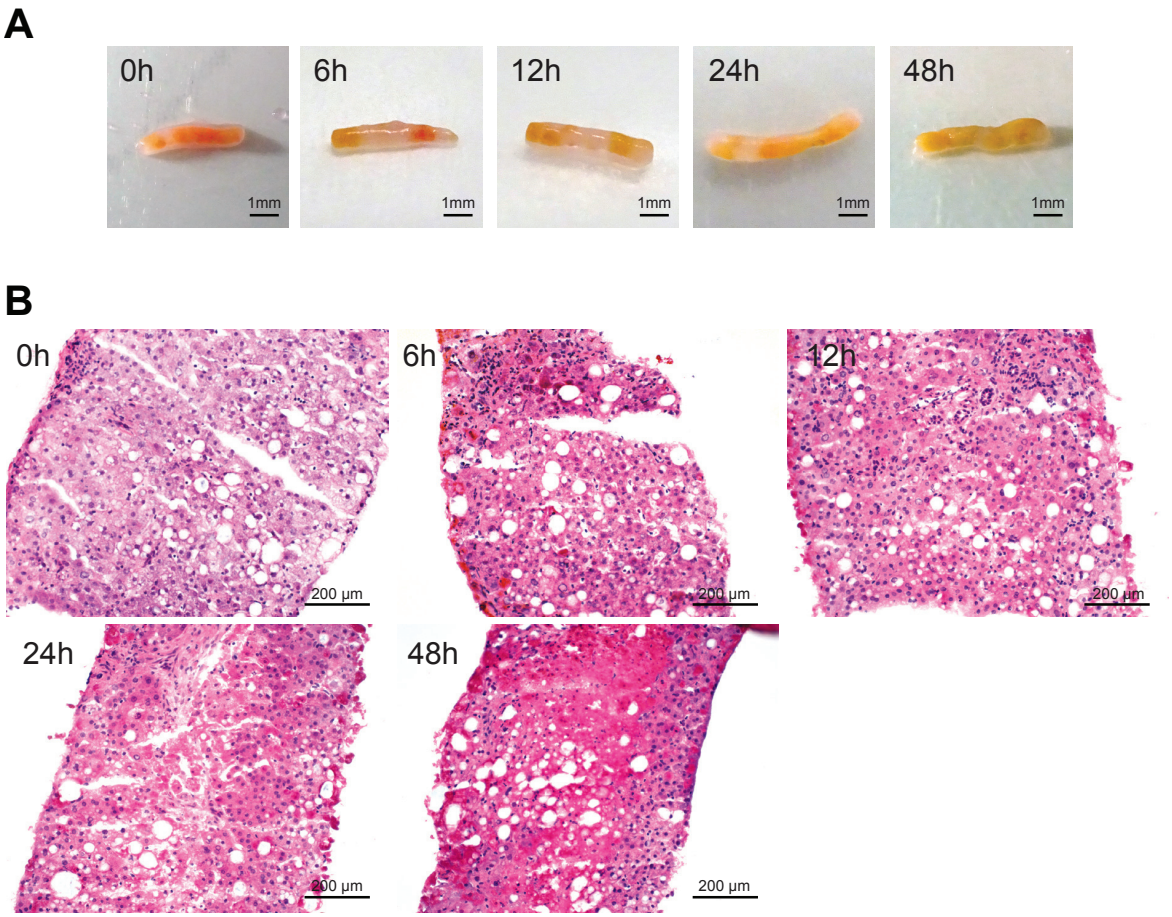

A
0h
6h
12h
24h
48h
1mm
B
0h
6h
12h
24h
48h
200 μm
C
0h
6h
24h
48h
NuclearID
DAPI
3.5%
7.1%
17.7%
37%
D
+poly(I:C) (6h)
NuclearID
DAPI
6.9%

**Figure S1. Macroscopic and microscopic biopsy integrity and cell viability during short-term *ex vivo* culture.** (A) Pieces of a fresh liver biopsy (D486, Table S2) were cultured for up to 48h without any stimulation and photographed at the indicated time points (scale bar, 1 mm) (B) Pieces of a fresh liver biopsy (D364, Table S2) were cultured for the indicated time points and embedded in OCT, cryosectioned and subjected to H&E staining. (C) Human liver biopsy pieces obtained from patients without viral infection were cultured *ex vivo* for the indicated time periods. Dead cells were stained by an additional incubation for 30 min in the presence of NUCLEAR-ID® Red/Green reagent. Biopsies were then embedded in OCT for cryosectioning. Consecutive cryosections were analyzed by fluorescence microscopy for visualization of dead cells (green, top panels) and following DAPI staining for nuclei (bottom panels, blue). Dashed white lines outline the edges of the biopsies. (D) Parallel human liver biopsy pieces were cultured *ex vivo* in the presence of 100 µg/mL poly(I:C) and processed for dead cell visualisation as in (C).

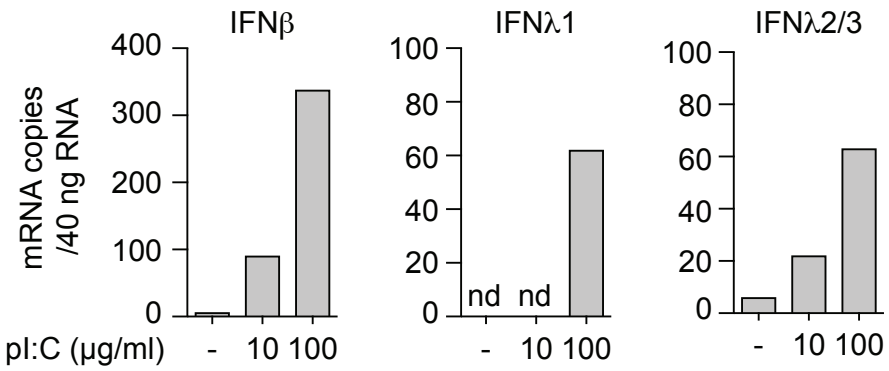



**Figure S2. Dose-dependent induction of IFN in poly(I:C) stimulated *ex vivo* cultured human liver biopsy tissue.** Fresh biopsy pieces of a patients without viral infection were incubated with two doses of poly(I:C) or left untreated for 3h and expression of IFN was analyzed by RT-QPCR. nd, not detected.

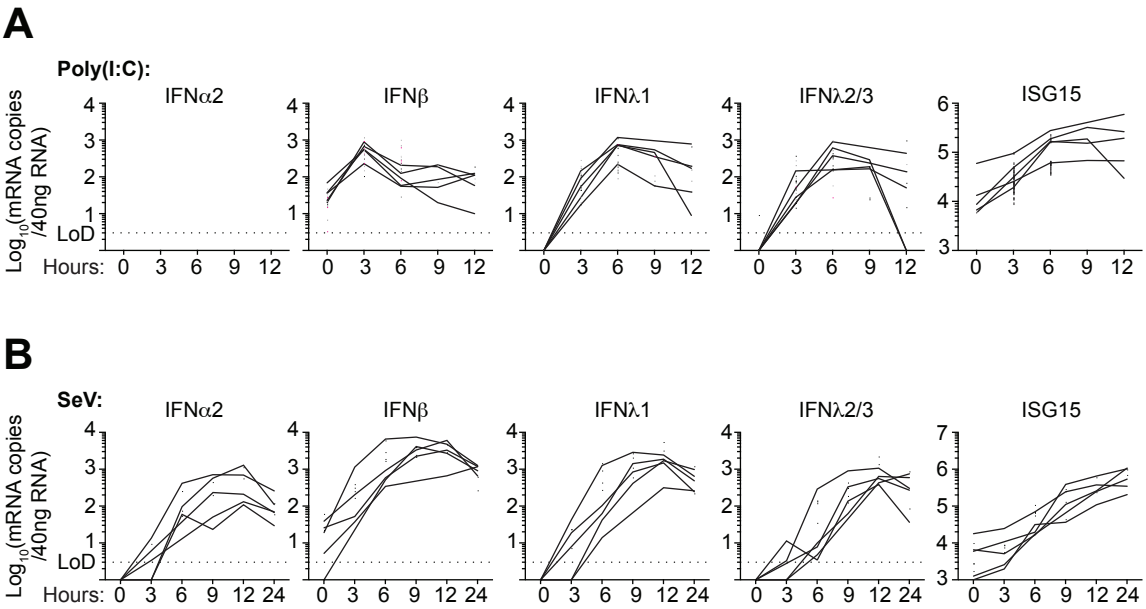



**Figure S3. IFN and ISG expression upon *ex vivo* stimulation of human liver biopsies with poly(I:C) or Sendai virus.** Fresh biopsies of patients without viral infection were cut into several pieces and immediately stimulated *ex vivo* with poly(I:C) (100µg/mL; n=6) for up to 12 hours (A) or with SeV (MOI ~10; n=5) (B) for up to 24 hours. Expression of IFNs and ISG15 mRNA was analyzed by RT-QPCR at the indicated time points. LoD, limit of detection. The following biopsies were used (C740, C917, C927, C956 and D042 in (A); D093, D116, D137, D149 and D166 in (B); Table S3).

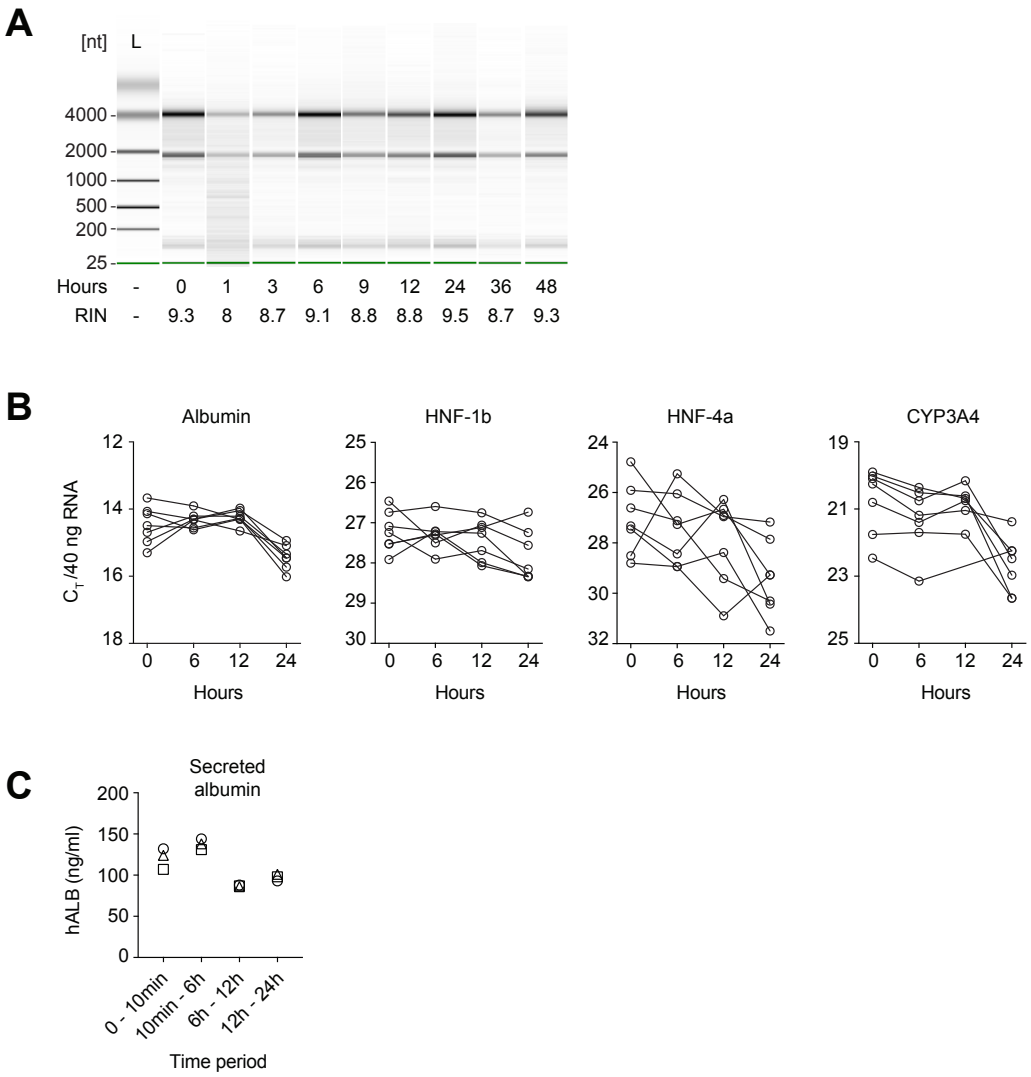



**Figure S4. RNA integrity and liver functions are preserved during short-term *ex vivo* culture.** (A) Total RNA extracted from human liver biopsy pieces without viral infection cultured *ex vivo* for the indicated time periods was analyzed using an Agilent 2100 BioAnalyzer as described in materials and methods. Each lane contains RNA from a different piece of the same biopsy. The same volume of RNA sample was loaded in each lane. RNA integrity numbers (RIN) are shown. L – RNA ladder; scale bar, 200µm. B) Liver biopsies of several patients (D060, D093, D116, D139, D166, D167, D183; Table S2 and S3) were cut into pieces and cultured for the indicated time before total RNA extraction. Expression of albumin, HNF-1β, HNF-4α and CYP3A4 mRNA was analyzed by RT-QPCR at the indicated time points. Data are shown as threshold cycle ($C_T$) values of corresponding RT-QPCR reactions. (C) Liver biopsies of three patients (D477, D478, D480) were washed two times, cut into pieces and maintained in culture as described. Supernatants were harvested after 10 min, 6, 12 and 24 hours albumin levels were measured by ELISA as described in materials and methods. The culture medium was changed after 10 min, 6 and 12 hours. The albumin measured in the supernatant was secreted during the indicated time intervals.

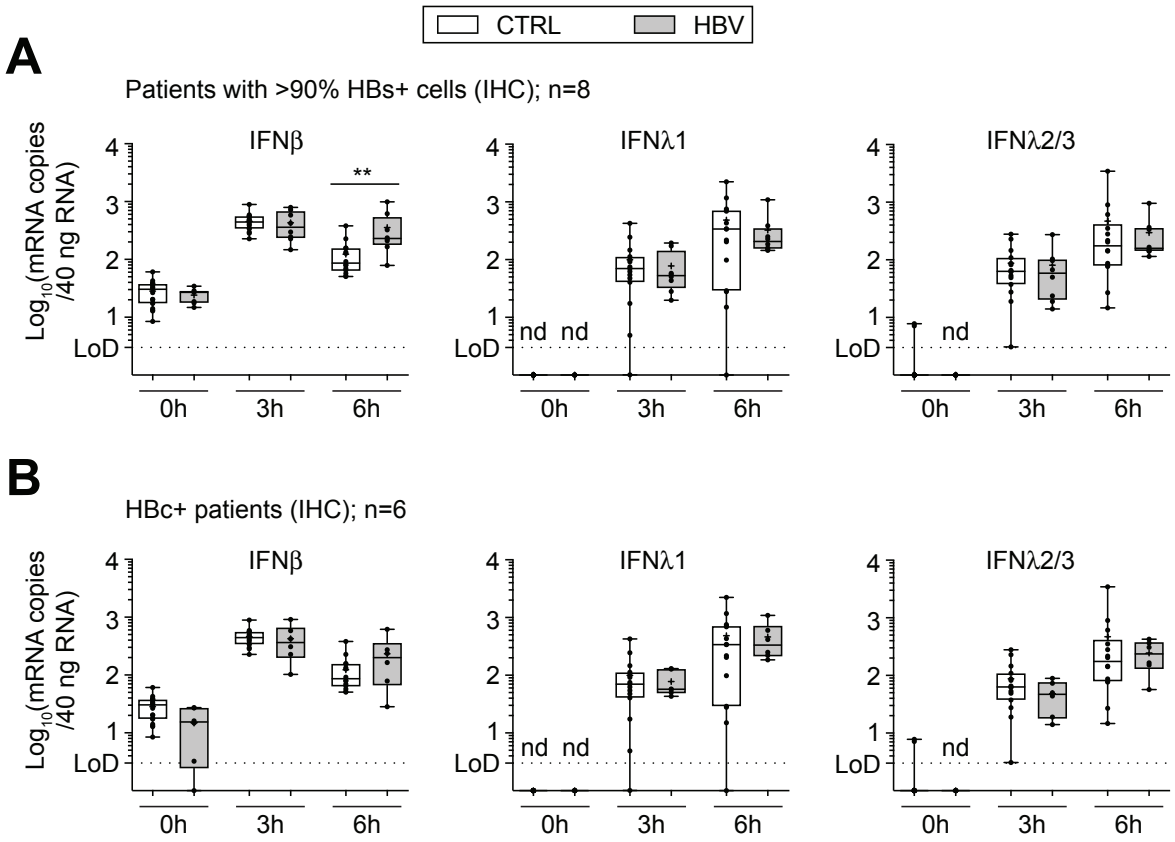



**Figure S5. Subgroup analysis of transcriptional induction of IFN in poly(I:C) treated *ex vivo* cultured biopsy tissues of HBV-infected (HBV) and uninfected (CTRL) patients.** The IFN gene expression analysis shown in Figure 3B was analysed in subsets of patients with (A) >90% of HBsAg-positive hepatocytes (by IHC) or (B) detectable HBcAg (by IHC). The group of 8 patients with >90% HBsAg positive cells included 1 patient with HBeAg+ infection, 2 with HBeAg+ hepatitis, 3 with HBeAg- infection, and 2 with HBeAg- hepatitis. The group of 6 patients with positive HBcAg staining included 1 patients with HBeAg+ infection, 1 with HBeAg+ hepatitis, 0 with HBeAg- infection, and 4 with HBeAg- hepatitis. Data display and statistical analysis was performed as described in Figure 3.

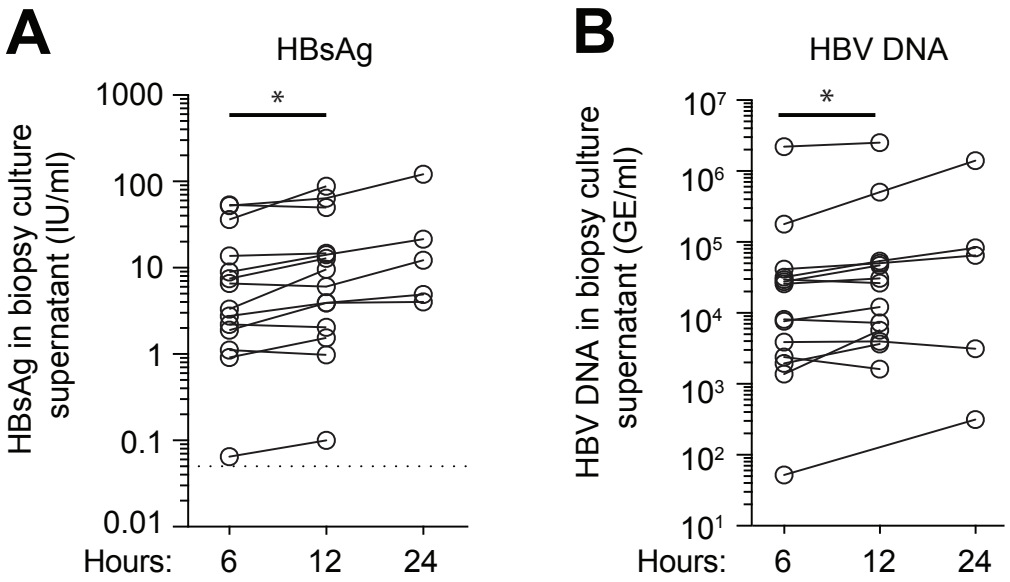



**Figure S6. HBV antigen and DNA production during *ex vivo* biopsy culture.** Culture supernatant from *ex vivo* cultured CHB liver biopsies (Table S2, asterisk) was collected at the indicated time points (6h, 12h, n=14; 24h, n=5) and was either directly used to measure HBsAg (A), or was subjected to HBV DNA extraction and quantification (B) as described in materials and methods. IU – international unit; GE – genome equivalent. Non-parametric Wilcoxon match-pairs signed rank test was used to compare gene expression between different time points (*$P$<.05).

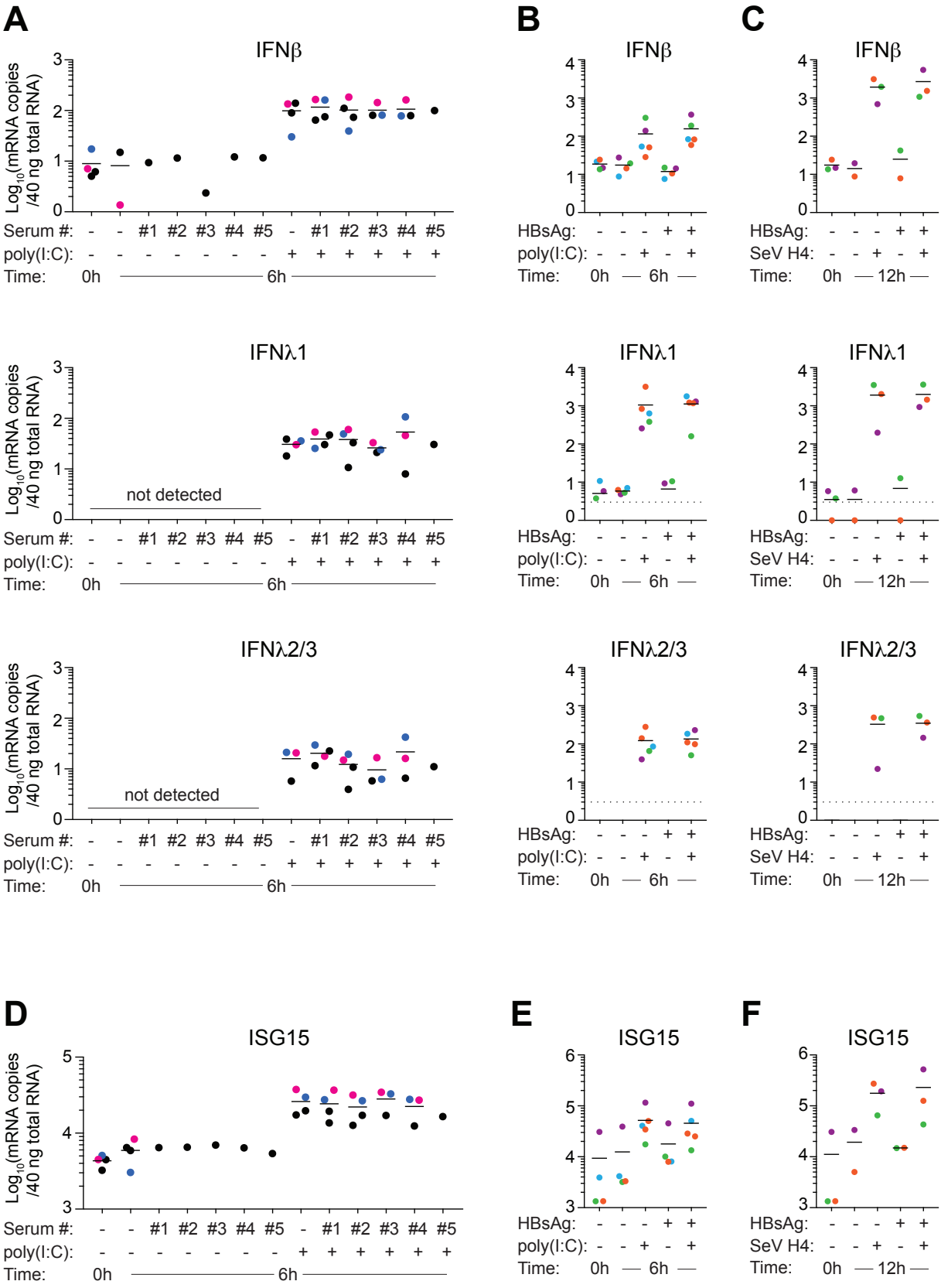
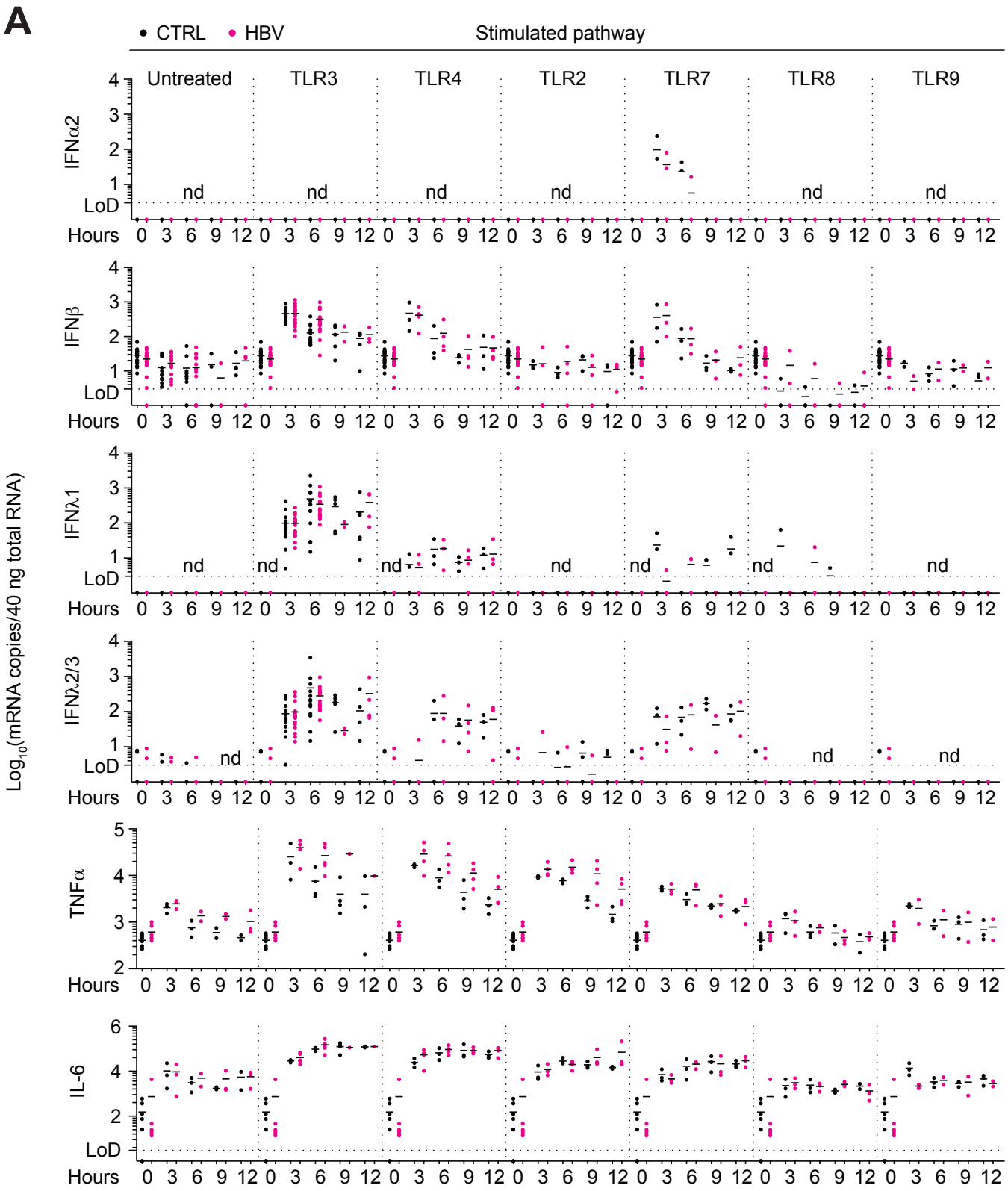

A
IFNβ
Log10(mRNA copies /40 ng total RNA)
Serum #: - - #1 #2 #3 #4 #5 - #1 #2 #3 #4 #5
poly(I:C): - - - - - - - + + + + + +
Time: 0h 6h
IFNλ1
not detected
IFNλ2/3
not detected
B
IFNβ
HBsAg: - - - + +
poly(I:C): - - + - +
Time: 0h 6h
IFNλ1
IFNλ2/3
C
IFNβ
HBsAg: - - - + +
SeV H4: - - + - +
Time: 0h 12h
IFNλ1
IFNλ2/3
D
ISG15
E
ISG15
F
ISG15

**Figure S7. Exposure to exogenous HBV antigens does not prevent poly(I:C)- and SeV-mediated IFN and ISG induction.** Interferon (A) and ISG (D) induction in liver biopsies (D437, D438, D439; Table S3) stimulated with 100 µg/ml poly(I:C) (6h) in the presence of 10% CHB patient serum (*Serum#, HBsAg (IU/ml), HBeAg pos/neg, Log viral load (IU/ml)*): #1, 748, pos, 9; #2, 5212, neg, 2.95; #3, 4941, neg, 6.23; #4, 6621, neg, 7.4; #5, 297, pos, <1.3). Interferon (B, C) and ISG (E, F) induction in liver biopsies (D445, D450, D459, D461, Tables S2 and S3) in the presence of 3.3 µg/ml recombinant HBsAg and stimulated with 100 µg/ml poly(I:C) (B, E) or infected with SeV (MOI~10) (C, F). Expression of IFN (A-C) and ISG15 (D-F) mRNA was analyzed by RT-QPCR at the indicated time points. Each color in the graphs represents an individual donor with duplicate samples for some donors and time points. Black lines show the mean. Dashed lines represent the limit of detection; nd, not detected.


A
CTRL
HBV
Stimulated pathway
Untreated
TLR3
TLR4
TLR2
TLR7
TLR8
TLR9
IFNα2
IFNβ
IFNλ1
IFNλ2/3
TNFα
IL-6
LoD
nd
Hours
0 3 6 9 12
Log10(mRNA copies/40 ng total RNA)

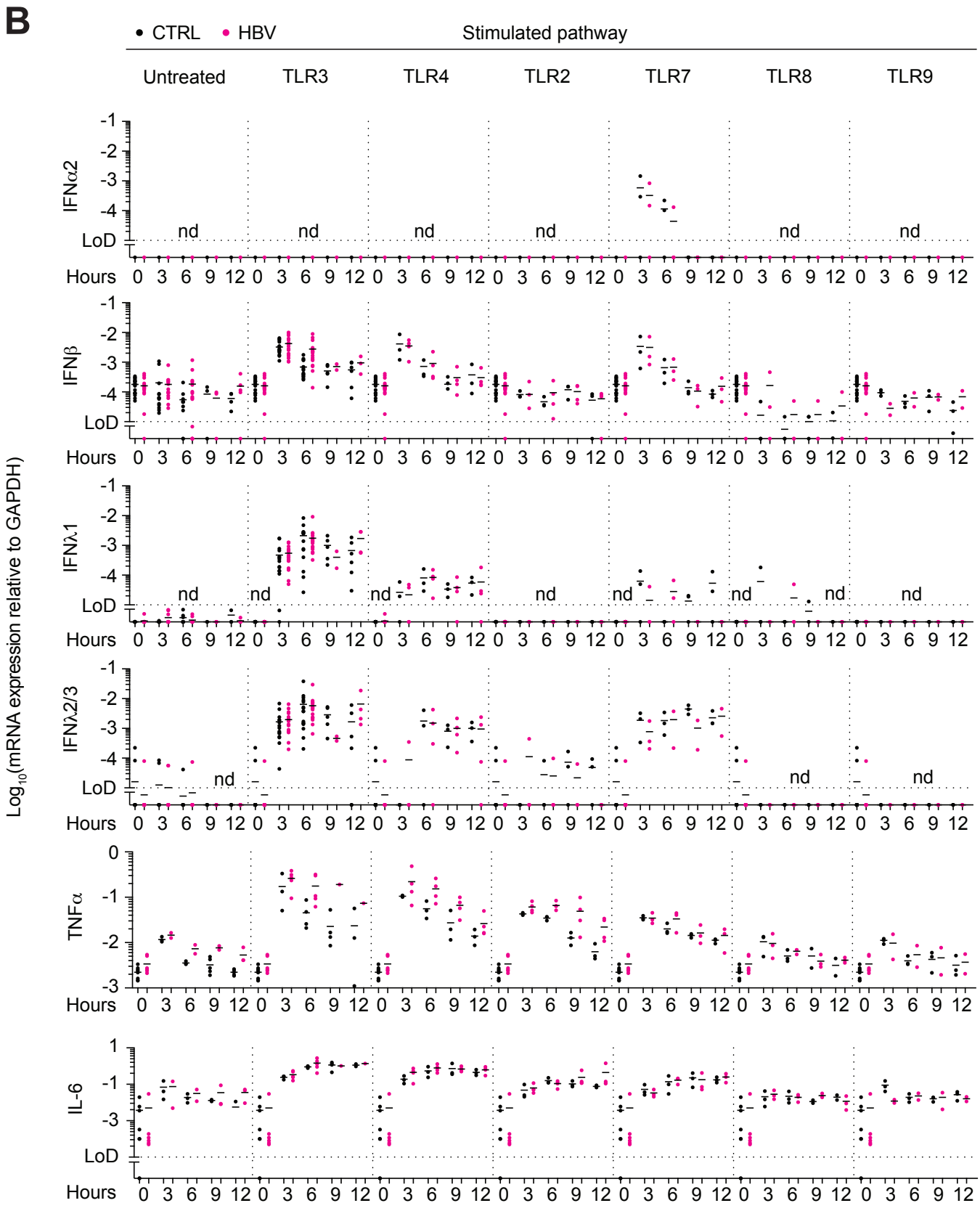

B
CTRL
HBV
Stimulated pathway
Untreated
TLR3
TLR4
TLR2
TLR7
TLR8
TLR9
IFNα2
IFNβ
IFNλ1
IFNλ2/3
TNFα
IL-6
LoD
nd
Hours
0 3 6 9 12
Log10(mRNA expression relative to GAPDH)

**Figure S8. Induction of IFNs and proinflammatory cytokines is not suppressed in the liver of HBV-infected patients upon *ex vivo* stimulation with different TLR ligands.** Fresh biopsies obtained from HBV infected patients (HBV, magenta) and uninfected control patients (CTRL, black) were cut into several pieces and stimulated with the TLR ligands poly(I:C) (TLR3), LPS (TLR4), PGN-SA (TLR2), R837 (TLR7), ssRNA (TLR8) and CpG DNA (TLR9) for stimulation of TLR3, TLR4, TLR2, TLR7, TLR8 and TLR9, respectively. Total RNA was isolated from the *ex vivo* cultured biopsy pieces at the indicated time points after TLR ligand addition and as a baseline control (0h) right before TLR ligand addition. Cytokine expression was analyzed by RT-qPCR and is shown as copy numbers per 40 ng of total RNA (A), or as relative levels normalized to GAPDH (B). Results are shown as dot-plots with a line showing the mean. N=11-19 (0, 3, 6h) for IFNs, N=3-8 (0, 3, 6h) for TNF$\alpha$ and IL-6; N=2-6 (9, 12h) for all. Dashed horizontal line represents the limit of detection by QPCR.

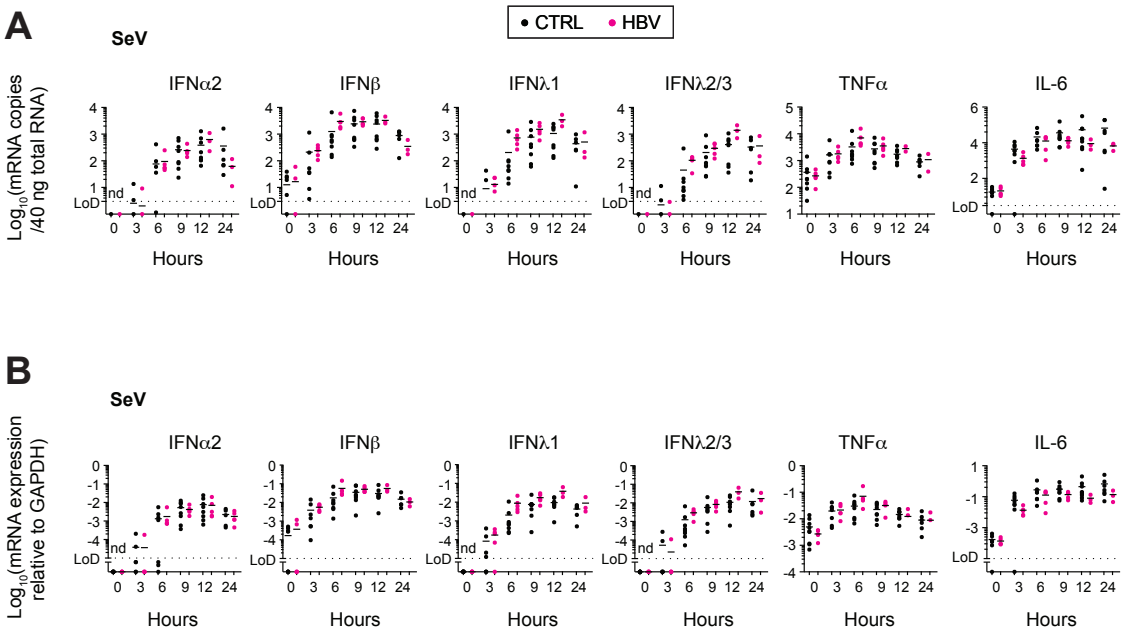



**Figure S9. Induction of IFN and proinflammatory cytokines is not suppressed in the liver of HBV-infected patients upon *ex vivo* stimulation with Sendai virus.** Fresh biopsies of HBV infected (HBV, magenta) and uninfected control (CTRL, black) patients were *ex vivo* cultured in the presence of SeV (MOI=10) for the indicated time periods and processed exactly as described in Figure S8. Expression levels are shown as copy numbers per 40 ng of total RNA (A), or as relative levels normalized to GAPDH (B).

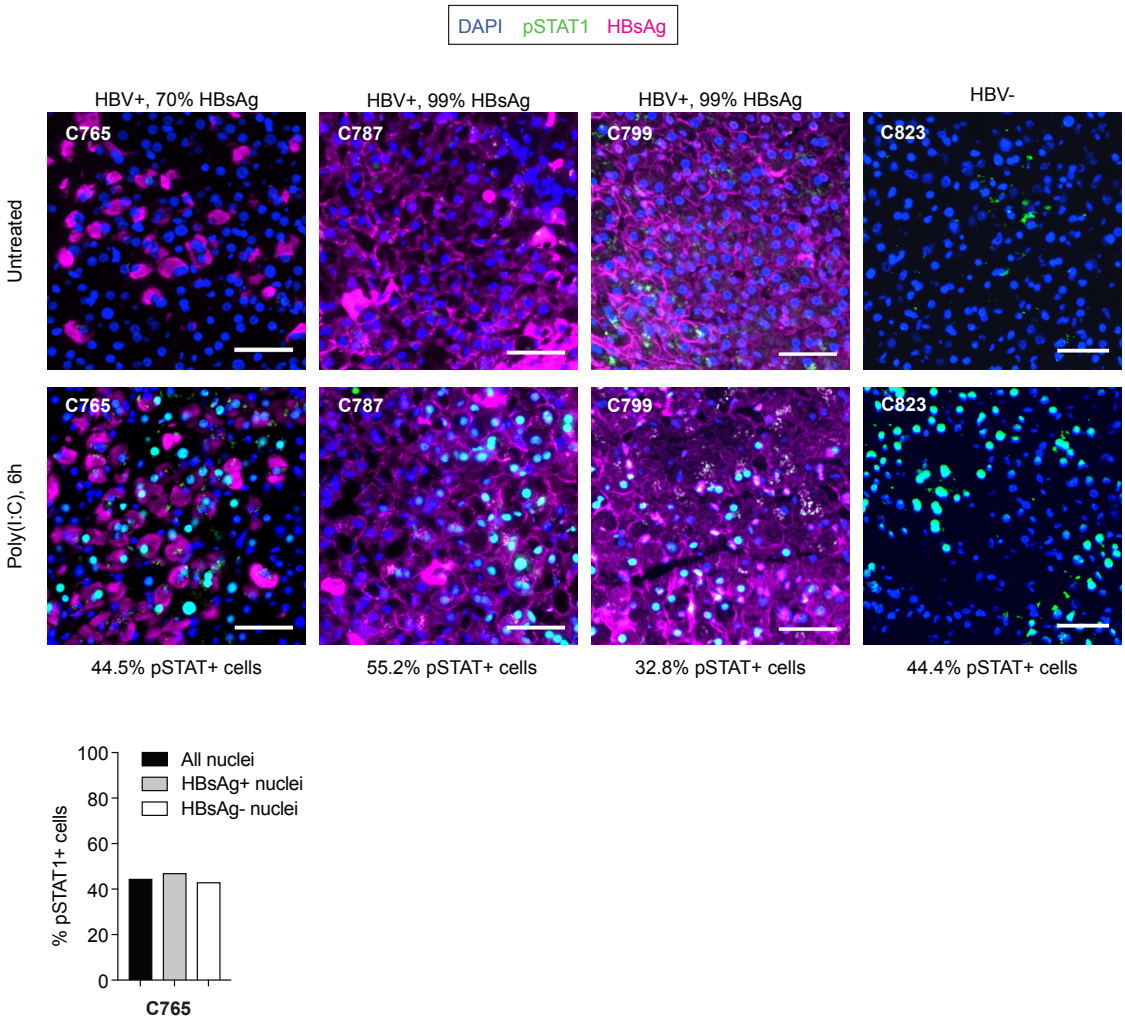



**Figure S10. IFN-signaling is not blocked in HBV positive hepatocytes.** Fresh liver biopsy pieces from highly HBV-viremic patients (Table S2, C765, C787 and C799) and an uninfected control patient (C823) were stimulated *ex vivo* with 100 µg/mL poly(I:C) or left untreated for 6 hours before embedding in OCT for cryosectioning. Cryosections were subjected to simultaneous immunofluorescence detection of HBsAg (magenta) and phospho(p)STAT1 (green). The fraction of HBsAg positive and pSTAT1 positive cells is shown at the top and bottom of the images, respectively. For sample C765, the percentage of pSTAT1 positive cells in HBsAg positive and HBsAg negative cells was counted separately for the entire section and shown in the diagram. Scale bar, 50 µm.

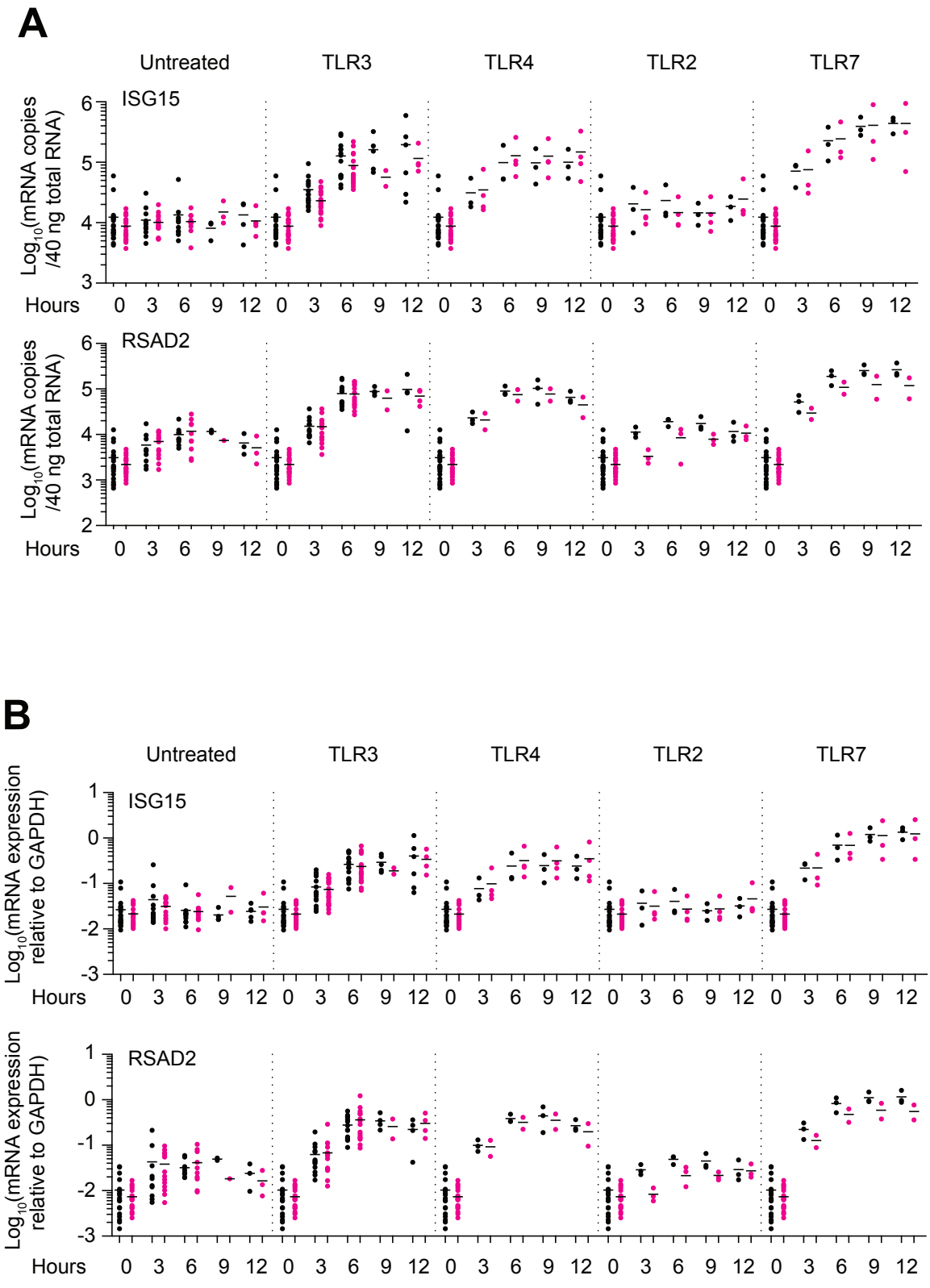

A
Untreated
TLR3
TLR4
TLR2
TLR7
ISG15
RSAD2
Log10(mRNA copies /40 ng total RNA)
Hours
0 3 6 9 12
B
Log10(mRNA expression relative to GAPDH)

**Figure S11. ISG induction is not suppressed in the liver of HBV-infected patients upon *ex vivo* stimulation with different TLR ligands.** Fresh biopsies obtained from HBV infected (HBV, magenta) and uninfected control (CTRL, black) patients were cut into several pieces and stimulated with the TLR ligands poly(I:C) (TLR3), LPS (TLR4), PGN-SA (TLR2) and R837 (TLR7). Total RNA was isolated from the *ex vivo* cultured biopsy pieces at the indicated time points after TLR ligand addition and as a baseline control (0h) right before TLR ligand addition. Cytokine expression was analyzed by RT-QPCR and is shown as copy numbers per 40 ng of total RNA (A), or as relative levels normalized to GAPDH (B). Results are shown as dot-plots with a line showing the mean. N=11-19 (0, 3, 6h) for IFNs, N=3-8 (0, 3, 6h) for TNF$\alpha$ and IL-6; N=2-6 (9, 12h) for all.

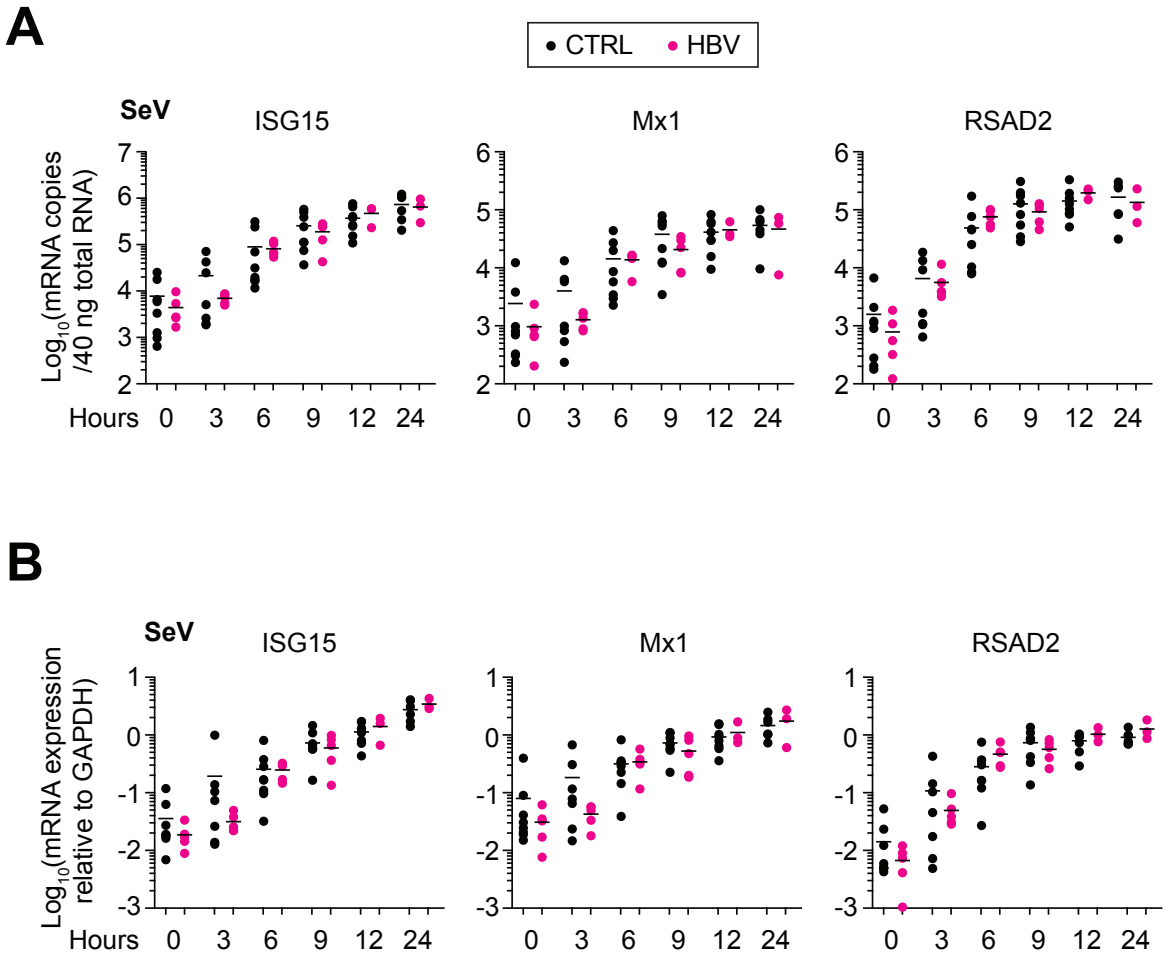



**Figure S12. ISG induction is not suppressed in the liver of HBV-infected patients upon *ex vivo* stimulation with SeV.** Fresh biopsies obtained from HBV infected (HBV, magenta) and uninfected control (CTRL, black) patients were cut into several pieces and stimulated with SeV (MOI=10). Total RNA was isolated from the *ex vivo* cultured biopsy pieces at the indicated time points after SeV addition and as a baseline control (0h) right before SeV addition. Cytokine expression was analyzed by RT-QPCR and is shown as copy numbers per 40 ng of total RNA (A), or as relative levels normalized to GAPDH (B). Results are shown as dot-plots with a line showing the mean. N=11-19 (0, 3, 6h) for IFNs, N=3-8 (0, 3, 6h) for TNF$\alpha$ and IL-6; N=2-6 (9, 12h) for all.

## 9.2 Appendix B. Supplementary information from section 5.3

### Supporting Information

### Hepatitis B Virus Replication Is Inhibited Downstream of Pre-genomic RNA in HBeAg-Negative Chronic Infection

Aleksei Suslov, Marie-Anne Meier, Sylvia Ketterer, Xueya Wang, Stefan Wieland and Markus H. Heim

**Supplementary Table 1**. Patient characteristics

| Biopsy ID | Comorbidities | Viral Load (IU/ml) | cccDNA (copies/cell) | pgRNA (copies/cell) | Replicative intermediates (copies/cell) | HBsAg+ staining (% cells) | HBcAg+ staining (% cells) | ISHAK grading | ALT (U/L) |
|---|---|---|---|---|---|---|---|---|---|
| **HBeAg-positive (HBe+)** | | | | | | | | | |
| B169 | | 1.09E+07 | 0.287 | 114 | 1711 | 70 | 0 | 7 | 181 |
| B345 | | 6.21E+03 | 0.005 | 3.37 | 1.45 | 90 | -[a] | 5 | 46 |
| B393 | | 2.13E+10 | - | 3540 | - | 90 | 40 | 3 | 111 |
| B880 | | >1.70E+08[b] | 9.5 | 259 | 3739 | 5 | 0 | 6 | 256 |
| C023 | | 6.44E+06 | - | 72 | 876 | 90 | 1 | 7 | 38 |
| C131 | | 1.13E+08 | - | 612 | 5153 | 80 | 10 | 13 | 204 |
| C143 | | >1.70E+08[b] | 14.4 | - | 16088 | 100 | 40 | 6 | 66 |
| C224 | | >1.70E+08[b] | 8.8 | 199 | 4929 | 100 | 2 | 8 | 529 |
| C357 | | 4.38E+09 | 1.82 | 1840 | 7276 | 50 | 40 | 12 | 567 |
| C537 | | 4.09E+08 | 2.53 | 669 | 1745 | 100 | 80 | 2 | 28 |
| C770 | | >1.70E+08[b] | 4.16 | 810 | 2775 | 99 | 90 | 2 | 23 |
| C787 | | >1.70E+08[b] | 4.96 | 275 | 4711 | 99 | 70 | 5 | 46 |
| C799 | | >1.70E+08[b] | - | 466 | - | 99 | 80 | 2 | 25 |
| C862 | | 4.08E+07 | 0.223 | 9.6 | 16.8 | 90 | 5 | 6 | 1572 |
| C921 | | 6.74E+03 | - | 1.68 | - | 95 | 0 | 2 | 61 |
| D190 | | 2.20E+06 | - | 3.11 | - | 100 | 5 | 1 | 52 |
| **HBeAg-negative chronic hepatitis B (ENCHB)** | | | | | | | | | |
| A661 | | 8.64E+04 | - | 5.75 | - | 5 | 0 | 9 | 111 |
| A990 | | 3.22E+07 | - | 70.9 | - | 5 | 0 | 7 | 406 |
| B109 | | 2.79E+03 | - | 0.363 | - | 30 | 0 | 1 | 20 |
| B115 | | 5.17E+04 | 1.20 | 2.62 | 180 | 40 | 0 | 6 | 96 |
| B139 | | 1.68E+05 | - | - | 27.8 | 70 | 0 | 13 | 560 |
| B163 | NASH | 7.00E+01 | - | 0.002 | - | 0 | 0 | - | 42 |
| B200 | | 4.96E+03 | 0.022 | - | - | 10 | 0 | 3 | 22 |
| B324 | | 1.62E+07 | 4.78 | 18.1 | 1669 | 85 | - | 4 | 97 |
| B335b | | 6.56E+04 | undet[c] | 6.46 | 3.65 | 80 | 0 | 3 | 76 |
| B347 | | 1.14E+04 | 0.298 | - | - | 10 | 10 | 1 | 33 |
| B365 | | 2.80E+05 | 1.43 | - | 16.7 | 50 | 0 | 4 | 58 |
| B645 | | 3.96E+03 | 0.003 | - | - | 10 | 0 | 3 | 23 |
| B651 | | 2.28E+05 | - | - | 22.1 | 70 | 0 | 4 | 105 |

**Supplementary Table 1** (continued)

| Biopsy ID | Comorbidities | Viral Load (IU/ml) | cccDNA (copies/cell) | pgRNA (copies/cell) | Replicative intermediates (copies/cell) | HBsAg+ staining (% cells) | HBcAg+ staining (% cells) | ISHAK grading | ALT (U/L) |
|---|---|---|---|---|---|---|---|---|---|
| B682 | | 2.46E+04 | 0.105 | - | 4.43 | 60 | 0 | 4 | 40 |
| B711 | | 3.78E+04 | undet | 0.292 | - | 70 | 0 | 2 | 98 |
| B775 | | 5.21E+03 | 0.081 | - | - | 30 | 0 | 2 | 52 |
| B906 | | 5.48E+03 | 0.076 | - | - | 80 | 0 | 10 | 29 |
| B908 | | 2.94E+03 | 0.120 | - | - | 30 | 0 | 3 | 17 |
| B914 | | 4.88E+02 | 0.232 | - | - | 50 | 0 | 3 | 69 |
| B922 | | 2.63E+05 | undet | 23.4 | 7.22 | 90 | 0 | 2 | 36 |
| B974 | | 4.09E+03 | 0.046 | - | - | 80 | 0 | 3 | 46 |
| C119 | | 2.63E+03 | 4.37 | 0.079 | 8.57 | 1 | 0 | 4 | 64 |
| C135 | NAFLD | 2.00E+01[d] | undet | 0.056 | - | 15 | 0 | 2 | 127 |
| C144 | | 2.60E+06 | 1.84 | 78.8 | 4940 | 40 | 5 | 5 | 116 |
| C148 | | 1.06E+02 | 0.002 | - | - | 40 | 0 | 3 | 50 |
| C162 | | 2.09E+05 | 2.51 | 2.75 | 30.9 | - | - | 5 | 26 |
| C241 | | 6.40E+01 | - | 0.749 | - | 5 | 0 | 2 | 110 |
| C254 | | 4.24E+05 | 0.212 | 6.96 | 41.5 | 5 | 0 | 6 | 106 |
| C268 | | 1.17E+06 | 0.140 | - | 14.9 | 3 | 1 | 5 | 151 |
| C286 | | 1.72E+04 | 0.016 | 0.752 | - | 1 | 0 | 3 | 54 |
| C296 | | 6.81E+04 | 4.58 | 8.32 | 23.8 | 100 | 0 | 8 | 20 |
| C309 | | 2.15E+05 | undet | 4.82 | 63.2 | 100 | 0 | 4 | 68 |
| C313 | | 8.13E+05 | 0.092 | 0.147 | 0.126 | 20 | 1 | 4 | 70 |
| C317 | | 1.80E+06 | undet | 1.20 | 428 | 70 | 3 | 2 | 86 |
| C325 | | 2.40E+03 | undet | 0.090 | - | 100 | 0 | 1 | 52 |
| C331 | | 3.55E+03 | 2.95 | 0.541 | - | 70 | 0 | 2 | 52 |
| C333 | | 4.80E+03 | 0.114 | 0.173 | - | 10 | 0 | 1 | 33 |
| C472 | | 4.10E+03 | 8.81 | - | - | 10 | 0 | 3 | 16 |
| C560 | | 4.98E+05 | 0.129 | 19.8 | 10.8 | 40 | 1 | 14 | 312 |
| C629 | | 3.17E+04 | 0.014 | - | 34.2 | 20 | 10 | 5 | 37 |
| C666 | | 7.53E+05 | 0.072 | 4.31 | 82.9 | 90 | 5 | 6 | 36 |
| C687 | | 1.56E+05 | 0.086 | 1.56 | 1.57 | 100 | 1 | 3 | 38 |
| C765 | | 6.02E+05 | 0.539 | 0.259 | 0.234 | 70 | 1 | 3 | 344 |

**Supplementary Table 1** (continued)

| Biopsy ID | Comorbidities | Viral Load (IU/ml) | cccDNA (copies/cell) | pgRNA (copies/cell) | Replicative intermediates (copies/cell) | HBsAg+ staining (% cells) | HBcAg+ staining (% cells) | ISHAK grading | ALT (U/L) |
|---|---|---|---|---|---|---|---|---|---|
| C786 | | 5.70E+01 | undet | 0.263 | - | 99 | 1 | 3 | 72 |
| C870 | | 2.05E+03 | - | 0.009 | - | 5 | 0 | 2 | 23 |
| C887 | | 3.85E+03 | - | 0.021 | - | 5 | 0 | 3 | 40 |
| C889 | | 1.00E+03 | - | 5.31 | - | 90 | 0 | 3 | 43 |
| C911 | | 1.71E+04 | - | 0.202 | - | 30 | 0 | 3 | 31 |
| C920 | | 4.00E+03 | 0.230 | 3.80 | - | 90 | 0 | 3 | 45 |
| C926 | | 2.57E+03 | - | 0.083 | - | 10 | 0 | 3 | 57 |
| C982 | | 1.50E+04 | - | 0.240 | - | 90 | - | 2 | 45 |
| D010 | | 5.84E+05 | - | 78.4 | - | 0 | 40 | 6 | 124 |
| D021 | | 2.92E+03 | - | 4.12 | - | 90 | 0 | 4 | 30 |
| D139 | | 1.21E+06 | 0.003 | 5.32 | 1.56 | 95 | 5 | 3 | 32 |
| D167 | | 2.13E+04 | - | 0.552 | - | 80 | 0 | 2 | 19 |
| D183 | | 4.60E+06 | - | 0.261 | - | 100 | 0 | 4 | 40 |
| **HBeAg-negative chronic infection (ENCI)** | | | | | | | | | |
| A922 | | 7.00E+02 | 0.011 | 0.027 | - | 20 | 0 | - | 13 |
| B067 | | 3.40E+01 | 0.010 | - | - | 10 | 0 | 2 | 26 |
| B071 | HIV (ART) | 1.20E+01[d] | 0.010 | 0.329 | 0.040 | 5 | 0 | 4 | 24 |
| B105 | | 1.20E+01[d] | undet | 2.55 | - | 50 | 0 | 0 | 19 |
| B323 | | 1.20E+01[d] | 0.003 | 0.013 | - | 10 | 0 | 3 | 29 |
| B987 | | 9.54E+02 | 0.023 | 1.07 | - | 90 | 0 | 4 | 31 |
| C030 | | 2.70E+01 | 0.000 | - | - | 0 | 0 | - | 24 |
| C064 | ASH | 2.00E+01[d] | 0.001 | - | - | 0 | 0 | - | 31 |
| C123 | | 1.36E+03 | 0.000 | 0.007 | - | 1 | 0 | 3 | 34 |
| C141 | | 2.37E+03 | 0.009 | - | - | 70 | 0 | 2 | 25 |
| C299 | | 7.62E+02 | 0.025 | 0.903 | - | 100 | 0 | 3 | 36 |
| C499 | ALD | 9.00E+01 | 0.001 | 0.016 | 0.011 | 1 | 0 | 8 | 25 |
| C660 | | 2.07E+02 | 0.002 | 0.018 | 0.015 | 0 | 0 | 4 | 28 |
| C692 | | 9.68E+02 | 0.002 | 0.093 | 0.0085 | 5 | 0 | 2 | 26 |
| C812 | | 5.80E+01 | 0.012 | 1.71 | - | 99 | 0 | 3 | 17 |
| C853 | | 1.32E+02 | - | 0.005 | - | 20 | 1 | 2 | 40 |

**Supplementary Table 1** (continued)

| Biopsy ID | Comorbidities | Viral Load (IU/ml) | cccDNA (copies/cell) | pgRNA (copies/cell) | Replicative intermediates (copies/cell) | HBsAg+ staining (% cells) | HBcAg+ staining (% cells) | ISHAK grading | ALT (U/L) |
|---|---|---|---|---|---|---|---|---|---|
| C912 | | 5.03E+02 | 0.145 | - | - | 99 | 0 | 2 | 21 |
| C968 | | 8.00E+01 | 0.024 | 0.140 | - | 90 | 0 | 3 | 8 |
| C979 | | 2.00E+01[d] | undet | undet | undet | 0 | - | - | 19 |
| D012 | | 1.39E+03 | - | 0.078 | - | 0 | 0 | 3 | 25 |
| D054 | | 4.64E+02 | 0.003 | 0.017 | 0.043 | 10 | 0 | 2 | 22 |
| D055 | | 1.92E+02 | 0.001 | 0.010 | 0.005 | 0 | 0 | - | 22 |
| D061 | NAFLD | 2.41E+03 | 0.001 | 0.029 | 0.009 | 30 | 0 | 4 | 44 |
| **Antiviral therapy[e-k] (AVT)** | | | | | | | | | |
| B850c[e] | | 2.00E+01[d] | 0.055 | - | - | 20 | 0 | 2 | 29 |
| C161[f] | | 2.00E+01[d] | 1.19 | - | 0.015 | 20 | 0 | 2 | 28 |
| C244[g] | | 2.00E+01[d] | 0.010 | 1.47 | - | 50 | 0 | 2 | 44 |
| C252[h] | | 2.00E+01[d] | - | 0.086 | - | 5 | 0 | 2 | 19 |
| C706[i] | | 2.00E+01[d] | 0.010 | 0.335 | 0.023 | 20 | 0 | 4 | 32 |
| C809[j] | | 2.00E+01[d] | 0.021 | 0.508 | 0.016 | 10 | 1 | 1 | 26 |
| D187[k] | | 2.00E+01[d] | - | 0.084 | - | 10 | 0 | 5 | 24 |

**Supplementary Table 2.** Primer sequences used in RT-QPCR experiments

| Target Gene | Primer direction | Sequence 5' --> 3' |
|---|---|---|
| HBV DNA / cccDNA | Forward | TGGCCAAAATTCGCAGTCCC |
| | Reverse | GATGAGGCATAGCAGCAGGATG |
| pgRNA | Forward | GAGTGTGGATTCGCACTCCTC |
| | Reverse | AGAAGAACTCCCTCGCCTCG |
| IMAP | Forward | TTTTCAGCTCCCAAGTGTCC |
| | Reverse | GCCGAGAGCAGGTAGCAGT |

# Figure S1

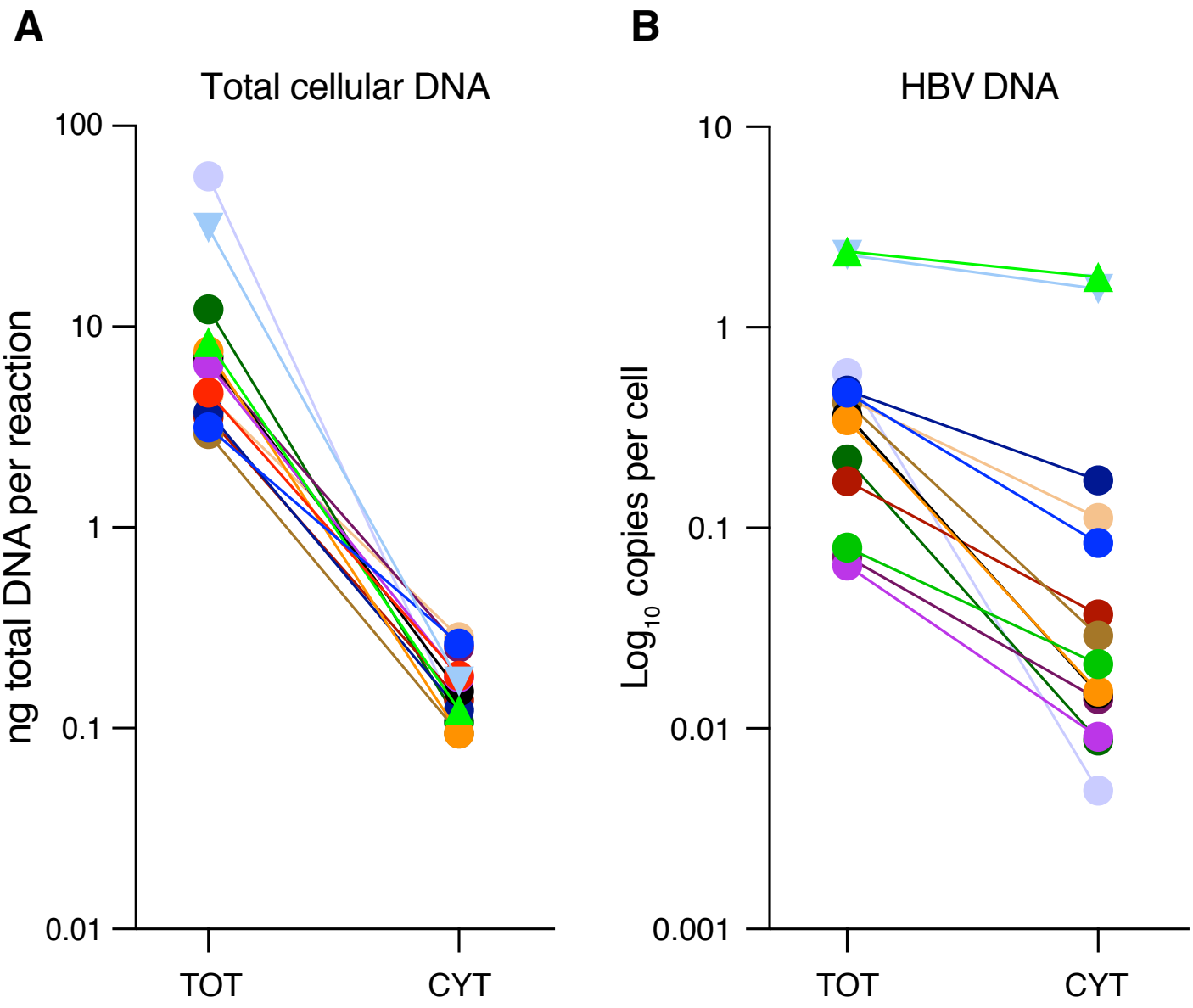



Figure S1. Integrated HBV DNA interferes with the measurement of replicative intermediates in the total DNA extract. HBV DNA was extracted from total lysates (TOT) or from the cytoplasmic fractions (CYT) of human liver biopsies as described in Materials and Methods and subjected to qPCR with either IMAP-specific primers to determine total human genomic DNA content (A) or with HBV specific primers to determine HBV DNA content (B). Individual colors designate individual patients/biopsies. Biopsies with total intrahepatic HBV DNA above 1 copy per cell are shown as triangles.