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Infection kinetics and impact of hepatitis B virus HBeAg negative strains on innate immunity gene expression in humanized mice

Dissertation

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A Introduction

A.1 The Hepatitis B Virus

A.1.1 Taxonomy, viral structure and replication circle

The human hepatitis B virus (HBV) is a small enveloped, partially double-stranded DNA virus. It is infecting human hepatocytes, leading to an acute or chronic form of hepatitis (Rods et al. 2007). The Australian antigen, found in the serum of an Australian Aboriginal, was first described in 1965 (Blumberg et al. 1965) and would later be identified as the hepatitis B surface antigen (HBsAg). In 1970 the viral particle itself, also called the Dane particle, could first be visualized using electron microscopy (Dane et al. 1970). Following cloning and sequencing of the HBV genome, HBV was associated with a number of related viruses found in mammals as well as birds. All of which belong to the family of hepadnaviridae (Galibert et al. 1979; Schaefer 2007). The family of hepadnaviridae contains two genera; the orthohepadnaviruses that infect mammals and the avihepadnaviruses infecting birds. In addition, besides the human- and chimpanzee specific hepatitis B virus, the orthohepadnaviruses include variants infecting woodchuck (WHV) (Galibert et al. 1982), ground squirrel (GSHV) (Seeger et al. 1984) as well as the woolly monkey (WMHBV) (Lanford et al. 1998). In addition, three unique hepadnavisues have been identified in bats that are able to infect human hepatocytes and are antigenically similar to the human hepatitis B virus (Drexler et al. 2013). Variants of the avihepadnaviruses can be found in Peking duck HBV (DHBV) (Mandart et al. 1984) and snow goose HBV (SGHBV) (Chang et al. 1999). Members of the hepadnaviridae all display similar characteristics in that they are highly species and tissue specific (Leenders et al. 1992), share a common genomic organization and their replication cycle involves the conversion of single-stranded RNA intermediates into double-stranded circular DNA (Nassal 2015). Since all hepadnaviridae utilize a viral reverse transcriptase as a means of converting RNA into DNA, they are considered to be distantly related to retroviruses (Nassal and Schaller 1996).

The HBV genome appears in form of partially double-stranded relaxed circular DNA (rcDNA) and contains approximately 3200 nucleotides in length. In

association with the HBV core protein (HBcAg), which encloses the rcDNA, it forms the viral nucleocapsid (Delius et al. 1983). While the rcDNA is circular in shape, it is not covalently closed. The negative strand is present in its full length, containing the complete genome (Nassal 2015). The positive strand only covers approximately two-thirds of the genome length. In addition, the 5´ end of the negative strand is covalently bound to the viral polymerase (P protein) (Gerlich and Robinson 1980; Datta et al. 2012).

The nucleocapsid is enveloped by an outer lipid membrane that is acquired during budding through the endoplasmic reticulum (ER). Imbedded into the outer membrane three distinct viral surface proteins (HBsAg) can be found; these are named the S- (small), the preS2- (middle) and the preS1- (large) protein (Nassal 2015). The DNA containing nucleocapsid together with the envelope form the viral particle (**figure A.1.1**). All three surface proteins are produced in large excess. Together with the host-derived lipid membrane, they form large quantities of so called subviral particles (SVPs), that are secreted from HBV infected cells (Dandri and Locarnini 2012). Consequently there are different structures of viral origin that can be identified. Besides the infectious Dane particle, two additional forms of subviral particles can be differentiated, they either form spheres or filaments of variable length but contain no viral genome (Glebe and Urban 2007). Although the precise role of the excess SVPs is not yet clear, they have been suggested to have a decoy-like function, absorbing host derived neutralizing antibodies and enabling the Dane particle to evade part of the host defense (Xu et al. 2009). Other possible functions that have been proposed, include the SVPs inducing a state of immune tolerance and thereby promoting further viral spreading (Dandri and Locarnini 2012).

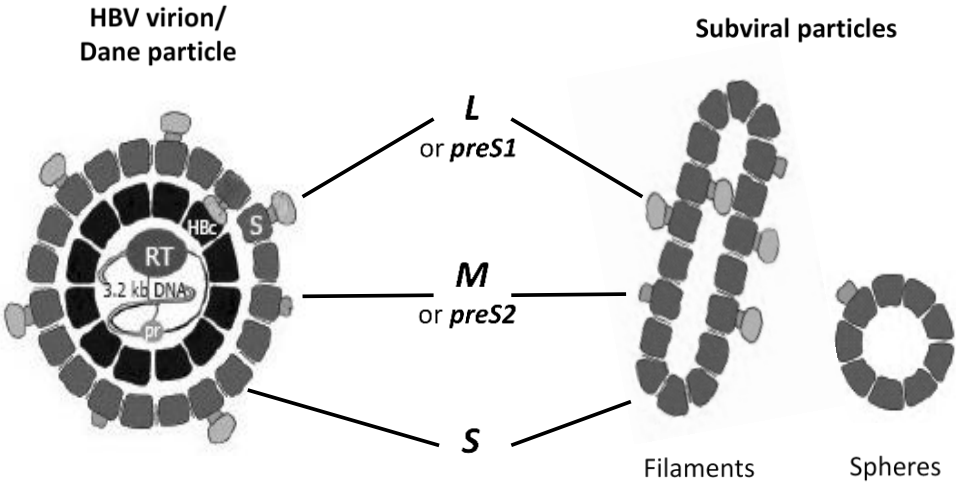

Figure A.1.1 ***Schematic display of the three viral structures***

The HBV virion contains the rcDNA and the core protein (black), while the subviral particles contain neither and appear as empty structures in the form of either filaments or spheres. The surface proteins L (preS1), M (pres2) and S (small) are present on the outer membrane of the HBV virion as well as all subviral particles (grey). (Adapted from Glebe and Urban 2007)

The length of the HBV genome averages at only 3200 nucleotides, consequently the HBV genome follows a compact organization in form of four open reading frames (ORFs) (**figure A.1.2**), with each nucleotide carrying coding information in one or even two overlapping ORF´s (Delius et al. 1983). In addition various regulatory elements overlap with areas containing coding information. The four ORF´s serve as templates for the transcription of the seven viral proteins, which can be divided into structural and non-structural proteins. Structural proteins are the three surface proteins as well as the core protein, whereas the HBeAg/precore protein, the X protein and the viral polymerase belong to the non-structural proteins (Nassal 2015; Block et al. 2007; Seeger and Mason 2000).

The PreS/S ORF functions as the template for the transcription of all three surface proteins (preS1, preS2 and S) (Dandri and Locarnini 2012) and overlaps in its entirety with the ORF encoding the viral polymerase. The PreS/S ORF includes three different start codons; as a consequence all three surface proteins share the common C-terminal domain of the S protein. The formation of the M- and the L- protein are achieved by expanding the protein by 55 and respectively 107 (or 118, varying among genotypes) amino acids at the N-terminus (Nassal 2015).

Transcription of the polyadenylated RNAs, being 3.5, 2.4, 2.1 and 0.7 kb in length respectively, is governed by the four viral promoters, the core, the PreS1 and PreS2, as well as the X. In addition transcription is further regulated by the two enhancement regions enhancer I and enhancer II (Makokha et al. 2019; Tong and Revill 2016). The HBV surface proteins are synthesized at the endoplasmic reticulum and embedded into the membrane, utilizing dedicated transmembrane domains. Formation of subviral particles, as well as envelopment of the nucleocapsid, is a result of budding from the post-ER pre-Golgi membrane, therefore HBV surface proteins are not present in the plasma membrane of hepatocytes (Bruss 2004).

The precore/core ORF encodes both the structural core protein as well as the nonstructural precore protein. The later undergoes posttranslational editing at the endoplasmic reticulum in the form of N- and C- terminal cleavage. The majority of the mature protein, now called the HBeAg, is secreted, but about 20% - 30% remain present in the cytosol of the infected hepatocyte (Lang et al. 2011). The core protein, which consist of 183 or 185 amino acids (varying between genotypes), forms the HBV capsid via self-assembly. The single core proteins form homodimers that are connected over a di-sulfide bridge. These core protein homodimers further self-assemble into the icosahedral shaped HBV capsids, which are either made up of 90 or 120 core protein dimers (Bruss 2004).

The X ORF functions as the template for the transcription of the X protein also named HBx (Seeger and Mason 2000). The primary function of the regulatory HBx protein is promotiong the transcription from the viral genome, which is present in form of the extrachromosomal covalently closed circular DNA (cccDNA). It thus plays a key role in the initiation and continuance of viral replication (Lucifora et al. 2011). One way of enhancing the transcription of viral proteins is archived by disabling the structural maintenance of chromosomes complex Smc5/6 (Smc) in its function as a restrictive factor, inhibiting the transcription of extrachromosomal DNA (Decorsière et al. 2016). The HBx protein has also been shown to compromise innate immune responses by down-regulating mitochondrial antiviral signaling, namely the retinoic acid inducible gene I (RIG-I) melanoma differentiation associated gene 5 (MDA5) pathway (Wei et al. 2010). In addition to its biological functions with regard to HBV replication and infection, the HBx protein has been described as a carcinogenic cofactor in

the development of hepatocellular carcinoma, as a late onset complication of chronic HBV (CHB) infection (Wang, M. et al. 2017; Dandri et al. 1996).

The fourth ORF, namely the pol ORF encodes the viral polymerase. It is the only enzyme encoded by the HBV genome and functions as an RNA dependent DNA-polymerase with an additional RNase activity (Wei and Peterson 1996). Structurally, the viral polymerase includes three functional domains: the terminal protein, the polymerase/reverse transcriptase domain and the RNase H domain. The terminal protein and the polymerase/reverse transcriptase region are separated by an additional spacer region (Nassal 2015). The terminal protein serves as the primer required for the reverse transcription of the viral pregenomic RNA (pgRNA) into the negative DNA strand (Zoulim and Seeger 1994).

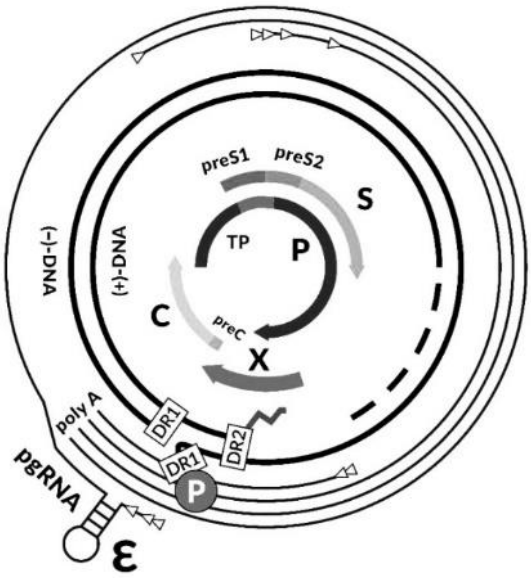

Figure A.1.2 ***Circular HBV Genome.***

Arrangement of the four open reading frames and their respective transcripts with regard to the rcDNA as it is found in infectious viral particles. The location of the four ORFs within the rcDNA (inner dark lines) is displayed by the arrows in the center. The resulting RNA transcripts are represented by the outer thin lines. As schematically shown, transcription is initiated at different promoter sites, but is terminated at a common polyadynelation site (adapted from Nassal et al. 2015).

Until today the life cycle of the hepatitis B virus is not understood in its entirety. It was found that HBV infection occurs at the basolateral membrane of the hepatocyte (Schulze et al. 2012). Here the attachment of the infectious particle onto the hepatocyte membrane is initiated by a non-cell type specific association

with heparin sulfate proteoglycans at the cell surface (Schulze et al. 2007), which is then followed by the cell type specific binding to the Na^+-taurocholate cotransporting polypeptide (NTCP). The NTCP is expressed at the basolateral membrane and exposed to the space of Disse, where it functions as a cotransporter for Na^+ and conjugated bile acids. The NTCP acts as the functional receptor for HBV, granting the virus its highly specific tropism (Yan et al. 2012). Therefore entry of HBV, as well as the hepatitis D virus (HDV), can be blocked by the lipopeptide Myrcludex B (MyrB), which corresponds to the N-terminal part of the L protein (Petersen et al. 2008; Volz et al. 2013). The entry mechanism that is utilized by HBV following binding to the cell membrane remains unclear. While NTCP-transfected human hepatoma cell lines do support HBV entry, NTCP-transfected mouse hepatocytes allow only HDV infection, but not HBV entry (Yan et al. 2013; Li et al. 2014). This suggests the need for additional species-specific factors that are essential for the infection with HBV. The high-affinity interaction between NTCP and the N terminal PreS1 region of the L-protein and likely other human hepatocyte specific factors, induces uptake of the infectious particle, possibly via endocytosis (Macovei et al. 2010; Nassal 2015). Viral capsids are then transported towards the nucleus, likely utilizing the hepatocyte microtubules system (Rabe et al. 2006). The interaction with nuclear transporter receptors induce the disassembly of the viral capsid and lead to the release of the rcDNA as well as viral core subunits (Schmitz et al. 2010; Kann et al. 2007). Steps that are required to convert rcDNA into cccDNA include the removal of the covalently attached viral polymerase, removal of the terminal redundant sequence at the negative strand, removal of the RNA primer as well as the repair of the gap at the positive strand and finally ligation of both DNA strands (Allweiss and Dandri 2017). Studies have shown that HBV is able to include host cell derived factors, like the DNA repair enzyme tyrosyl-DNA-phosphodiesterase or the DNA polymerase κ into its life cycle (Königer et al. 2014; Qi et al. 2016). Nevertheless the process of cccDNA formation is still not well understood. The cccDNA interacts with histone and non-histone proteins within the nucleus, forming a nonintegrated minichromosome, that approximates cellular chromatin (Bock et al. 2001; Newbold et al. 1995). The newly formed cccDNA minichromosome then serves as template from where HBV RNA transcription takes place, utilizing the preexisting cellular transcription machinery (Levrero et al. 2009). As most regulating elements within the HBV genome contain binding sites for several host

cell derived transcription factors, cccDNA expression is under the regulative control of the viral HBx protein as well as hepatocyte specific transcription factors (Lucifora et al. 2011; Tang and McLachlan 2001; Quasdorff et al. 2008).

Pregenomic RNA as well as all other mRNAs are transported into the cytoplasm, where translation takes place. In addition, the pgRNA is packaged into the core particle and converted into rcDNA. The secondary epsilon (ε) structure, present at the 5´and 3´ end of the pgRNA, enables binding of the viral polymerase and thereby acts as the initiation signal for pgRNA packaging (Bartenschlager and Schaller 1992). Transcription of pgRNA therefore is a determining factor of the rate of viral replication. The core particle is then enveloped and is released via the exosome pathway by budding through multivesicular bodies (**figure A.1.3**) (Tong and Revill 2016). Secretion of sub viral particles is achieved utilizing the general secretin pathway (Prange 2012; Hoffmann et al. 2013).

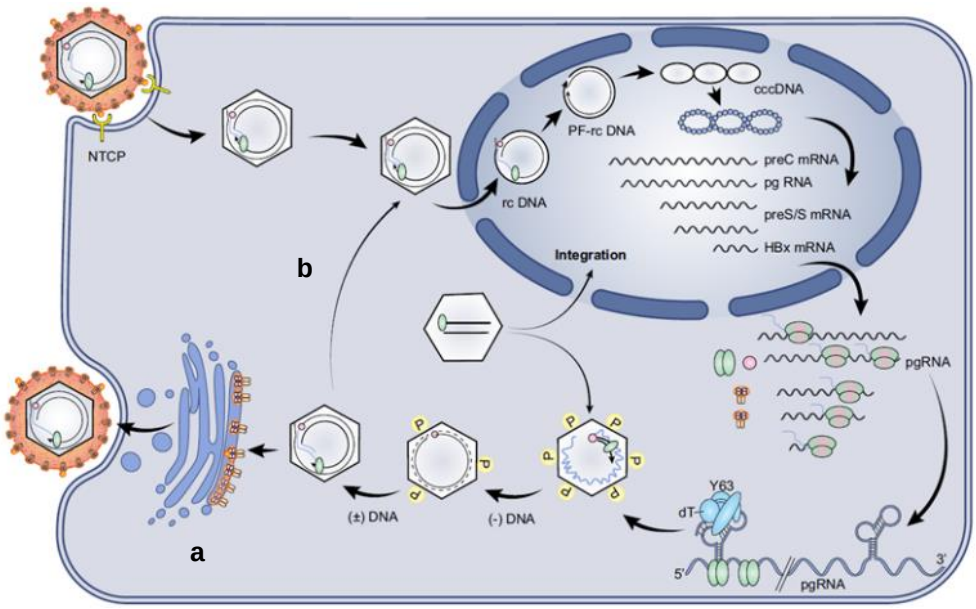

Figure A.1.3 ***HBV replication cycle.***

Specific binding to hepatocytes through the Na^+ - taurocholate cotransporting polypeptide (NTCP), followed by uncoating, and transport of the HBV capsid towards the nucleus. The rcDNA is transformed into the cccDNA, which is the extrachromosomal template for HBV gene expression. pgRNA is packaged inside the core particle and reverse transcribed, formig the rcDNA. Mature core particles can either be enveloped and released as virions (**a**), or be transported to the nucleus to repeatedly generate cccDNA (**b**) (adapted from Tong et al. 2016).

A.1.2 The HBeAg/precore protein

The precore/core open reading frame serves as a template for the transcription of pgRNA as well as the precore RNA. It contains two in-frame initiation codons, from where two co-terminal HBV proteins are translated. Utilizing the second AUG, the core protein is translated from the pgRNA (Tong and Revill 2016). The precore RNA, from which the precore protein is transcribed, is collinear with regard to the pgRNA but is extended at the 5´ end, allowing for the translation from the precore AUG codon, which is the upstream AUG (Seeger and Mason 2015). The first 19 amino acids of the precore protein resemble a signaling peptide that directs the precore protein to the endoplasmic reticulum, where the N-terminal signaling peptide is cleaved of. Further post-translational modification is performed within the Golgi compartment. As a result the C-terminal arginine-rich sequence is cleaved of, converting the precore protein into the HBeAg that is then secreted (Alexopoulou and Karayiannis 2014; Tong and Revill 2016).

The HBeAg/precore protein seems neither to be required for entry of human hepatocytes nor the establishment of HBV infection. The HBeAg/precore protein has proposed to dampen the innate as well as the adaptive immune response towards HBV infection. It could thereby promote the establishment of CHB (Walsh and Locarnini 2012). The HBeAg was for example shown to potentially impair innate immunity related cell signaling cell signaling in Huh-7 cells (Locarnini et al. 2005). Also Toll-like receptor 2 (TLR2) expression on hepatocytes as well as Kupffer cells in liver biopsies of CHB patients appeared to be reduced in HBeAg positive patients, compared to HBeAg negative patients. Toll-like receptor 2 signaling, stimulated by the TLR2 ligand Pam3Cys also appeared to be decreased in peripheral blood monocytes (PBMCs) (Visvanathan et al. 2007). With regard to the adaptive immune response the HBeAg has been associated with immune evasion, acting as a HBcAg decoy and depleting T-helper cells in utero (Chen et al. 2005). Overall the accurate function of the HBeAg/precore protein is unclear and remains subject of investigation.

The HBeAg negative chronic hepatitis, characterized by the lack of HBeAg, is associated with necroinflamation, liver fibrosis and a low rate of spontaneous remission. Patients often harbor viral variants that contain a precore and/or basal

core promoter mutation that abolishes or significantly decimates HBeAg production. HBeAg negative chronic hepatitis patients have shown to respond poorly towards interferon therapy when compared to HBeAg positive chronic infection. Studies have also linked the lack of HBeAg and the presence of the G1896A precore mutation to a more severe course of infection with increased rates of fulminant hepatitis or the development of hepatocellular carcinoma (Kim et al. 2016; EASL 2017).

A.1.3 Precore/core region mutation in HBV

Over the course of chronic infection with HBV, selection pressure promotes the occurrence of random point mutations within the HBV genome. Within individuals chronically infected with HBV, a number of mutations in different genomic regions of the HBV genome have been described. Such mutations include for example lamivudine or other antiviral resistance mutations in the Pol region, substitutions, insertions or deletions in the precore/core region as well as in the preS1 or preS2 regions (Tipples et al. 1996; Locarnini 2003). These mutations may provide the virus with means of escaping immune as well as therapeutic pressure and contribute to viral persistence. Over the course of HBV infection, these viral variants are able to replace the initial viral population within the individual patient at specific phases during the chronic HBV infection (Günther 2006). Since HBV wild type strains have not been replaced, it is assumed that they are better suited to the initial or immune tolerant phase of HBV infection (Datta et al. 2012).

Clinical outcome in CHB patients is related to viral as well as host factors. Increased rates of viral replication, in certain viral variants, have been associated with a more severe outcome CHB patients (Hasegawa et al. 1994). Other viral factors, that accelerate the development of liver disease, include HBV genotype differences, mutations in the HBV precore and core promoter region as well as the absence of the precore porotein/ HBeAg (Ozasa et al. 2006). Over the course of CHB, viral variants that display either an abolished or at least impaired expression of the precore protein/ HBeAg, are found to be favored and, in case

of the PC/BCP double mutation, display higher viral activity and increased viral loads (Volz et al. 2007; Huang et al. 2006).

The precore protein is translated from the precore mRNA (preC RNA), while the core protein is translated form the slightly shorter pregenomic mRNA. Transcription of both RNAs is initiated by the core promoter. The core promoter consists of the basal core promoter (BCP) and an upstream regulatory sequence (URS) (**figure A.1.4**). The BCP region contains the cis-acting regulatory elements for the preC as well as the pregenomic transcripts and also includes the direct repeat 1 (DR1) (Kramvis and Kew 1999). The upstream regulatory sequence overlaps with the enhancer II element (EN2) and contains cis-acting elements, modulating BCP activity. Over the span of the URS and the BCP, numerous sequences motifs can be found that enable the interaction with liver-specific transcription factors, such as the hepatocyte nuclear factor 3 (HNF-3) and hepatocyte nuclear factor 4 (HNF-4), the TATA binding protein (TBP), the peroxisome proliferator activated receptor α (PPAR α), the retinoid X receptor α (RXRα) and the chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) (Li and Ou 2001; Yu and Mertz 1996). Interaction with these trans-acting elements enable HBV to regulate and differentiate the transcription of preC and pregenomic RNA. For example, in case of the two HNF-4 binding sites within the core promoter, the primary transcription factor for binding site 1 is HNF-4, while binding site 2 also interacts with other transcription factors like RXRα, COUP-TF1, PPARα, and human testicular receptor 2 (TR2). Interaction of binding site 2 with HNF-4 and TR2 suppresses preC RNA transcription, while binding with PPARc/RXRa heterodimers stimulates pregenomic RNA transcription. Other trans-acting elements, like COUP-TF1 suppresses transcription of preC as well as pregenomic RNA (Raney et al. 1997; Yu and Mertz 1997; Li and Ou 2001).

Numerous mutations have been reported within the precore/core region, not all of which affect the HBeAg status. Mutations within the BCP region affect preC RNA at the transcriptional level, while mutations within the precore region may introduce stop codons or cause frameshifts and thereby disrupt preC RNA translation (Kim et al. 2016). The double mutation A1762T/G1764A within the BCP region reduces the core promoter activity and thereby reduces preC RNA transcription, but does not abolish production of the precore protein completely (Okamoto et al. 1994; Buckwold et al. 1996). The BCP double mutation has been

described to display upregulated pregenomic RNA transcription and in turn a higher rate of viral replication (Baumert et al. 1996; Tsai et al. 2009).

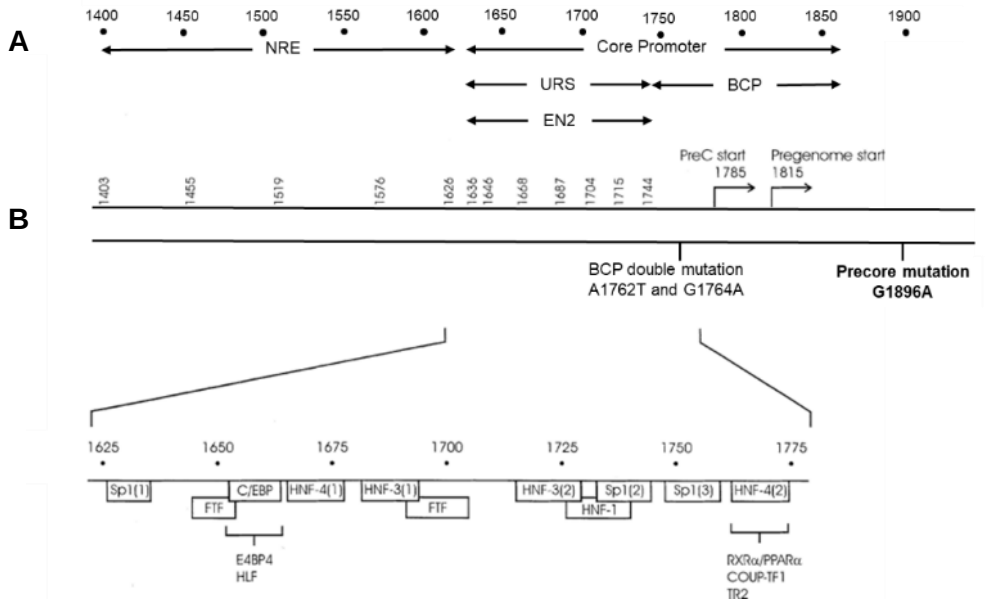

Figure A.1.4 ***Precore/core promoter region***

A: Schematic display of the basic core promoter (BCP), the upstream regulatory sequence (URS) and the enhancer II element (EN2). **B:** Schematic display of the precore mutation G1896A, the BCP double mutation as well as the binding sites of hepatocyte specific transcription factors (adapted from Molla et al. 2002).

The most common mutation that is resulting in an HBeAg negative phenotype, is the point mutation G1896A within the precore region. This nonsense mutation introduces the stop codon TAG previously TGG (Trp-28) and disrupts preC RNA translation (Okamoto et al. 1990; Carman et al. 1989). As a result HBeAg production is completely abolished and seroconversion from HBeAg to anti-HBeAg antibody is thereby prompted. The G1896A mutation is located within the epsilon (ε) structure, a highly conserved secondary structure of the pregenomic RNA, acting as a packaging signal and binding site for the viral polymerase (Bartenschlager and Schaller 1992). The ε structure consists of two paired (upper and lower stem) and two unpaired regions (loop and bulge). In the HBV wild type, the affinity between U1858 and G1896 within the lower stem is increased by the G1896A substitution (**figure A.1.5**) (Ito et al. 2018). Since nucleotide 1858 is HBV genome specific, mutation G1896A is more commonly found in HBV genotypes, that display T1858, such as genotype D, and is less likely to be found in genotypes

displaying C1858. In genotype A, sub-genotypes C1, F2, F3, and genotype H, the preexisting base pair of C1858 – G1896 within the stem-loop structure is disrupted and the resulting C1858 – A1896 does not benefit the ε structure (Li et al. 1993; Lok et al. 1994; Norder et al. 2003).

The definite clinical significance of the preocore stop codon mutation G1896A, as well as the BCP double mutation A1762T/G1764A, are still not fully determined. The precore mutation G1896A has been implicated to lead to a more severe course of CHB and was associated with the development of fulminant hepatitis (Omata et al. 1991; Liang et al. 1991; Hasegawa et al. 1991; Kosaka et al. 1991; Friedt et al. 1999). The BCP double mutation has also been linked to development of fulminant hepatitis (Sato et al. 1995; Kusakabe et al. 2009), liver cirrhosis (Tseng et al. 2015) and an increased risk of hepatocellular carcinoma in CHB patients (Baptista et al. 1999; Kao et al. 2003; Chou et al. 2008). Other studies suggest that mutations in the precore/core region do not play a predominant role in the development of fulminant hepatitis or acute on chronic liver failure (Laskus et al. 1993; Sterneck et al. 1996; Gao et al. 2017).

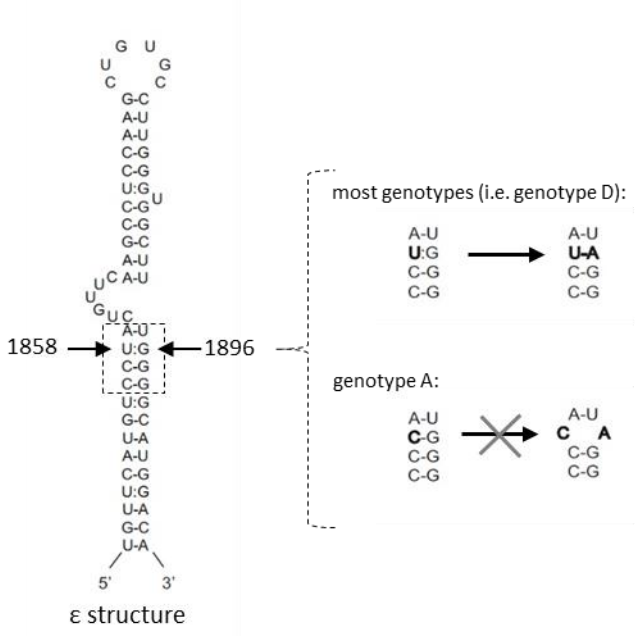

Figure A.1.5 ***Encapsidation signal in form of the stem loop structure***

A: Schematic display of the stem loop structure. A secondary RNA structure consisting two paired (upper and lower stem) and two unpaired regions (loop and bulge). B: the G1896A mutation leading to an increased affinity between U1858 and G1896 in i.e. genotype D, while disrupting the preexisting pair of C-G in i.e. genotype A (adapted from Ito et al. 2018).

A.1.4 Epidemiology and genotypes

Regardless of the availability of an effective vaccination, infection with the hepatitis B virus still remains a global health burden. Chronic HBV infection remains endemic in many countries worldwide and represents a major cause of chronic liver-disease, especially in sub-Saharan African countries. HBV and HCV infection account for as much as two thirds of global cases of liver cirrhosis.

The World Health Organization (WHO) estimates 257 million people worldwide to be HBsAg seropositive. In 2015 the WHO attributed 887.000 annual deaths to either the infection with HBV or its complications, namely liver cirrhosis and hepatocellular carcinoma. It is estimated that among patients with liver cirrhosis globally, HBV prevalence exceeds 40%. Worldwide roughly 2 billion people show evidence of past or present infection with HBV (WHO 2015).

The estimation of chronic hepatitis B infection prevalence is based upon seroprevalence studies for the hepatitis B surface antigen (HBsAg) in the general population. The global prevalence of chronic hepatitis B infection was first systematically estimated by Schweitzer et al. after a pooled analysis of country-level HBsAg seroprevalence in 2015. HBsAg seroprevalence averages at 3,61% of the global population but differs notably among the six WHO regions. Average HBsAg seroprevalence ranges from 8,8% in the African region and 5,26% in the Western Pacific region, 3,01% in the Eastern Mediterranean region, to 2,06% in the European region, 1,90% in the South East Asian region and 0,81% in the Region of the Americas (Ott et al. 2012). It is important to note that HBsAg seroprevalence may be much higher in endemic regions with prevalence levels that far exceed the WHO region average e.g. South Sudan (22,38%), Niger (15,48%), Somalia (14,77%) or Vietnam (10,79%) (Schweitzer et al. 2015).

Since 2015 modelled estimates of chronic hepatitis B prevalence have been published by the WHO, the Institute for Health Metrics and Evaluation (IHME) and the CDA Foundation/Polaris Observatory (CDA). Global chronic hepatitis B prevalence estimated ranges from 3.5% to 5.6% but differs notably between high prevalence areas e.g. central Asia and Sub-Saharan Africa and low prevalence areas such as the Americas as well as Western Europe (**figure A.1.6**). Overall estimates of chronic hepatitis B prevalence published by Schweitzer et al., the

WHO, the IHME or the CDA appear to be comparable despite variance in methodology and HBeAg seroprevalance data source (Schmit et al. 2021).

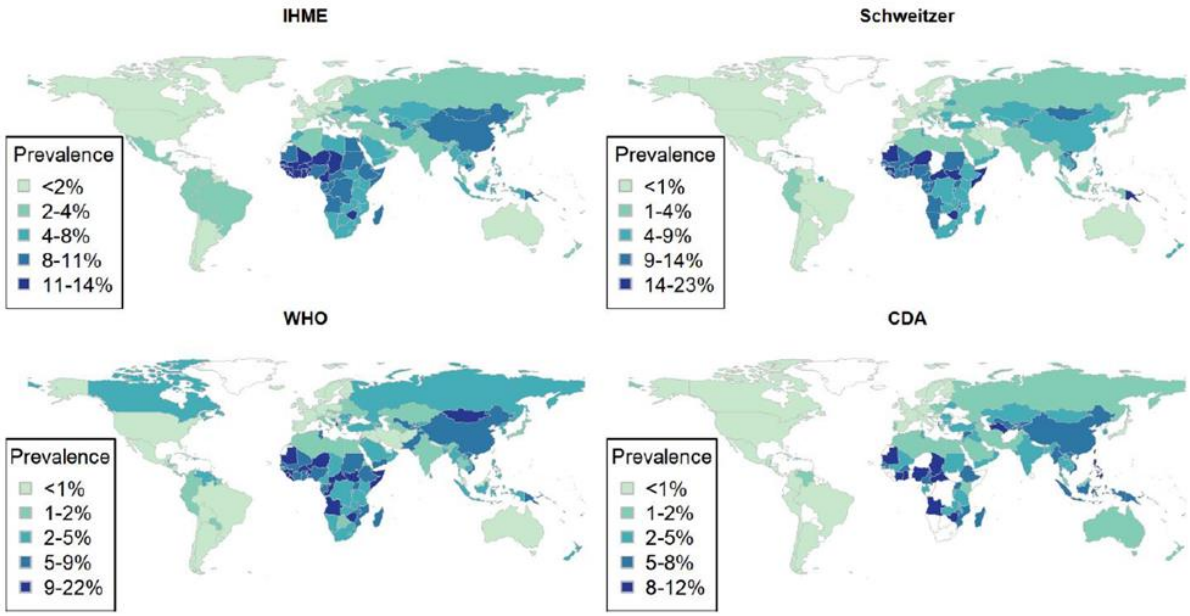

Figure A.1.6 ***Global estimates of chronic hepatitis B prevalence***

Estimates of chronic hepatitis B prevalence published by the Institute for Health Metrics and Evaluation (IHME), Schweitzer et al, the World Health Organization (WHO) and the CDA Foundation (CDA). While estimated global prevalence of chronic hepatitis B ranges from 3.5% to 5.6%, prevalence notably differs between high prevalence regions e.g. Sub-Saharan Africa and low prevalence regions e.g. Western Europa and the Americas. Estimated global distribution is comparable among all four studies (Schmit et al. 2021).

The lack of proofreading activity within the DNA polymerase and consequentially the miss-incorporation of nucleotides, has led to the development of multiple HBV genotypes and sub-genotypes. Genotypes differ in more the 8% of their genome, while subtypes differ in at least 4% (Guirgis et al. 2010). Today 10 defined genotypes (A-J) as well as several subtypes have been described (Lin and Kao 2017). HBV genotypes are distinctively geographically distributed over the world (**figure A.1.7**) (Shi et al. 2013).

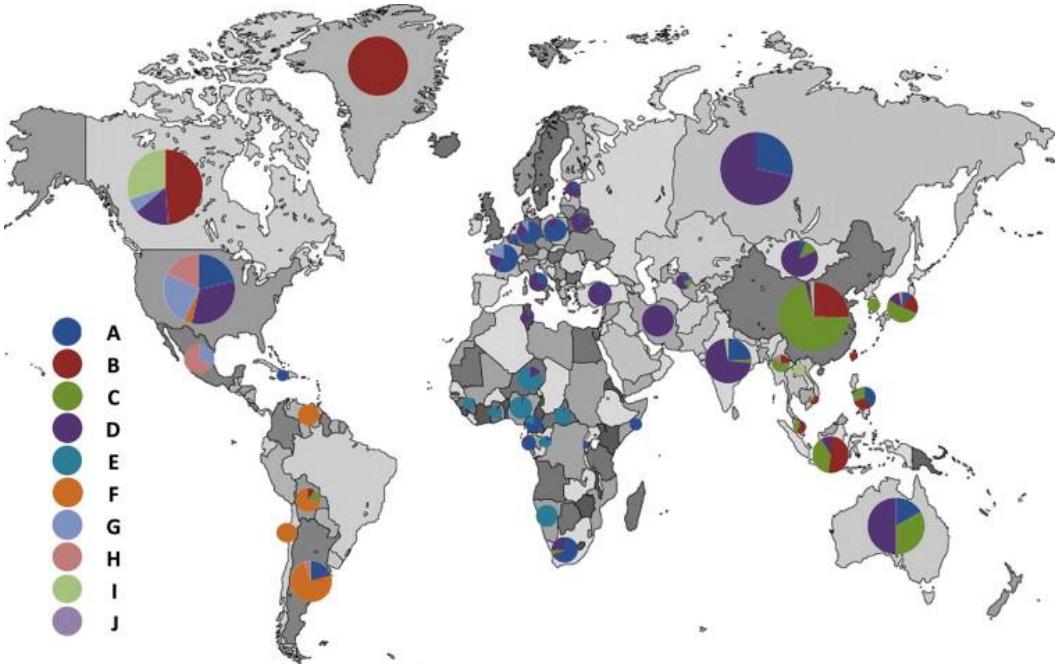

Figure A.1.7 ***Global distribution of major HBV genotypes***

HBV genotype A is predominantly found in Europe alongside with genotype D. In Asia genotype C and B are most commonly found. In the Americas are genotype A, B, D and F are the common genotypes. Note that size of each circle does not represent the prevalence of HBV infection (Shi et al. 2013).

Genotype A is predominantly present in Africa, Northwestern Europe and the United States. Genotype B as well as C are commonly found in the Asia-Pacific region. Genotype D is mainly found in the Mediterranean, the Middle East, Central Asia and India. Genotype E is mostly restricted to Western Africa and genotype F to South and Central America. Genotype G has been found in the United States, France and Germany. Genotype H is prevalent in Central America (Liaw et al. 2010). Genotype I was found in Vietnam and Laos and the most recent genotype J was discovered in Japan (Sunbul 2014). Genotypes distribution has been correlated with horizontal versus vertical/perinatal modes of transmission (Kao and Chen 2002). In highly endemic regions, like the Asia-Pacific region, where perinatal transmission plays a predominate role, genotype B and C are more prevalent in comparison to regions like Europe, where HBV prevalence is lower and horizontal modes of transmission play a more important role and genotype A is more prevalent (Kao and Chen 2002). Nevertheless whether mode of transmission impacts genotype distribution remains to be determined by further studies.

A.1.5 Course of infection, diagnosis and treatment

Transmission of HBV occurs parenterally through infected body fluids as well as blood. In low prevalence areas sexual transmission of HBV causes up to 65% of HBV infections, followed by i.v. needle sharing among drug users, causing up to 20% of HBV transmissions. In areas with a higher incidence of HBV carriers (e.g. South-east Asia and Africa), perinatal transmission of HBV is more common and plays a predominant role as means of transmission. Incubation period ranges from 30 days to up to 180 days, after which HBV infection can results in several different courses of infection. In most cases, up to 65% in immunocompetent adults, HBV infection is eliminated asymptomatically. In 30% of the cases, HBV infection results in acute hepatitis and may lead to fulminant hepatitis with a mortality rate of up to 1% in hospitalized patients (Herold 2018).

Overall more than 90% of acute HBV infections are cleared spontaneously. In general, development of chronic HBV infection (CHB), with persistence of HBsAg or viral replicative activity over more 6 months is rare. Importantly the quota at which acute HBV progresses into CHB differs greatly depending on age, at which HBV infection has first occurred. Viral persistence occurs in >90% of perinatal infections. Rates decline with older age and viral persistence is only observed in 5% of immunocompetent adults (Chu et al. 1989; Chang 2008). Prolonged viral persistence may lead to chronic hepatitis resulting in liver cirrhosis and hepatocellular carcinoma (HCC). Higher levels of viremia in CHB patients have been associated with an increased risk of developing HCC (Chen et al. 2006).

Generally, in the case of viral persistence, CHB naturally progresses through four phases (**figure A.1.8**). Notable patients do not inevitably undergo all phases, or have to proceed through them chronologically. In addition, alternative variations of CHB progression have been suggested (Thomas and Liang 2016).

Nevertheless the typical four phases are (EASL 2017): The HBeAg-positive chronic HBV infection, previously named the immune tolerant phase. The HBeAg-positive chronic hepatitis B, previously immune clearance phase. The HBeAg-negative chronic HBV infection, previously labelled immune control or inactive carrier phase and the HBeAg-negative chronic hepatitis B, previously named immune escape phase. The HBeAg-positive chronic HBV infection (immune tolerant phases) is characterized by HBsAg being detectable in high

concentrations (~10^5 IU/mL), high levels of HBV DNA (>20000 IU/mL), positive HBeAg serum status, regular levels of alanine aminotransferase (ALT) and only minimal inflammation or fibrosis within liver tissue. Especially in the case of perinatal HBV infection, the immune tolerant phase may last over 20 years with the absence of actual liver damage. Immune modulation might be a result of HBsAg associated functional deficiency in HBs-specific B-cell. In addition T-cell exhaustion and depletion might be caused through constant antigen exposure of T-cells to the abundantly present HBsAg, presented by hepatocytes and professional antigen presenting cells (Bertoletti and Ferrari 2016; Le Bert et al. 2020).

Infection in immunocompetent adults, typically does not result in a prolonged immune tolerance phases (Bertoletti and Kennedy 2015). The HBeAg-positive chronic hepatitis B (immune clearance phase) is initiated by the loss of immune tolerance. In this phase HBsAg levels decline and HBV DNA may fluctuate. Elevated ALT levels indicate inflammatory activity within the liver. In addition, the immune activity pressures the virus, which can result in the development of viral variants containing precore or basal core promoter mutation. In this phase, successful HBeAg seroconversion can result in the control of HBV replication. Alternatively HBV may circumvent immune clearance and establish HBeAg-negativ CHB (Dandri and Locarnini 2012). The The HBeAg-negative chronic HBV infection (immune control/low or not-replicative phase) is characterized by low viral loads, minimal HBsAg levels as well as regular ALT levels. Achievement of HBsAg seroconversion during this inactive phase, before the age of 50 and the onset of liver cirrhosis, is associated with a vastly improved outcome, compared to patients over the age of 50, displaying manifested liver cirrhosis or co-infection with HCV. Spontaneous HBsAg seroclearance in patients is rare and has only a yearly rate of 0.5%-1%. Reactivation of HBV infection after HBeAg seroconversion occurs in about one third of CHB cases. These patients display a HBeAg negative CHB. Commonly, precore or/and basal core promoter mutations can be identified in these patients.

This HBeAg-positive chronic HBV infection (immune escape phase) is characterized by increased levels of viremia as well as ALT and is associated with a greater risk of developing liver cirrhosis and hepatocellular carcinoma (Trépo et al. 2014). HBV infection in patients that display persistently detectable HBV DNA

levels in serum or liver, but lack detectable HBsAg, has been defined as occult HBV infection (OBI) (Allain et al. 2009).

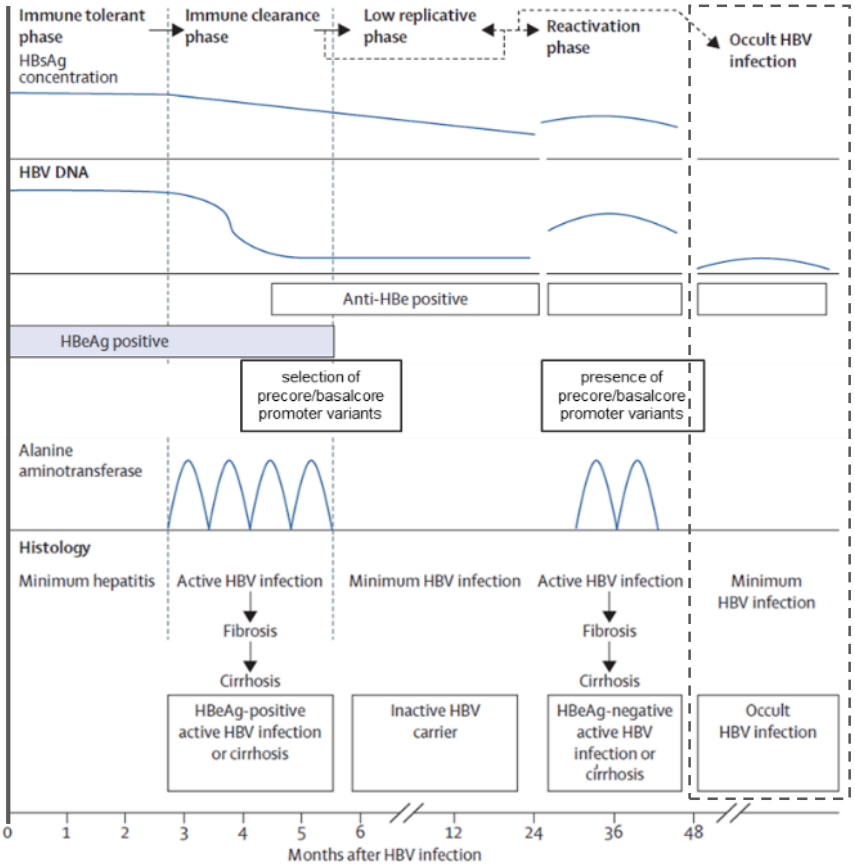

Figure A.1.8 ***Phases of chronic infection with hepatitis B***

Phases of chronic HBV infection schematically shown with corresponding clinical parameters (adapted from Trépo et al. 2014).

Diagnosis of HBV infection and distinguishment between acute HBV and reactivation is centered on the serologic detection of anti-HB-IgM, anti-HBc-IgG, HBsAg, anti -HBs, HBeAg, anti -HBe as well as quantification of HBV DNA. In case of an acute HBV infection, HBsAg can be detected prior to onset of hepatitis and is a marker for active HBV transcription. Failed HBsAg seroconversion after 6 months is an indicator of viral persistence. HBeAg can be detected in acute as well as in chronic HBV (Herold 2018). In acute HBV infection, anti-HBc-IgM is the predominant immunoglobulin over anti-HBc-IgG, but may also be detected during exacerbation in CHB (Maruyama et al. 1994). Usually, low levels of anti-HBc-Ag in combination with high levels of HBV DNA suggest a reactivation rather than

acute HBV infection (Ichai and Samuel 2019). In addition, positive anti-HBsAg but negative anti-HBc-IgG suggest prior HBV vaccination.

Therapy of acute HBV infection should be primarily symptomatic, since 95% of HBV infections are self-limiting. Co-infections with e.g. HIV, HCV or HDV should be ruled out. Liver damage can be assed via ALT and AST levels. Anti-viral therapy is only indicated in patients with severe liver damage and aims to suppress HBV replication and prevent further hepatic inflammation (Trépo et al. 2014).

Indication for the treatment of CHB arises mainly from the combination of three criteria and is generally independent of HBeAg status in patients. These criteria include the serum levels of HBV DNA as well as ALT levels and the severity of liver tissue damage. In the absence of liver cirrhosis, patients exceeding HBV DNA levels of >2,000 IU/ml serum and ALT levels of the twice upper limit of normal (ULN) (~40 IU/L) should be treated if liver biopsy shows signs of at least moderate fibroses. If viral titers are >20,000IU/ml and ALT levels are above 2xULN, treatment may be started without a liver biopsy. If ALT levels are within the ULN, treatment may still be indicated if HBV DNA levels are >2000 IU/ml and patients display at least moderate liver fibrosis. Today two main treatment options can be considered in the treatment of CHB. The usage of nucleoside/nucleotide analogues (NAs) or the treatment with IFNα – pegylated IFNα in that case. The currently available and in Europe approved NAs for the treatment of CHB include adefovir dipivoxil (ADV) , entecavir (ETV), lamivudine (LAM), telbivudine (TBV), tenofovir alafenamide (TAF) and tenofovir disoproxil fumarate (TDF). NAs are associated with either a high or a low barrier against HBV resistance. NAs classified as displaying a low barrier against HBV resistance include ADV, LAM and TBV, while NAs with high barrier to HBV resistance include ETV, TAF as well as TDF. Antiviral treatment with a NA associated with high barrier to resistance (i.e., ETV, TDF, TAF) carries the advantages of a favourable safety profile (compared to IFNα treatment) alongside with an expectable high long-term antiviral efficiency that leads to HBV DNA levels below the limit of detection in the vast majority of compliant patients. NAs may be safely applied in any HBV infected patient group including patients that display decompensated liver disease, liver transplants, extrahepatic manifestations, acute hepatitis B or severe chronic HBV exacerbation. In addition NAs are the sole option to prevent HBV

reactivation in patients under immunosuppression. Treatment with pegylated IFNα aims to induce immunological control with HBeAg and HBsAg loss and has a finite extent of treatment. Disadvantages of pegylated IFNα treatment include severe side effects in patients as well as a high variability virological and serological response. It is also contraindicated in patients with preexisting decompensated liver cirrhosis as well as during pregnancy (Lee and Banini 2019; EASL 2017). IFN suppresses the epigenetic transcription of cccDNA as well as it induces the expression of interferon stimulated genes (Belloni et al. 2012; Allweiss et al. 2014) and modulates natural killer cell activity (Kakimi et al. 2000). Nucleoside/nucleotide analogues treatment involves lifelong treatment. Inhibition of the viral polymerase suppresses viral replication (Grimm et al. 2011).

Anti-viral therapy is only able to control HBV replication, since neither antiviral therapy is able to eradicate the presence of cccDNA and thereby clear the infection entirely (Perrillo 2006).

A.2 Innate immune response towards viral infection

A.2.1 Pathogen-associated molecular patterns recognition and Toll-like receptor signaling

The innate immunity serves as a rapid response towards pathogens infecting tissue as well as prompting the adaptive immune response via presentation of pathogens by either dendritic or other antigen-presenting cells (Abbas et al. 2018). Pathogen recognition within the innate immune system is accomplished by the recognition of conserved and recurring pathogen-associated molecular patterns (PAMPs). PAMPs include for example nucleic acids and nucleotides, polysaccharides, lipoproteins and glycolipids. They are recognized by so called pattern-recognition receptors (PRRs). PRRs are a diverse group of receptors, capable of cell-intrinsic as well as cell extrinsic pathogen recognition. PRRs make use of distinct ligand-recognition domains, like leucine-rich repeats, C-type lectin domains and various nucleic acid–binding domains to allow for PAMP detection (Iwasaki and Medzhitov 2015). PRRs involved in the detection of viral infection include Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) as well as nucleotide-binding oligomerization domain receptors (NODs) (Takeuchi and Akira 2009). Following PAMP recognition in viral infection PRR downstream signaling leads to the induction of type I interferon (IFN) and other inflammatory cytokines, including interleukins (ILs) as well as tumor necrosis factors (TNF). This subsequently invokes an inflammatory state within the infected tissue (Riera Romo et al. 2016; Takeuchi and Akira 2010). In case of the TLR-family, downstream signaling is initiated by the recruitment of the Toll/IL-1 receptor (TIR) domain-containing proteins, such as the myeloid differentiation factor 88 (MyD88) (Akira et al. 2006b). The diverse family of the TLRs can be characterized based on their location. TLR3, TLR7, TLR8, TLR9, and TLR10 are localized within the endosome, whereas TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are localized at the cell surface (Newton and Dixit 2012). In addition to the previously mentioned MyD88, there are four additional TIR domain-containing proteins involved in TLR downstream signaling. These include the MyD88 adapter-like (TIRAP/Mal), the Sterile-alpha and Armadillo motif-containing protein (SARM), the TIR domain-containing adaptor inducing IFN-b (TRIF) and the TRIF-related adaptor molecule (TRAM). Structurally, the TLRs consists of an extracellular N-terminal leucine-rich repeat domain, a transmembrane domain

and the intracellular TIR domain (Takeda et al. 2003). PAMP recognition is achieved via ligand binding at the N-terminal leucine-rich repeat domain, upon which TLRs dimerize and undergo conformational changes, allowing for TIR-domain-containing adaptor molecules to be recruited (Akira et al. 2006a).

Depending on the adaptor molecules of either MyD88 or TRIF, TLR-downstream signaling follows two distinct pathways, that can either be described as MyD88 or TRIF dependent (**figure A.2.1**) (Takeuchi and Akira 2010; Chen and Jiang 2013). In case of the MyD88 dependent pathway as a first step MyD88 is bridged to the TLR via TIRAP/Mal (Verstak et al. 2009). In addition to the TIR domain, MyD88 also contains a death domain (DD), which is used to interact with the Interleukin-1 (IL-1) receptor-associated kinase 4 (IRAK4) and IRAK1 to form a MyD88-IRAK4-IRAK1 complex (Lin et al. 2010). IRAK4 and IRAK1 are serin/threonine kinases and two of four none members of the Interleukin-1 (IL-1) receptor-associated kinase (IRAK) family (Janssens and Beyaert 2003). Following MyD88 IRAK4 interaction, IRAK1 is activated by IRAK4, autophosphorylated and dissociates from MyD88 (Kollewe et al. 2004). IRAK1 then associates with the TNF receptor associated factor 6 (TRAF6), leading to the K63-linked polyubiquitination of TRAF6 as well as the Mitogen-activated protein kinase kinase 7 (MAP3K7) or TAK1. TAK1 forms a complex with its regulatory subunits TGF-beta activated kinase 1 binding protein 1 (TAB1), TAB2 and TAB3 (Conner et al. 2006). These interact with the polyubiquitin chain generated previously and lead to the activation of TAK1. TAK1 then activates two different pathways. On one side, the IκB kinase (IKK) complex is phosphorylated and in turn phosphorylates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is inhibited by the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα). Phosphorylation is allowing NF-κB to translocate into the nucleus, inducing pro inflammatory gene expression. On the other side TAK1 also activates a number of different mitogen-activated protein kinases (MAPKs), which also results an upregulation of pro inflammatory gene expression (Kawasaki and Kawai 2014). The MyD88 dependent pathway is utilized by all known TLRs with the exception of TLR3. TLR3 and also TLR4 make use of the TRIF dependent signaling pathway in order to accomplish downstream signaling and INF1 induction (Newton and Dixit 2012). In case of TLR4 the additional adaptor protein TRAM is needed to initiate TRIF dependent signaling. TLR3 has no need for additional adaptors and is able to directly interact with TRIF.

TRIF is able to interact with TRAF6 as well as TRAF3. TRAF interaction induces NF-κB signaling similar to MyD88 dependent signaling. In contrast to MyD88 signaling, TRIF additionally recruits the protein receptor interacting protein-1 (RIP-1), leading to an enhanced NF-κB reaction (Cusson-Hermance et al. 2005). TRAF3 associates with the TANK binding kinase-1 (TBK1) and IKK-I. This allows for the phosphorylation of the interferon regulatory factor 3 (IRF3) and IRF7 and thereby their dimerization and translocation into the nucleus. Here INF1 expression is induced (Thompson et al. 2011). TRAM interaction with TLR2 might also allow for IFN1 induction as a response towards viral infection by TLR2 (Stack et al. 2014).

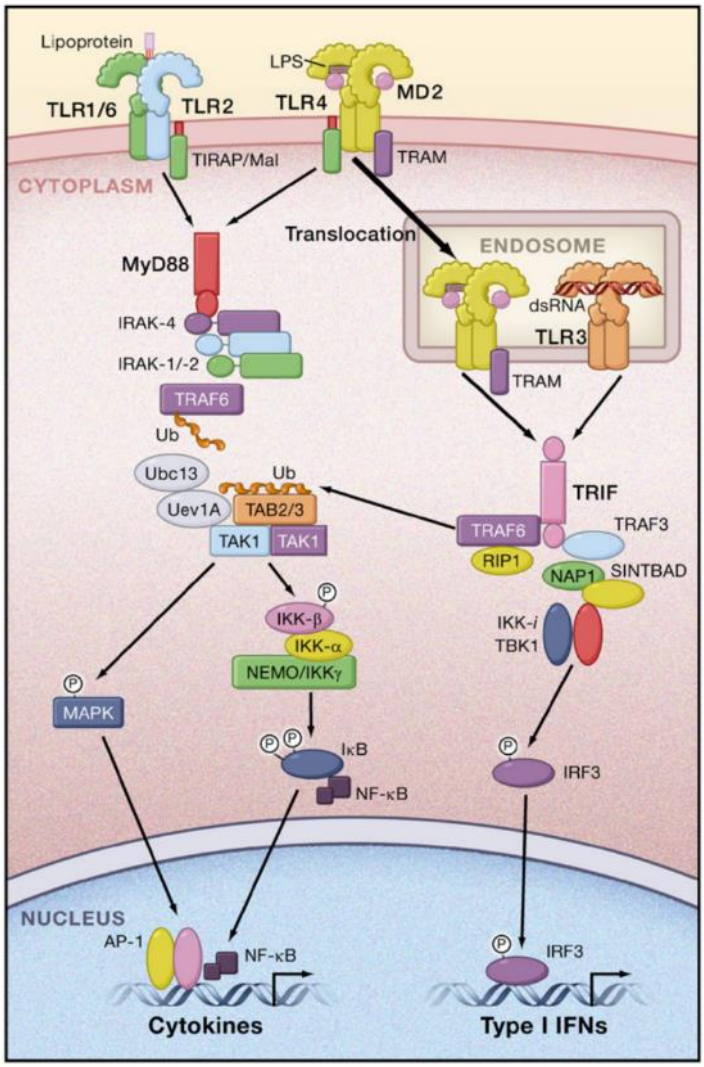

Figure A.2.1 ***TLR signaling pathways***

MyD88 dependent downstream signaling of TLR2, TLR4 and TRIF dependent downstream signaling of TLR3 and TLR4 (Takeuchi and Akira 2010).

A.2.2 Type 1 Interferon signaling inducing Interferon stimulated gene expression

Interferons (IFNs) function as a first line of defense in viral infection. As a response towards viral infection, IFNs promote an antiviral state in infected tissue. This is reflected in the expression of IFN-stimulated genes (ISGs) (Katze et al. 2002). Interferons are divided into three classes: type 1, type 2 and type 3. Type 1 interferons (IFN1) include IFN-α, β, as well as the subtypes ε, κ, and ω. All type 1 interferons utilize the universally expressed heterodimeric IFN receptor, composed of the interferon-alpha/beta receptor alpha chain 1 and 2 (IFNAR1 and IFNAR2), to initiate IFN signaling (**figure A.2.2**) (Chow and Gale 2015).

IFN1 activates the JAK-STAT signaling pathway. Upon IFN1 binding, Tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) are activated. Phosphorylation within the receptor leads to the formation of a phosphotyrosine-based motif, that recruits the signal transducer and activator of transcription 1 and 2 (STAT1/STAT2). STAT1 and STAT2 are then phosphorylated and form a heterodimer. Cytoplasmic IRF9 is able to bind to the STAT1-STAT2 heterodimer, forming the IFN-stimulated gene factor 3 (ISGF3) complex. After translocation of the ISGF3 into the nucleus, it binds to IFN-stimulated response elements (ISREs) and induces ISG transcription (Green et al. 2018). ISGs, acting as antiviral agents, include for example MX1, OAS, ISG15 or ISG20 (Raftery and Stevenson 2017; Schoggins and Rice 2011).

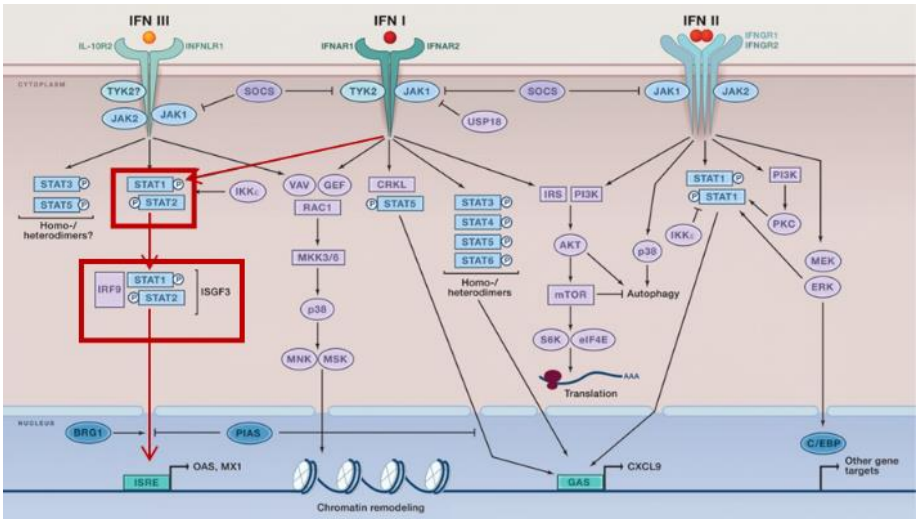

Figure A.2.2 ***Interferon signaling***

Type I interferons active the JAK-STAT signaling pathway (highlighted red) leading to ISG induction (adapted from Chow et al. 2015).

A.2.3 Lack of INF response in HBV infection

The acute infection with HBV induces barely any detectable type 1 IFN response in patients (Dunn et al. 2009). In vivo studies showed that HBV does not induce an IFN response in infected chimpanzees (Wieland et al. 2004), while HCV did lead to an IFN triggered ISG induction (Su et al. 2002). This demonstrates, that while having a similar tropism, HBV and HCV are met by a vastly different innate immune response (Wieland and Chisari 2005). Chronically infected human liver chimeric uPA/SCID/beige mice also displayed only a weak ISG induction as a response to HBV mono infection, while ISG induction, as well as human cytokine expression, was elevated, in HBV/HDV co-infected mice. This demonstrates that HBV mono infection fails to induce an inflammatory antiviral state in infected human hepatocytes compared to HBV/HDV co-infection (Giersch et al. 2015). While the circulating NK cells count in patients appeared to be elevated in early phases of HBV infection (Fisicaro et al. 2009), NK cell effector function was impaired with increasing viral loads (Dunn et al. 2009). It was shown, that NK cell function may be suppressed by immunemodulatory cytokines like IL10 in CHB, it also has been shown that NK cells are able to retain their cytotoxic function (Peppa et al. 2010). In addition NK cells have been suggested to be involved in liver damage during HBV reactivation in CHB. Overall the role of NK cells in CHB remains uncertain (Bertoletti and Ferrari 2013). Nevertheless since HBV replication is often not detectable within the early phases of infection, the innate immune response has been assumed to be an important factor in controlling viral replication in patients (Morikawa et al. 2016).

A.3 HBV Infection Models

A.3.1 In vitro and In vivo models

The narrow host range as well as the tissue tropism of HBV impose specific requirements upon in vitro as well as in vivo infection models and have hindered the study and full understanding of a number of aspects in the infection and replication cycle of HBV. As the natural target of infection, primary human hepatocytes (PHHs) are considered to be the gold standard in the in vitro study of HBV and its interaction with hepatocytes. While they are capable of supporting the entire replication cycle of HBV (Rijntjes et al. 1988; Rumin et al. 1996), PHHs in cell culture only have a limited life span. PHHs do not replicate but dedifferentiate, losing their biological characterization as a hepatocyte within weeks after planting (Elaut et al. 2006; Thomas and Liang 2016). The only immune competent non-human primates fully susceptible to HBV infection are chimpanzees (Barker et al. 1973). The chimpanzee model for HBV has therefore played an important role in the study of HBV pathogenesis as well as the vaccine and drug development (Wieland 2015). The challenging laboratory logistics, high costs, regulatory restrictions as well as ethical considerations limit the use of the chimpanzee model as an in vivo model for HBV infection.

To overcome the limitations in the PHH cell culture and the chimpanzee model, a number of different in vitro as well as in vivo models have been developed.

Besides PHHs, there have been a number of cell culture based models utilized in the study of HBV. As an alternative to PHHs, tree shrew hepatocytes can be isolated and infected with HBV in vitro (Glebe et al. 2003). Hepatocyte-like cells (HEP) derived from pluripotent stem cells have also been demonstrated to be viable for infection with HBV (Si-Tayeb et al. 2010; Shlomai et al. 2014).

HepG2, derived from a hepatoblastoma, and Huh7, derived from a hepatocellular carcinoma, are two hepatoma derived cell lines commonly utilized in the study of HBV. Both cell lines lack the NTCP receptor and are therefore not susceptible to HBV infection, but viral expression can be achieved via transfection of an cloned HBV DNA over length construct, that serves as a template for transcription, mimicking the function of the cccDNA (Sureau et al. 1986; Tsurimoto et al. 1987). Expression of the needed HBV receptor can be accomplished via NTCP-

transfection, this in turn allows for the infection with HBV within the HepG2 or Huh7 hepatoma cell lines (Yan et al. 2012; Ni et al. 2014).

The HepaRG cell line is a bi-potent liver progenitor cell, derived from a liver tumor in association with chronic hepatitis C virus infection. HepaRG cells differentiate either into hepatocyte-like cells or develop into bile duct epithelium-like cells. Differentiated HepRG (dHepRG) cells have been shown to support initial HBV infection as well as subsequent replication cycles. Nevertheless there is no subsequent viral spreading as a result of HBV-DNA expression within these in vitro systems (Gripon et al. 2002; Hantz et al. 2009).

Besides the previously mentioned chimpanzee model, a number of different HBV in vivo models have been described. The infection of ducks or woodchucks with the HBV related hepadnaviruses duck hepatits B virus (Mason et al. 1980) and the woodchuck hepatitis virus respectively (Summers et al. 1978), have made it possible to study the pathogenesis of chronic hepadnavirus infection, development of HCC as well as develop antiviral therapy in animal models (Mason 2015).

The tree shrew species Tupaia bulangeri is the only non-primate susceptble to the infection with HBV (Köck et al. 2001). In addition to its in vitro application the tree shrew model can also be utilized in vivo. Infection of neonate Tupaia results in chronic HBV infection with moderate levels of viremia and in some cases in the development of liver fibroses as well as HCC. Infection of adult animals only leads to low and transient levels of viral replication within a mild acute course of infection (Walter 1996; Yang et al. 2015).

Mice, being a well-characterized small laboratory animal, are naturally not susceptible to the infection with HBV. In order to study HBV in a mouse model, transgenic mice that are expressing either the entire HBV genome or selected HBV proteins have been developed. These models allow for the study of the cellular and humoral immune response against the virus but are incapable of clearing HBV and cccDNA is generally not detected in transgenic mice. The adenoviral vector based HBV transduction (Ad-HBV) in mice primarily depicts the acute self-limiting infection with HBV. The hydrodynamic injection model makes use of a rapid injection of a large volume containing naked HBV DNA into animals via the tail vein. This results in significant liver damage and alanine transaminase (ALT) elevation shortly after injection but leads to the uptake of the HBV DNA

within the liver and subsequently to transient gene expression in hepatocytes of immunocompetent mice (Allweiss and Dandri 2016). Nevertheless, virions produced in these models are not able to (re)infect murine hepatocytes, rendering assessment of for example cccDNA formation, mechanisms of cell entry and intrahepatic spreading impossible. Even in the hNTCP transgenic knock-in mice, murine hepatocytes remain resistant to HBV infection. In order to circumvent these restrictions and better understand the HBV lifecycle, mouse models based on the transplantation of human hepatocytes have been developed (Dandri and Petersen 2017).

A.3.2 Human liver chimeric mice

The liver is a highly regenerative tissue, capable of restoring hepatocyte function as well as liver structure even after severe liver damage or extensive surgery (Standring and Ananad 2016). These properties allow for the transplantation of PHHs into rodents, where PHHs would then reform liver tissue resembling the properties of a human liver. Studies demonstrated that isolated PHHs are able to form liver-like constructs within the dorsal fascia, (Jirtle et al. 1980) the spleen (Kusano and Mito 1982) as well as within the intraperitoneal cavity (Demetriou et al. 1986) when transplanted into rats.

In order to translate this concept into a mouse model, in which human hepatocytes are engrafted and able to expand forming, a liver-like structure and maintain their characteristics as human hepatocytes, two basic conditions have to be met.

First: immune response to the transplanted xenogenic hepatocytes has to be eliminated in order to counteract clearance of the transplanted hepatocytes. Second: endogenous liver damage needs to be induced within the mouse liver to create space as well as a regenerative stimulus for the transplanted hepatocytes to repopulate the murine liver structure.

In order to establish a murine model for HBV production, immortalized hepatocytes were transfected with a full-length HBV genome and transplanted into mice with combined immunodeficiency (Rag-2 deficiency) via intrasplenic injection. Rag-2 mice lack the ability to form mature T- and B- lymphocytes due

to the deletion of the Rag-2 gene encoding the recombination activating gene 2 protein. HBV as well as HBsAg could be detected in transplanted mice (Brown et al. 2000).

The mutation in the Prkdc gene, encoding a DNA repair complex protein, leads to lack of B- and T- lymphocytes and consequential to severe combined immunodeficiency (SCID). After transplantation of PHHs into the kidney capsule of mice harboring the SCID-mutation, transplanted PHHs were demonstrated to be susceptible to HBV infection. Although, count of hepatocytes transplanted subcutaneously into the kidney capsule slowly declined over 3 – 6 months, the full HBV lifecycle was supported within the xenogenic liver tissue (Ohashi et al. 2000). These models demonstrate the possibility of engraftment of human hepatocytes into immunodeficient mice as well as their capability of supporting HBV infection or HBV genome expression.

To provide the necessary room within the parenchymal structure as well as a potent stimulus to promote human hepatocyte engraftment and repopulation, an intrinsically driven murine liver damage was introduced to the HBV mouse model. This can be achieved by the hepatotoxic albumin promoter driven expression of urokinase-type plasminogen activator (uPA). Alb-uPA transgenic mice were first made use of in the study of neonatal bleeding disorders (Heckel et al. 1990). It was shown that transgene-deficient cells progenitor cells within the uPA transgenic mice were able to selectively proliferate and reconstitute liver tissue effectively (Sandgren et al. 1991). Transplanted PHH in uPA mice displayed comparable properties in replacing diseased murine liver tissue (Rhim et al. 1994).

Alternatively murine hepatocyte failure can be induced via the use of fumaryl acetoacetat hydrolase (Fah) deficient mice. Fah deficiency ($Fah^{-/-}$) results in the accumulation of hepatotoxic intermediates from the tyrosine catabolism, resulting in murine liver injury (Kelsey et al. 1992). $Fah^{-/-}$ mice have been successfully engrafted, repopulated with PHHs and infected with HBV (Bissig et al. 2007; Azuma et al. 2007; He et al. 2010).

Using transgenic uPA mice crossbred with immunodeficient Rag-2 mice, Dandri et al. demonstrated that transplantation of PHHs resulted in liver repopulation of up to 15%, indicating that human hepatocytes had undergone 6 to 7 cell doublings. Moreover transplanted mice were susceptibly to HBV infection,

developing a stable HBV infection in the repopulated hepatocytes within 8 week after HBV infection (Dandri et al. 2001). PHH transplantation in uPA/SCID mice was done to establish a suitably mouse model for the infection with the hepatitis C virus. While Alb-uPA heterozygous mice only displayed minimal engraftment of PHHs, homozygous Alb-uPA mice where repopulated by PHHs, replacing up to 50% of the murine liver (Mercer et al. 2001). Since then, the chronic mono infection with HBV as well as HCV in homozygous uPA/SCID mice has been demonstrated and characterized (Meuleman et al. 2005), followed by the HBV/HDV co-infection in homozygous uPA/SCID/beige mice in 2012 (Lutgehetmann et al. 2012).

In addition to the lack of functional B- and T- lymphocytes in SCID mice, SCID/beige mice also lack natural killer cells due to the beige (bg^J) mutation (Roder and Duwe 1979). Crossbreeding of homozygous uPA mice with SCID/beige mice results in uPA/SCID/beige mice (Lütgehetmann et al. 2011).

Experiments in this doctoral thesis were performed using the human chimeric uPA/SCID/beige mouse model (**figure A.3.1**) (Dandri and Petersen 2012). Three to four week old uPA/SCID/beige mice were transplanted with cryopreserved human hepatocytes via intrasplenic injection. To quantify repopulation of human hepatocytes in the murine liver parenchyma levels of human serum albumin were determined 8 weeks post PHH engraftment. uPA/SCID/beige mice that displayed appropriate albumin levels were inoculated with either HBV wild-type or the HBV G1896A precore variant. Viremia was determined in blood samples over the course of 12 weeks, after which mice were sacrificed, livers were collected and intrahepatic measurements were performed.

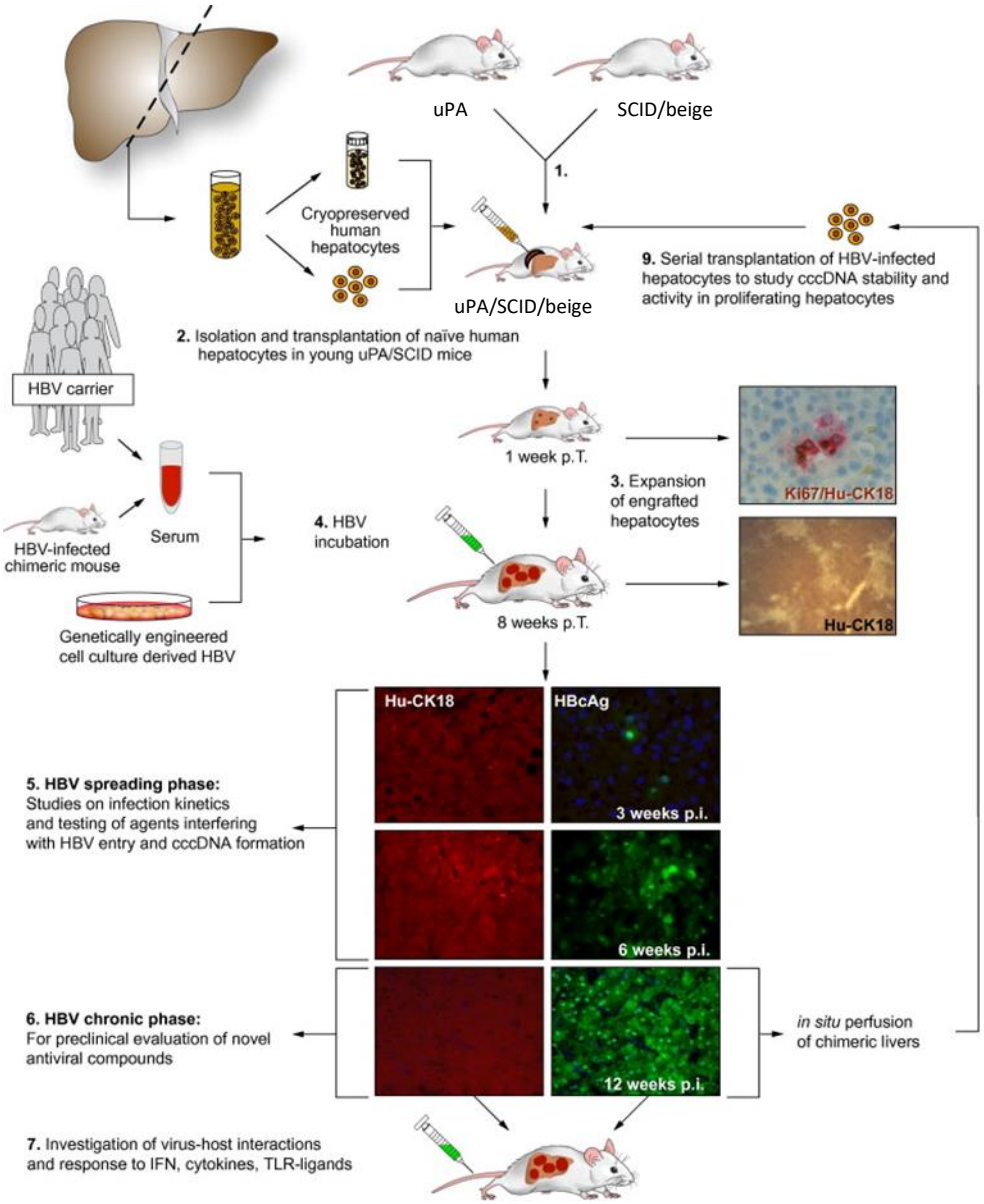

Figure A.3.1 ***uPA/SCID/beige mouse model***

Human liver chimeric mice are generated by crossbreeding uPA mice with SCID/beige. After engraftment and liver repopulation mice are inoculated with HBV (adapted from Dandri et al. 2012).

A.4 Aim of work

The innate immune system poses the first line of defense against potential pathogens and plays an essential role in the activation of an adequate adaptive immune response. The mechanisms by which HBV is able to circumvent an effective immune response are yet poorly defined (Walsh and Locarnini 2012; Suslov et al. 2018). Therefore, further knowledge of the interactions occurring between HBV, the infected hepatocytes and the host immune response is key to understand the mechanisms modulating immune responses and evasion strategies adopted by the hepatitis B virus. Such knowledge may also allow the development of new treatment options aiming at the cure of HBV. This study aims to investigate the role of HBeAg/precore protein on HBV activity and on the intrinsic innate responses of infected human hepatocytes. In particular, the study is designed to examine in vivo the effect of the absence of HBeAg/precore protein in primary human hepatocytes infected with the most common HBeAg negative HBV variant.

The HBeAg/precore protein has been described to promote immune evasion by acting as a HBcAg decoy and by depleting T-helper cells in utero(Chen et al. 2005). Downregulation of genes involved in innate immunity and cell signaling, potentially caused by the HBeAg/precore protein, were shown in Huh-7 cells (Locarnini et al. 2005). TLR2 expression on hepatocytes as well as on Kupffer cells in liver biopsies of CHB patients was shown to be lower in HBeAg positive patients, compared to HBeAg negative patients. In the presence of HBeAg/precore protein, TLR2 signaling, stimulated by the TLR2 ligand Pam3Cys also appeared to be impaired in peripheral blood monocytes (PBMCs) (Visvanathan et al. 2007). In addition it was described that TLR2-, TLR3-, TIRAP/Mal-, TRAM- and TRIF-induction of NF-κB pathways can be inhibited in the presence of the HBeAg/precore protein in HEK293 cells (human embryonic kidney 293 cell line) (Lang et al. 2011). All these different lines of evidence indicate that HBeAg can induce a state of immune tolerance in HBV infection (Dandri and Locarnini 2012).

In this study, we employed human liver chimeric uPA/SCID/beige mice to comparatively characterize infection kinetics and replication activity of the HBV genotype D wild type and its G1896A, HBeAg negative, variant **(specific aim 1)**.

Moreover, we examined the expression of innate immunity related genes in vivo to determine whether or not the HBeAg/precore protein can directly impact the intrinsic innate responses of the human hepatocytes **(specific aim 2)**. It has been suggested that the HBeAg might be able to disrupt TLR2 related signaling by co-localizing with TIR-domain containing elements of the TLR-signaling pathway. To evaluate whether the HBeAg/precore protein is able to hinder the induction of the TLR2 signaling pathway, we treated mice that were either infected with wild type HBV or with the G1896A HBeAg negative variant with the TLR2 agonist Pam3Cys and determined virological as well as immune-related changes **(specific aim 3)**.

B Material and Methods

B.1 Instruments

Table B.1.1 ***Instruments***

Instrument	Manufacturer	Country
ABI Prism 377 automated sequencer	Applied Biosystems	USA
Absorbance Microplate Reader ELx808	BioTek	USA
ChemiDoc XRS Imaging Station	BioRAD Laboratories	USA
Chemiluminescent Microparticle Immunoassay Architect	Abbott Laboratories	USA
Centrifuge Galaxy Mini	VWR	USA
Centrifuge MiniSpin	Eppendorf	Germany
Centrifuge 5415R	Eppendorf	Germany
Centrifuge 5417C	Eppendorf	Germany
Data Analysis Software KC4	BioTek	USA
Geneious Bioinformatics Software	Biomatters Ltd	New Zealand
GraphPad Prism 5 Software	GraphPad	USA
Light Cycler Software 3.5	Roche Diagnostics	Switzerland
Lightcycler 1.5 Real-time PCR System	Roche Diagnostics	Switzerland
Microscope Biorevo BZ-9000	Keyence	Japan
Mini Trans-Blot Electrophoretic Transfercell	BioRAD Laboratories	USA
ND-1000 spectrophotometer	NanoDrop Technologies	USA
Power Supply PAC300	BioRAD Laboratories	USA
QuantityOne Software	BioRAD Laboratories	USA
QubitFluorometer 2.0	Invitrogen™ (Life Technologies GmbH)	USA
Thermomixer compact	Eppendorf	Germany
Thermocycler iCycler	Biorad	Germany
Thermocycler Veriti 96-well fast	Applied Biosystems	USA
ViiA™ 7 System	Life Technologies GmbH	Germany
ViiA™ 7 Software	Life Technologies GmbH	Germany
Vortexer MS2 Minishaker	IKA	Germany
Vortexer Reax Top	Heidolph	Germany

B.2 Material

B.2.1 General reagents

Table B.2.1 ***General reagents***

Reagents	Manufacturer	Country
Acetone	Th. Geyer GmbH & Co	Germany
Ammonium acetate	Sigma-Aldrich	USA
Anchored-oligo(dT) primer (cDNA)	Hoffmann-La Roche	Switzerland
Aqua ad inectabilia	B. Braun Melsungen AG	Germany
ATP 100mM	Biozym Scientific GmbH	Germany
AW1 buffer	Qiagen	Netherlands
AW2 buffer	Qiagen	Netherlands
Buffer AL	Qiagen	Netherlands
Dako Mounting Medium	Dako	Denmark
Desoxynucleotide mix (cDNA)	Hoffmann-La Roche	Switzerland
DNA Master (HybProbe)	Hoffmann-La Roche	Switzerland
DNase I stock solution (DNAse Kit)	Qiagen	Netherlands
Ethanol 100%	Th. Geyer GmbH & Co	Germany
Ethanol 75%	Th. Geyer GmbH & Co	Germany
Extraction solution	Sigma-Aldrich	USA
Flurophore tyramide	Perkin Elmer	USA
GelRed	GeneON GmbH	Germany
Glycogen	Hoffmann-La Roche	Switzerland
Hepatocyte Wash Medium	Invitrogen™ (Life Technologies GmbH)	USA
Hoechst 33258	Hoechst AG	Germany
HRP Conjugated Goat anti-Human Albumin Detection Antibody A80-129P	Bethyl Laboratories	USA
Hydrogen peroxide	Merck	Germany
Isoflurane	Baxter International	USA
Isopropyl alcohol	Baxter International	USA
LightCycler FastStart DNA Master SYBR Green I	Hoffmann-La Roche	Switzerland
Magnesium chloride	Hoffmann-La Roche	Switzerland
Methanol	Th. Geyer GmbH &Co	Germany
Pam3Cys-SKKKK	EMC microcollections GmbH	Germany

Phosphate buffered saline	Invitrogen™ (Life Technologies GmbH)	USA
ProtectorRNase inhibitor	Hoffmann-La Roche	Switzerland
Protein Precipitation Reagent	Epicentre	USA
Proteinase K	Epicentre	USA
QIAGEN Protease	Qiagen	Netherlands
Quant-iT buffer	Invitrogen™ (Life Technologies)	USA
Quant-iT reagent Invitrogen™	Invitrogen™ (Life Technologies)	USA
RDD buffer	Qiagen	Netherlands
Reaction buffer	Epicentre	USA
REDExtract-N-Amp PCR Reaction mix	Sigma-Aldrich	USA
Red-Taq Polymerase	Sigma	USA
RLT buffer	Qiagen	Netherlands
RNAse free water	Qiagen	Netherlands
RPE buffer	Qiagen	Netherlands
RW1 buffer	Qiagen	Netherlands
Sodium chloride	Carl Roth GmbH & Co	Germany
Sodium chloride solution 0,9%	B. Braun Melsungen AG	Germany
Standard Quant-it	Invitrogen™ (Life Technologies GmbH)	USA
Sulfuric acid	Carl Roth GmbH & Co	Germany
TaqMan™ Fast Advanced Master Mix	Applied Biosystems	USA
TaqMan™ Fast Virus 1-Step Master Mix	Applied Biosystems	USA
Transcriptor Reverse Transcriptase	Hoffmann-La Roche	Switzerland
Transcriptor RT Reaction Buffer	Hoffmann-La Roche	Switzerland
Tris acetate EDTA buffer	Sigma	USA
Uracil-DNA glycosylase	Hoffmann-La Roche	Switzerland

B.2.2 Kits

Table B.2.2 ***Kits***

Kits	Manufacturer	Country
ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit	Applied Biosystems	USA
Architect HBeAg assay	Abbott Ireland Diagnostics	Ireland
Architect HBsAg assay	Abbott Ireland Diagnostics	Ireland
MasterPure DNA Purification Kit	Epicentre	USA
MinElute PCR Purification Kit	Qiagen	Netherlands
QIAmp MinElute Virus Spin Kit	Qiagen	Netherlands
QIAquick Gel Extraction Kit	Qiagen	Netherlands
RNeasy RNA Mini Kit	Qiagen	Netherlands
Taqman Gene Expression Assays	Applied Biosystems	USA
Transcriptor First Strand cDNA Synthesis Kit	Hoffmann-La Roche	Switzerlan
TSA Fluorescein System Perkin	Elmer	Germany

B.2.3 Viral variants HBW wt and HBV G1896A precore variant

The viral variants used in this study to inoculate human liver chimeric uPA/SCID/beige mice were provided by the group of Prof. Dr. Glebe at the Justus-Liebig-Universität, Gießen. Both viruses are cell culture derived from a 1.5 HBV construct, based on the pCH-9/309 plasmid. Both viruses only differ at the position 1896 of the HBV genotype D genome. In the HBV precore variant the G1896A mutation is introduced leading to a stop codon. Prior to this study, viruses were shown to successfully infect Tupaia hepatocytes and express HBeAg according to viral variant.

B.3 Methods

B.3.1 Generation of humanized uPA/SCID/beige mice

To generate human liver chimeric mice, uPA transgenic mice (Jackson Laboratories, ME, USA) were crossed with SCID/beige mice (Taconic Farms, Denmark, EU). Three to four week old homozygous uPA/SCID/beige mice were injected intrasplenically with $1x10^6$ thawed viable human hepatocytes, isolated from one liver specimen obtained from a reduced-sized liver transplant. All mice in this study were transplanted with hepatocytes obtained from one single donor. In order to estimate rate of liver repopulation in uPA/SCID/beige mice, human serum albumin concentrations in mouse sera were determined via the Human Albumin Quantification Set (Bethyl Laboratories, Biomol GmbH, Hamburg, Germany). In addition human liver cell content was determined histologically, by staining of the human marker CK18, as well as by determining the amount of present beta-globin via real-time PCR in a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA). Mice were sacrificed at time points indicated in the results, liver specimens were cryo-conserved in chilled isopentane and stored at -80°C in order to perform histological and molecular analyses (Allweiss et al. 2014).

To evaluate the influence of the HBeAg/precore protein upon expression of intrinsic innate immunity genes as well as viral kinetics, groups of uninfected mice, HBV wild type (HBV wt) infected mice and HBV G1896A precore variant (HBV precore) were compared. To further assess the impact of the HBeAg/precore protein with regard to disruption of TLR-signaling, HBV wt infected mice and HBV precore variant infected mice and uninfected mice were treated with the TLR1/TLR2 agonist Pam3Cys-SKKKK (EMC microcollections GmbH, Germany). Treated mice were compared to untreated HBV wt, untreated HBV precore infected mice and uninfected untreated controls.

Mice were housed under specific pathogen-free conditions in accordance with institutional guidelines under approved protocols. Procedures were approved by the Ethical Committee of the city and state of Hamburg and according to the principles of the Declaration of Helsinki.

B.3.2 Viral infection with the HBV wild type or the HBV G1896A precore variant

Transplanted uPA/SCID/beige, displaying adequate levels of human liver chimerisam, received a single intraperitoneal injection, containing $1x10^7$ HBV DNA genome equivalents. Mice were either infected with cell culture derived HBV genotype D wild type or its cell culture derived G1896A variant. In addition to infection with cell culture derived viral particles, mice were alternatively inoculated with mouse serum (containing $1x10^7$ HBV DNA genome equivalents), derived from either HBV wild type or HBV G1896A variant infected mice.

B.3.3 Virological measurements and intrahepatic quantification

Viral HBV DNA was extracted from serum samples of 5µl using the QIAmp MinElute Virus Spin Kit (Qiagen, Netherlands). To quantify HBV DNA, real-time PCR was performed in a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, USA) using published HBV-specific primers and Taqman-hybridization probes (**table B.3.1**). In order to establish a standard curve, needed for quantification, known references of cloned HBV DNA were amplified in parallel (Loeb et al. 2000; Volz et al. 2007).

DNA was extracted from liver specimens with the MasterPure DNA Purification Kit (Epicentre, USA). RNA was extracted utilizing the RNeasy RNA Mini Kit (Qiagen, Netherlands). Kits were used according to manufacturers` instructions. Additionally, in case of cccDNA analysis, purified DNA was treated with 20 IU of plasmid-safe DNAase1 (Epicentre, USA), to enhance cccDNA fraction. Quantification of intrahepatic HBV DNA was performed via real-time PCR in a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, USA), utilizing HBV specific primers, fluorescence hybridization probes **(table B.3.1)** and the TaqMan™ Fast Advanced Master Mix (Applied Biosystems, USA). Real-time PRC was performed under the following conditions: Initial step at 95°C for 20 seconds; 40 cycles at 95°C for one second and 60 °C for 20 seconds. Known references were amplified in parallel to enable quantification.

To determine intrahepatic levels of HBV RNA, real-time PCR (ViiA™ 7 Real-Time PCR System) using HBV specific primers and fluorescence hybridization probes as well as the TaqMan™ Fast Virus 1-Step Master Mix was performed. One Step real-time PRC was performed under the following conditions: Intrahepatic purified RNA was denatured for ten minutes for 95°C, cooled down to -4°C and subsequently revers transcribed at 50°C for five minutes. Reverse transcriptase was inactivated at 95°C for 20 seconds. Amplification was performed in 40 cycles under following conditions: initial step 95°C for 20 seconds, 40 cycles at 95°C for 3 seconds and 60 °C for 30 seconds. HBV specific primers and probes were used (Malmström et al. 2012). Values were normalized using human GAPDH transcription.

Table B.3.1 ***HBV specific primers and hybridization probes***

Primer/probe	**Sequence**
HBV tot. Fw	CTCGTGGTGGACTTCTCTC
HBV tot. Rv	CAGCAGGATGAAGAGGAA
HBV ccc Fw	CTCCCCGTCTGTGCCTTCT
HBV ccc Rv	GCCCCAAAGCCACCCAAG
HBV pregenomic Fw	GGAGTGTGGATTCGCACTCCT
HBV pregenomic Rv	AGATTGAGATCTTCTGCGAC
HBV tot. FL probe	CACTCACCAACCTCCTGTCCTCCAA
HBV tot. LC probe	TGTCCTGGTTATCGCTGGATGTGTCT
HBV ccc FL probe	GTTCACGGTGGTCTCCATGCAACGT
HBV ccc LC probe	AGGTGAAGCGAAGTGCACACGGACC
HBV pg FL probe	GAGGCAGGTCCCCTAGAAGA
HBV pg LC probe	ACTCCCTCGCCTCGCAGAC

B.3.4 Intrahepatic transcription of innate immunity related genes

RNA was purified from liver specimens as described above. 1µg of total purified RNA was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Switzerland) with oligo-dT primers according to manufacturers` instructions. To determine transcription levels of innate immunity related genes in human liver chimeric uPA/SCID/beige mice or SCID/beige mice, real-time PCR was performed in a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, USA). To distinguish between transcription levels in human hepatocytes that are repopulating the murine liver structure and murine transcription of residual hepatocytes and nonparenchymal liver cells, primers and probes that do not cross-react and specifically recognize human or murine transcripts respectively were used (Taqman Gene Expression Assays, Applied Biosystems, USA) (**table B.3.2** and **table B.3.3**). Real-time PCR was performed under the following conditions: Initial step at 95°C for 20 seconds followed by 40 cycles at 95°C for one second and 60 °C for 20 seconds. The mean of hGAPDH and hRPL30 or mActb and mEef2 transcription was used to normalize gene transcription.

Table B.3.2 ***Taqman Gene Expression Assays – human genes***

Human gene	**Assay ID**
hCXCL10/IP10	Hs00171042_m1
hEIF2AK2	Hs00169345_m1
hGAPDH	Hs99999905_m1
hHLA-E	Hs03045171_m1
hIFNA1	Hs00855471_g1
hIFNAR1	Hs01066116_m1
hIFNB1	Hs00277188_s1
hIL6	Hs00985639_m1
hIL6ST	Hs01006739_m1
hISG15	Hs00192713_m1
hISG20	Hs00158122_m1
hMX1	Hs00895608_m1
hMYD88	Hs00182082_m1
hOAS1	Hs00973637_m1
hRIG1/hDDX58	Hs01061432_m1
hRPL30	Hs00265497_m1
hSOCS3	Hs02330328_s1

hSTAT1	Hs01013989_m1
hTAP1	Hs00388675_m1
hTGFB1	Hs00171257_m1
hTLR2	Hs01014511_m1
hTLR3	Hs00152933_m1
hTNF	Hs99999043_m1

Table B.3.3 ***Taqman Gene Expression Assays – murine genes***

Munriene gene	**Assey ID**
mActb	Mm00607939_s1
mCasp1	Mm00438023_m1
mCxcl10	Mm00445235_m1
mIfnb1	Mm00439552_s1
mIl6	Mm00446190_m1
mMill2	Mm00464004_m1
mMx1	Mm01217998_m1
mTgfb1	Mm00441724_m1
mTlr2	Mm0042346_m1
mTlr3	Mm00442346_m1
mTnfa	Mm00443258_m1
mTrem1	Mm01278455_m1

B.3.5 Immunofluorescence

Immunofluorescence staining of the HBcAg and the human keratin 18 (CK18) was performed using 12µm cryostat sections of human chimeric mouse livers. Sections were fixed with acetone and washed with TN-buffer. Blocking of the endogenous peroxidase was performed using a 0,4% hydrogen peroxide phosphate-buffered saline solution. Liver section were incubated with mouse anti-CK18 (1:400; Dako, Denmark) and rabbit anit-core (1:2000; Dako, Denmark) at 4°C over night. Specific signals were visualized using the Alexa Fluor secondary antibody goat anti-mouse 546- labeled (Invitrogen, USA) and the secondary

antibody AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA). The TSA Fluorescein System (Perkin Elmer, Germany) was used to enhance HBcAg staining. Nuclear staining was achieved by Hoechst 33258 (1:20000, Invitrogen, Germany). Sections were mounted with fluorescent mounting media (Dako, Denmark) and analyzed by the fluorescence microscope BZ9000 (Keyence, Japan) using the same settings for different experimental groups (Allweiss et al. 2014).

B.3.6 Detection of HBeAg in mouse serum

Qualitative detection of HBeAg in mouse serum of infected mice was performed using the Abbott ARCHITECT HBeAg assay (Abbott Ireland Diagnostics, Ireland) according to manufacturer's instructions.

B.3.7 Treatment with Pam3Cys-SKKK

Stably HBV infected as well as uninfected human liver chimeric uPA/SCID/beige mice as well as SCID/beige mice were treated with the TLR1/TLR2 agonist Pam3Cys-SKKKK (EMC microcollections GmbH, Germany). Mice, treated with Pam3Cys, received an intraperitoneal injection of 0.67µg of Pam3Cys per gram mouse body weight. Pam3Cys-SKKKK was reconstituted in endotoxin-free water, stored and further diluted following manufacturers` recommendations.

B.3.8 Statistics

Statistical analysis was performed using the GraphPad Prism 5 software. For group-wise comparisons the nonparametric Mann-Whitney U test was applied. P values < 0.05 were considered statistically significant.

C Results

C.1 Hepatitis B virus wild type and G1896A precore variant infection and innate immune responses

HBV infection fails to induce a strong inflammatory response in patients, chimpanzees or mice (Wieland et al. 2004; Dunn et al. 2009; Giersch et al. 2015). To assess whether the HBeAg/precore protein impacts viral kinetics or the intrinsic innate immune response in human hepatocytes in chronic HBV infection, mice displaying adequate levels of human liver chimerism were inoculated with cell culture derived genotype D wild type hepatitis B virus (n=8) or its HBV precore variant containing the G1896A precore mutation (n=6). PHHs were derived from a single donor. Blood samples were drawn at multiple time points to monitor development of viremia over the course of 12 weeks. Stable chronically infected mice were sacrificed after 12 weeks and liver specimens were obtained in order to examine intrahepatic gene expression related to innate immunity as well as intrahepatic viral parameters.

C.1.1 Viremia and HBeAg

In order to investigate differences in viral infection kinetics between HBV wild type and the HBV precore variant infection, human liver chimeric uPA/SCID/beige mice were inoculated with $1x10^7$ HBV DNA wt genome equivalents or $1x10^7$ HBV DNA precore variant genome equivalents respectively. HBV DNA genome equivalents/ml serum in HBV wild type and in HBV precore variant infected mice were determined after 3, 6, 9 and 12 weeks of infection. The overall development of viral loads over the course of 12 weeks was comparable with previous studies (Volz et al. 2013) and displayed no significant difference between mice infected with HBV wild type or the HBV precore variant (**figure C1.1**). HBV wild type infected mice showed median viral titers of $1.2x10^5$ HBV DNA genome equivalents/ml at three weeks, $3.8x10^6$ HBV DNA genome equivalents/ml at six weeks, $3.3x10^7$ HBV DNA genome equivalents/ml at nine and $2.8x10^8$ HBV DNA genome equivalents/ml at 12 weeks of infection (solid bar). HBV precore variant infected mice showed median viral titers of $4.4x10^4$ HBV DNA genome

equivalents/ml at three weeks, $1.9x10^7$ copies DNA/ml at six weeks, $2.2x10^8$ HBV DNA genome equivalents/ml at nine and $3.1x10^8$ copies DNA/ml at 12 weeks of infection (non solid bar). HBeAg quantification was performed to ensure absence of HBeAg in HBV precore variant infected mice. As expected HBV precore infected mice showed no evidence of HBeAg in the serum (**figure C.1.2**).

Mice that were inoculated with infectious serum derived from either HBV wt or HBV G1896A infected mice also displayed similar development of viral loads over the course of 12 weeks of infection regardless of HBeAg status. These data show that both cell cultured and passaged virions infected mice with similar efficacy and led to the development of similar spreading kinetics.

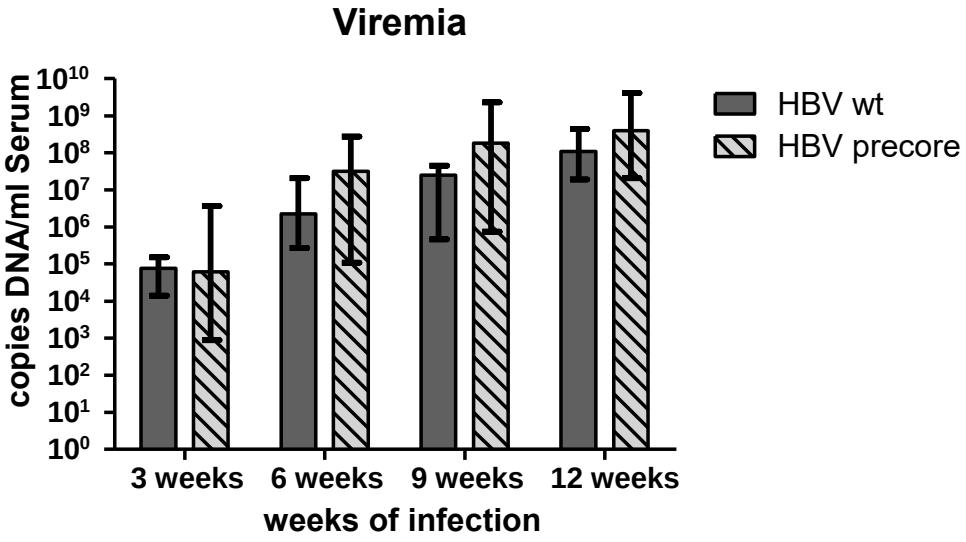

Figure C.1.2 ***Development of viremia over the course of infection***

Course of viremia over the course of 12 weeks of infection with HBV wild type or HBV precore variant. Copies of HBV DNA/ml serum did only display negligible differences between HBeAg positive (n=8) and HBeAg negative (n=6) mice. Columns represent median with range.

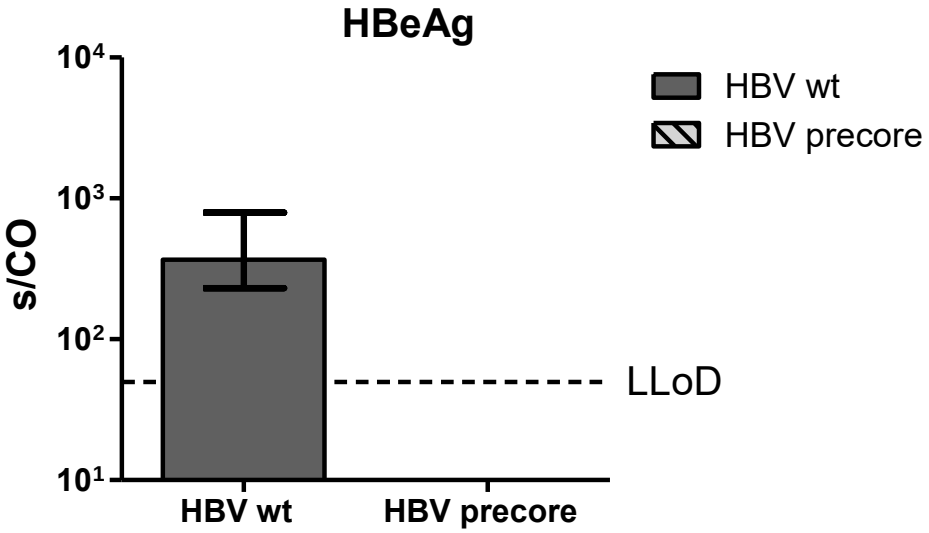

Figure C.1.1 ***HBeAg levels in infected mice***

HBeAg levels in serum remained undetected in HBV precore infected mice, ensuring the absence of HBeAg in mice infected with the G1896A precore variant. HBeAg in precore variant infected mice remained undetectable in all cases. Columns represent median with range.

C.1.2 Intrahepatic viral parameters

To compare intrahepatic viral activity and viral spreading of the HBV wt and the HBV precore variant, mice were sacrificed and liver specimens were obtain after 12 weeks of infection. RNA and DNA was extracted to determine intrahepatic viral parameters. Intrahepatic HBV DNA measurements are relative to the amount of human β-globin DNA copies. This enables estimation of the amount of viral DNA per human hepatocyte. Viral RNA measurements were normalized utilizing the RNA transcription of the housekeeper genes GAPDH and RPL30.

The total amount of HBV DNA, which includes HBV rcDNA as well as cccDNA, per human hepatocyte did not differ between HBV wild type (median $1.7x10^2$) and HBV precore variant (median $3.08x10^2$) infected mice, indicating that intrahepatic viral spreading is independent of HBeAg/precore protein presence (**figure C.1.3**). Expression of pregenomic RNA, which is an essential intermediate of viral replication, also was comparable among HBV wt (median 1.44) and HBV precore variant (median 1.83) infected mice (**figure C.1.4**). This indicates that on a quantitative scale, HBV production per human hepatocyte does not seem to be influenced by the HBeAg/precore protein.

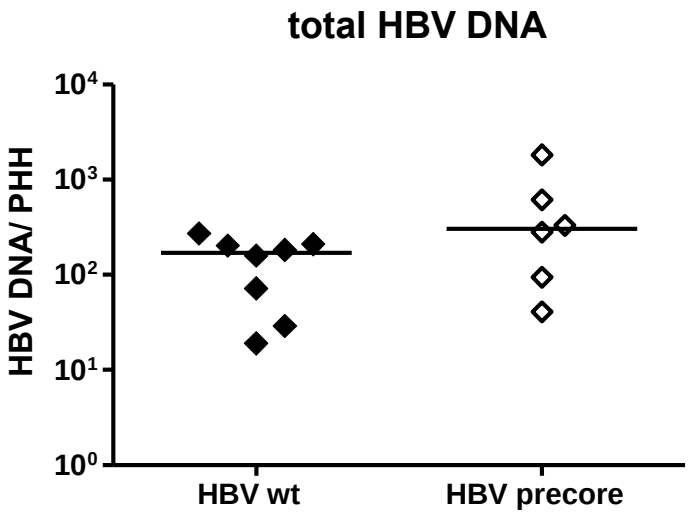

Figure C.1.3 ***Intrahepatic HBV DNA***

Total intrahepatic amount of HBV DNA relative to human β-Globin and thereby per human hepatocyte in infected mice appeared to be comparable in mice infected with the HBV wild type and the HBV precore variant (p=0.1079). Every dot represents a single mouse. Lines depict the corresponding median.

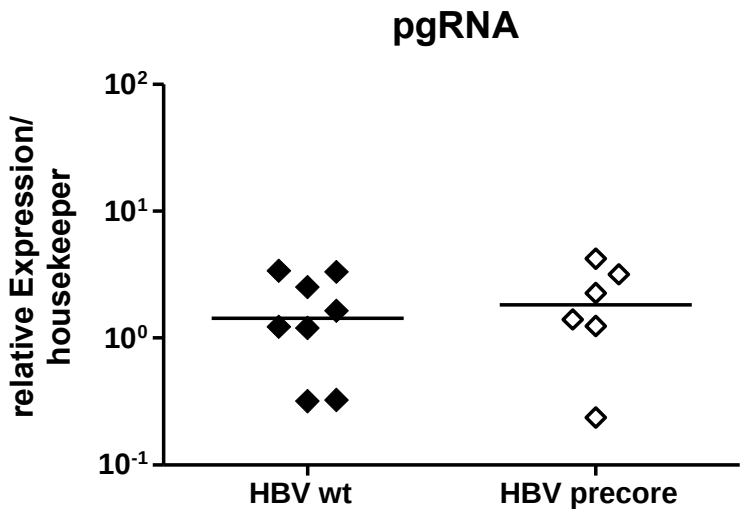

Figure C.1.4 ***pgRNA in HBV wt and HBV precore variant infected mice***

Intrahepatic amount of HBV pregenomic RNA transcription relative to human housekeeper genes. No difference between HBV wild type and HBV precore variant infected mice was detected (p=0.7546). Every dot represents a single mouse. Lines depict the corresponding median.

The HBV cccDNA is a key factor in the life cycle of HBV. Establishment of at least one copy of cccDNA within a target cell is quintessential for the replication cycle of HBV and marks the successful establishment of an infection. Studies have shown that formation of cccDNA in human hepatocytes in the initial phase of HBV infection is only accomplished in a minority of the targeted cellls and increases over the course of infection (Volz et al. 2013).

After 12 weeks of infection, intrahepatic copies of cccDNA/PHH in samples that had undergone plasmid safe ATP-dependent DNAse digestion (PSD) as well as samples that were not PSD treated were analyzed. In samples that did not undergo PSD digestion cccDNA/PHH was measured at a median of 3.13 copies of cccDNA per human hepatocyte in mice infected with the HBV wt and at 3.75 copies of cccDNA per human hepatocyte in HBV precore infected mice (data not shown). In samples that were PSD treated HBV wild type infected mice displayed median copies of 0.152 cccDNA per cell and HBV precore variant infected mice displayed 0.256 copies per human hepatocyte (**figure C.1.5**). Intrahepatic amounts of cccDNA per human hepatocyte in purified DNA that had undergone

PSD digestion appeared to be lower overall but no significant difference between HBV wt and HBV precore variant infection was noted. This indicates that formation and intrahepatic amplification of the cccDNA minichromosome did not differ substantially between mice infected with the HBV wild type or the HBV precore variant.

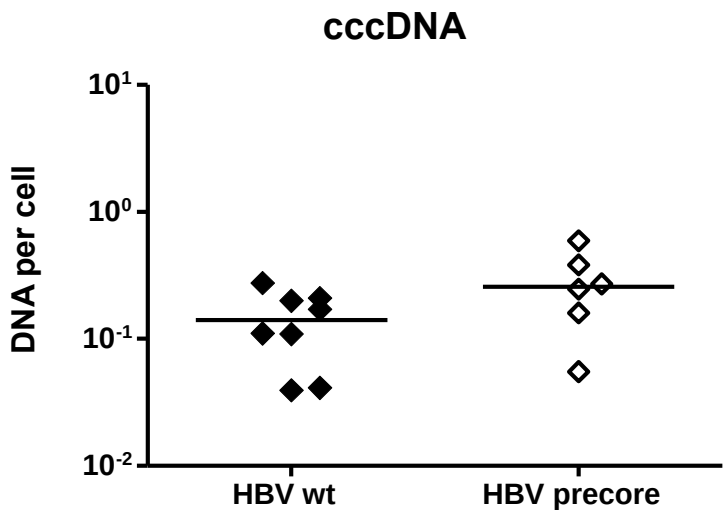

Figure C.1.5 ***cccDNA per human hepatocyte*** (after PSD digestion)

Intrahepatic amount of HBV cccDNA per human hepatocyte did not significantly differ between HBV wild type and HBV precore variant infection (p=0.1419). Every dot represents a single mouse. Lines depict the corresponding median.

As shown in **figure C.1.4**, relative expression of pgRNA did not vary significantly between HBV wt infeceted mice and HBV precore variant infected mice. The pgRNA serves as bi-cistronic mRNA for the expression of the core and P protein as well as being packaged and converted into rcDNA later on. The amount of pgRNA transcribed per cccDNA minichromosome therefore could be a valuable indicator of infectious viral activity and viral protein bio synthesizes in infected liver tissue.

Interestingly, the amount of pgRNA that is transcribed per cccDNA minichromosome/PHH appeared to be significantly lower in human liver tissue infected with the HBV precore variant (median = 10.73) compared to pgRNA per cccDNA minichromosome/PHH in HVB wt (median = 6.41) infected liver specimen (p=0.0127) (**figure C.1.6**). Transcription of pgRNA is essential for viral replication and a determining factor for the HBV replication rate. The amount of pgRNA transcribed from each individual copy of the cccDNA minichromosome represents one key element that plays into the overall efficiency of intrahepatic HBV replication since the pgRNA molecule is the precursor of the rcDNA molecule within the newly formed viral particles.

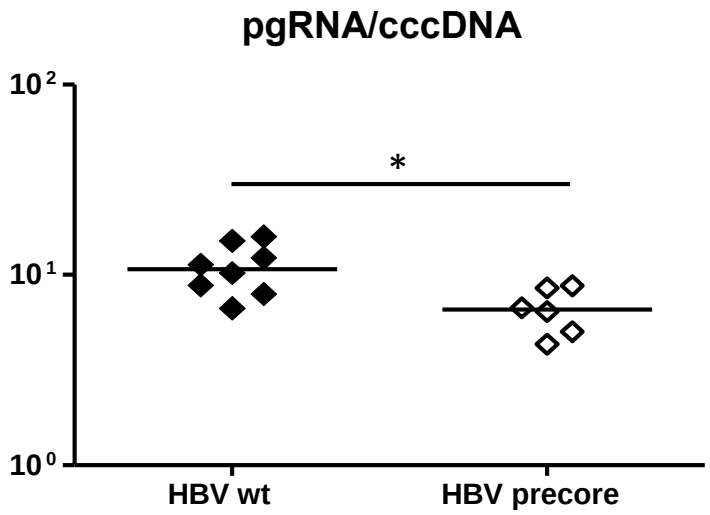

Figure C.1.6 ***pgRNA per cccDNA/PHH***

Intrahepatic pregenomic RNA transcription per cccDNA/human hepatocyte (with applied PSD digestion) appeared to be significantly lower in mice infected with the HBV precore variant compared to mice infected with HBV wt (p=0.0127). (Every dot represents a single mouse. Lines depict the corresponding median).

The rcDNA is a reference point to estimate the amount of newly formed HBV genomes. Therefore the amount of rcDNA/cccDNA **(figure C.1.7)** may represent the rate of viral activity or efficiency of viral replication from the cccDNA minichromosome template. Our data showed that rcDNA/cccDNA was not significantly influenced by the G1896A mutation but displayed a higher variance in HBV precore variant infected mice compared to HBV wt (p=0.2824).

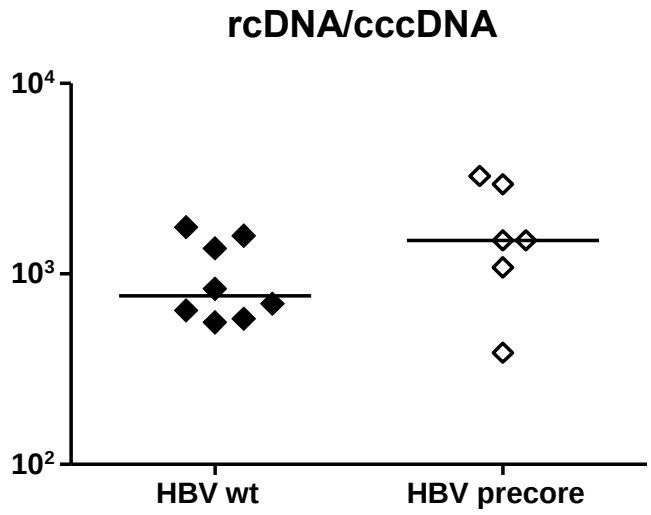

Figure C.1.7 ***rcDNA per cccDNA***

Intrahepatic amount of HBV rcDNA/cccDNA (after PSD digestion) was not significantly increased in HBV G1896A infected mice but displayed a higher variance compared to HBV wt (p=0.2824). Every dot represents a single mouse. Lines depict the corresponding median.

These differences may reflect a more efficient packaging of pgRNA which is then reverse transcribed. Thus, in line with structural differences within the epsilon region of pgRNA determined in the HBV G1896A variant, slightly but significantly lowering intrahepatic pgRNA levels. Even though, our rcDNA/cccDNA data do not show significant higher levels of rcDNA/cccDNA in mice infected with the HBV G1896A variant it might be possible that more efficient packaging and transcription of pgRNA leads to reduced levels of pgRNA/cccDNA/PHH in HBV G1896A infection.

C.1.3 Intracellular distribution of core antigen in HBV infected human hepatocytes

Immunofluorescence staining of the HBcAg and the human keratin 18 (CK18) was performed in liver tissue of mice infected with HBV wt or HBV precore variant after 12 week of infection to evaluate the impact of the HBeAg/precore protein on core antigen distribution within infected primary human hepatocytes. CK18 staining (red) is utilized to distinguish human hepatocytes within the murine human chimeric liver of uPA/SCID/beige mice. As shown in **figure C.1.8** CK18 staining visualizes and ensures a sufficient repopulation with PHHs of the uPA/SCID/beige mouse liver. Exemplary **figure C.1.8 A** is showing CK18 staining of humanized areas within a liver section in red whereas murine areas remain dark (example marked with star). As expected, HBV infection exclusively takes hold in human hepatocytes as shown in **figure C.1.8 B**. Areas repopulated with human hepatocytes display the presence of the core antigen (green) and adjacent predominantly murine areas display core antigen only in isolated PHHs (nuclear staining shown in blue).

HBcAg distribution in HBV wt and HBV precore variant infected mice after 12 weeks of infection was not observed to be altered by the lack or presence of the HBeAg/precore protein when comparing HBcAg staining in infected liver sections. Overall core antigen distribution was in accordance with previous studies (Allweiss et al. 2014). As shown exemplary in **figure C.1.8 C-D** (HBV precore variant) and **figure C.1.8 E-F** (HBV wt), the core antigen is present in infected human hepatocytes and appears to accumulate at the cell nucleus. The HBeAg or the precore mutation G1896A does not to have effect on core antigen distribution within HBV infected human hepatocytes.

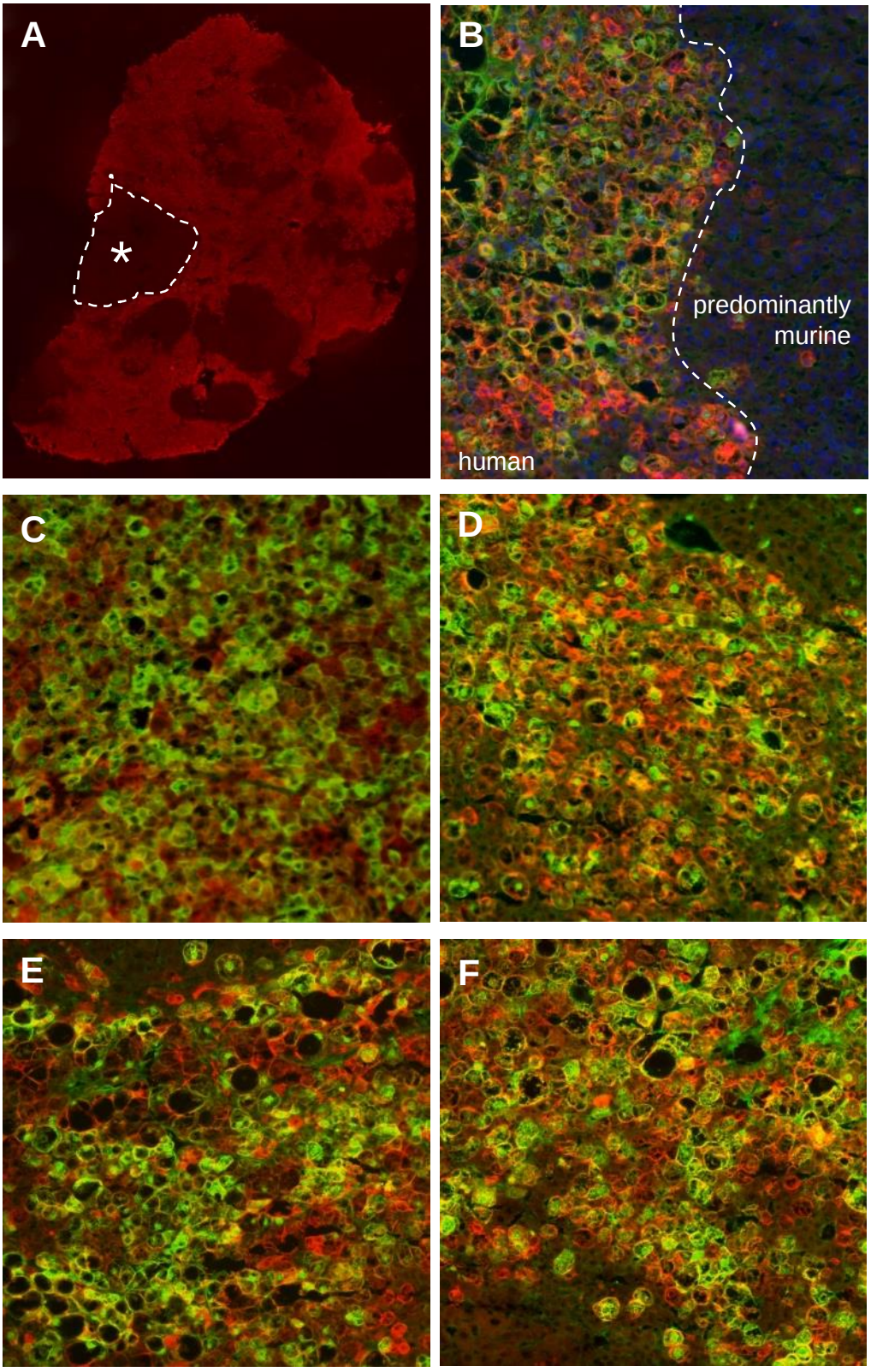

Figure C.1.8 ***Immunofluorescence staining of CK18 and HBV core antigen***

A: CK18 staining (red) showing repopulated and remnant murine (*) areas within a uPA/SCID/beige mouse liver. **B:** CK18 (red) HBcAg (green) staining within a repopulated area adjacent to predominantly remaining murine liver (nucleoli shown blue). **C-D:** CK18 (red) and HBcAg (green) in HBV precore variant infected mice. **E-F:** CK18 (red) and HBcAg (green) in HBV wt infected mice.

C.1.4 Innate immunity gene transcription in human liver chimeric uPA/SCID/beige mice

As mentioned and shown in previous studies, the induction of expression of innate immunity related genes in livers of HBV mono infected human liver chimeric uPA/SCID/beige mice is weak. In contrast to HBV mono infection, it has been demonstrated that induction of innate immunity genes appears stronger in HBV/HDV co-infected mice (Giersch et al. 2015). To investigate whether the HBeAg/precore protein influences the transcription levels of innate immunity related genes, mice where infected with either HBV genotype D wt (n=8) or its corresponding G1896A precore variant (n=7). Mice were sacrificed after 12 weeks of infection and liver specimens were collected in order to assess transcription of innate immunity genes. Gene transcription levels are shown relative to expression levels of housekeeping genes (mean of hGAPDH and hRPL30 or mEef2 and mActb) and were determined via real-time PCR utilizing human-specific or murine-specific primers and probes to differentiate between murine and human gene expression in liver tissue.

In order to differentiate between the innate immune response towards HBV within the infected human hepatocytes and the surrounding murine liver tissue consisting of remnant murine hepatocytes as well as non-parenchymal cells (e.g. Kupffer cells), human-specific primers not cross-reacting with murine sequences were utilized to determine induction of innate immunity related genes exclusively in human hepatocytes. While murine-specific primers were used to determine induction of expression of innate immunity related genes in surrounding murine liver tissue that may still be affected by HBV production in human hepatocytes.

C.1.4.1 Transcription of genes involved in PAMP recognition

Toll-like receptors (TLRs) are expressed in innate immune cells like macrophages or dendritic cells as well as non-immune cells. In case of a viral infection these receptors are able to recognize PAMPs such are di- and triacyl lipoproteins (TLR2) or dsRNAs (TLR3) (Newton and Dixit 2012; Lang et al. 2011).

To evaluate the effect of the HBeAg/precore protein upon expression of genes involved in PAMP recognition, transcription of human TLR2, TLR3, MYD88 and retinoic acid-inducible gene 1 (RIG1) as well as murine mTlr2 and mTlr3 were quantified. TLR2 and TLR3 are major PAMP-recognition receptors within the innate immune response (Aderem and Ulevitch 2000). MYD88 functions as an adaptor protein in the TLR and the interleukin-1 signaling pathway (Akira et al. 2006b) and RIG1 acts as a sensor for viral RNA (Sayed et al. 2017).

Transcription of human TLR2 (**figure C.1.9)** was not significantly increased as a result of HBV infection but appeared to be slightly elevated in HBW wt infection ($p=0.0946$) as well as in HBV precore variant infection ($p=0.2409$) in comparison to uninfected mice. There was no significant difference between HBV wt and HBV precore variant infection ($p=0.3969$). Similarly, human TLR3 transcription (**figure C.1.9)** was not significantly increased in HBV infected mice but appeared slightly increased in HBW wt infection ($p=0.0603$) as well as in HBV precore variant infection ($p=0.0641$). No difference between HBV wt and HBV precore variant infection ($p=0.7789$) was shown.

Relative transcription of human MYD88 and human RIG1 appear significantly enhanced as a result of HBV infection (**figure C.1.10**).

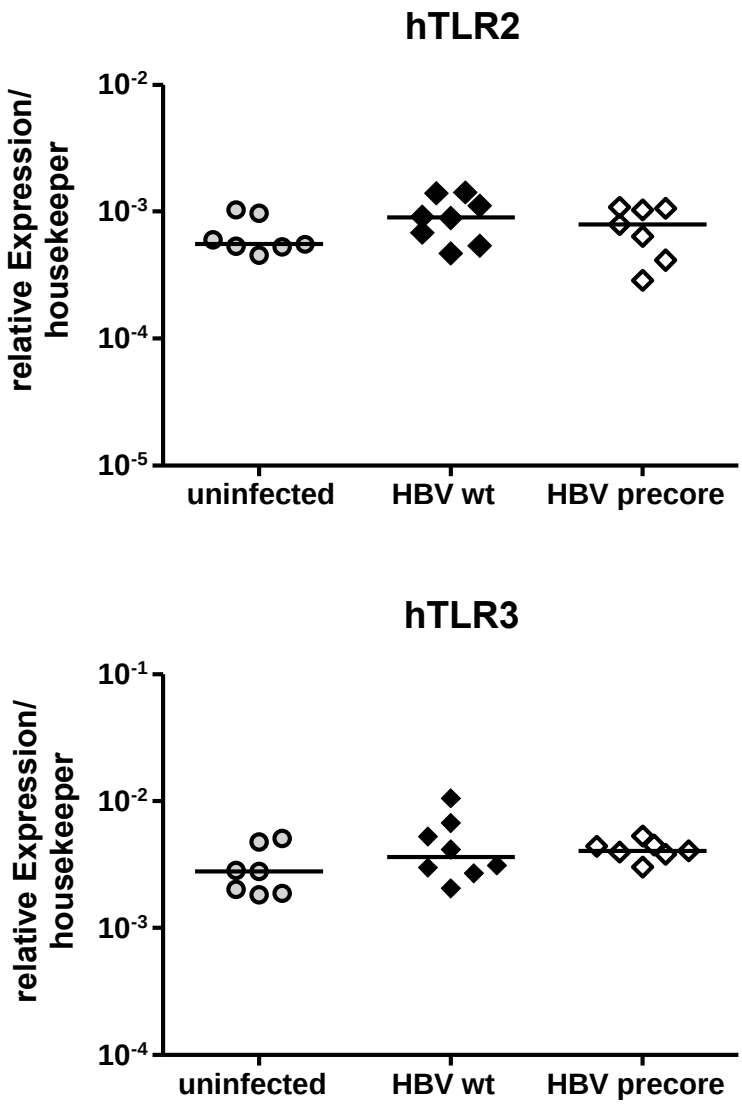

Figure C.1.9 ***Transcription of TLR2 and TLR3***

Transcription of human TLR2 and human TLR3 appeared to be slightly elevated in infected mice but did not display differences between mice infected with HBV wild type and HBV precore variant infected mice. TLR2 transcription was not significantly increased as a result of HBV wt infection (p=0.0946) or HBV precore infection (p=0.2409).Transcription of TLR3 was not significantly elevated HBV wt infection (p=0.0603) or HBV precore infection (p=0.0641). Every dot represents a single mouse. Lines depict the corresponding median.

While human MYD88 transcription (**figure C.1.10**) was significantly increased in HBV wt infected mice (p= 0.0016) as well as in HBV precore infected mice (p=0.0107) when compared to the uninfected mice. However, the lack of precore protein expression did not lead to further enhancement of MYD88 transcription (p=0. 5358). In contrast, the enhancement appeared slightly less pronounced in HBV precore variant infected mice (*) than in mice infected with HBV wt (**). Relative transcription of human RIG1 (**figure C.1.10**) was significantly induced in HBV wt (p= 0.0047) and HBV precore variant infection (p= 0.0087) but our data show no significant difference between HBV wt and HBV precore variant infection (p=1.0).

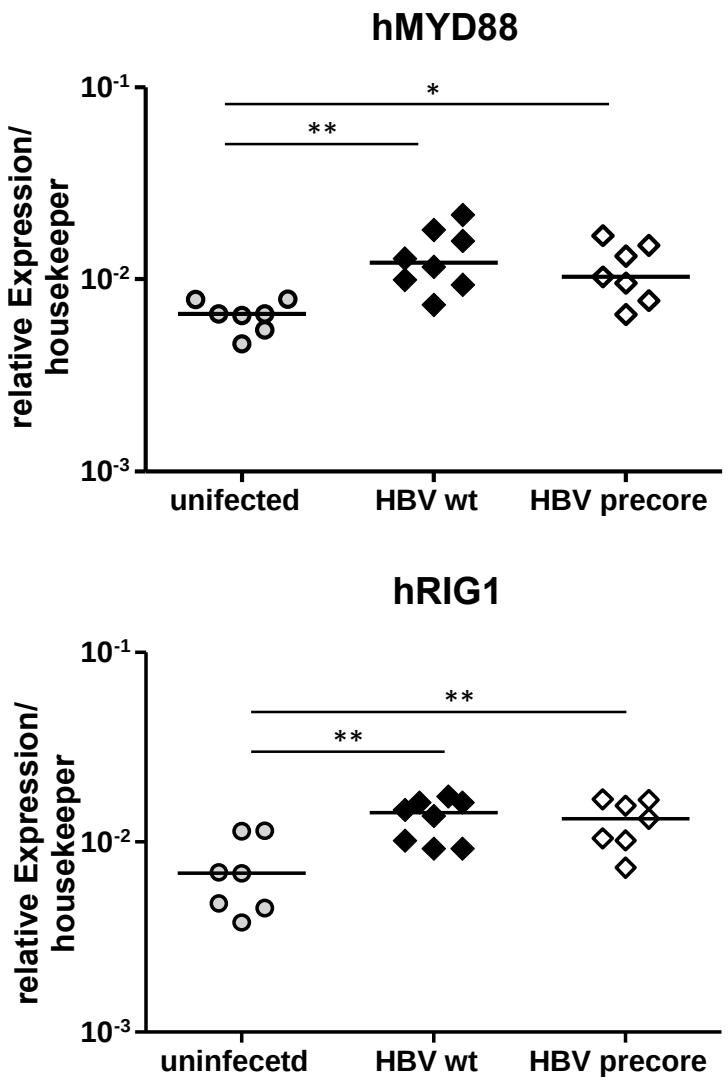

Figure C.1.10 ***Transcription of human MYD88 and RIG1***

Transcription of human MYD88 (HBV wt p=0.0016 and HBV precore p=0.0107) and human RIG1 (HBV wt p=0.0047 and HBV precore p=0.0087) was significantly induced in infected mice, but did not display differences between mice infected with HBV wild type or the HBV precore variant. Every dot represents a single mouse. Lines depict the corresponding median.

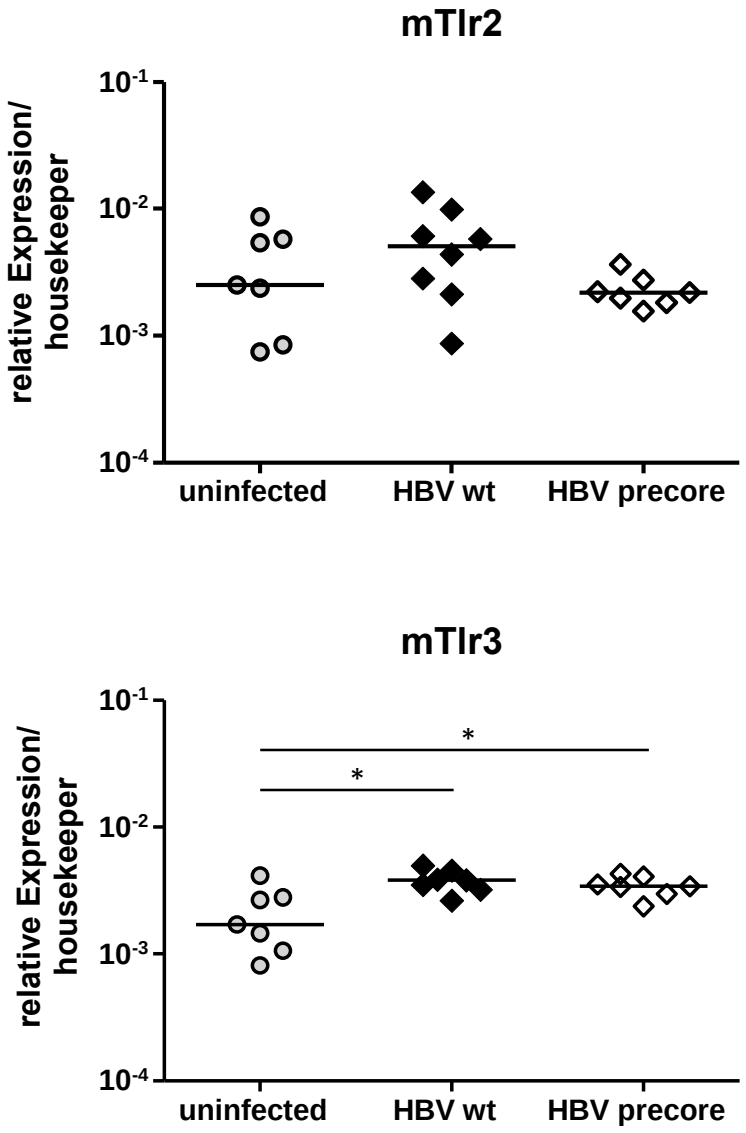

Figure C.1.11 ***Transcription of murine Tlr2 and Tlr3***

Relative murine Tlr2 transcription was not significantly induced in HBV wt (p=0.1405) or HBV precore variant infection (p=0.2279). Transcription of murine Tlr3 was significantly induced as a result of infection with HBV wt (p=0,0131) and HBV precore variant (p=0,0189) regardless of HBeAg status (p=0.4557). The HBeAg status did not have a significant impact. Every dot represents a single mouse. Lines depict the corresponding median.

Although median transcription levels of murine Tlr2 (**figure C.1.11**) appears to be elevated in comparison with uninfected mice, higher levels of variance were observed in uninfected and HBV wt infected mice. No significant induction or impact of the HBeAg/precore protein was shown. Murine Tlr3 transcription (**figure C.1.11**) was significantly increased in HBV infected mice (p=0.0131) and HBV precore variant infected mice (p=0.0189). The HBeAg status did not lead to difference in relative transcription of mTlr3.

C.1.4.2 Transcription of interferon stimulated genes

As part of the innate immune response towards pathogens, induction of expression of a number of genes is interferon driven. Expression of these interferon stimulated genes (ISGs) reflect part of the inflammatory response in viral infection (Katze et al. 2002). As described, HBV infection is not met by a strong IFN response. ISG induction was shown to be weak in HBV wt mono infected human liver chimeric uPA/SCID/beige mice (Wieland et al. 2004; Giersch et al. 2015). To assess the impact of the HBeAg/precore protein with respect to the expression of ISGs and other innate immunity related genes in human liver chimeric uPA/SCID/beige mice, gene transcription was compared between an uninfected control group, HBV wt infected group and a HBV precore variant infected group. HBeAg expression is abolished in the HBV precore variant infected group. Therefore, an upregulation of gene transcription in HBV precore variant infected mice in comparison to HBV wt infected mice, would indicate an immunomodulation function of the HBeAg/precore protein with regard to ISG expression. Relative gene transcription is shown in **figure C.1.12**, **figure C.1.13**, **figure C.1.14**, **figure C.1.15** and **figure C.1.16**.

MX dynamin like GTPase 1 (MX1) functions as a guanosine triphosphate-metabolizing protein, which is part of the cellular antiviral response. It antagonizes the replication process of several RNA but also DNA viruses. It is induced by type 1 and type 2 interferons (Shi et al. 2017). OAS1 is induced by interferons and promotes the activation of the latent RNase L, which results in viral RNA degradation (Liu et al. 2017).

Transcription levels of human MX1 (**figure C.1.12**) were not induced in HBV infected mice (HBV wt p=0.1405; HBV precore variant p=0.3552). Transcription of human OSA1 (**figure C.1.12**) was significantly induced in HBV wt infected mice (p=0.0030) and also appeared to be elevated in HBV precore variant infected mice but displayed a higher variance (p=0.1043). In both cases our data did not show a significant difference in transcription levels as a result of the HBeAg status.

ISG15 encodes the ubiquitin-like protein ISG15 and is activated by interferon α and interferon β. It is part of the antiviral innate immune response and is involved in cell signaling, either conjugation to its target or functioning as a free unconjugated protein (Sooryanarain et al. 2017). USP18 encodes a ubiquitin-specific peptidase that specifically cleaves ISG15, regulating an interferon stimulated inflammatory response (Puente et al. 2003). Transcription of human ISG15 (**figure C.1.13**) was not significantly induced in HBV infection (HBV wt p=0.1159; HBV precore variant p=0.1914). The HBeAg status did not have a significant impact (p=0.6943). HBV infection did not induce transcription of USP18 (**figure C.1.13**) significantly (HBV wt p=0.1984; HBV precore variant p=0.4024). The lack of HBeAg/precore protein did not impact transcription of USP18 significantly (p=0.2319).

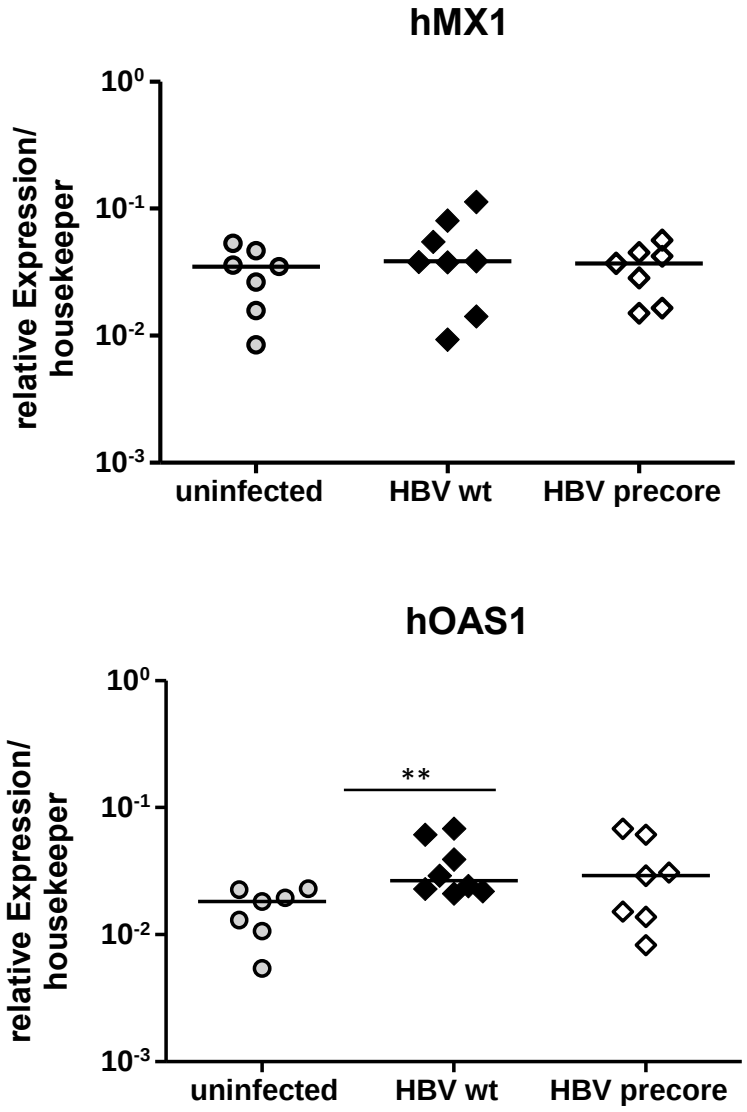

Figure C.1.12 ***Transcription human MX1 and OAS1***

Intrahepatic transcription of human MX1 was not induced as a result of HBV infection. Transcription of human OAS1h was significantly induced in HBV wt infected mice (p=0.0030) and appeared to be increased in HBV precore variant infection, nevertheless it displayed a higher variance (p=0.1043) compared to HBV wt infection. The lack of the HBeAg/precore protein did not have a significant impact in both cases. Every dot represents a single mouse. Lines depict the corresponding median.

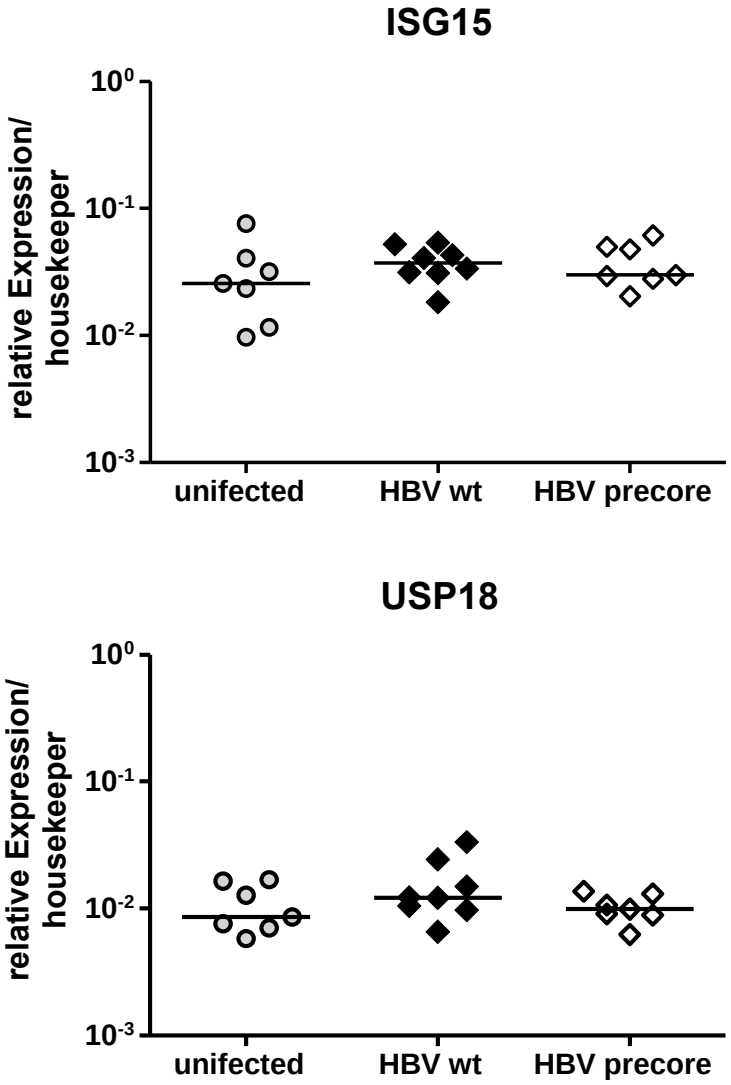

Figure C.1.13 ***Transcription of human ISG15 and USP18***

Intrahepatic transcription of human ISG15 as well as human USP18 did not appear to be induced in HBV infected mice and the HBeAg status did not influence transcription levels significantly. Every dot represents a single mouse. Lines depict the corresponding median.

Interferon stimulated exonuclease gene 20 (ISG20) is an interferon induced antiviral exoribonuclease, that primarily targets single stranded RNA and displays antiviral activity towards e.g. HCV and HBV (Leong et al. 2016). The interferon α and β receptor subunit 1, encoded by IFNAR1, serves as a type 1 membrane protein and forms one of the two chains of the interferon α/β receptor (Lutfalla et al. 1992). Signal transducer and activator of transcription 1 (STAT1) mediates the cellular signaling and response to interferons and other cytokines as well as growth factors via the JAK/STAT kinase signaling pathway (Liu et al. 1998). SOCS3 encodes the suppressor of cytokine signaling 3, which is part of a negative feedback system, regulating interferon and cytokines signaling through the JAK/STAT pathway (Gao et al. 2018).

Human ISG20 transcription (**figure C.1.14**) was not induced in HBV infection. IFNAR1 transcription (**figure C.1.14**) appeared to be elevated in HBV infected mice but was not significantly increased (HBV wt p=0.1159; HBV precore variant p=0.1043). ISG20 transcription as well as IFNAR1 levels remained unaffected by the HBeAg status.

Transcription of human STAT1 (**figure C.1.15**) was significantly induced in mice infected with the HBV precore variant infection (p=0.0364). STAT1 transcription appeared elevated in HBV wt infection (p=0.0760) but displayed a higher variance compared to uninfected and HBV precore infected mice. The lack of HBeAg/precore protein did not further enhance STAT1 transcription (p=0.8665). Transcription of SOCS3 (**figure C.1.15**) appeared induced as a result of HBV infection but was not significantly increased in HBV wt (p=0.0946) or HBV precore variant (p=0.01474). The HBeAg status had no effect (p=0.8518).

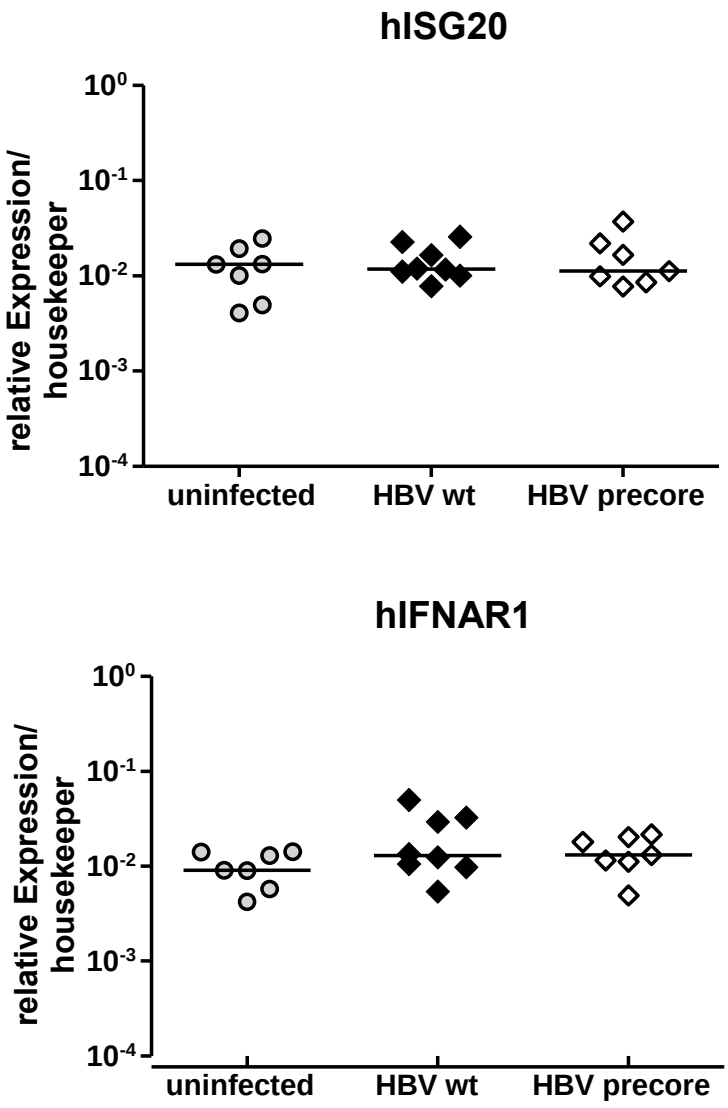

Figure C.1.14 ***Transcription of human ISG20 and IFNAR1***

Intrahepatic transcription of human ISG20 was not increased in HBV infection. Transcription of human IFNAR1 appears to be elevated HBV infected mice compared to uninfected mice but was not increased significantly (HBV wt p=0.1159; HBV precore variant p=0.1043). Transcription levels remained unaffected by the HbeAg status. Every dot represents a single mouse. Lines depict the corresponding median.

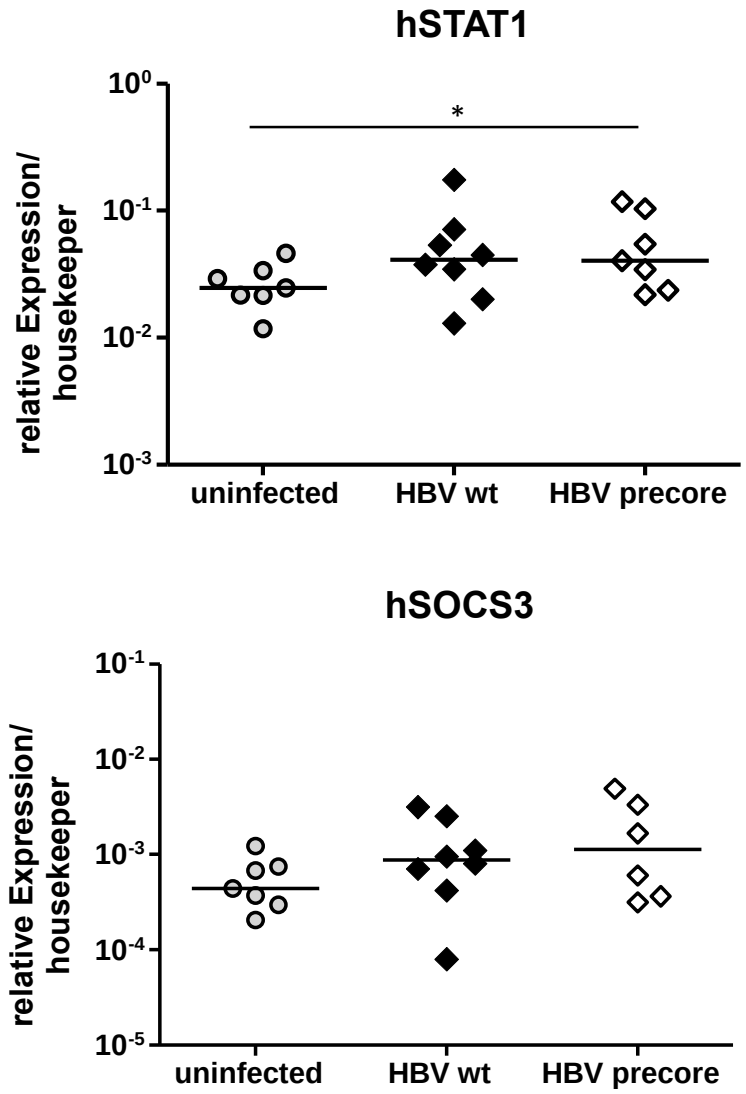

Figure C.1.15 ***Transcription of human STAT1 and SOCS3***

Transcription of human STAT1 was significantly induced in HBV precore variant infection (p=0.0364) and appeared elevated in HBV wt infection although it displayed a higher variance (p=0.0760). The lack of HBeAg/precore protein did not further enhance STAT1 transcription (p=0.8665). Transcription of human SOCS3 appeared increased as a result of HBV infection but was not significant in HBV wt (p=0.0946) or HBV precore variant (p=0.01474). HBeAg status had no effect (p=0.8518). Every dot represents a single mouse. Lines depict the corresponding median.

Inflammatory caspases 1 is protease that cleaves precursor proteins of a number of cytokines involved in inflammatory response processes (e.g. interleukin-1 and interleukin-18). It has also been shown to cleave proteins involved in innate immune signaling like Mal and TRIF. Its activity is mediated by the inflammasome complex which, after assembly, binds to the binds to pro-caspase-1 (Wang, Y. et al. 2017).

Murine transcription of mMx1 **(figure C.1.16)** was significantly increased in HBV wt infection (p=0.0145) as well as HBV precore variant infection (p=0.087) compared to uninfected mice. Transcription of mCasp1 (**figure C.1.16**) was also significantly induced both in HBV wt infection (p=0.0185) and HBV precore variant infection (p=0.087). In both cases HBeAg status did not have a significant impact on transcription levels.

The ISG induction, as a response towards HBV infection was overall observed to be weak. These findings are in line with previous studies. Interestingly only weak or even no elevated levels of ISG transcription were found regardless of the lack of the HBeAg/precore protein in HBV G1896A precore variant infected mice when compared to HBV wild type infected mice. This suggests, that the effect of the HBeAg/precore protein with regard to ISG suppression might be negligible in the context of HBV infection in human liver chimeric uPA/SCID/beige mice.

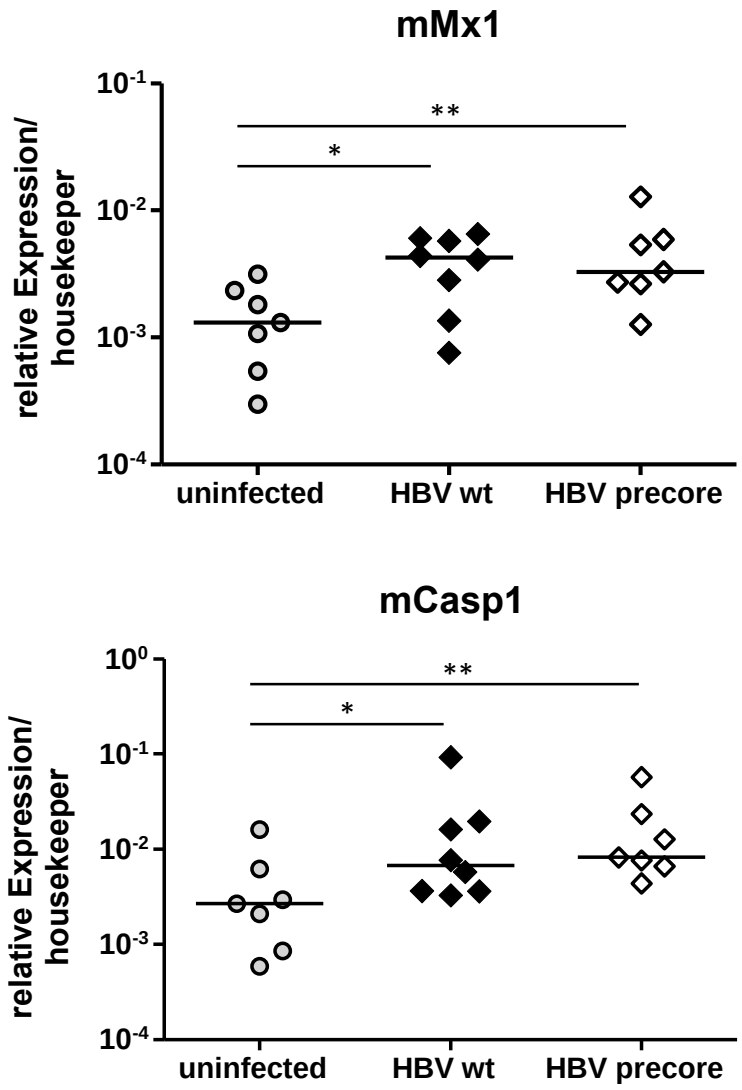

Figure C.1.16 ***Transcription of murine mMx1 and mCasp1***

Relative transcription of murine Mx1 (HBV wt p=0.0145 and HBV precore p=0,0087) and murine Casp1 (HBV wt p=0,0185 and HBV precore p=0.0087) was significantly increased in mice infected with HBV compared to uninfected controls but did not differ between the HBV wt infection and the HBV precore variant infection (mMx1 p=0.9551; mCasp1 p=0.4634). Every dot represents a single mouse. Lines depict the corresponding median.

C.1.4.3 Transcription of cytokines and genes related to antigen presentation

In addition to gene expression related to PAMP recognition and subsequent signaling cascades, expression of other cytokines as well as genes related to antigen presentation and cell signaling is essential in the antiviral inflammatory response. Relative gene expression levels of cytokines as well as genes involved in antigen presentation are shown in **figure C.1.17**, **figure C.1.18**, **figure C.1.19**, **figure C.1.20** and **figure C.1.21**.

The human leukocyte antigen (HLA) is part of the major histocompatibility complex, an antigen-presenting complex that is able to display pathogen derived peptides on antigen-presenting cells for recognition as part of the immune response towards pathogen infected cells, e.g. CD8-positive T cells via the T-cell receptor. The HLA Class I Histocompatibility Antigen, Alpha Chain E (HLA-E) is a heterodimer consisting of a light chain and a heavy chain that is anchored in the cell membrane. Its function relates to cell recognition of antigen presentation by natural killer cells (NK cells) (Araújo et al. 2018).

Human HLA-E transcription was not induced in HBV wild type infected mice or in HBV precore variant infected mice (**figure C.1.17**).

The antigen peptide transporter 1 (TAP1) takes part in the transport of antigens from the cytoplasm to the endoplasmic reticulum for association with MHC class 1 molecules (Gaudet and Wiley 2001). Transcription of human TAP1 (**figure C.1.17**) was significantly induced by HBV wt infection ($p=0.003$) as well as HBV precore variant infection $p=0.0006$). Nevertheless the HBeAg status did not significantly alter TAP1 transcription in infected mice ($p=0.2810$).

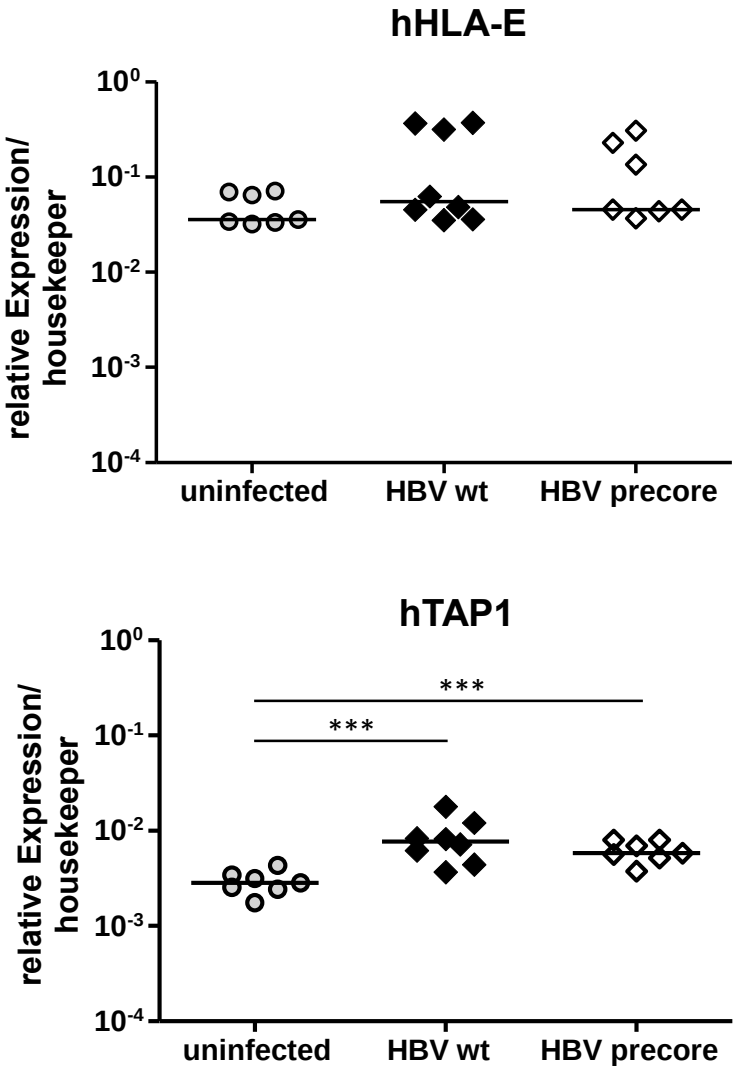

Figure C.1.17 ***Expression of human HLA-E and TAP1***

Transcription of human HLA-E was not impacted by HBV infection or HBeAg status. Transcription of TAP1 was significantly induced as a result of HBV infection both in the case of HBV wt (p=0.0003) as well as HBV precore variant (p=0.0006). TAP1 transcription was not significantly impacted by the HBeAg status (p=0.2810). Every dot represents a single mouse. Lines depict the corresponding median.

The Interleukin-6 receptor subunit beta, encoded by IL6ST, functions as a signal-transducing molecule for, among others, interleukin 6. Binding leads to homodimerization followed by the activation of the Janus kinases (Hibi et al. 1990). The TGFβ gene encodes the multifunctional transforming growth factor beta 1 protein, that regulates or is involved in a multitude of immune function as well as differentiation and growth of various cell types (Gleizes et al. 1996).

Human TGFβ transcription **(figure C.1.18)** was significantly induced in mice infected with the HBV wt (p=0.0103) as well as the HBV precore variant (p=0.0131). No significant impact of the HBeAg/precore protein was shown (p=0.1893). IL6ST transcription (**figure C.1.18**) also appeared to be significantly induced in HBV wt (p=0.0070) and HBV precore variant infected mice (p=0.0189). Again no significant impact of the HBeAg/precore protein was shown (p=0.6022).

C-X-C motif chemokine 10 (CXCL10) is a pro inflammatory chemokine, that is also involved in differentiation, as well as activation of peripheral immune cells (Romagnani et al. 2001).

Relative expression of human CXCL10 (**figure C.1.19**) was also significantly induced in HBV infected mice, when compared to the uninfected control group (HBV wt p=0.0006; HBV precore variant p=0.0087). But CXCL10 expression was not altered by the HBeAg status in infected mice (p=0.1520). Notably there was an overall higher variance in uninfected mice.

Analogous to human transcription, median murine transcription levels of mCxcl10 (**figure C.1.20**) appeared to be elevated in infected mice, but did display an overall higher variance (HBV wt p=0.0603; HBV precore variant p=0.1297). There was no impact of the HBeAg status (p=0.6126).

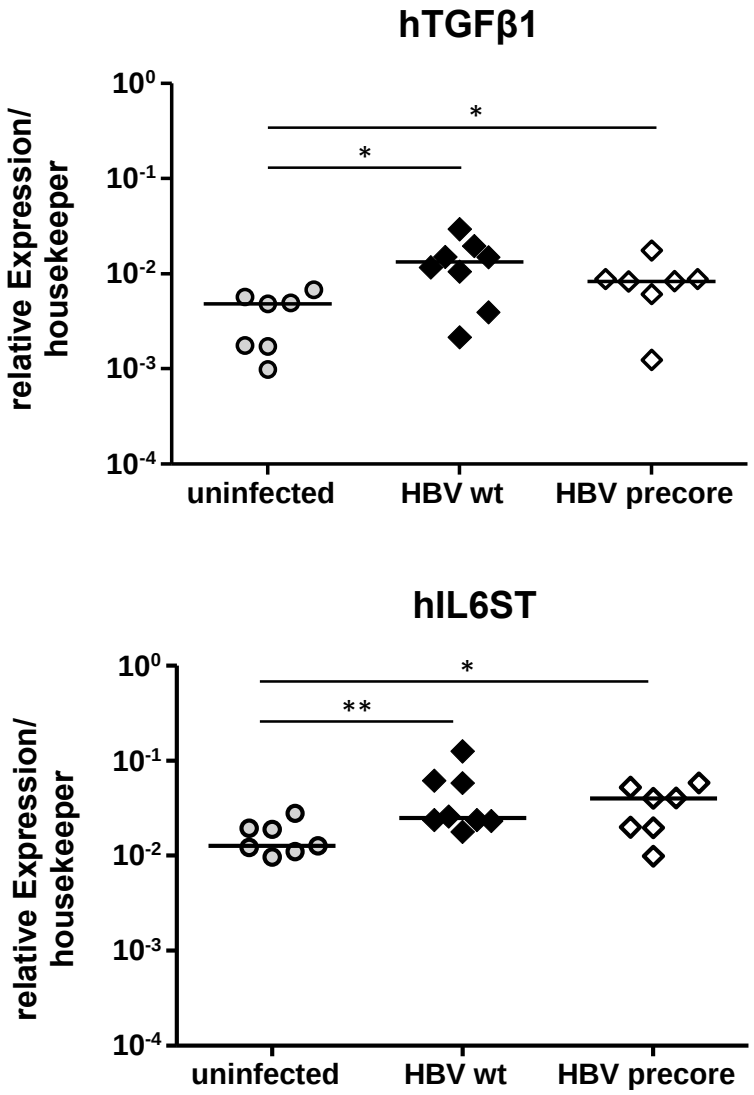

Figure C.1.18 ***Expression of human TGFβ1 and IL6ST***

Relative expression of human TGFβ1 (HBV wt p=0.0103 and HBV precore p=0.0131) and human IL6ST (HBV wt p=0.0070 and HBV precore p=0.0189) was significantly elevated in infected mice. Significant induction of relative gene expression was found to independent of the HBeAg status. Every dot represents a single mouse. Lines depict the corresponding median.

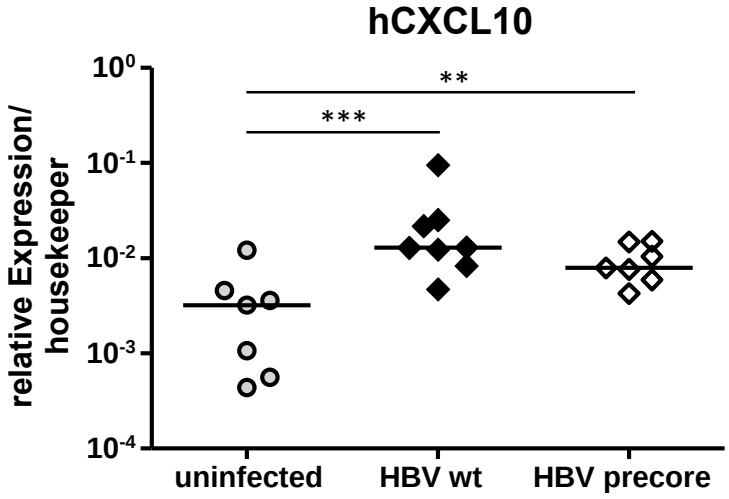

Figure C.1.19 ***Expression of human CXCL10***

Relative expression of human CXCL10 was significantly induced in HBV wt infected mice (p=0.0006) and HBV precore variant infected mice (p=0.0087 CXCL10 expression appeared to be independent of the HBeAg status (p=0.1520). Every dot represents a single mouse. Lines depict the corresponding median.

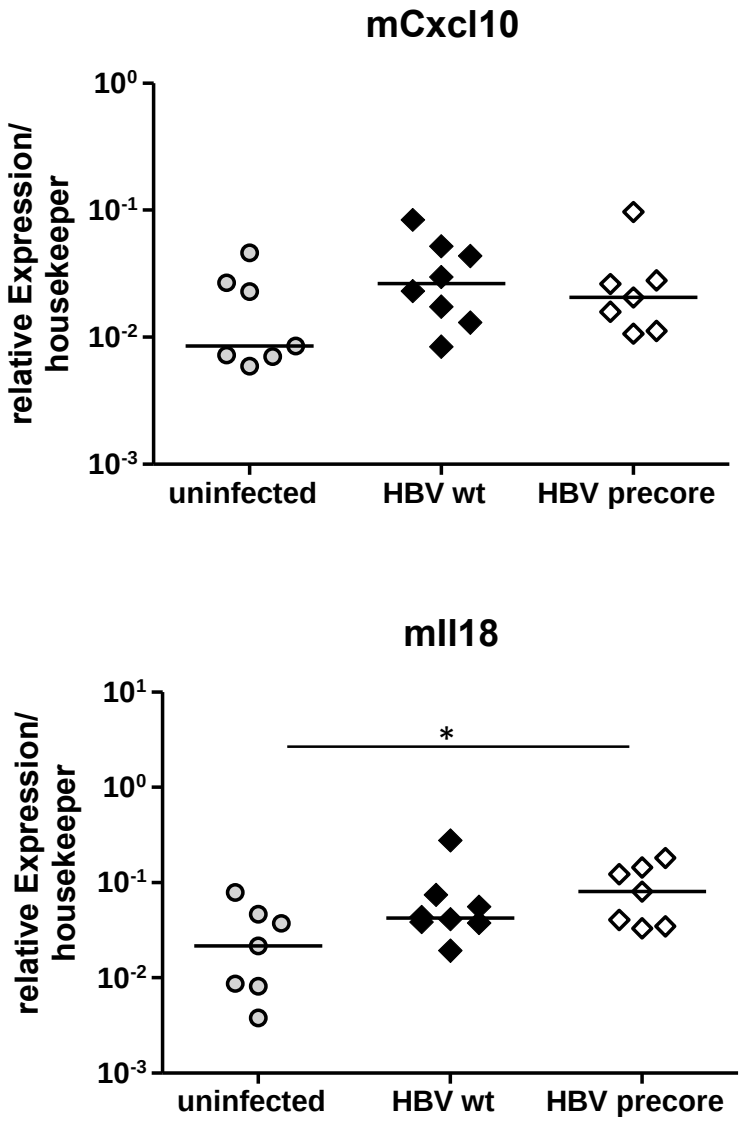

Figure C.1.20 ***Transcription of murine mCxcl1 and mIl18***

Median transcription of murine Cxcl10 appeared to elevated but is not significantly induced (HBV wt p=0.0603; HBV precore variant p=0.1297). Murine Il18 appeared to be elevated in mice infected with HBV wt (p=0.0603) and was significantly induced in mice infected with the HBV precore variant (p=0.0189). No effect of the HBeAg was shown. Every dot represents a single mouse. Lines depict the corresponding median.

Murine Interleukin-18, which is encoded by mIl18, is part of the IL-1 cytokine family and functions as a pro inflammatory cytokine. It is, among other functions, involved in T-cell and NK-cell immune response (Kashiwamura et al. 2002). Transcription of murine Il18 (**figure C.1.20**) appeared to be elevated in HBV wt infected mice and was significantly induced in HBV precore variant infected mice (p=0.0189). Our data does not show a significant impact of the absence of the HBeAg/precore protein (p=0.3063).

Murine Mill2 encodes the MHC I-like leukocyte 2 protein, that belongs to a group of non-classical MHC class I molecules that occurs in mice (Kajikawa et al. 2018). In contrast to other determined transcription levels, transcription of murine Tgfβ and murine Mill2 appeared to be lower in HBV infected mice when compared to uninfected controls. Only mMill2 transcription in HBV precore variant infected mice was significantly lowered (p=0.0379). Expression was not significantly influenced by the lack or presence of the HBeAg/precore protein (**figure C.1.21**). Notable mTgfβ1 as well as mMill2 displayed a high overall variance. As to how this effect is related to the HBV infection or to the HBeAg status remains uncertain.

In general, relative murine transcription levels tended to display a wider variance, when compared to human transcription levels in HBV infected human liver chimeric uPA/SCID/beige mice.

Overall human innate immunity gene transcription was slightly enhanced in infected mice, but no further enhancement was detected in the absence of the HBeAg/precore protein. In accordance with findings in relative ISG transcription, the effect of the HBeAg/precore protein with regard to transcription of cytokines as well as genes related to antigen presentation appeared to be insignificant in human liver chimeric uPA/SCID/beige mice infected with HBV.

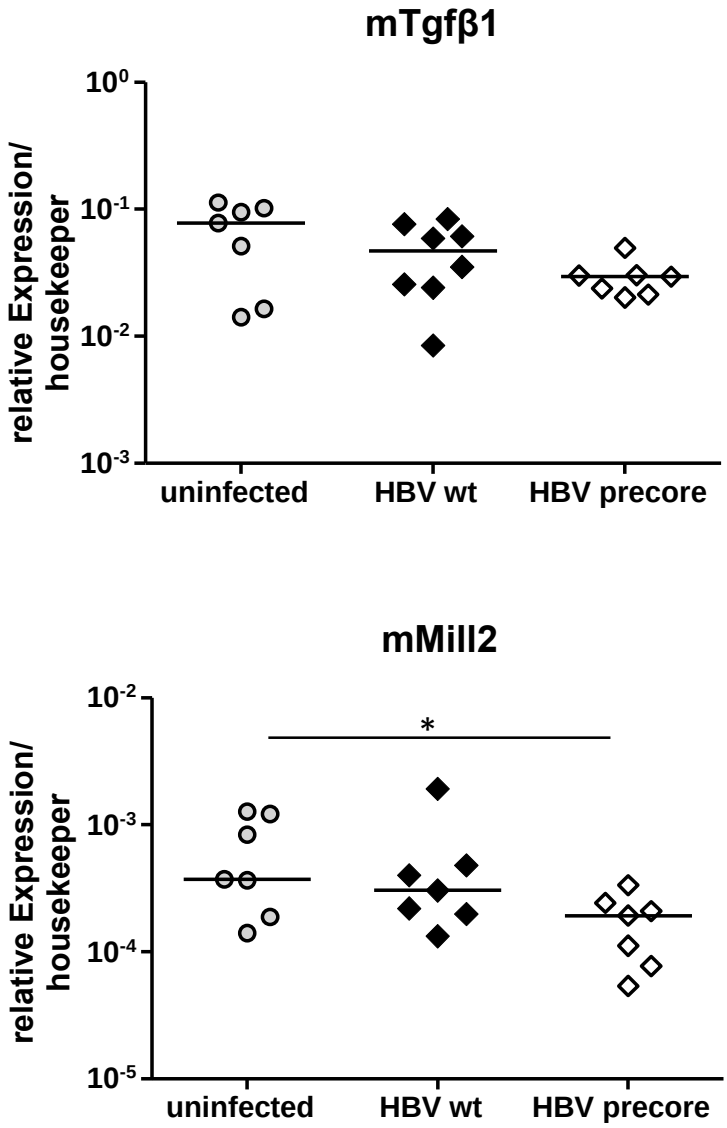

Figure C.1.21 ***Transcription of murine Mill2 and mTgfβ1***

Relative transcription of murine Mill2 and murine Tgfβ1 appeared to be slightly reduced in HBV infected mice. Transcription of mMill2 seemed to be significantly reduced in HBV precore infected mice (p=0.0379). HBeAg status had no significant effect on murine transcription. Every dot represents a single mouse. Lines depict the corresponding median.

C.2 TLR2 signaling induction via Pam3Cys

The induction of an inflammatory response in human hepatocytes, in the absence of NK cells and B- or T- lymphocytes, was observed to be weak and overall barely exceeded gene transcription observed in uninfected human liver chimeric uPA/SCID/beige mice transplanted with primary human hepatocytes of the same donor. The observations in this study are in accordance with previous studies. Interestingly the HBeAg/precore protein did not seem to hinder induction of gene transcription in HBV wt infected mice when compared to the relative transcription levels of HBV precore variant infected mice that lack the HBeAg/precore protein.

The HBV infection by itself might not provide a sufficient stimulus for an inflammatory response and the consequential upregulation of innate immunity related gene transcription in human hepatocytes at least in a system lacking adaptive immune responses.

In order to trigger the induction of innate immune responses in humanized mice, TLR2 signaling was stimulated using the TLR2 ligand Pam3Cys (Kumar et al. 2009). Mice were treated with the TLR1/2 agonist Pam3Cys and received 0,667 µg/g of mouse body weight as an intraperitoneal injection. Pam3cys was either administrated as a single dose treatment or repeatedly administrated in 24h intervals over the course of four days. Mice were sacrificed at different time points post Pam3Cys treatment as indicated in results and liver specimens were collected to determine intrahepatic RNA expression.

TLR2 signaling has been described to appear hindered in the presence of the HBeAg/precore protein (Visvanathan et al. 2007). Co-localizing of the HBeAg/precore protein to the TIR domain amino acid motive in the adapter molecules of TIRAP/Mal and TRAM was suggested to displays TIRAP/Mal - MyD88 interaction and in turn disrupt TLR2 signaling in HEK293 cells (human embryonic kidney 293 cell line) (Lang et al. 2011). Therefore, Pam3Cys administration was employed to assess the impact of HBeAg/precore protein in hindering the induction of TLR2 signaling in vivo.

C.2.1 Kinetics of TLR2 induction in murine hepatocytes and non-parenchymal cells

To evaluate the influence of the HBeAg/precore protein on TLR2 signaling related gene expression, mice were treated with the TLR2 agonist Pam3Cys. In order to treat HBV infected humanized uPA/SCID/beige mice the kinetics of TLR2 induction had to be first estimated. To do so SCID/beige mice were treated with 0.67µg/g mouse body weight of Pam3Cys. Mice were sacrificed at two hours (n=3), four hours (n=3), eight hours (n=3) as well as 24 (n=3) hours post single dose treatment and murine gene transcription of TLR-signaling target genes (mTlr2, mIfnβ, mTnfα, mIL6, mTgfβ and mCxcl10) were determined in collected liver specimens. In addition, the induction of gene transcription following repeated administration of Pam3Cys was evaluated in SCID/beige mice. Mice received Pam3Cys injections in 24 hour intervals over the course of four days and were sacrificed at either eight hours or 24 hours after the last Pam3Cys injection.

A clear induction of TLR2 signaling related murine gene transcription was observed in treated mice when compared to an untreated control group. Murine gene transcription is displayed relative to the expression of the murine housekeeper genes mEef2 and mActb. Overall, murine gene expression of all mentioned murine genes was notable induced at two hours post single dose treatment with Pam3Cys and declined with increased time post treatment. In the case of mTlr2 and mCxcl10, Pam3Cys treatment lead to a strong induction of gene expression within two hours when compared to the baseline expression in the untreated control group, followed by a decline of transcription levels, eventually returning to baseline expression after 24 hours post single dose treatment. Murine transcription of mTgfβ was not induced that prominently, but displayed a similar enhancement kinetic with murine gene transcription eventually aligning with baseline gene expression after 24 hours post treatment (**figure C.2.1**). Transcription of mIfnβ, mTnfα and mIL6 appeared to be below the lower limit of detection within the untreated control group but were detected in treated mice at two, four and eight hours post single dose treatment. In case of mIfnβ and mTnfα murine gene expression was still detectably after 24 hours post treatment, whereas murine expression of mIL6 returned to being below the lower limit of detection at 24 hours post single dose treatment (**figure C.2.2**).

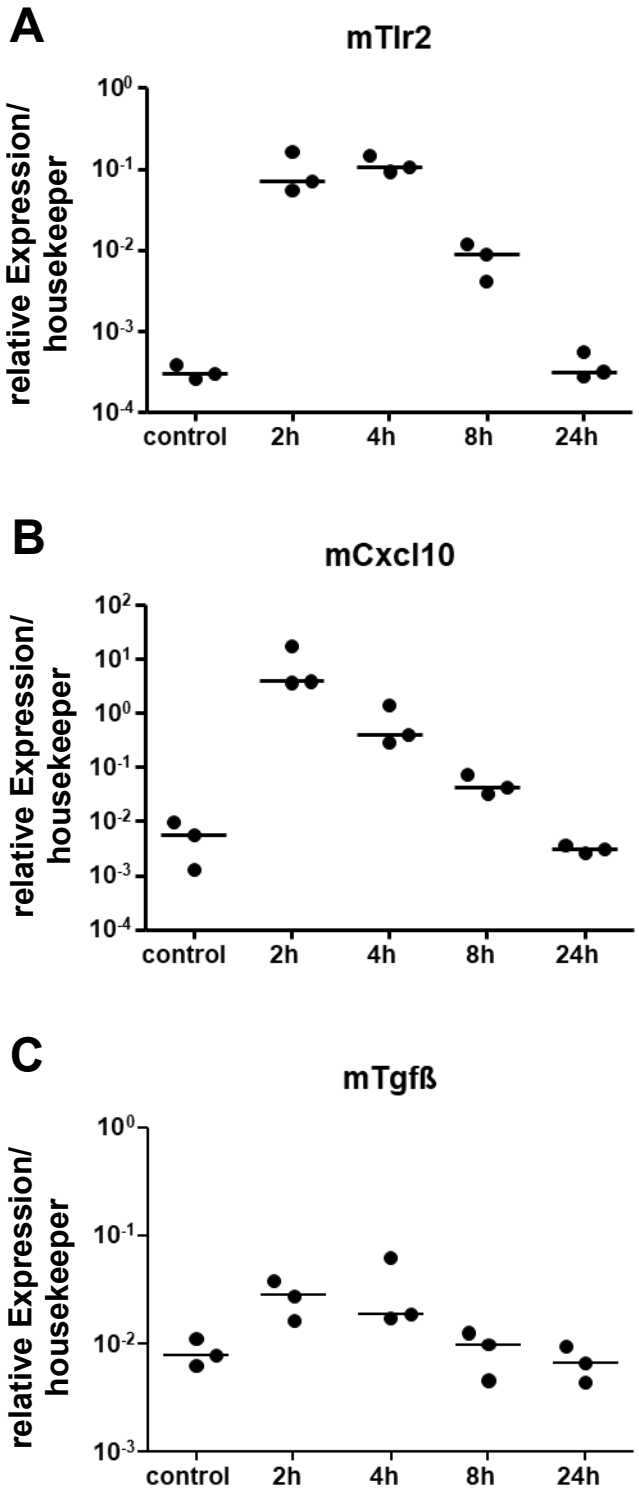

Figure C.2.1 ***Murine gene expression post Pam3Cys treatment***

Relative expression of (**A**) murine Tlr2, (**B**) murine Cxcl10 and (**C**) murine Tgfβ1 at 2 hours, 4 hours, 8 hours and 24 hours post treatment with the TLR1/TLR2 agonist Pam3Cys. Every dot represents a single mouse. Lines depict the corresponding median.

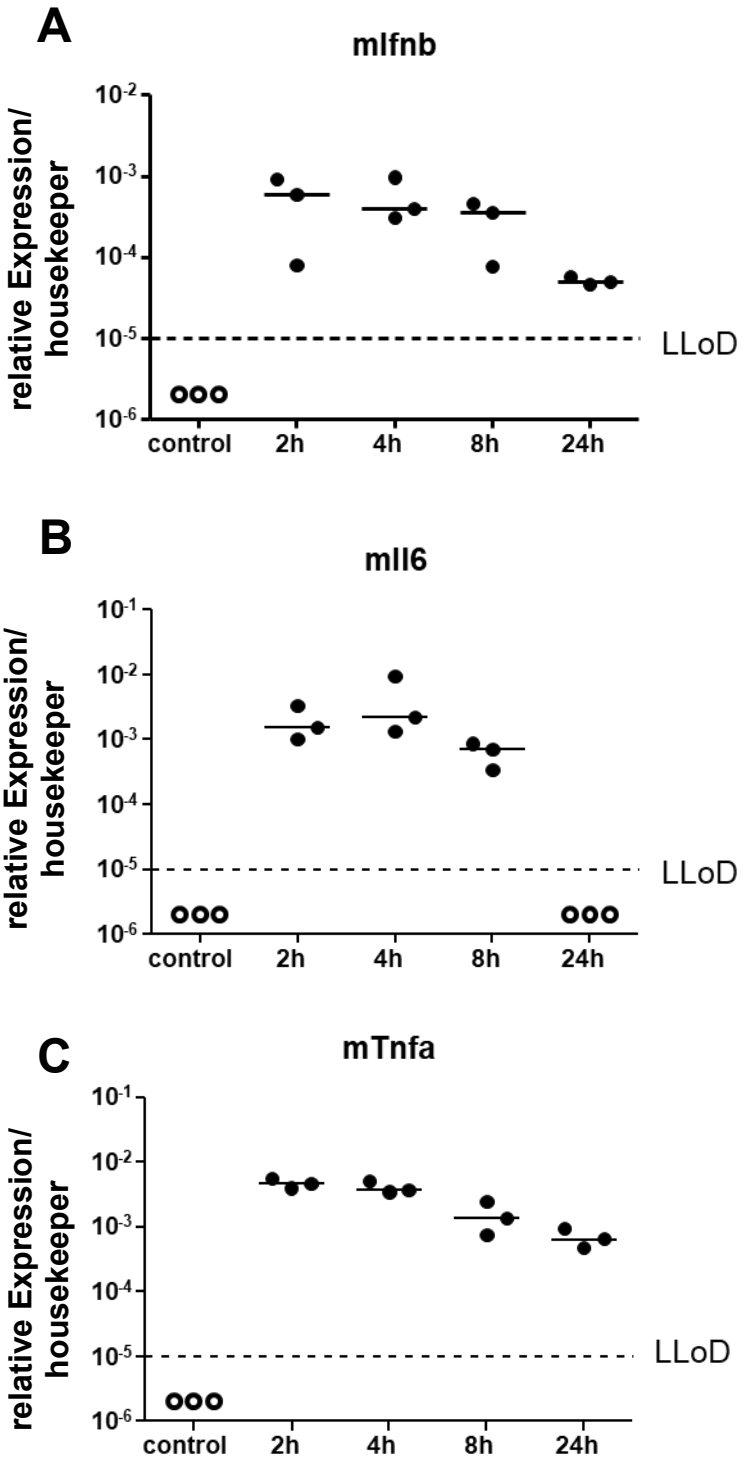

Figure C.2.2 ***Murine gene expression post Pam3Cys treatment***

Relative expression of (**A**) murine Ifnβ, (**B**) murine Il6 and (**C**) murine Tnfα at 2 hours, 4 hours, 8 hours and 24 hours post treatment with the TLR1/TLR2 agonist Pam3Cys. Every dot represents a single mouse. Lines depict the corresponding median.

Induction of gene expression in comparison to single dose Pam3Cys treatment followed different patterns shown in **figure C.2.3** and **figure C.2.4**. In case of mTlr2, interval treatment lead to a clear induction of mTlr2 expression, that did not return to base line levels of expression after 24 hours post treatment as it was shown in a single dose treatment setting. Similar, transcription of mIl6 was noticeably induced in Pam3Cys treated mice, enhancement was maintained during treatment and even did not return to baseline levels after 24 hours. Murine gene expression of murine Tgfβ displayed a similar pattern. Expression levels did not return to base line levels 24 hours post last Pam3Cys administration like in single dose treated mice. Transcription of murine Ifnβ as well as murine Tnfα was noticeably induced by Pam3Cys treatment and, similar to single dose treatment, did not return to base line levels of gene expression even 24 hours after the final Pam3Cys dose injection. Interestingly mCxcl10 displayed a trend of expression levels that appeared comparable to gene transcription induced by single dose treatment.

These results demonstrate that the induction of murine TLR2 signaling related gene transcription, utilizing Pam3Cys as a TLR1/TLR2 agonist, is efficient and follows distinct gene expression kinetics. Moreover this serves as prove of principle that Pam3Cys treatment leads to a clearly distinguishable – in this case murine - induction of TLR2 related signaling gene expression. Based on the above results, the time points of two hours, four hours and eight hours post single dose Pam3Cys treatment were chosen to investigate the impact of the HBeAg/precore protein with regard to TLR2-signaling related gene expression.

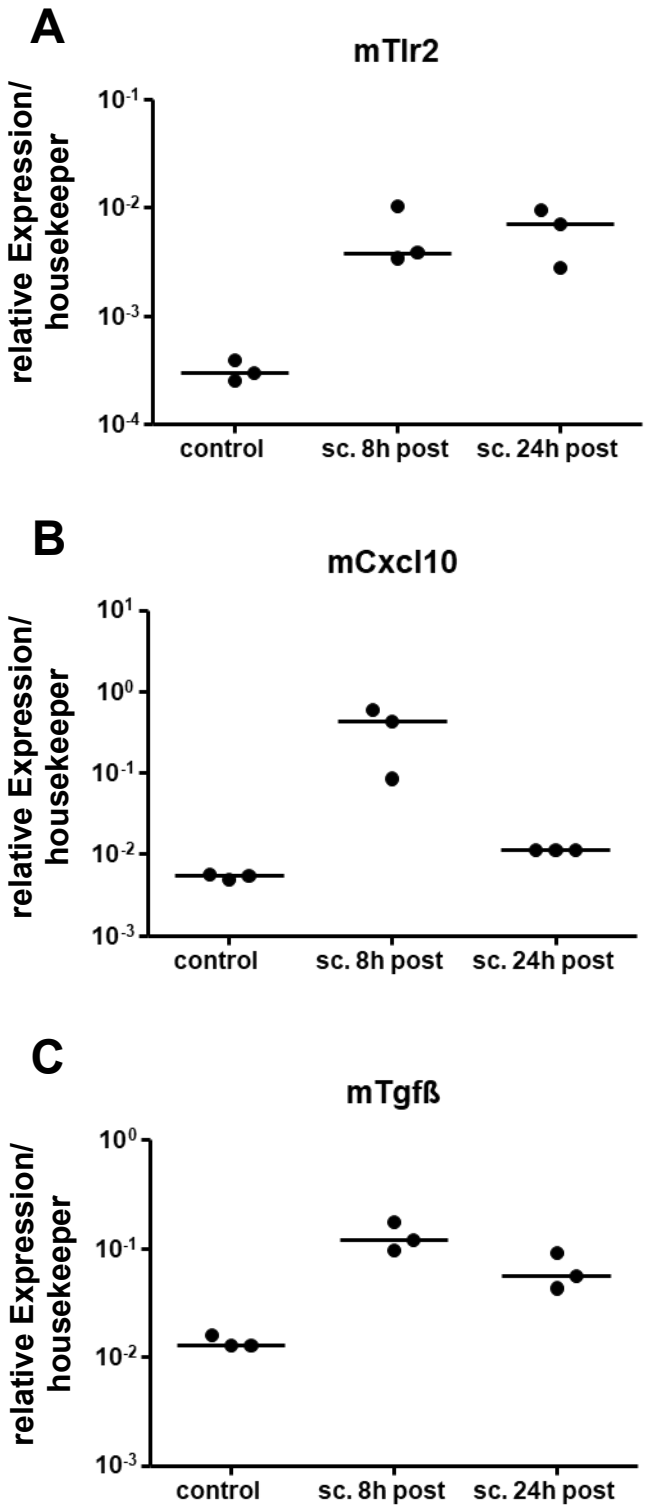

Figure C.2.3 ***Murine gene expression following interval treatment with Pam3Cys***

Relative expression of (**A**) murine Tlr2, (**B**) murine Cxcl10 and (**C**) murine Tgfβ at 8 hours and 24 hours after four succeeding doses of the TLR1/TLR2 agonist Pam3Cys compared to gene expression of an untreated control group. Every dot represents a single mouse. Lines depict the corresponding median.

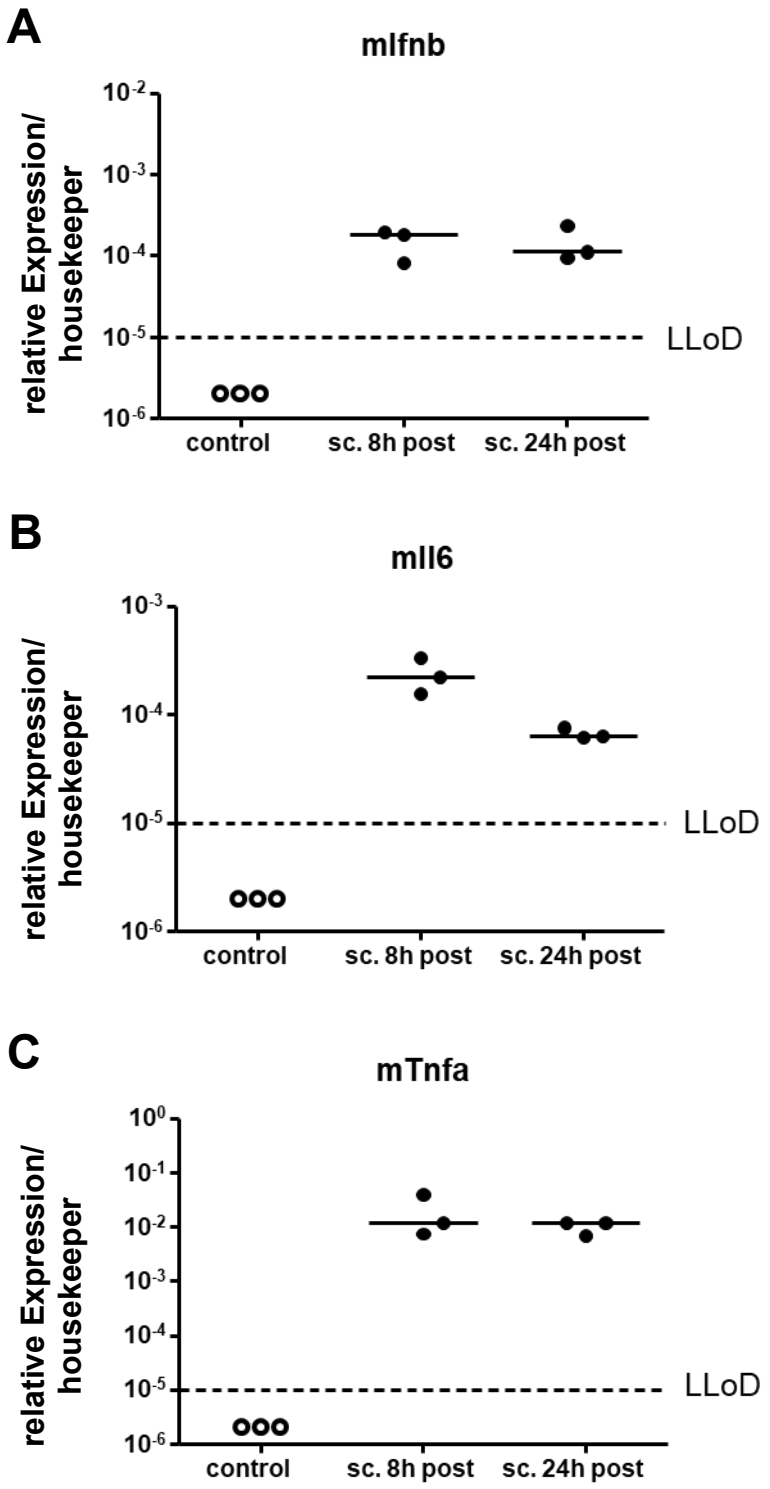

Figure C.2.4 ***Murine gene expression following interval treatment with Pam3Cys***

Relative expression of (**A**) murine Ifnβ, (**B**) murine Il6 and (**C**) murine Tnfα at 8 hours and 24 hours after four succeeding doses of the TLR1/TLR2 agonist Pam3Cys compared to gene expression of an untreated control group. Every dot represents a single mouse. Lines depict the corresponding median.

C.2.2 Gene expression following Pam3Cys treatment in HBV wild type or G1896A precore variant infected human liver chimeric uPA/SCID/beige mice

As demonstrated, Pam3Cys treatment successfully induced expression of innate immunity gene in murine hepatocyte and non-parenchymal liver cells in SCID/beige mice. Since HBV infection does not sufficiently induce transcription of innate immunity genes itself, regardless of HBeAg status, an additional stimulus of innate immunity gene expression might be needed to detect a potential influence of the HBeAg/precore protein with regard to innate immune response. Pam3Cys treatment might therefore provide the possibly necessary additional induction of innate immunity gene transcription, in order to observe an effect of the lack of the HBeAg/precore protein in HBV precore variant infected mice. Similar to SCID/beige mice, HBV wt and HBV precore variant infected mice were treated with Pam3Cys (0,667 µg/g mouse body weight) and sacrificed 2 hours, 4 hours and 8 hours post Pam3Cys injection. As described above intrahepatic transcription was determined in liver specimens collected from sacrificed mice and is displayed as relative gene transcription to housekeeper genes (mean of hGAPDH and hRPL30 or mEef2 and mActb).

Untreated control group consists of untreated uninfected mice (n=7), untreated HBV wt infected mice (n=10) and untreated HBV precore variant infected mice (n=8). At two hours post Pam3Cys treatment uninfected mice (n=2), HBV wt infected mice (n=3) and HBV precore variant infected mice (n=3) where analyzed. At four hours post Pam3Cys treatment uninfected mice (n=3), HBV wt infected mice (n=1), HBV precore variant infected mice (n=2) and at eight hours post Pam3Cys treatment uninfected mice (n=3), HBV wt infected mice (n=2) and HBV precore variant infected mice (n=3) were analyzed. It is important to note that the sample size in Pam3Cys treated HBV infected human liver chimeric uPA/SCID/beige mice, that was analyzed in this study, does not permit statistical analyses but allowed us to identify a possible trend.

C.2.3 Transcription of innate immunity genes following Pam3Cys treatment in HBV infected mice

Similar to Pam3Cys inducted gene expression in SCID/beige mice, expression levels of murine Tlr2 and murine Cxcl10 in human liver chimeric uPA/SCID/beige mice appeared to be noticeably induced in Pam3Cys treated mice. Induction kinetics were found to be similar to Pam3Cys induction in SCID/beige mice, with elevated levels of transcription as early as 2 hours post injection, that were reverting back to baseline levels of transcription over the course of 8 hours (**figure C.2.5**). Noticeably, induced transcription levels appear to be comparable across HBV infected and uninfected mice and also did not indicate a suppression of gene expression in HBV wt infected mice.

Interestingly and in contrast to murine transcription, transcription of TLR2 in human hepatocytes appeared not to be induced by Pam3Cys treatment, regardless of HBV infection or HBeAg status. CXCL10 transcription on the contrary appeared to be induced similar to murine transcription of Cxcl10. But also displayed no indication of diminished gene transcription due to the presence of the HBeAg/precore protein. The lack of induction of human TLR2 transcription, independent of HBV infection or HBeAg status, could point to a generally reduced capability of human hepatocytes to express TLR2 in comparison to e.g. non-parenchymal liver cells (**figure C.2.6**).

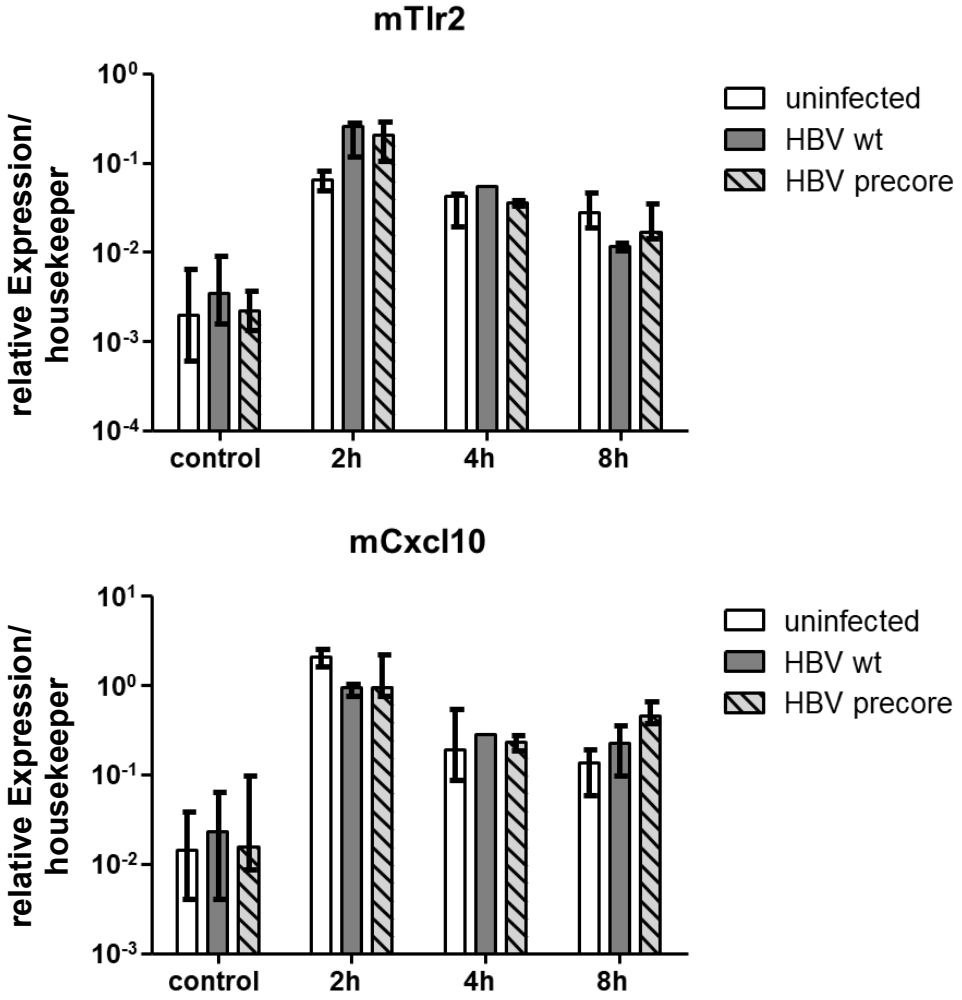

Figure C.2.5 ***Pam3Cys induced murine expression in HBV infected mice***

Relative expression of murine Tlr2 and murine Cxcl10 at 2 hours, 4 hours and 8 hours post Pam3Cys treatment was comparable to expression kinetics in uninfected SCID/beige mice and did not appear to be influenced by HBV infection or the lack of the HBeAg. Bars depict median relative expression with range as indicated.

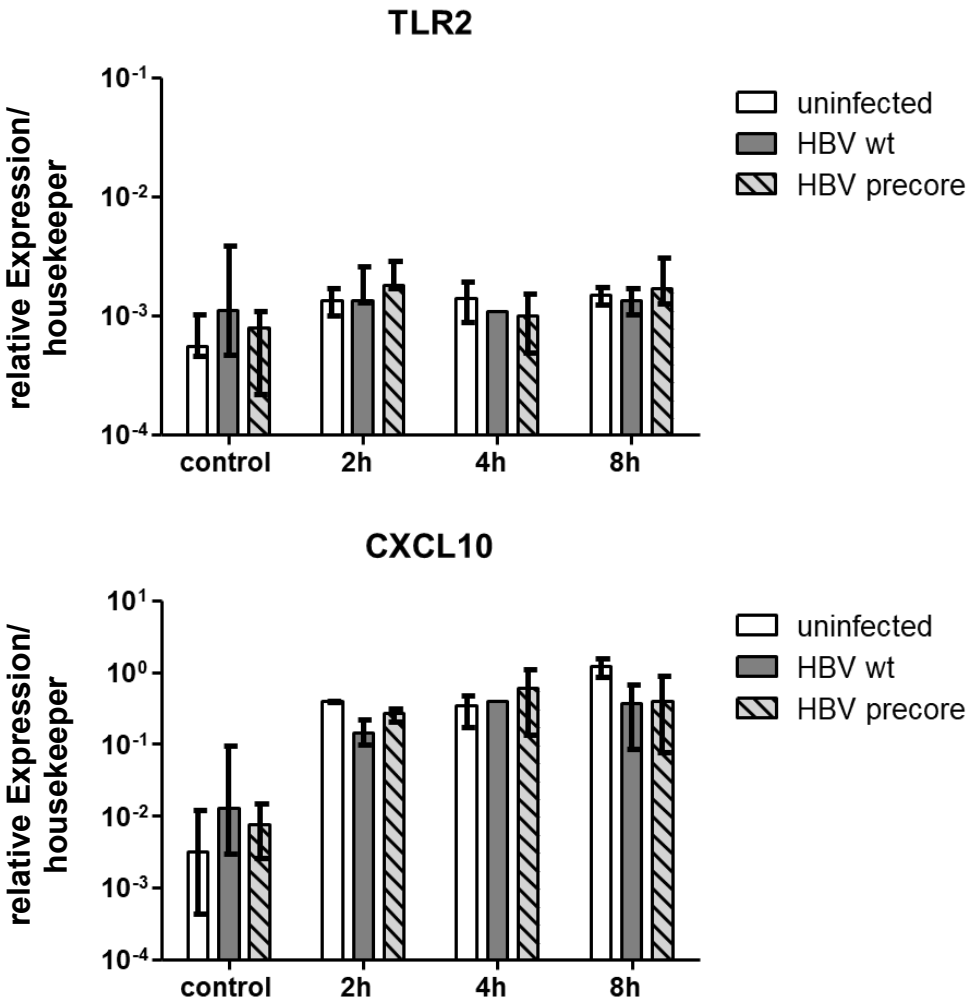

Figure C.2.6 ***Pam3Cys induced human expression in HBV infected mice***

Relative expression of human TLR2 and human CXCL10 was noticeably induces at 2 hours, 4 hours and 8 hours post Pam3Cys treatment but seemed to be unaffected by the HBV infection or the lack of the HBeAg. Bars depict median relative expression with range as indicated.

Murine induction of mTnfα transcription as well as mIl6 transcription in human liver chimeric uPA/SCID/beige mice displayed a similar overall pattern as Pam3Cys treatment did in SCID/beige mice. Importantly, human transcription of TNFα and IL6 was not detectable in liver tissue following Pam3Cys treatment at any time point. This points to the inability of human hepatocyte to express TNFα or IL6 and suggests that murine transcription of mTnfα and mIl6 might be derived from non-parenchymal liver cells rather than residual murine hepatocytes (**figure C.2.7**).

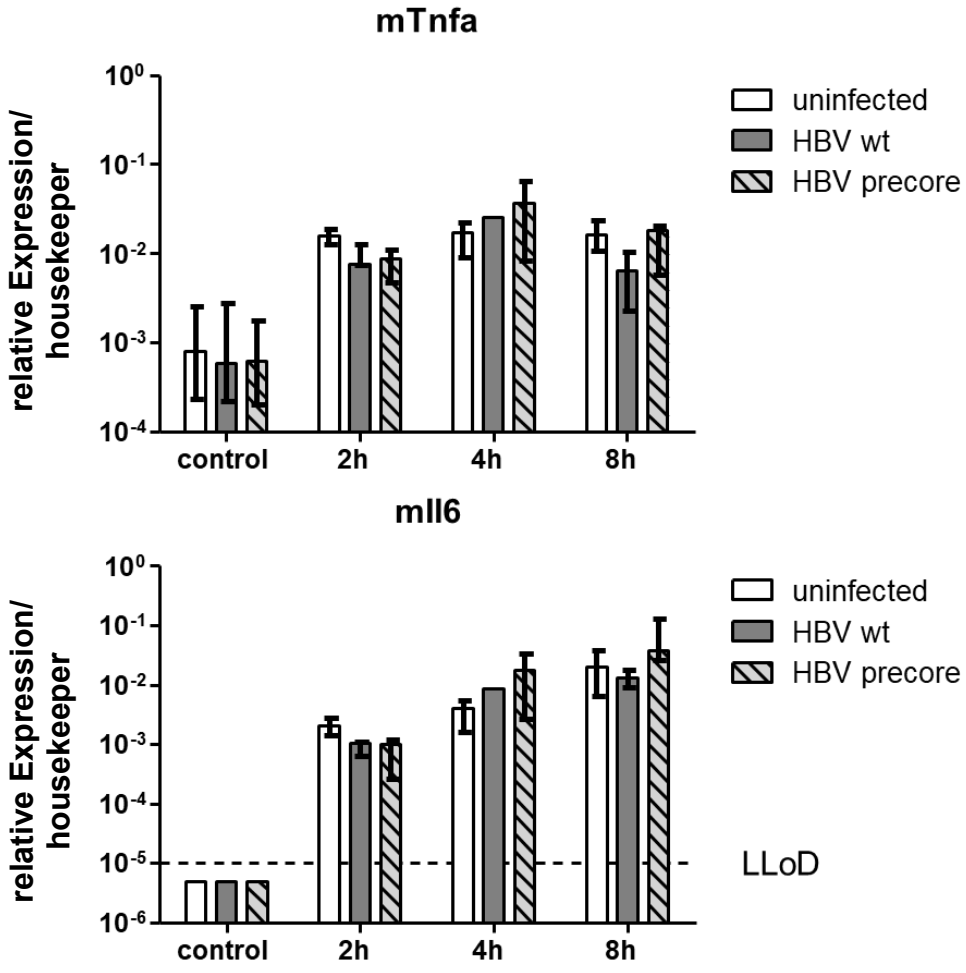

Figure C.2.7 ***Pam3Cys induced murine expression in HBV infected mice***

Relative expression of murine Tnfα and murine Il6 at 2 hours, 4 hours and 8 hours post Pam3cys treatment was comparable to expression kinetics in uninfected SCID/beige mice and did not appear to be affected by HBV infection or the lack of the HBeAg. Bars depict median relative expression with range as indicated.

Expression of murine Tgfβ as well as human TGFβ in human liver chimeric uPA/SCID/beige mice did not appear to be induced following Pam3Cys treatment. This appears to be in line with the weak induction of mTgfβ induction observed in SCID/beige mice.

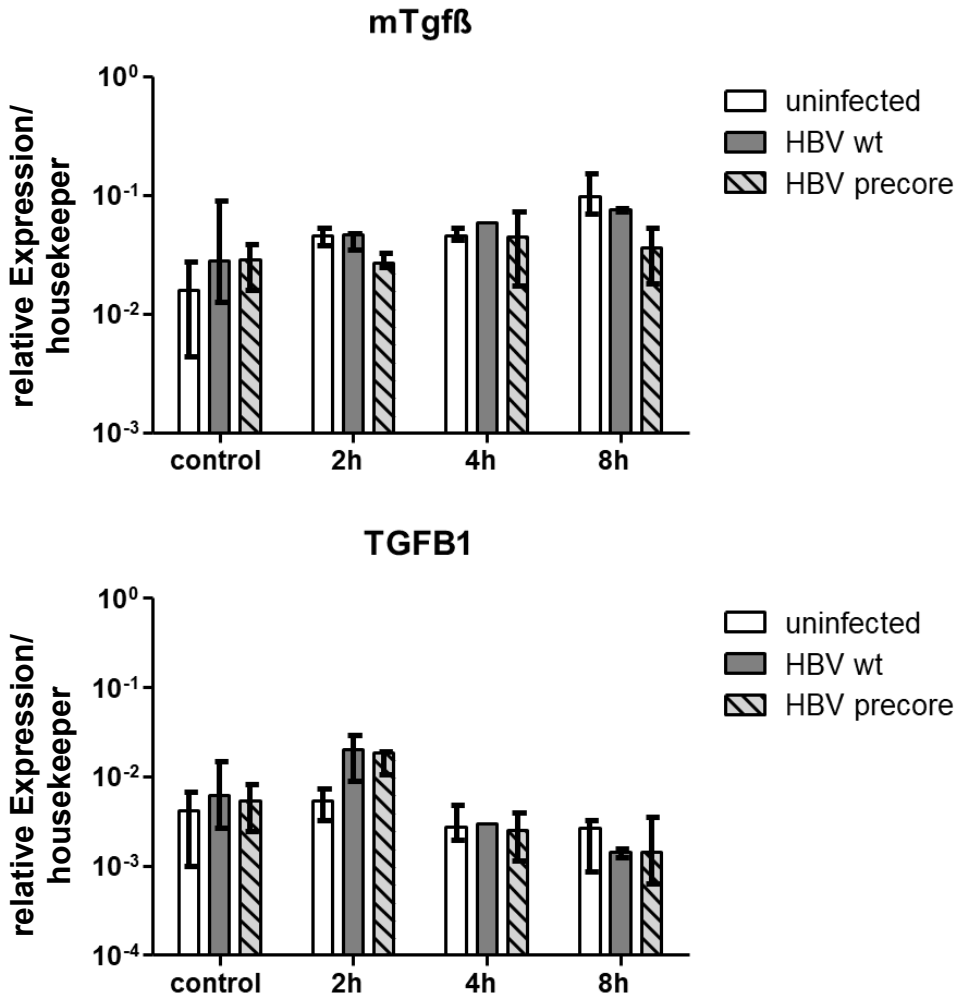

Figure C.2.8 ***Pam3Cys induced TGFβ expression in HBV infected mice***

Relative expression of murine Tgfβ and human TGFβ at 2 hours, 4 hours and 8 hours post Pam3cys treatment displayed similar expression kinetics as murine Tgfβ expression in uninfected SCID/beige mice. Murine and human expression did not appear to be affected by HBV infection or the lack of the HBeAg overall. Bars depict median relative expression with range as indicated.

Pam3Cys treatment also lead to an apparent induction of TAP1 as well as ISG20 in uninfected mice as well as HBV infected mice (**figure C.2.9**). The presence of the HBeAg/precore protein did not appear to dampen Pam3Cys induced expression of TAP1 or ISG20 in human hepatocytes.

In contrast to TAP1 and ISG20, expression of ISG15, STAT1 and OAS1 seemed to be rather unaffected by Pay3Cys treatment, regardless of HBV infection or HBeAg status and a clear induction could not be observed (**figure C.2.10**).

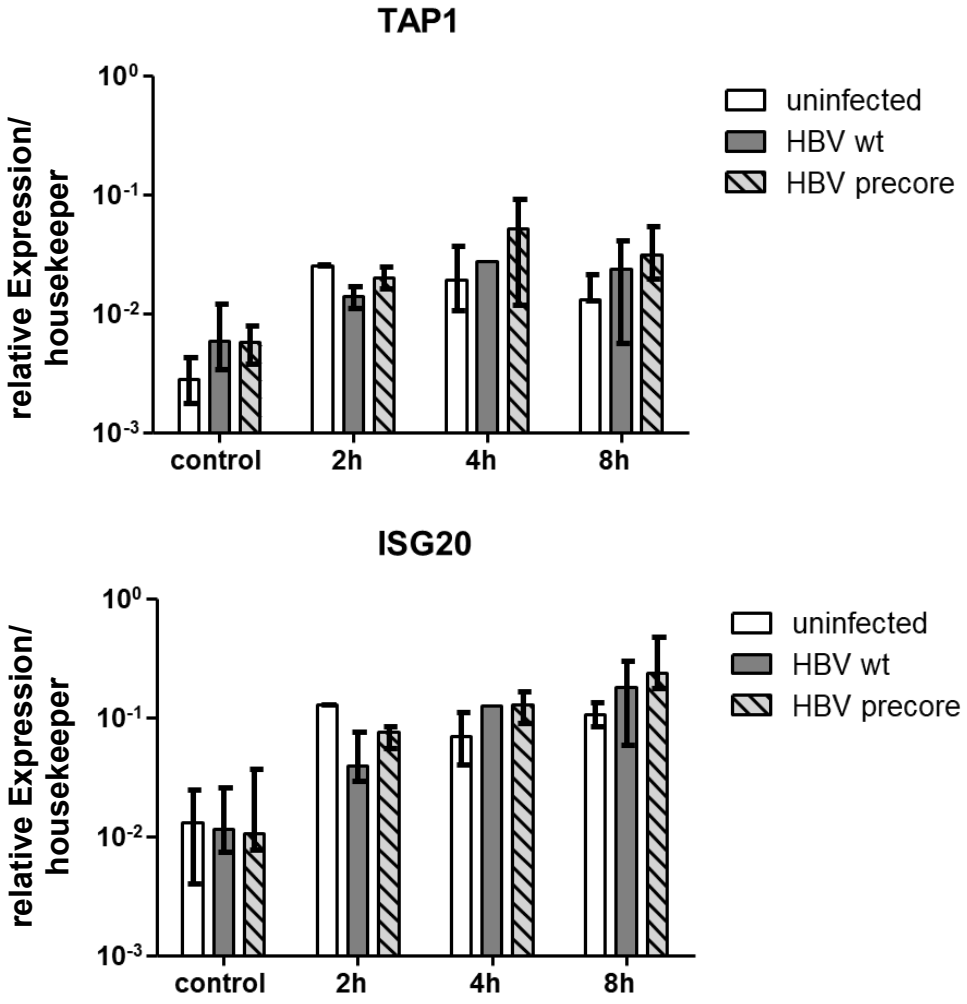

Figure C.2.9 ***Pam3Cys induced human expression in HBV infected mice***

Relative expression of human TAP1 and human ISG20 was noticeably induced at 2 hours, 4 hours and 8 hours post Pam3Cys treatment but did not appear dampened by the HBV infection or promoted by the lack of the HBeAg overall. Bars depict median relative expression with range as indicated.

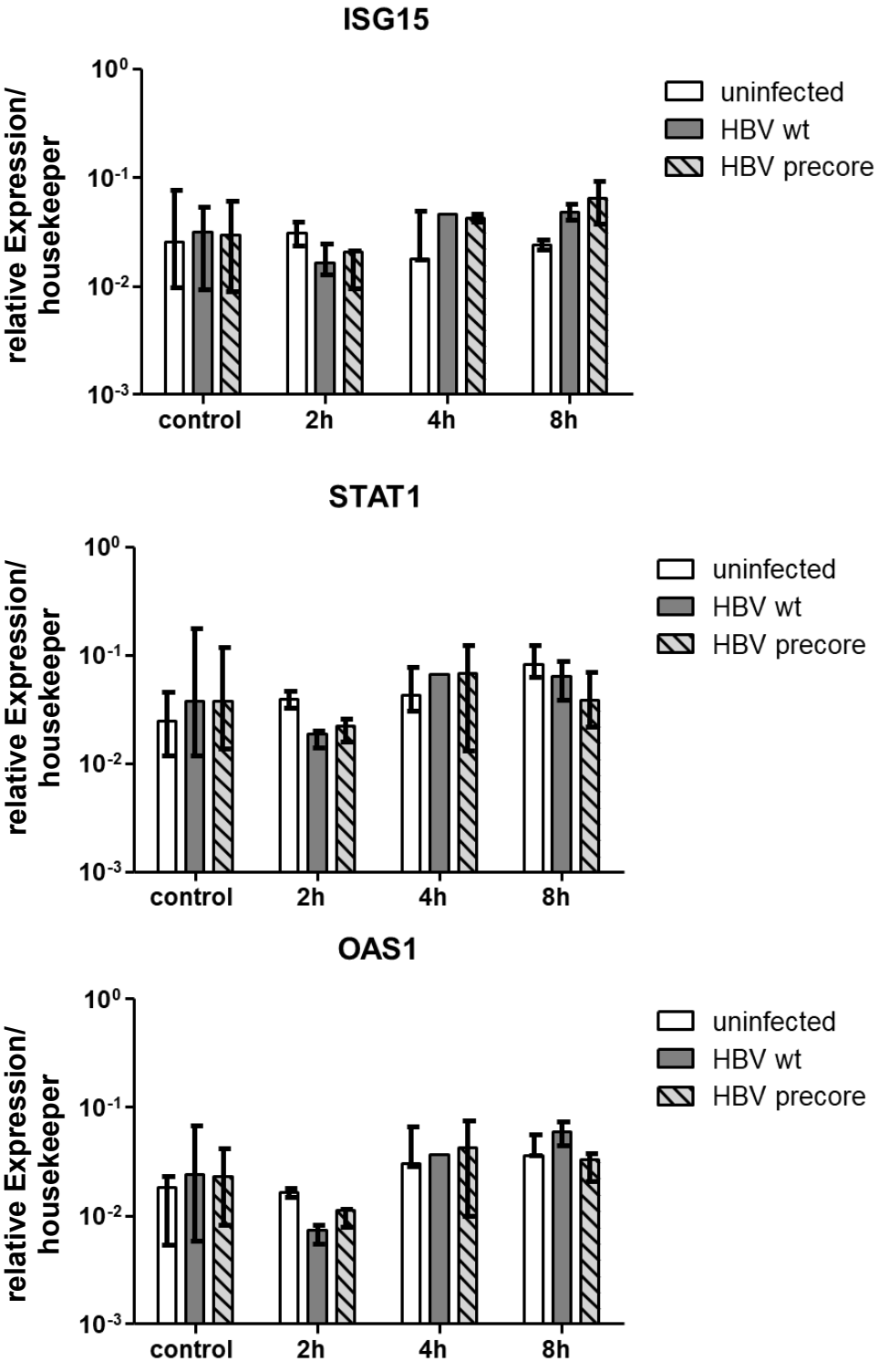

Figure C.2.10 ***Pam3Cys induced human expression in HBV infected mice***

Relative expression of human ISG15, human STAT1 and human OAS1 appeared not to be induced at 2 hours, 4 hours and 8 hours post Pam3Cys regardless of HBV infecetion or HBeAg status. Bars depict median relative expression with range as indicated.

Alongside with ISG15, STAT1 and OAS1 expression levels of HLA-E, MAX1, USP18 and MDY88 did not appear to be induced in human hepatocytes following Pam3Cys treatment of human liver chimeric uPA/SICD/beige mice (data not shown). Overall, induction of murine as well as human gene expression could be observed in liver tissue following Pam3Cys treatment. A distinct induction of gene expression of murine genes of e.g. mTlr2, mCxcl10 as well as mTnfα and mIl6 could be observed. Human gene transcription was indeed induced in some cases, like CXCL10, ISG15 and TAP1. But also displayed no induction in a number of different human innate immunity related genes. A distinct impact of the presence of the HBeAg/precore protein or even HBV infection as a whole, could neither be shown in murine expression nor human gene expression levels.

C.2.4 Intrahepatic viral parameters in Pam3Cys treated HBV infected human liver chimeric uPA/SCID/beige mice

Intrahepatic viral parameters were determined in liver specimens of HBV infected mice two hours, four hours and eight hours post single dose of Pam3Cys.

HBV wt infected mice sacrificed at two hours post Pam3Cys treatment had median viral titers of $8,46x10^6$ copies DNA/ml and HBV precore variant infected mice sacrificed at two hours post Pam3Cys treatment exhibited median viral titers of $5,09x10^6$ copies DNA/ml. Viral titer of HBV wt infected mouse sacrificed at four hours post Pam3Cys treatment was at $1,32x10^7$ copies DNA/ml and a median titer $4,57x10^9$ in HBV precore variant infected mice respectively. HBV wt infected mice sacrificed at eight hours post Pam3Cys injection displayed median viral titers of $4,14x10^9$ copies DNA/ml, while HBV precore variant infected mice displayed median viral titers of $2,79x10^9$ copies DNA/ml.

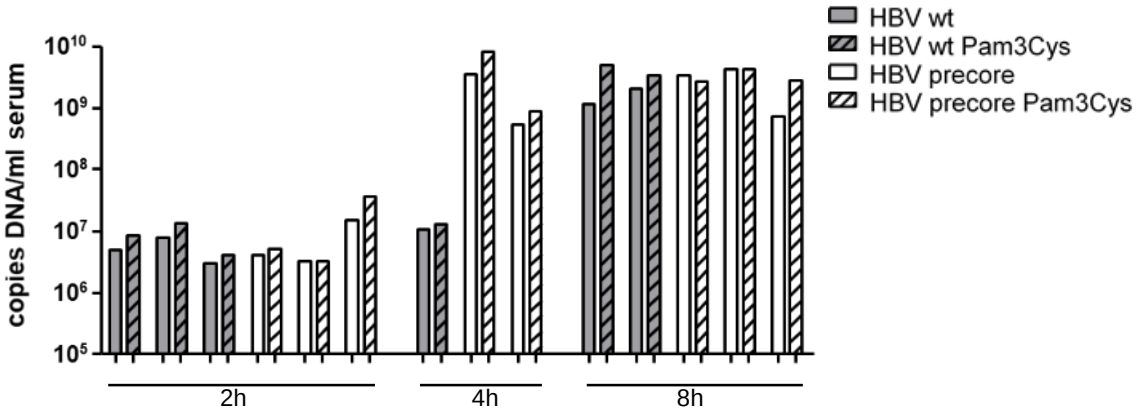

Figure C.2.11 ***Viral titer pre and post pam3Cys***

Viral titers of each individual mouse pre and post Pam3Cys dose at two hours, four hours and eight hours. There was no reduction in viral titer due to Pamy3Cys administration regardless of HBeAg status. Every bar represents a single mouse.

While the range of viral titers varied among mice treated with Pam3Cys in this setting, viral titers were not reduced as a result of Pam3Cys injection (**figure C.2.11**).

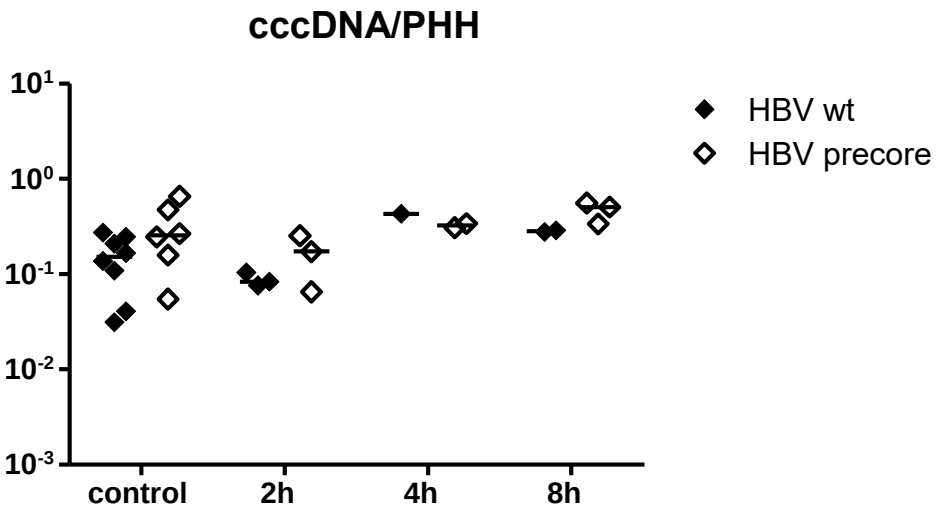

Figure C.2.12 ***cccDNA per human hepatocyte (with PSD)***

cccDDNA per human hepatocyte does not appear to be reduced when compared to an untreated control. Every dot represents a single mouse. Lines depict the corresponding median.

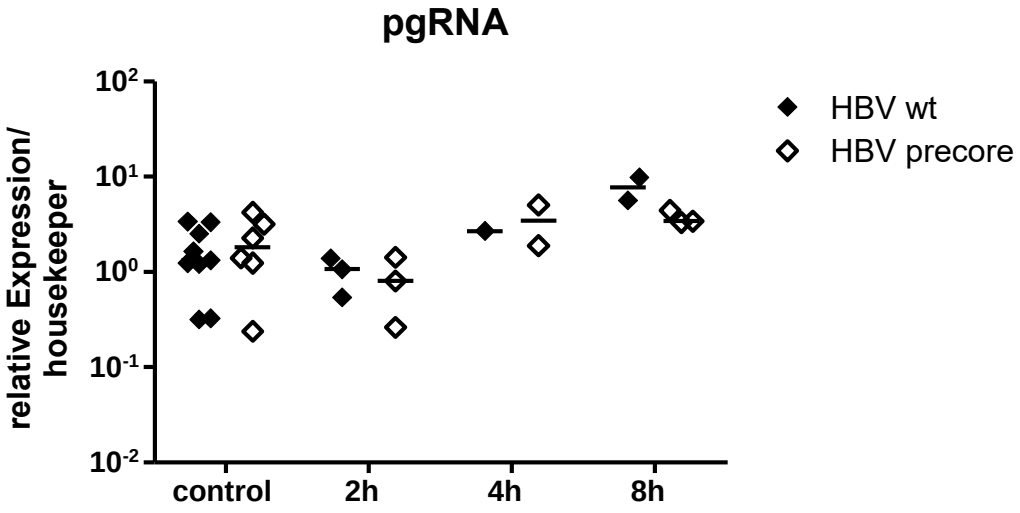

Figure C.2.13 **pgRNA in mice treated with Pam3Cys**

Relative expression of pgRNA was elevated in individual mice in correspondence to viral titers but did not appeared to be hindered by Pam3Cys treatment overall. Every dot represents a single mouse. Lines depict the corresponding median.

Levels of cccDNA/PHH (**figure C.2.12**) overall did not seem to be reduced compared to the untreated control. Intrahepatic cccDNA per human hepatocyte appeared to be lower in mice displaying lower viral loads in serum. This reflects a higher degree of intrahepatic spreading in mice displaying higher viral titers which therefore displayed a higher overall output of viral particles. As in untreated HBV infected mice, amount of cccDNA did not appear to be affected by the G1896A precore mutation

Relative expression of pgRNA (**figure C.2.13**) was not hindered by Pam3Cys treatment but displayed noticeable variant in correspondence to viral titers in mice sacrificed in this experimental setting.

Figure C.2.14 shows rcDNA/cccDNA to approximate viral replicative activity relative to established hepatic viral spreading. Interestingly rcDNA per cccDNA might be reduced in mice immediately after Pam3Cys treatment. Mice sacrificed at two hours post Pam3Cys administration might display lower levels of rcDNA/cccDNA compared to untreated mice. Level of rcDNA/cccDNA appeared to return to baseline at four and eight hours post Pam3Cys administration.

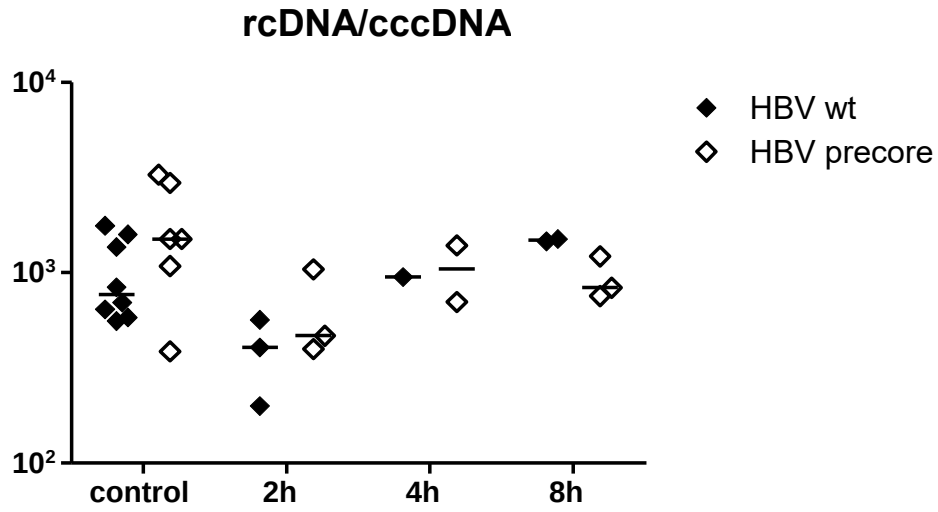

Figure C.2.14 ***rcDNA/cccDNA (with PSD)***

As in untreated infected mice, rcDNA/cccDDNA was unaffected by the presence or lack of the HBeAg. Every dot represents a single mouse. Lines depict the corresponding median.

Whether a Pam3Cys-induced Toll-like receptor signaling related innate immune response is hindering viral replicative activity needs further investigation. Pam3Cs administration did not seem to effect viral titers and an impact of the HBeAg/precore protein was not noted in this experimental setting.

D Discussion

D.1 Impact of the HBeAg/precore protein in HBV infection

In this study we intended to further investigate the function of the HBeAg/precore protein within the infectious cycle of HBV and its impact upon the intrinsic innate immune response of human hepatocytes toward infection with HBV. Previous studies have reported that the HBeAg/precore protein of the hepatitis B virus counteracts the innate immune response and have suggested that the function of the HBeAg lies within the interference with TLR related signaling (Locarnini et al. 2005; Visvanathan et al. 2007; Lang et al. 2011). In order to evaluate the function of the HBeAg/precore protein with regard to the intrinsic innate immune response of infected hepatocytes, human liver chimeric uPA/SCID/beige mice were infected with either the HBV wt or the HBV G1896A variant.

The findings in this study demonstrate that HBV is able to successfully establish chronic HBV infection in human hepatocytes within human liver chimeric uPA/SCID/beige mice, regardless of the presence of the HBeAg/precore protein. Infection kinetics and viral spreading seem only marginally affected by the G1896A mutation and the resulting lack of the HBeAg/precore protein.

With regard to the impact of the HBeAg/precore protein upon intrinsic innate immunity response of human hepatocytes the results of this study demonstrate that HBV infection overall only triggers a weak innate immunity response, independent of the HBeAg status. We could show that, in contrast to previous in vitro studies, the HBeAg/precore protein does not hinder the intrinsic innate immunity pathways of the infected human hepatocytes in vivo, although further investigation is needed to assess whether the HBeAg/precore protein may interfere with HBV recognition in non-parenchymal liver cells as well as other cellular components of the innate and the adaptive immune response.

D.1.1 Establishment and persistence of HBV infection in uPA/SCID/beige mice in relation to the HBeAg/precore protein status

There is conflicting data from in vitro studies on the capacity of the HBV G1896A variant to replicate in comparison to wild type HBV. Higher HBV DNA yields have been demonstrated in HepG2 and Huh7 cells transfected with a plasmid construct that included the G1896A stop codon compared to transfection of a HBV wt-like plasmid, but it has also been shown that the G1896A mutation does not lead to increased amounts of secreted viral particles in transfected Huh7 cells. In Huh7 cells transfection of the HBV G1896A variant was associated with higher amounts of pgRNA/cccDNA as well as higher amounts of precoreRNA/cccDNA while amounts of secreted viral particles remained comparable.(Scaglioni, P. P. et al. 1997; Lamberts et al. 1993; Samal et al. 2015).

The findings in this study demonstrate that HBV is able to successfully establish chronic HBV infection in human hepatocytes within human liver chimeric uPA/SCID/beige mice regardless of the presence of the HBeAg/precore protein.

As show in figure C.1.1 serum levels of viral DNA in human liver chimeric uPA/SCID/beige mice over the course of infection appear similar, if not slightly higher in the HBV G1896A variant. Intrahepatic viral parameters confirm the successful formation of cccDNA (figures C.1.5) in human hepatocytes by both viral variants. Levels of intrahepatic HBV DNA (figure C.1.3) as well as pgRNA transcription (figure C.1.4) demonstrated an active replication of HBV within human hepatocytes. Production of functional and infectious viral particles could be demonstrated by successful establishment of HBV infection in mice inoculated with infectious serum derived from either mice previously infected with the HBV wild type or the HBV G1896A variant respectively. These findings confirm that the establishment of HBV infection and production of further infectious viral particles is independent of presence of the HBeAg/precore protein.

In our study we found that the amount of pgRNA/cccDNA/human hepatocyte appeared to be slightly increased in HBV wild type. This may reflect a more efficient packaging of pgRNA which is then reverse transcribed and thereby results in lower levels of pgRNA per cccDNA minichromosome in the HBV G1896A mutant. Combined with the observation that overall intrahepatic viral parameters and viral kinetics were comparable among the two groups, this

suggests that the lower rate of pgRNA detected in cells infected with the precore variant is due to the presence of the G1896A mutation. We think that intracellular pgRNA loads are lower because the packaging, reverse transcription and secretion are likely more efficient in the HBV G1896A mutant than in HBV wt. Although viremia are similar, with a very weak tendency to be higher in HBV G1896A infection, rcDNA and cccDNA levels are also slightly higher in HBV G1896A, suggesting that the very efficient if not higher replication efficiency might support a faster spreading of the G1896A mutant because of its altered epsilon structure (ε structure). It has been proposed that the G1896A point mutation leads to a stronger interaction between U1858 and A1896 within the 28th codon of the pgRNA. This might stabilizes the ε structure of the pgRNA, thereby increasing the efficiency of encapsulation of pgRNA (Ito et al. 2018). In line with the findings in our study, this offers an explanation for the lower pgRNA amounts despite high levels of infectious particles.

In conclusion HBV viral kinetics and intrahepatic activity seem slightly increased in mice infected with the HBV precore variant in the absence of NK cells and B- or T- lymphocytes in human liver chimeric uPA/SCID/beige mice. Our results are in line with the previous findings, although the overall differences in vivo are not dramatic in a system where HBV wt already replicates at high levels. We conclude that the G1896A mutation might lead to an increased efficiency of pgRNA packaging. These findings suggest that the altered epsilon structure - as a result of the G1896A mutation - supports replication fitness in human hepatocytes. Discrepancies between previous in vitro findings and in vivo results in this study may also be a result of the limitations of the in vitro models utilized in previous studies. Transfection of over-length HBV plasmids in HepG2 or Huh7 cell culture systems might not fully be able to depict the HBV replication cycle and therefore lead to discrepancies between previous in vitro findings and in vivo results in this study.

D.1.2 Impact of the HBeAg/precore protein on innate immunity related gene transcription

Acting as a first line of defense, the innate immune system plays a fundamental role in the control of foreign pathogens. The efficient induction of an interferon driven inflammatory response is considered a hallmark of adequate antiviral immunity. As previously described, HBV infection by itself does not trigger a strong innate immune response (Wieland et al. 2004; Dunn et al. 2009). The reason behind the apparent lack of a sufficient innate immune response towards HBV infection remains unclear. Different models of HBV immune-modulating activity have been proposed. Among these, it has been suggested that the HBeAg/precore protein may possess the capability to actively down-regulate the expression of innate immunity genes or to hinder their induction. It has been proposed that the HBeAg/precore protein might be able to suppress TLR expression as shown in patient derived monocytes and hepatocytes. The precore protein has also been shown to disrupt TLR-related signaling in vitro via co-localizing with the TIR-domain of the adapter molecules TIRAP/Mal and TRAM, displacing the TIRAP/Mal – MyD88 interaction (Lang et al. 2011; Visvanathan et al. 2007; Locarnini et al. 2005). To assess the impact of the HBeAg/precore protein, transcription levels of innate immunity related genes were compared among mice infected with HBV genotype D wild type and the corresponding HBV G1896A precore mutation variant. Aim of this study was to investigate whether the lack of the HBeAg/precore protein in HBV infected human liver chimeric uPA/SCID/beige mice would lead to different expression levels of certain innate immunity related genes in human hepatocytes in vivo. It has been previously described that gene expression related to innate immunity response was reduced in Huh7 cells in the presence of the precore protein. Huh7 cells were transfected with a 1.3 over length HBV plasmid in which core/precore transcription was controlled by a tetracycline responsive promoter. It has been found that in Huh7 cells expressing the precore protein 43 out of 45 genes examined, were down regulated up to 11-fold (Locarnini et al. 2005). In another study Visvanathan et al. evaluated the impact of the HBeAg within liver biopsies and whole blood samples of patients with either HBeAg positive (9 patients) or HBeAg negative (12 patients) CHB. Samples from patients with steatosis served as diseases controls in that study. In addition CHB patient derived PBMCs were stimulated via TLR2-ligandes

after being exposed to cell culture supernatant, derived from Huh-7 cells expressing either the precore or core protein in a tetracycline responsive expression system. The study described a reduced TLR2 expression in hepatocytes of HBeAg positive CHB patients when compared to HBeAg negative CHB or steatosis patients, while TLR4 expression was not impacted by CHB infection. Moreover, TLR2 expression was increased in HepG2 cells after transduction with precore negative recombinant HBV baculoviruses in comparison to transduction with precore positive recombinant HBV baculoviruses. Pam3Cys stimulation of PBMCs and whole blood samples indicated a reduced expression of TNF-α as well as Il-6 in PBMCs and lower expression TNF-α and TLR2 in whole blood samples of patients with HBeAg positive CHB. It was concluded that the lack of HBeAg/precore protein is associated with an up-regulation of the TLR2 signaling pathway, demonstrating a link between the HBeAg/precore protein and down-regulation of the innate immune response towards HBV infection (Visvanathan et al. 2007). As a follow up Lang et al. observed that the precore protein co-localizes with TIR-domain containing proteins Mal and TRAM. Co-localization of the precore protein and TIR domain containing proteins was shown in HEK293 cells co-transfected with plasmids expressing the HBeAg/precore protein and Flag-tagged TIR proteins Mal or TRAM. Co-immunoprecipitation was performed to demonstrate a possible interaction between the precore protein and TIR-containg proteins via a precore specific N-terminal sequence, which has been found to be similar to the TIR motif. Utilizing Huh7 cells or HEK293 cells it was shown that the precore protein might be able to disrupt Pam3Cys induced signaling via TIR:TIR disruption in Huh7/HEK293 cells. HEK293 cells were co-transfected with TIR-containing proteins as well as HBeAg/precore protein expressing plasmids. It was shown that TLR2, Mal, TRAM as well as TRIF dependent signaling was impaired with increasing dose of co-transfected HBeAg/precore protein expressing plasmids. The interaction of the HBeAg/precore protein with the TIR domain was suggested as a mechanism, which enables the HBeAg to actively function as an immune modulatory element by disrupting TLR2 innate immunity related signaling (Lang et al. 2011).

Based upon these in vitro studies, we would have expected to observe a regulatory effect of the HBeAg/precore protein in HBV wt infected uPA/SCID/beige mice in comparison with mice infected with the HBV G1896A

variant, in which the HBeAg/precore protein is missing. Since the HBeAg/precore protein has been suggested to disrupt TIR-domain dependent signaling, we expected to observe an up-regulated innate immunity response in human hepatocytes infected with the HBV G1986A variant because precore expression is abolished. In that case, no TIR:TIR disruption should take place, which could translate into a clearly distinguishable increased innate immune response in hepatocytes, reflected by an up-regulation in innate immunity related gene expression.

Our in vivo data show that regardless of the lack of the non-structural HBeAg/precore protein, HBV does not elicit an enhancement of the intrinsic antiviral response in primary human hepatocytes in vivo. In line with previous studies (Giersch et al. 2015), primary human hepatocytes overall did not respond with a strong inflammatory response towards HBV infection in human liver chimeric uPA/SCID/beige mice. This was reflected in a weak or lack of induction of most innate immunity related gene expression here analyzed. Out of all innate immunity related genes reviewed, merely the relative expression levels of MYD88, RIG1, TAP1, CXCL10, TGFB1 and IL6ST proved to be significantly induced in HBV infected mice (**table D.1.1**).

Table D.1.1 ***Gene expression significantly induced by HBV infection***

	uninfected	HBV wt		HBV G1896A	
	rel. median expression	rel. median expression	**fold induction**	rel. median expression	**fold induction**
MYD88	6.62E-03	1.22E-02	**1.84**	1.03E-02	**1.56**
RIG1	6.86E-03	1.39E-02	**2.03**	1.33E-02	**1.94**
TAP1	2.84E-03	7.66E-03	**2.70**	5.84E-03	**2.06**
CXCL10	3.23E-03	1.28E-02	**3.97**	7.93E-03	**2.45**
TGFB1	4.18E-03	1.32E-02	**3.17**	8.32E-03	**1.99**
IL6ST	1.27E-02	6.00E-02	**4.71**	4.02E-02	**3.16**

The slightly lower fold induction shown in HBV G1896A infection might be due to faster packaging of pgRNA which could provide the G1896A variant with a slightly more efficient way to circumvent immune recognition in human hepatocytes.

In addition, transcription levels of TLR2, STAT1 and SOCS3 appeared to be elevated in mice infected with HBV, but were not significantly induced by HBV infection regardless of HBeAg status (**table D.1.2**).

Table D.1.2 ***Gene expression likely to be induced by HBV infection***

	uninfected	HBV wt		HBV G1896A	
	rel. median expression	rel. median expression	**Fold induction**	rel. median expression	**Fold induction**
TLR2	5.54E-04	9.04E-04	**1.63**	7.90E-04	**1.43**
STAT1	2.47E-02	4.11E-02	**1.66**	4.05E-02	**1.64**
SOCS3	4.39E-04	7.55E-04	**1.72**	6.03E-04	**1.37**

Induction of innate immunity genes, even if significant, remained weak. These findings consolidate the assumption that HBV is able to avoid recognition from the innate immune response.

An immune-modulatory effect of the HBeAg/precore protein in human hepatocytes in the absence of an adaptive immune response, e.g. via the proposed disruption of the TLR-signaling, was clearly not shown. Our data show that HBV does not trigger an enhancement of intrinsic innate immune response in human hepatocytes in vivo – regardless of the lack or presence of the HBeAg/precore protein.

Hepatoma derived cell lines such as Huh7 might not fully be able to display the effects of TLR signaling since it has been previously described that Huh7 cells fail to respond towards ligand induced TLR signaling with an activation of NF-κB. (Preiss et al. 2008). In addition, the in vitro models mentioned above are unable to support the full infection and replication cycle of HBV and make use of transfection to simulate HBV protein expression. Disruption of ligand stimulated TIR-dependent signaling in HEK293 cells via co-transfection of an HBeAg expression plasmid was also dose dependent. This might suggest that the co-localization of intracellular precore protein with TIR-domain containing proteins might be negligible under physiological conditions, where concentration of intracellular precore protein might be significantly lower than in a cell culture system. In addition overexpression of plasmids in hepatoma derived cell cultures

does not represent the HBV infection and the intrinsic innate immune response of human hepatocytes accurately. The human liver chimeric uPA/SCID/beige mice in vivo model supports real infection of primary human hepatocytes and therefore much more accurately depicts the intrinsic innate immune response of human hepatocytes towards HBV infection.

It is also important to notice that the impact of proper parenchymal organization and presence of non-parenchymal liver cells, such as Kupffer cells, within an infectious model is hard to quantify and might indeed influence the innate immune response of hepatocytes toward HBV infection. The discrepancy in results from previous in vitro studies to our data shown in this study might therefore be a reflection of the limitations of the different in vitro HBV infection models employed.

With regard to the impact of HBeAg/precore protein, we conclude that the HBeAg/precore protein, in contrast to previously shown in vitro data, does not alter innate immunity related gene expression in human hepatocytes and therefore its absence does not lead to a baseline induction of ISGs which could facilitate recognition of HBV infection by the innate immune system.

D.1.3 Pam3Cys induction of innate immunity genes in HBV infected mice

As discussed above, this in vivo study showed that there is no HBeAg-mediated active downregulation of the intrinsic innate immune response of PHHs in HBV infection. Additionally and in line with previous studies (Wieland et al. 2004; Wieland and Chisari 2005; Dunn et al. 2009; Giersch and Dandri 2015; Giersch et al. 2015) we found that HBV infection did not trigger a strong innate immunity responds in infected human hepatocytes. This lack of induction of innate immunity genes as a response to HBV infection in PHHs is not HBeAg dependent. In order to evaluate the impact of the HBeAg/precore protein upon TIR-depended signaling following TLR2 stimulation in vivo, we utilized Pam3Cys stimulation as described earlier in B.3.7 and C.2.

When comparing overall murine and human gene transcription within Pam3Cys stimulated liver tissue, induction of murine innate immunity related genes appeared to be strong, while induction of human innate immunity related genes overall appeared to be weak. Transcription levels of mTlr2, mIfnb, mCxcl10, mTnfa and mIl6 where clearly elevated following Pam3Cys treatment. While transcription of human TAP1 and human CXCL10 did appear to be induced via Pam3Cys treatment, transcription of human TLR2 and human TGFβ was not further induced and transcription of human TNFα and human IL6 remained under the lower limit of detection, even after Pam3Cys treatment. The discrepancy in induction of human and murine gene transcription within liver tissue is likely due to the different cell type analyzed, which are known to express different levels of TLRs.

When determining human gene transcription, isolated RNA is derived only form human hepatocytes present in the human chimeric liver. Conversely, when analyzing murine gene transcription, RNA is derived from residual murine hepatocytes as well as murine non-parenchymal liver cells (e.g. Kupffer cells and sinusoidal endothelial cells) which are known to play a key role in the local innate immune response (Knolle et al. 1995). Following Pam3Cys treatment, induction of innate immunity related gene transcription in these murine non-parenchymal liver cells could disproportional overshadow transcription levels in murine hepatocytes. For example a severe hepatitis as a result of strong TLR2 driven antiviral response of macrophages has been demonstrated in mice infected with

hepatotrophic mouse hepatitis virus type 3 (Bleau et al. 2016). Gene transcription in murine hepatocytes could thus be considerable lower, approximating transcription levels in human hepatocytes, and could be masked by the induction of gene transcription in murine non-parenchymal liver cells. In addition, overall TLR2 expression in human hepatocytes might be considerable lower than average expression of TLR2 in non-parenchymal liver cells. Human hepatocytes have been shown to express TLR2 and display induced CXCL10 transcription after Pam3Cys stimulation in vitro (Luangsay and Ait-Goughoulte et al. 2015). Nevertheless the in vivo density of TLR2 expression on human hepatocytes in comparison to non-parenchymal cells and their capability to up regulate innate immunity related gene transcription in response to PAMP recognition remain in part unclear (Berzsenyi et al. 2011; Hari et al. 2019).

This raises the question whether and to what extent TLR2 signaling contributes to an intrinsic anti-viral response to HBV infection in human hepatocytes in human liver chimeric uPA/SCID/beige mice. It has been described that TLR-1/2 ligand stimulation in HepaRG cell lead to lower amounts of intracellular HBV DNA as well as decreased secretion of HBsAg and HBeAg (Luangsay and Ait-Goughoulte et al. 2015). Preliminary, we could show no such effect in vivo as a result of Pam3cys treatment. Although a limited number of mice was used, our study indicates that TLR2 stimulation is not able to induce a substantial antiviral effect in vivo in a system lacking human immune cells. TLR2 mediated signaling appears to be mainly triggered in murine NPCs, therefore further characterization of the cross talk between murine NPCs and PHHs as well as characterization of the expression and functionality of TLR2 in PHHs an NPCs is needed to evaluate the function of TLR2 signaling in HBV infection and broaden the understanding to what extent the human liver chimeric mouse model can be used for such studies.

D.2 Final conclusions

The findings in this study suggest that the regulatory role of the HBeAg/precore protein, as it has been proposed with regard to expression of innate immunity related genes, appears to be negligible in the context of HBV infection in primary human hepatocytes in vivo.

While the HBeAg/precore protein might be able to co-localizing with the TIR domain of adapter molecules in the TLR-signaling pathway or suppress TLR expression, this does not lead to an impaired gene transcription in human hepatocytes in vivo. We are able to show that, in the setting of human liver chimeric uPA/SCID/beige mice infected with HBV, the presence of the HBeAg/precore protein or its lack appears to be inconsequential with respect to the intrinsic innate immunity response in human hepatocytes. This was reflected in the expression of innate immunity related genes in human hepatocytes being unaffected by the HBeAg status. HBV infection and production of newly formed and functional viral particles was demonstrated regardless of the lack of the non-structural precore protein. We showed that pgRNA content appears to be slightly lower in HBV G1896A infected human hepatocytes indicating, in line with in vitro studies, that that pgRNA packaging may be more efficient. The weak antiviral response of human hepatocytes toward HBV infection in general, as it also was shown in this study, is unlikely to be associated with the HBeAg/precore protein.

The limitations of the human liver chimeric uPA/SCID/beige mouse model naturally restrict the ability to evaluate the influence of the adaptive immune response towards HBV infection. Since human liver chimeric uPA/SCID/beige mice lack B- and T- lymphocytes as well as natural killer cells, it is impossible to evaluate the effect of the HBeAg/precore protein upon these component of an antiviral response in HBV infection.

The induction of innate immunity related genes observed in murine NPCs, both as a result of HBV infection as well as Pam3Cys exposure may indicate that TLR-related recognition and signaling is predominantly active in NPCs like Kupffer cells in comparison to PHHs. However, it remains to be investigated whether NPC induction could trigger PHH responses, since species-specific barriers may limit the cross-talk between these cell components in the system used here. Excluding the interaction of HBV with other cellular components of the antiviral immune

response, we could provide in vivo evidence that the absence of the precore protein within the infected PHHs does not alter the capacities of these cells to sense HBV infection.

With regard to its function, studies have suggested a number of different possible mechanisms: Ranging from inhibition of interferon α signaling (Christen et al. 2007) to the lack of HBV recognition, in which HBV is described as a “stealth virus” (Luangsay and Gruffaz et al. 2015; Cheng et al. 2017). To summarize, mechanism that have been suggested recently in order to approach the apparent lack of an strong immune response toward HBV infection can be roughly classified into active immune modulation, immune response evasion or complete lack of recognition by PAMP recognition receptors. A recent study demonstrated that IFN or ISG expression in liver specimens from CHB patients was not elevated when compared to expression levels in control patients. Nevertheless stimulation of TLR3 with poly(I:C) prompted an IFN response and induced ISG expression in liver specimens derived from CHB patients. Indicating that, while CHB did not lead to an innate immune response, the innate immune response is not actively suppressed in infected liver tissue. In line with the results in vivo data demonstrated in this stud it was concluded that, rather than interfering with the innate immune response at all, HBV appears to be invisible to the recognition by PAMP receptors (Suslov et al. 2018).

In conclusion, the HBeAg/precore protein does not actively suppress innate immunity in human hepatocytes and its absence does not lead to higher levels of innate immunity responses, providing further evidence that HBV can bypasss or avoid immune recognition in infected cells. However, the contribution of secreted HBeAg to counteract the innate and adaptive immune responses deserve further investigations.

E Abstract

The HBeAg/precore protein of the hepatitis B virus has been reported to counteract the innate immune response in vitro (Locarnini et al. 2005; Visvanathan et al. 2007; Lang et al. 2011). Nevertheless, the role of HBeAg in the HBV life cycle, in vivo, and its capacity to modulate the expression of antiviral innate signaling pathways remains unclear. Previous studies have described an immune modulatory effect of the HBeAg/precore protein in vitro, such an effect has not yet been demonstrated in vivo. The aim of this study was to elucidate the role of HBeAg/precore protein in HBV infection kinetics, replicative activity and expression of innate immunity genes in vivo in human liver chimeric uPA/SCID/beige mice. UPA/SCID/beige mice were infected with cell culture derived HBV genotype D wild type or its corresponding G1896A precore mutation variant lacking HBeAg expression. Viral titers were evaluated over the course of 12 weeks. Intrahepatic viral parameters as well as transcription levels of innate immune response related genes were determined after 12 weeks of infection. In addition, mice were treated with the TLR1/TLR2 agonist Pam3Cys to evaluate the impact of the HBeAg/precore protein on TLR signaling. Infection kinetics did not differ substantially, although we detected significantly lower amounts of pgRNA/cccDNA/PHH, suggesting higher packaging capacities of the G1896A mutant. In line with previous findings, induction of innate immune response was weak in infected hepatocytes regardless of HBeAg status. Surprisingly the precore protein did not contribute to disrupt infection recognition or transcription of innate immunity related genes in HBV-infected primary human hepatocytes in human liver chimeric uPA/SCID/beige mice. Stimulation of the TLR2 pathway also had a weak impact on PHHs and, consequently, did not affect HBV activity HBV-infected PHHs in immune deficient human liver chimeric mice.

Das Precore-Protein/HBeAg des Hepatitis B Virus wurde in der Vergangenheit mit der Herunterregulation der angeborenen Immunantwort in Zusammenhang gebracht (Locarnini et al. 2005; Visvanathan et al. 2007; Lang et al. 2011). Dennoch ist die Rolle, die das HBeAg/Precore-Protein im HBV-Replikationszyklus spielt, sowie die Fähigkeit, Einfluss auf die anti-viralen Signalkaskaden zu nehmen immer noch nicht abschließend verstanden. In

vorangegangene in vitro Untersuchungen wurde ein immunmodulierender Effekt des HBeAg beschrieben, in vivo wurde ein solcher Effekt in Infektionsmodellen bisher nicht beschrieben. Ziel dieser Arbeit war es, in vivo den Ablauf der HBV-Infektion, die Replikationsaktivität von HBV sowie die Genexpression im Bezug auf die angeborene Immunantwort in humanisierten Mäusen zu untersuchen, in denen die Expression des HBeAg aufgrund der G1896A-Precore-Mutation fehlt. Dazu wurden humanisierte uPA/SCID/beige Mäuse mit dem HBV (Genotyp D Wildtyp) und der korrespondierenden G1896A Precore Mutationsvariante infiziert. Virale Titer wurden über einen von Zeitraum von 12 Wochen ausgewertet. Intrahepatische virale Parameter, sowie die Transkription von Genen der angeborenen Immunantwort, wurden nach 12 wöchiger Infektion bestimmt. Zusätzlich wurden Mäuse mit dem TLR1/TLR2 Agonisten Pam3Cys behandelt, um den Einfluss des HBeAg auf den TLR-Signalweg zu beurteilen. Die Untersuchung der Infektionskinetik legte keine Unterschiede zwischen dem HBV-Wildtyp und der HBV Precorevariante offen. In Einklang mit früheren Untersuchungen stellte sich die Reaktion der angeborenen Immunantwort in infizierten Tieren virusvariantenübergreifend als schwach dar. Entgegen der Erwartungen trägt das HBeAg nicht zur Reduktion der Erkennung oder der Transkription von Genen der angeborenen Immunantwort in HBV-infizierten humanen Hepatozyten in humanisierten uPA/SCID/beige Mäusen bei.

F List of Abbreviations

Ad-HBV	adenoviral vector based HBV
ADV	adefovir dipivoxil
ALT	alanine aminotransferase
ASHV	arctic squirrel hepatitis virus
AST	aspartate aminotransferase
BCP	basal core promoter
Casp1	caspase 1
cccDNA	covalently closed circular DNA
CDA	CDA Foundation/Polaris Observatory
CHB	chronic hepatitis B
COUP-TF1	chicken ovalbumin upstream promoter transcription factor 1
CXCL10	C-X-C Motif Chemokine Ligand 10
DD	death domain
DHBV	duck hepatitis B virus
DNA	deoxyribonucleic acid
DR1	direct repeat 1
EN2	enhancer II element
ER	endoplasmic reticulum
ETV	Entecavir
Fah	fumaryl acetoacetat hydrolase
GSHV	ground squirrel hepatitis virus
HBcAg	hepatitis core antigen
HBeAg	hepatitis b envelope antigen
HBsAg	hepatitis b surface antigen
HBV	hepatitis B virus
HBV wt	hepatitis B virus wild type
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis D virus
HIV	human immunodeficiency viruses
HLA-E	Major Histocompatibility Complex, Class I, E
HNF-3	hepatocyte nuclear factor 3
HNF-4	hepatocyte nuclear factor 4

IFN1	type 1 interferon
IFNAR	interferon-alpha/beta receptor alpha chain
IFNAR1	Interferon Alpha And Beta Receptor Subunit 1
IFNα	interferon alpha
IgG	immunoglobulin G
IgM	immunoglobulin M
IHME	Institute for Health Metrics and Evaluation
IKK	IκB kinase
IL	interleukin
IL28	interleukine 18
IL6ST	interleukin 6 Signal Transducer
IRAK	interleukin-1 (IL-1) receptor-associated kinase
IRF	interferon regulatory factor
ISG	interferon stimulated genes
ISG15	interferon stimulated gene 15
ISG20	interferon stimulated exonuclease gene 20
ISGF3	IFN-stimulated gene factor 3
ISRE	to IFN-stimulated response element
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
JAK1	Janus kinase 1
LAM	Lamivudine
MAP3K7 / TAK1	mitogen-activated protein kinase kinase 7
MAPK	mitogen-activated protein kinase
MDA5	melanoma differentiation associated gene 5
Mill2	MHC I-like leukocyte 2
mRNA	messenger RNA
MX1	MX dynamin like GTPase 1
MyD88	myeloid differentiation factor 88
MyrB	Myrcludex B
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
NOD	nucleotide-binding oligomerization domain receptor
NTCP	Na^+-taurocholate cotransporting polypeptide

OAS1	2'-5'-oligoadenylate synthetase 1
OBI	occult HBV infection
ORF	open reading frame
Pam3Cys	Pam3Cys-Ser-(Lys)4 trihydrochloride
Pam3Cys-SKKKK	Synthetic Pam3Cys analogue
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood monocyte
PCR	Polymerase chain reaction
PEG-IFN	pegylated interferon alpha
pgRNA	pregenomic RNA
PHH	primary human hepatocyte
PPAR α	peroxisome proliferator activated receptor α
preC mRNA	precore messenger RNA
PRR	pattern-recognition receptor
PSD	plasmid safe digestion
Rag-2	recombination activating gene 2
rcDNA	relaxed circular DNA
RIG1	retinoic acid-inducible gene 1
RIP-1	protein receptor interacting protein-1
RLR	retinoic acid-inducible gene-1 like receptor
RNA	ribonucleic acid
rtPCR	Real time polymerase chain reaction
RXRα	retinoid X receptor α
SARM	sterile-alpha and armadillo motif-containing protein
SCID	severe combined immunodeficiency
SGHBV	snow goose hepatitis B virus
Smc	structural maintenance of chromosomes complex
SOCS3	suppressor of cytokine signaling 3
STAT	signal transducer and activator of transcription
SVP	subviral particle
TAB1	TGF-beta activated kinase 1 binding protein 1
TANK	TRAF family member-associated NF-kappa B activator
TAP1	Transporter 1, ATP Binding Cassette Subfamily B
TBK1	TANK binding kinase-1

TAF	tenofovir alafenamide
TBP	TATA binding protein
TBV	Telbivudine
TDF	tenofovir disoproxil fumarate
TGFB1	Transforming Growth Factor Beta 1
TIR	Toll/IL-1 receptor
TIRAP / Mal	MyD88 adapter-like
TLR	toll-like receptor
TNF	tumor necrosis factor
TR2	human testicular receptor 2
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-b
TYK2	Tyrosine kinase 2
ULN ALT	Upper limit of normal ALT
uPA	urokinase-type plasminogen activator
URS	upstream regulatory sequence
USP18	ubiquitin specific peptidase 18
WHV	woodchuck hepatitis B virus
WHO	world health organization
WMHBV	woolly monkey hepatitis B virus

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I Bibliography

Abbas AK, Lichtman AH, Pillai S. Cellular and molecular immunology. Philadelphia, PA: Elsevier; 2018.

Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature. 2000;406(6797):782–7. doi:10.1038/35021228.

Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006a;124(4):783–801. doi:10.1016/j.cell.2006.02.015.

Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006b;124(4):783–801. doi:10.1016/j.cell.2006.02.015.

Alexopoulou A, Karayiannis P. HBeAg negative variants and their role in the natural history of chronic hepatitis B virus infection. World J Gastroenterol. 2014;20(24):7644–52. doi:10.3748/wjg.v20.i24.7644.

Allain J-P, Belkhiri D, Vermeulen M, Crookes R, Cable R, Amiri A, et al. Characterization of occult hepatitis B virus strains in South African blood donors. Hepatology. 2009;49(6):1868–76. doi:10.1002/hep.22879.

Allweiss L, Dandri M. Experimental in vitro and in vivo models for the study of human hepatitis B virus infection. J Hepatol. 2016;64(1 Suppl):31. doi:10.1016/j.jhep.2016.02.012.

Allweiss L, Dandri M. The Role of cccDNA in HBV Maintenance. Viruses 2017. doi:10.3390/v9060156.

Allweiss L, Volz T, Lütgehetmann M, Giersch K, Bornscheuer T, Lohse AW, et al. Immune cell responses are not required to induce substantial hepatitis B virus antigen decline during pegylated interferon-alpha administration. J. Hepatol. 2014;60(3):500–7. doi:10.1016/j.jhep.2013.10.021.

Araújo RC, Dias FC, Bertol BC, Silva DM, Almeida PH, Teixeira AC, et al. Liver HLA-E Expression Is Associated with Severity of Liver Disease in Chronic Hepatitis C. J Immunol Res. 2018;2018:2563563. doi:10.1155/2018/2563563.

Azuma H, Paulk N, Ranade A, Dorrell C, Al-Dhalimy M, Ellis E, et al. Robust expansion of human hepatocytes in Fah−/−/Rag2−/−/Il2rg−/− mice. Nat Biotechnol. 2007;25:903 EP -. doi:10.1038/nbt1326.

Baptista M, Kramvis A, Kew MC. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology. 1999;29(3):946–53. doi:10.1002/hep.510290336.

Barker LF, Chisari FV, McGrath PP, Dalgard DW, Kirschstein RL, Almeida JD, et al. Transmission of type B viral hepatitis to chimpanzees. J Infect Dis. 1973;127(6):648–62. doi:10.1093/infdis/127.6.648.

Bartenschlager R, Schaller H. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J. 1992;11(9):3413–20.

Baumert TF, Rogers SA, Hasegawa K, Liang TJ. Two core promotor mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest. 1996;98(10):2268–76. doi:10.1172/JCI119037.

Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, et al. IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized

mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. J Clin Invest. 2012;122(2):529–37. doi:10.1172/JCI58847.
Bertoletti A, Ferrari C. Innate and adaptive immune responses in chronic hepatitis B virus infections: Towards restoration of immune control of viral infection. Postgrad Med J. 2013;89(1051):294–304. doi:10.1136/postgradmedj-2011-301073rep.
Bertoletti A, Ferrari C. Adaptive immunity in HBV infection. J. Hepatol. 2016;64(1 Suppl):S71-S83. doi:10.1016/j.jhep.2016.01.026.
Bertoletti A, Kennedy PT. The immune tolerant phase of chronic HBV infection: new perspectives on an old concept. Cell Mol Immunol. 2015;12(3):258–63. doi:10.1038/cmi.2014.79.
Berzsenyi MD, Roberts SK, Preiss S, Woollard DJ, Beard MR, Skinner NA, et al. Hepatic TLR2 & TLR4 expression correlates with hepatic inflammation and TNF-α in HCV & HCV/HIV infection. J Viral Hepat. 2011;18(12):852–60. doi:10.1111/j.1365-2893.2010.01390.x.
Bissig K-D, Le TT, Woods N-B, Verma IM. Repopulation of adult and neonatal mice with human hepatocytes: A chimeric animal model. PNAS. 2007;104(51):20507–11. doi:10.1073/pnas.0710528105.
Bleau C, Burnette M, Filliol A, Piquet-Pellorce C, Samson M, Lamontagne L. Toll-like receptor-2 exacerbates murine acute viral hepatitis. Immunology. 2016;149(2):204–24. doi:10.1111/imm.12627.
Block TM, Guo H, Guo J-T. Molecular virology of hepatitis B virus for clinicians. Clin Liver Dis. 2007;11(4):685-706, vii. doi:10.1016/j.cld.2007.08.002.
Blumberg BS, ALTER HJ, VISNICH S. A "New" Antigen in Leukemia Sera. JAMA. 1965;191:541–6.
Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, Zentgraf H. Structural organization of the hepatitis B virus minichromosome. J Mol Biol. 2001;307(1):183–96. doi:10.1006/jmbi.2000.4481.
Brown JJ, Parashar B, Moshage H, Tanaka KE, Engelhardt D, Rabbani E, et al. A long-term hepatitis B viremia model generated by transplanting nontumorigenic immortalized human hepatocytes in Rag-2-deficient mice. Hepatology. 2000;31(1):173–81. doi:10.1002/hep.510310126.
Bruss V. Envelopment of the hepatitis B virus nucleocapsid. Virus Res. 2004;106(2):199–209. doi:10.1016/j.virusres.2004.08.016.
Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol. 1996;70(9):5845–51.
Carman WF, Hadziyannis S, Mcgarvey MJ, Jacyna MR, Karayiannis P, Makris A, Thomas HC. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. The Lancet. 1989;334(8663):588–91. doi:10.1016/S0140-6736(89)90713-7.
Chang M-H. Natural history and clinical management of chronic hepatitis B virus infection in children. Hepatol Int. 2008;2(Supplement 1):28–36. doi:10.1007/s12072-008-9050-9.
Chang SF, Netter HJ, Bruns M, Schneider R, Frölich K, Will H. A new avian hepadnavirus infecting snow geese (Anser caerulescens) produces a significant fraction of virions containing single-stranded DNA. Virology. 1999;262(1):39–54. doi:10.1006/viro.1999.9844.

Chen M, Sallberg M, Hughes J, Jones J, Guidotti LG, Chisari FV, et al. Immune Tolerance Split between Hepatitis B Virus Precore and Core Proteins. Journal of Virology. 2005;79(5):3016–27. doi:10.1128/JVI.79.5.3016-3027.2005.

Chen C-J, Yang H-I, Su J, Jen C-L, You S-L, Lu S-N, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA. 2006;295(1):65–73. doi:10.1001/jama.295.1.65.

Chen H, Jiang Z. The essential adaptors of innate immune signaling. Protein Cell. 2013;4(1):27–39. doi:10.1007/s13238-012-2063-0.

Cheng X, Xia Y, Serti E, Block PD, Chung M, Chayama K, et al. Hepatitis B virus evades innate immunity of hepatocytes but activates cytokine production by macrophages. Hepatology. 2017;66(6):1779–93. doi:10.1002/hep.29348.

Chou Y-C, Yu M-W, Wu C-F, Yang S-Y, Lin C-L, Liu C-J, et al. Temporal relationship between hepatitis B virus enhancer II/basal core promoter sequence variation and risk of hepatocellular carcinoma. Gut. 2008;57(1):91–7. doi:10.1136/gut.2006.114066.

Chow KT, Gale M. SnapShot: Interferon Signaling. Cell. 2015;163(7):1808-1808.e1. doi:10.1016/j.cell.2015.12.008.

Christen V, Duong F, Bernsmeier C, Sun D, Nassal M, Heim MH. Inhibition of alpha interferon signaling by hepatitis B virus. Journal of Virology. 2007;81(1):159–65. doi:10.1128/JVI.01292-06.

Chu CM, Liaw YF, Pao CC, Huang MJ. The etiology of acute hepatitis superimposed upon previously unrecognized asymptomatic HBsAg carriers. Hepatology. 1989;9(3):452–6.

Conner SH, Kular G, Peggie M, Shepherd S, Schüttelkopf AW, Cohen P, van Aalten DMF. TAK1-binding protein 1 is a pseudophosphatase. Biochem J. 2006;399(3):427–34. doi:10.1042/BJ20061077.

Cusson-Hermance N, Khurana S, Lee TH, Fitzgerald KA, Kelliher MA. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem. 2005;280(44):36560–6. doi:10.1074/jbc.M506831200.

Dandri M, Schirmacher P, Rogler CE. Woodchuck hepatitis virus X protein is present in chronically infected woodchuck liver and woodchuck hepatocellular carcinomas which are permissive for viral replication. Journal of Virology. 1996;70(8):5246–54.

Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, Sommer G, et al. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology. 2001;33(4):981–8. doi:10.1053/jhep.2001.23314.

Dandri M, Locarnini S. New insight in the pathobiology of hepatitis B virus infection. Gut. 2012;61 Suppl 1:17. doi:10.1136/gutjnl-2012-302056.

Dandri M, Petersen J. Chimeric mouse model of hepatitis B virus infection. J Hepatol. 2012;56(2):493–5. doi:10.1016/j.jhep.2011.05.037.

Dandri M, Petersen J. Animal models of HBV infection. Best Pract Res Clin Gastroenterol. 2017;31(3):273–9. doi:10.1016/j.bpg.2017.04.014.

Dane DS, Cameron CH, Briggs M. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet. 1970;1(7649):695–8.

Datta S, Chatterjee S, Veer V, Chakravarty R. Molecular biology of the hepatitis B virus for clinicians. J Clin Exp Hepatol. 2012;2(4):353–65. doi:10.1016/j.jceh.2012.10.003.

Decorsière A, Mueller H, van Breugel PC, Abdul F, Gerossier L, Beran RK, et al. Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. Nature. 2016;531(7594):386–9. doi:10.1038/nature17170.

Delius H, Gough NM, Cameron CH, Murray K. Structure of the hepatitis B virus genome. J Virol. 1983;47(2):337–43.

Demetriou AA, Levenson SM, Novikoff PM, Novikoff AB, Chowdhury NR, Whiting J, et al. Survival, organization, and function of microcarrier-attached hepatocytes transplanted in rats. PNAS. 1986;83(19):7475–9. doi:10.1073/pnas.83.19.7475.

Drexler JF, Geipel A, König A, Corman VM, van Riel D, Leijten LM, et al. Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes. PNAS. 2013;110(40):16151–6. doi:10.1073/pnas.1308049110.

Dunn C, Peppa D, Khanna P, Nebbia G, Jones M, Brendish N, et al. Temporal Analysis of Early Immune Responses in Patients With Acute Hepatitis B Virus Infection. Gastroenterology. 2009;137(4):1289–300. doi:10.1053/j.gastro.2009.06.054.

EASL. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J. Hepatol. 2017;67(2):370–98. doi:10.1016/j.jhep.2017.03.021.

Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, Rogiers V. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. Curr Drug Metab. 2006;7(6):629–60.

Fisicaro P, Valdatta C, Boni C, Massari M, Mori C, Zerbini A, et al. Early kinetics of innate and adaptive immune responses during hepatitis B virus infection. Gut. 2009;58(7):974–82. doi:10.1136/gut.2008.163600.

Friedt M, Gerner P, Lausch E, Trübel H, Zabel B, Wirth S. Mutations in the basic core promotor and the precore region of hepatitis B virus and their selection in children with fulminant and chronic hepatitis B. Hepatology. 1999;29(4):1252–8. doi:10.1002/hep.510290418.

Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature. 1979;281(5733):646–50. doi:10.1038/281646a0.

Galibert F, Chen TN, Mandart E. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: Comparison with the hepatitis B virus sequence. J Virol. 1982;41(1):51–65.

Gao S, Joshi SS, Osiowy C, Chen Y, Coffin CS, Duan Z-P. Chronic hepatitis B carriers with acute on chronic liver failure show increased HBV surface gene mutations, including immune escape variants. Virol. J. 2017;14(1):203. doi:10.1186/s12985-017-0870-x.

Gao Y, Zhao H, Wang P, Wang J, Zou L. The roles of SOCS3 and STAT3 in bacterial infection and inflammatory diseases. Scand J Immunol. 2018;88(6):e12727. doi:10.1111/sji.12727.

Gaudet R, Wiley DC. Structure of the ABC ATPase domain of human TAP1, the transporter associated with antigen processing. EMBO J. 2001;20(17):4964–72. doi:10.1093/emboj/20.17.4964.

Gerlich WH, Robinson WS. Hepatitis B virus contains protein attached to the 5' terminus of its complete DNA strand. Cell. 1980;21(3):801–9.

Giersch K, Dandri M. Hepatitis B and Delta Virus: Advances on Studies about Interactions between the Two Viruses and the Infected Hepatocyte. J Clin Transl Hepatol. 2015;3(3):220–9. doi:10.14218/JCTH.2015.00018.

Giersch K, Allweiss L, Volz T, Helbig M, Bierwolf J, Lohse AW, et al. Hepatitis Delta co-infection in humanized mice leads to pronounced induction of innate immune responses in comparison to HBV mono-infection. J Hepatol. 2015;63(2):346–53. doi:10.1016/j.jhep.2015.03.011.

Glebe D, Urban S. Viral and cellular determinants involved in hepadnaviral entry. World J Gastroenterol. 2007;13(1):22–38.

Glebe D, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH. Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. J Virol. 2003;77(17):9511–21. doi:10.1128/jvi.77.17.9511-9521.2003.

Gleizes PE, Beavis RC, Mazzieri R, Shen B, Rifkin DB. Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1. J. Biol. Chem. 1996;271(47):29891–6. doi:10.1074/jbc.271.47.29891.

Green R, Ireton RC, Gale M. Interferon-stimulated genes: New platforms and computational approaches. Mamm Genome. 2018;29(7-8):593–602. doi:10.1007/s00335-018-9755-6.

Grimm D, Thimme R, Blum HE. HBV life cycle and novel drug targets. Hepatol Int. 2011;5(2):644–53. doi:10.1007/s12072-011-9261-3.

Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A. 2002;99(24):15655–60. doi:10.1073/pnas.232137699.

Guirgis BSS, Abbas RO, Azzazy HME. Hepatitis B virus genotyping: Current methods and clinical implications. Int J Infect Dis. 2010;14(11):e941-53. doi:10.1016/j.ijid.2010.03.020.

Günther S. Genetic variation in HBV infection: Genotypes and mutants. J Clin Virol. 2006;36 Suppl 1:S3-S11.

Hantz O, Parent R, Durantel D, Gripon P, Guguen-Guillouzo C, Zoulim F. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. J Gen Virol. 2009;90(Pt 1):127–35. doi:10.1099/vir.0.004861-0.

Hari P, Millar FR, Tarrats N, Birch J, Quintanilla A, Rink CJ, et al. The innate immune sensor Toll-like receptor 2 controls the senescence-associated secretory phenotype. Sci Adv. 2019;5(6):eaaw0254. doi:10.1126/sciadv.aaw0254.

Hasegawa K, Huang JK, Wands JR, Obata H, Liang TJ. Association of hepatitis B viral precore mutations with fulminant hepatitis B in Japan. Virology. 1991;185(1):460–3.

Hasegawa K, Huang J, Rogers SA, Blum HE, Liang TJ. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. J. Virol. 1994;68(3):1651–9.

He Z, Zhang H, Zhang X, Xie D, Chen Y, Wangensteen KJ, et al. Liver xeno-repopulation with human hepatocytes in Fah-/-Rag2-/- mice after pharmacological immunosuppression. Am J Pathol. 2010;177(3):1311–9. doi:10.2353/ajpath.2010.091154.

Heckel JL, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell. 1990;62(3):447–56.
Herold G. Innere Medizin 2019: Eine vorlesungsorientierte Darstellung : unter Berücksichtigung des Gegenstandskataloges für die Ärztliche Prüfung : mit ICD 10-Schlüssel im Text und Stichwortverzeichnis. Köln: Herold Gerd; 2018.
Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T. Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell. 1990;63(6):1149–57. doi:10.1016/0092-8674(90)90411-7.
Hoffmann J, Boehm C, Himmelsbach K, Donnerhak C, Roettger H, Weiss TS, et al. Identification of α-taxilin as an essential factor for the life cycle of hepatitis B virus. J. Hepatol. 2013;59(5):934–41. doi:10.1016/j.jhep.2013.06.020.
Huang Y-H, Wu J-C, Chang T-T, Sheen I-J, Huo T-I, Lee P-C, et al. Association of core promoter/precore mutations and viral load in e antigen-negative chronic hepatitis B patients. J Viral Hepat. 2006;13(5):336–42. doi:10.1111/j.1365-2893.2005.00688.x.
Ichai P, Samuel D. Management of Fulminant Hepatitis B. Curr Infect Dis Rep. 2019;21(7):25. doi:10.1007/s11908-019-0682-9.
Ito K, Yoneda M, Sakamoto K, Mizokami M. Virological and Clinical Characteristics of Hepatitis B Virus Genotype A. J Gastroenterol. 2018;53(1):18–26. doi:10.1007/s00535-017-1367-5.
Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. Nat Immunol. 2015;16(4):343–53. doi:10.1038/ni.3123.
Janssens S, Beyaert R. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. Mol Cell. 2003;11(2):293–302.
Jirtle RL, Biles C, Michalopoulos G. Morphologic and histochemical analysis of hepatocytes transplanted into syngeneic hosts. Am J Pathol. 1980;101(1):115–26.
Kajikawa M, Ose T, Fukunaga Y, Okabe Y, Matsumoto N, Yonezawa K, et al. Structure of MHC class I-like MILL2 reveals heparan-sulfate binding and interdomain flexibility. Nat Commun. 2018;9(1):4330. doi:10.1038/s41467-018-06797-8.
Kakimi K, Guidotti LG, Koezuka Y, Chisari FV. Natural Killer T Cell Activation Inhibits Hepatitis B Virus Replication in Vivo. J Exp Med. 2000;192(7):921–30.
Kann M, Schmitz A, Rabe B. Intracellular transport of hepatitis B virus. World J Gastroenterol. 2007;13(1):39–47. doi:10.3748/wjg.v13.i1.39.
Kao J-H, Chen D-S. Global control of hepatitis B virus infection. The Lancet Infectious Diseases. 2002;2(7):395–403. doi:10.1016/S1473-3099(02)00315-8.
Kao J-H, Chen P-J, Lai M-Y, Chen D-S. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology. 2003;124(2):327–34. doi:10.1053/gast.2003.50053.
Kashiwamura S-i, Ueda H, Okamura H. Roles of interleukin-18 in tissue destruction and compensatory reactions. J Immunother. 2002;25 Suppl 1:S4-11.

Katze MG, He Y, Gale Jr M. Viruses and interferon: A fight for supremacy. Nature Reviews Immunology. 2002;2:675 EP -. doi:10.1038/nri888.
Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol. 2014;5:461. doi:10.3389/fimmu.2014.00461.
Kelsey G, Ruppert S, Schedl A, Schmid E, Thies E, Schütz G. Multiple effects on liver-specific gene expression in albino lethal mice caused by deficiency of an enzyme in tyrosine metabolism. J Cell Sci Suppl. 1992;16:117–22.
Kim H, Lee S-A, Do SY, Kim B-J. Precore/core region mutations of hepatitis B virus related to clinical severity. World J Gastroenterol. 2016;22(17):4287–96. doi:10.3748/wjg.v22.i17.4287.
Knolle P, Löhr H, Treichel U, Dienes HP, Lohse A, Schlaack J, Gerken G. Parenchymal and nonparenchymal liver cells and their interaction in the local immune response. Z Gastroenterol. 1995;33(10):613–20.
Köck J, Nassal M, MacNelly S, Baumert TF, Blum HE, Weizsäcker F von. Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus. J Virol. 2001;75(11):5084–9. doi:10.1128/JVI.75.11.5084-5089.2001.
Kollewe C, Mackensen A-C, Neumann D, Knop J, Cao P, Li S, et al. Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. J Biol Chem. 2004;279(7):5227–36. doi:10.1074/jbc.M309251200.
Königer C, Wingert I, Marsmann M, Rösler C, Beck J, Nassal M. Involvement of the host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA persistence reservoir of hepatitis B viruses. PNAS. 2014;111(40):E4244-53. doi:10.1073/pnas.1409986111.
Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, et al. Fulminant hepatitis B: Induction by hepatitis B virus mutants defective in the precore region and incapable of encoding E antigen. Gastroenterology. 1991;100(4):1087–94. doi:10.5555/uri:pii:001650859190286T.
Kramvis A, Kew MC. The core promoter of hepatitis B virus. J Viral Hepat. 1999;6(6):415–27.
Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. Biochem. Biophys. Res. Commun. 2009;388(4):621–5. doi:10.1016/j.bbrc.2009.08.062.
Kusakabe A, Tanaka Y, Mochida S, Nakayama N, Inoue K, Sata M, et al. Case-control study for the identification of virological factors associated with fulminant hepatitis B. Hepatol Res. 2009;39(7):648–56. doi:10.1111/j.1872-034X.2009.00519.x.
Kusano M, Mito M. Observations on the fine structure of long-survived isolated hepatocytes inoculated into rat spleen. Gastroenterology. 1982;82(4):616–28.
Lamberts C, Nassal M, Velhagen I, Zentgraf H, Schröder CH. Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. J. Virol. 1993;67(7):3756–62.
Lanford RE, Chavez D, Brasky KM, Burns RB, Rico-Hesse R. Isolation of a hepadnavirus from the woolly monkey, a New World primate. Proc Natl Acad Sci U S A. 1998;95(10):5757–61.
Lang T, Lo C, Skinner N, Locarnini S, Visvanathan K, Mansell A. The hepatitis B e antigen (HBeAg) targets and suppresses activation of the toll-like receptor

signaling pathway. J. Hepatol. 2011;55(4):762–9. doi:10.1016/j.jhep.2010.12.042.
Laskus T, Persing DH, Nowicki MJ, Mosley JW, Rakela J. Nucleotide sequence analysis of the precore region in patients with fulminant hepatitis B in the United States. Gastroenterology. 1993;105(4):1173–8.
Le Bert N, Gill US, Hong M, Kunasegaran K, Tan DZM, Ahmad R, et al. Effects of Hepatitis B Surface Antigen on Virus-Specific and Global T Cells in Patients With Chronic Hepatitis B Virus infection. Gastroenterology. 2020;159(2):652–64. doi:10.1053/j.gastro.2020.04.019.
Lee HM, Banini BA. Updates on Chronic HBV: Current Challenges and Future Goals. Curr Treat Options Gastroenterol 2019. doi:10.1007/s11938-019-00236-3.
Leenders WPJ, Hertogs K, Moshage H, Yap SH. Host and tissue tropism of hepatitis B virus. Liver. 1992;12(2):51–5. doi:10.1111/j.1600-0676.1992.tb00556.x.
Leong CR, Funami K, Oshiumi H, Mengao D, Takaki H, Matsumoto M, et al. Interferon-stimulated gene of 20 kDa protein (ISG20) degrades RNA of hepatitis B virus to impede the replication of HBV in vitro and in vivo. Oncotarget. 2016;7(42):68179–93. doi:10.18632/oncotarget.11907.
Levrero M, Pollicino T, Petersen J, Belloni L, Raimondo G, Dandri M. Control of cccDNA function in hepatitis B virus infection. J Hepatol. 2009;51(3):581–92. doi:10.1016/j.jhep.2009.05.022.
Li J, Ou JH. Differential regulation of hepatitis B virus gene expression by the Sp1 transcription factor. J Virol. 2001;75(18):8400–6. doi:10.1128/jvi.75.18.8400-8406.2001.
Li H, Zhuang Q, Wang Y, Zhang T, Zhao J, Zhang Y, et al. HBV life cycle is restricted in mouse hepatocytes expressing human NTCP. Cell. Mol. Immunol. 2014;11(2):175–83. doi:10.1038/cmi.2013.66.
Li JS, Tong SP, Wen YM, Vitvitski L, Zhang Q, Trépo C. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. J Virol. 1993;67(9):5402–10. doi:10.1128/JVI.67.9.5402-5410.1993.
Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med. 1991;324(24):1705–9. doi:10.1056/NEJM199106133242405.
Liaw Y-F, Brunetto MR, Hadziyannis S. The natural history of chronic HBV infection and geographical differences. Antivir Ther. 2010;15 Suppl 3:25–33. doi:10.3851/IMP1621.
Lin S-C, Lo Y-C, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. Nature. 2010;465(7300):885–90. doi:10.1038/nature09121.
Lin C-L, Kao J-H. Natural history of acute and chronic hepatitis B: The role of HBV genotypes and mutants. Best Pract Res Clin Gastroenterol. 2017;31(3):249–55. doi:10.1016/j.bpg.2017.04.010.
Liu B, Liao J, Rao X, Kushner SA, Chung CD, Chang DD, Shuai K. Inhibition of Stat1-mediated gene activation by PIAS1. PNAS. 1998;95(18):10626–31. doi:10.1073/pnas.95.18.10626.

Liu X, Xing H, Gao W, Di Yu, Zhao Y, Shi X, et al. A functional variant in the OAS1 gene is associated with Sjögren's syndrome complicated with HBV infection. Sci Rep. 2017;7(1):17571. doi:10.1038/s41598-017-17931-9.

Locarnini S. Hepatitis B viral resistance: Mechanisms and diagnosis. J Hepatol. 2003;39:124–32. doi:10.1016/S0168-8278(03)00318-0.

Locarnini S, Shaw T, Dean J, Colledge D, Thompson A, Li K, et al. Cellular response to conditional expression of the hepatitis B virus precore and core proteins in cultured hepatoma (Huh-7) cells. J Clin Virol. 2005;32(2):113–21. doi:10.1016/j.jcv.2004.10.002.

Loeb KR, Jerome KR, Goddard J, Huang M, Cent A, Corey L. High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system. Hepatology. 2000;32(3):626–9. doi:10.1053/jhep.2000.9878.

Lok AS, Akarca U, Greene S. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. Proceedings of the National Academy of Sciences. 1994;91(9):4077–81. doi:10.1073/pnas.91.9.4077.

Luangsay S, Gruffaz M, Isorce N, Testoni B, Michelet M, Faure-Dupuy S, et al. Early inhibition of hepatocyte innate responses by hepatitis B virus. J Hepatol. 2015;63(6):1314–22. doi:10.1016/j.jhep.2015.07.014.

Luangsay S, Ait-Goughoulte M, Michelet M, Floriot O, Bonnin M, Gruffaz M, et al. Expression and Functionality of Toll- and RIG-like receptors in HepaRG Cells. J. Hepatol. 2015. doi:10.1016/j.jhep.2015.06.022.

Lucifora J, Arzberger S, Durantel D, Belloni L, Strubin M, Levrero M, et al. Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. J Hepatol. 2011;55(5):996–1003. doi:10.1016/j.jhep.2011.02.015.

Lutfalla G, Gardiner K, Proudhon D, Vielh E, Uzé G. The structure of the human interferon alpha/beta receptor gene. J. Biol. Chem. 1992;267(4):2802–9.

Lutgehetmann M, Mancke LV, Volz T, Helbig M, Allweiss L, Bornscheuer T, et al. Humanized chimeric uPA mouse model for the study of hepatitis B and D virus interactions and preclinical drug evaluation. Hepatology. 2012;55(3):685–94. doi:10.1002/hep.24758.

Lütgehetmann M, Bornscheuer T, Volz T, Allweiss L, Bockmann J-H, Pollok JM, et al. Hepatitis B virus limits response of human hepatocytes to interferon-α in chimeric mice. Gastroenterology. 2011;140(7):2074. doi:10.1053/j.gastro.2011.02.057.

Macovei A, Radulescu C, Lazar C, Petrescu S, Durantel D, Dwek RA, et al. Hepatitis B virus requires intact caveolin-1 function for productive infection in HepaRG cells. J Virol. 2010;84(1):243–53. doi:10.1128/JVI.01207-09.

Makokha GN, Abe-Chayama H, Chowdhury S, Hayes CN, Tsuge M, Yoshima T, et al. Regulation of the Hepatitis B virus replication and gene expression by the multi-functional protein TARDBP. Sci Rep. 2019;9(1):8462. doi:10.1038/s41598-019-44934-5.

Malmström S, Larsson SB, Hannoun C, Lindh M. Hepatitis B viral DNA decline at loss of HBeAg is mainly explained by reduced cccDNA load--down-regulated transcription of PgRNA has limited impact. PLoS One. 2012;7(7):e36349. doi:10.1371/journal.pone.0036349.

Mandart E, Kay A, Galibert F. Nucleotide sequence of a cloned duck hepatitis B virus genome: Comparison with woodchuck and human hepatitis B virus sequences. J Virol. 1984;49(3):782–92.
Maruyama T, Schödel F, Iino S, Koike K, Yasuda K, Peterson D, Milich DR. Distinguishing between acute and symptomatic chronic hepatitis B virus infection. Gastroenterology. 1994;106(4):1006–15.
Mason WS, Seal G, Summers J. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J Virol. 1980;36(3):829–36.
Mason WS. Animal models and the molecular biology of hepadnavirus infection. Cold Spring Harb Perspect Med 2015. doi:10.1101/cshperspect.a021352.
Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 2001;7(8):927–33. doi:10.1038/90968.
Meuleman P, Libbrecht L, Vos R de, Hemptinne B de, Gevaert K, Vandekerckhove J, et al. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. Hepatology. 2005;41(4):847–56. doi:10.1002/hep.20657.
Morikawa K, Shimazaki T, Takeda R, Izumi T, Umumura M, Sakamoto N. Hepatitis B: Progress in understanding chronicity, the innate immune response, and cccDNA protection. Ann Transl Med. 2016;4(18):337. doi:10.21037/atm.2016.08.54.
Nassal M, Schaller H. Hepatitis B virus replication-an update. J Viral Hepat. 1996;3(5):217–26. doi:10.1111/j.1365-2893.1996.tb00047.x.
Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 2015. doi:10.1136/gutjnl-2015-309809.
Newbold JE, Xin H, Tencza M, Sherman G, Dean J, Bowden S, Locarnini S. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. J Virol. 1995;69(6):3350–7.
Newton K, Dixit VM. Signaling in Innate Immunity and Inflammation. Cold Spring Harbor Perspectives in Biology. 2012;4(3):a006049-a006049. doi:10.1101/cshperspect.a006049.
Ni Y, Lempp FA, Mehrle S, Nkongolo S, Kaufman C, Fälth M, et al. Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology. 2014;146(4):1070–83. doi:10.1053/j.gastro.2013.12.024.
Norder H, Arauz-Ruiz P, Blitz L, Pujol FH, Echevarria JM, Magnius LO. The T(1858) variant predisposing to the precore stop mutation correlates with one of two major genotype F hepatitis B virus clades. J Gen Virol. 2003;84(Pt 8):2083–7. doi:10.1099/vir.0.19034-0.
Ohashi K, Marion PL, Nakai H, Meuse L, Cullen JM, Bordier BB, et al. Sustained survival of human hepatocytes in mice: A model for in vivo infection with human hepatitis B and hepatitis delta viruses. Nat. Med. 2000;6(3):327–31. doi:10.1038/73187.
Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, et al. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J. Virol. 1990;64(3):1298–303.

Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, et al. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol. 1994;68(12):8102–10.

Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med. 1991;324(24):1699–704. doi:10.1056/NEJM199106133242404.

Ott JJ, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: New estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine. 2012;30(12):2212–9. doi:10.1016/j.vaccine.2011.12.116.

Ozasa A, Tanaka Y, Orito E, Sugiyama M, Kang J-H, Hige S, et al. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. Hepatology. 2006;44(2):326–34. doi:10.1002/hep.21249.

Peppa D, Micco L, Javaid A, Kennedy PTF, Schurich A, Dunn C, et al. Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog. 2010;6(12):e1001227. doi:10.1371/journal.ppat.1001227.

Perrillo RP. Therapy of hepatitis B -- viral suppression or eradication? Hepatology. 2006;43(2 Suppl 1):S182-93. doi:10.1002/hep.20970.

Petersen J, Dandri M, Mier W, Lutgehetmann M, Volz T, Weizsacker F von, et al. Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. Nat Biotechnol. 2008;26(3):335–41. doi:10.1038/nbt1389.

Prange R. Host factors involved in hepatitis B virus maturation, assembly, and egress. Med. Microbiol. Immunol. 2012;201(4):449–61. doi:10.1007/s00430-012-0267-9.

Preiss S, Thompson A, Chen X, Rodgers S, Markovska V, Desmond P, et al. Characterization of the innate immune signalling pathways in hepatocyte cell lines. J Viral Hepat. 2008;15(12):888–900. doi:10.1111/j.1365-2893.2008.01001.x.

Puente XS, Sánchez LM, Overall CM, López-Otín C. Human and mouse proteases: A comparative genomic approach. Nat Rev Genet. 2003;4(7):544–58. doi:10.1038/nrg1111.

Qi Y, Gao Z, Xu G, Peng B, Liu C, Yan H, et al. DNA Polymerase κ Is a Key Cellular Factor for the Formation of Covalently Closed Circular DNA of Hepatitis B Virus. PLoS Pathog. 2016;12(10):e1005893. doi:10.1371/journal.ppat.1005893.

Quasdorff M, Hösel M, Odenthal M, Zedler U, Bohne F, Gripon P, et al. A concerted action of HNF4alpha and HNF1alpha links hepatitis B virus replication to hepatocyte differentiation. Cell. Microbiol. 2008;10(7):1478–90. doi:10.1111/j.1462-5822.2008.01141.x.

Rabe B, Glebe D, Kann M. Lipid-mediated introduction of hepatitis B virus capsids into nonsusceptible cells allows highly efficient replication and facilitates the study of early infection events. J Virol. 2006;80(11):5465–73. doi:10.1128/JVI.02303-05.

Raftery N, Stevenson NJ. Advances in anti-viral immune defence: Revealing the importance of the IFN JAK/STAT pathway. Cell Mol Life Sci. 2017;74(14):2525–35. doi:10.1007/s00018-017-2520-2.

Raney AK, Johnson JL, Palmer CN, McLachlan A. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. J Virol. 1997;71(2):1058–71.

Rhim JA, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Replacement of diseased mouse liver by hepatic cell transplantation. Science. 1994;263(5150):1149–52. doi:10.1126/science.8108734.

Riera Romo M, Pérez-Martínez D, Castillo Ferrer C. Innate immunity in vertebrates: An overview. Immunology. 2016;148(2):125–39. doi:10.1111/imm.12597.

Rijntjes PJ, Moshage HJ, Yap SH. In vitro infection of primary cultures of cryopreserved adult human hepatocytes with hepatitis B virus. Virus Res. 1988;10(1):95–109.

Roder J, Duwe A. The beige mutation in the mouse selectively impairs natural killer cell function. Nature. 1979;278(5703):451–3. doi:10.1038/278451a0.

Rods J, Benhamou J-P, Blei AT, Reichen J, Rizzetto M. Textbook of Hepatology. Oxford, UK: Blackwell Publishing Ltd; 2007.

Romagnani P, Annunziato F, Lazzeri E, Cosmi L, Beltrame C, Lasagni L, et al. Interferon-inducible protein 10, monokine induced by interferon gamma, and interferon-inducible T-cell alpha chemoattractant are produced by thymic epithelial cells and attract T-cell receptor (TCR) alphabeta+ CD8+ single-positive T cells, TCRgammadelta+ T cells, and natural killer-type cells in human thymus. Blood. 2001;97(3):601–7. doi:10.1182/blood.v97.3.601.

Rumin S, Gripon P, Le Seyec J, Corral-Debrinski M, Guguen-Guillouzo C. Long-term productive episomal hepatitis B virus replication in primary cultures of adult human hepatocytes infected in vitro. J Viral Hepat. 1996;3(5):227–38. doi:10.1111/j.1365-2893.1996.tb00048.x.

Samal J, Kandpal M, Vivekanandan P. Hepatitis B "e" antigen-mediated inhibition of HBV replication fitness and transcription efficiency in vitro. Virology. 2015;484:234–40. doi:10.1016/j.virol.2015.06.011.

Sandgren EP, Palmiter RD, Heckel JL, Daugherty CC, Brinster RL, Degen JL. Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. Cell. 1991;66(2):245–56.

Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med. 1995;122(4):241–8. doi:10.7326/0003-4819-122-4-199502150-00001.

Sayed N, Ospino F, Himmati F, Lee J, Chanda P, Mocarski ES, Cooke JP. Retinoic Acid Inducible Gene 1 Protein (RIG1)-Like Receptor Pathway Is Required for Efficient Nuclear Reprogramming. Stem Cells. 2017;35(5):1197–207. doi:10.1002/stem.2607.

Scaglioni, P. P. et al. Biologic Properties of Hepatitis B Viral Genomes with Mutations in the Precore Promoter and Precore Open Reading Frame. Virology. 1997;233:374–81.

Schaefer S. Hepatitis B virus taxonomy and hepatitis B virus genotypes. World J Gastroenterol. 2007;13(1):14. doi:10.3748/wjg.v13.i1.14.

Schmit N, Nayagam S, Thursz MR, Hallett TB. The global burden of chronic hepatitis B virus infection: comparison of country-level prevalence estimates from four research groups. Int J Epidemiol. 2021;50(2):560–9. doi:10.1093/ije/dyaa253.

Schmitz A, Schwarz A, Foss M, Zhou L, Rabe B, Hoellenriegel J, et al. Nucleoporin 153 arrests the nuclear import of hepatitis B virus capsids in the nuclear basket. PLoS Pathog. 2010;6(1):e1000741. doi:10.1371/journal.ppat.1000741.

Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. Curr Opin Virol. 2011;1(6):519–25. doi:10.1016/j.coviro.2011.10.008.

Schulze A, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology. 2007;46(6):1759–68. doi:10.1002/hep.21896.

Schulze A, Mills K, Weiss TS, Urban S. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. Hepatology. 2012;55(2):373–83. doi:10.1002/hep.24707.

Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: A systematic review of data published between 1965 and 2013. The Lancet. 2015;386(10003):1546–55. doi:10.1016/S0140-6736(15)61412-X.

Seeger C, Ganem D, Varmus HE. Nucleotide sequence of an infectious molecularly cloned genome of ground squirrel hepatitis virus. J Virol. 1984;51(2):367–75.

Seeger C, Mason WS. Hepatitis B Virus Biology. Microbiol Mol Biol Rev. 2000;64(1):51–68.

Seeger C, Mason WS. Molecular biology of hepatitis B virus infection. Virology. 2015;479-480:672–86. doi:10.1016/j.virol.2015.02.031.

Shi W, Zhang Z, Ling C, Zheng W, Zhu C, Carr MJ, Higgins DG. Hepatitis B virus subgenotyping: History, effects of recombination, misclassifications, and corrections. Infect Genet Evol. 2013;16:355–61. doi:10.1016/j.meegid.2013.03.021.

Shi X, Jiao B, Chen Y, Li S, Chen L. MxA is a positive regulator of type I IFN signaling in HCV infection. J Med Virol. 2017;89(12):2173–80. doi:10.1002/jmv.24867.

Shlomai A, Schwartz RE, Ramanan V, Bhatta A, Jong YP de, Bhatia SN, Rice CM. Modeling host interactions with hepatitis B virus using primary and induced pluripotent stem cell-derived hepatocellular systems. Proc Natl Acad Sci U S A. 2014;111(33):12193–8. doi:10.1073/pnas.1412631111.

Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, et al. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology. 2010;51(1):297–305. doi:10.1002/hep.23354.

Sooryanarain H, Rogers AJ, Cao D, Haac MER, Karpe YA, Meng X-J. ISG15 Modulates Type I Interferon Signaling and the Antiviral Response during Hepatitis E Virus Replication. J Virol 2017. doi:10.1128/JVI.00621-17.

Stack J, Doyle SL, Connolly DJ, Reinert LS, O'Keeffe KM, McLoughlin RM, et al. TRAM is required for TLR2 endosomal signaling to type I IFN induction. J. Immunol. 2014;193(12):6090–102. doi:10.4049/jimmunol.1401605.

Standring S, Ananad N, editors. Gray's anatomy: The anatomical basis of clinical practice ; [get full access and more at ExpertConsult.com]. 41st ed. Philadelphia, Pa.: Elsevier; 2016.

Sterneck M, Günther S, Santantonio T, Fischer L, Broelsch CE, Greten H, Will H. Hepatitis B virus genomes of patients with fulminant hepatitis do not share a specific mutation. Hepatology. 1996;24(2):300–6. doi:10.1002/hep.510240203.

Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, et al. Genomic analysis of the host response to hepatitis C virus infection. PNAS. 2002;99(24):15669–74. doi:10.1073/pnas.202608199.

Summers J, Smolec JM, Snyder R. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc Natl Acad Sci U S A. 1978;75(9):4533–7. doi:10.1073/pnas.75.9.4533.

Sunbul M. Hepatitis B virus genotypes: Global distribution and clinical importance. World J Gastroenterol. 2014;20(18):5427–34. doi:10.3748/wjg.v20.i18.5427.

Sureau C, Romet-Lemonne J-L, Mullins JI, Essex M. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell. 1986;47(1):37–47. doi:10.1016/0092-8674(86)90364-8.

Suslov A, Boldanova T, Wang X, Wieland S, Heim MH. Hepatitis B Virus Does Not Interfere With Innate Immune Responses in the Human Liver. Gastroenterology. 2018;154(6):1778–90. doi:10.1053/j.gastro.2018.01.034.

Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003;21:335–76. doi:10.1146/annurev.immunol.21.120601.141126.

Takeuchi O, Akira S. Innate immunity to virus infection. Immunol Rev. 2009;227(1):75–86. doi:10.1111/j.1600-065X.2008.00737.x.

Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805–20. doi:10.1016/j.cell.2010.01.022.

Tang H, McLachlan A. Transcriptional regulation of hepatitis B virus by nuclear hormone receptors is a critical determinant of viral tropism. Proceedings of the National Academy of Sciences. 2001;98(4):1841–6. doi:10.1073/pnas.98.4.1841.

Thomas E, Liang TJ. Experimental models of hepatitis B and C - new insights and progress. Nat Rev Gastroenterol Hepatol. 2016;13(6):362–74. doi:10.1038/nrgastro.2016.37.

Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA. Pattern recognition receptors and the innate immune response to viral infection. Viruses. 2011;3(6):920–40. doi:10.3390/v3060920.

Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology. 1996;24(3):714–7. doi:10.1002/hep.510240340.

Tong S, Revill P. Overview of hepatitis B viral replication and genetic variability. J. Hepatol. 2016;64(1 Suppl):S4-S16. doi:10.1016/j.jhep.2016.01.027.

Trépo C, Chan HLY, Lok A. Hepatitis B virus infection. The Lancet. 2014;384(9959):2053–63. doi:10.1016/S0140-6736(14)60220-8.

Tsai A, Kawai S, Kwei K, Gewaily D, Hutter A, Tong DR, et al. Chimeric constructs between two hepatitis B virus genomes confirm transcriptional impact of core promoter mutations and reveal multiple effects of core gene mutations. Virology. 2009;387(2):364–72. doi:10.1016/j.virol.2009.03.002.

Tseng T-C, Liu C-J, Yang H-C, Chen C-L, Yang W-T, Tsai C-S, et al. Higher proportion of viral basal core promoter mutant increases the risk of liver cirrhosis in hepatitis B carriers. Gut. 2015;64(2):292–302. doi:10.1136/gutjnl-2014-306977.

Tsurimoto T, Fujiyama A, Matsubara K. Stable expression and replication of hepatitis B virus genome in an integrated state in a human hepatoma cell line transfected with the cloned viral DNA. PNAS. 1987;84(2):444–8. doi:10.1073/pnas.84.2.444.

Verstak B, Nagpal K, Bottomley SP, Golenbock DT, Hertzog PJ, Mansell A. MyD88 adapter-like (Mal)/TIRAP interaction with TRAF6 is critical for TLR2- and TLR4-mediated NF-kappaB proinflammatory responses. J. Biol. Chem. 2009;284(36):24192–203. doi:10.1074/jbc.M109.023044.

Visvanathan K, Skinner NA, Thompson AJV, Riordan SM, Sozzi V, Edwards R, et al. Regulation of Toll-like receptor-2 expression in chronic hepatitis B by the precore protein. Hepatology. 2007;45(1):102–10. doi:10.1002/hep.21482.

Volz T, Lutgehetmann M, Wachtler P, Jacob A, Quaas A, Murray JM, et al. Impaired intrahepatic hepatitis B virus productivity contributes to low viremia in most HBeAg-negative patients. Gastroenterology. 2007;133(3):843–52. doi:10.1053/j.gastro.2007.06.057.

Volz T, Allweiss L, Ben MBarek M, Warlich M, Lohse AW, Pollok JM, et al. The entry inhibitor Myrcludex-B efficiently blocks intrahepatic virus spreading in humanized mice previously infected with hepatitis B virus. J Hepatol. 2013;58(5):861–7. doi:10.1016/j.jhep.2012.12.008.

Walsh R, Locarnini S. Hepatitis B precore protein: Pathogenic potential and therapeutic promise. Yonsei Med J. 2012;53(5):875–85. doi:10.3349/ymj.2012.53.5.875.

Walter E. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. Hepatology. 1996;24(1):1–5. doi:10.1053/jhep.1996.v24.pm0008707245.

Wang, Y., Ning, X., Gao, P., Wu, S., Sha, M., Lv, M., et al. Inflammasome Activation Triggers Caspase-1-Mediated Cleavage of cGAS to Regulate Responses to DNA Virus Infection. Immunity. 2017;46(3):393–404. doi:10.1016/j.immuni.2017.02.011.

Wang, M., Xi, D., Ning, Q. Virus-induced hepatocellular carcinoma with special emphasis on HBV. Hepatol Int. 2017;11(2):171–80. doi:10.1007/s12072-016-9779-5.

Wei X, Peterson DL. Expression, Purification, and Characterization of an Active RNase H Domain of the Hepatitis B Viral Polymerase. J. Biol. Chem. 1996;271(51):32617–22. doi:10.1074/jbc.271.51.32617.

Wei C, Ni C, Song T, Liu Y, Yang X, Zheng Z, et al. The hepatitis B virus X protein disrupts innate immunity by downregulating mitochondrial antiviral signaling protein. J Immunol. 2010;185(2):1158–68. doi:10.4049/jimmunol.0903874.

WHO. Guidelines for the Prevention Care and Treatment of Persons with Chronic Hepatitis B Virus Infection. Geneva: World Health Organization; 2015.

Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. PNAS. 2004;101(17):6669–74. doi:10.1073/pnas.0401771101.

Wieland SF. The chimpanzee model for hepatitis B virus infection. Cold Spring Harb Perspect Med 2015. doi:10.1101/cshperspect.a021469.

Wieland SF, Chisari FV. Stealth and cunning: Hepatitis B and hepatitis C viruses. Journal of Virology. 2005;79(15):9369–80. doi:10.1128/JVI.79.15.9369-9380.2005.

Xu Y, Hu Y, Shi B, Zhang X, Wang J, Zhang Z, et al. HBsAg inhibits TLR9-mediated activation and IFN-alpha production in plasmacytoid dendritic cells. Mol Immunol. 2009;46(13):2640–6. doi:10.1016/j.molimm.2009.04.031.

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife. 2012;1:e00049. doi:10.7554/eLife.00049.

Yan H, Peng B, He W, Zhong G, Qi Y, Ren B, et al. Molecular determinants of hepatitis B and D virus entry restriction in mouse sodium taurocholate cotransporting polypeptide. J Virol. 2013;87(14):7977–91. doi:10.1128/JVI.03540-12.

Yang C, Ruan P, Ou C, Su J, Cao J, Luo C, et al. Chronic hepatitis B virus infection and occurrence of hepatocellular carcinoma in tree shrews (Tupaia belangeri chinensis). Virol. J. 2015;12:26. doi:10.1186/s12985-015-0256-x.

Yu X, Mertz JE. Promoters for synthesis of the pre-C and pregenomic mRNAs of human hepatitis B virus are genetically distinct and differentially regulated. J Virol. 1996;70(12):8719–26.

Yu X, Mertz JE. Differential regulation of the pre-C and pregenomic promoters of human hepatitis B virus by members of the nuclear receptor superfamily. J Virol. 1997;71(12):9366–74.

Zoulim F, Seeger C. Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase. J. Virol. 1994;68(1):6–13.

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