Hepatitis D virus infection in humanized uPA/SCID mice: Characterization of virus-host interactions, persistence and impact of antiviral treatments

Dissertation
to obtain the academic degree of
Doctor of Natural Sciences (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
of the Free University Berlin

by
Katja Giersch
from Berlin

2014

This doctoral dissertation was supervised by Prof. Dr. Maura Dandri and performed at the I. Department of Internal Medicine, University Medical Center Hamburg-Eppendorf, in Hamburg, Germany from November 2011 until September 2014.

1. Referee: Prof. Dr. M. Melzig
2. Referee: Prof. Dr. M. Dandri

Disputation: February 24th 2015

Danke!

Mein besonderer Dank geht an Prof. Maura Dandri, die ein wertvolles Mausmodell nach Hamburg gebracht und eine großartige Forschergruppe am Universitätsklinikum Hamburg-Eppendorf aufgebaut hat und somit diese Dissertation möglich machte. Ich danke Prof. Maura Dandri und Marc Lütgehetmann für die Ausschreibung des spannenden Themas der Arbeit, für die außergewöhnliche Betreuung und Unterstützung während der letzten drei Jahre und für die Möglichkeit, die zahlreichen Kongresse besuchen zu können. Ich möchte mich darüber hinaus für das Vertrauen und die Freiräume beim Anfertigen dieser Arbeit bedanken. Auch ohne Tassilo Volz, der unermüdlich wissenschaftliche und nicht-wissenschaftliche Fragen beantwortete, würde es diese Arbeit nicht geben.

Danke an die gesamte Arbeitsgruppe für die gute Zusammenarbeit, Hilfsbereitschaft und die tolle Atmosphäre inner- und außerhalb des Labors. Insbesondere danke ich Lena Allweiss für den wissenschaftlichen Austausch und für das mehrmalige Korrekturlesen dieser Arbeit, Claudia Dettmer und Gerlinde Apitzsch für ihre unermüdliche Hilfe im Labor, Anne Groth für die intensive Betreuung der Mäuse, Lida Mancke und Martina Helbig für die Vorarbeiten und Etablierung des HDV-Mausmodells und den vielen anderen Doktoranden, die so viel Spaß in unsere Gruppe gebracht haben (Oliver Bhadra, Sofia Gass, Nicola Oehler, Jan-Lukas Braunschmidt, Maria Homs, Janine Kah).

Besonders bedanken möchte ich mich bei Prof. Matthias Melzig von der Freien Universität Berlin für die Unterstützung und Beratung während meiner gesamten Promotionszeit.

Abschließend bin ich meiner Familie dankbar, dass sie mein wissenschaftliches Interesse gefördert und mich auf all meinen Wegen unterstützt hat.

Abbreviations

AAT	alpha antitrypsin
Alb	albumin
ADAR	adenosine deaminase acting on RNA
ADV	adefovir
ALT	alanine aminotransferase
APOBEC	apolipoprotein B mRNA editing enzymes, catalytic polypeptide-like
AST	aspartate aminotransferase
BCA	bicinchoninic acid
CARD	caspase activation and recruitment domain
Casp8	caspase 8
CD	cluster of differentiation
Dapi	4', 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
cccDNA	covalently closed circular DNA
CK18	cytokeranin 18
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
ERGIC	ER-Golgi intermediate compartment
ESCRT	endosomal sorting complexes required for transport
ETV	entecavir
Fah	fumarylacetoacetate hydrolase
FDA	food and drug administration
Fw	forward
GAPDH	glyceraldehyde-3- phosphate dehydrogenase
GAS	IFN-γ-activated sequence
GTP	guanosine triphosphate
h..	human
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBIG	hepatitis B immune globulin

HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBxAg	hepatitis B x antigen
HCV	hepatitis C virus
HDAg	hepatitis Delta antigen
HDV	hepatitis Delta virus
HLA	HLA class I histocompatibility antigen
HAS	human serum albumin
HepaRG	a human hepatocellular carcinoma cell line
HepG2	a human hepatocellular carcinoma cell line
HSPA6	heat shock 70 kDa protein 6
Hu(-CK18)	human (cytokeratin 18)
Huh7	a human hepatocellular carcinoma cell line
IFN	interferon
IFNAR1/2	interferon α/ß receptors ½
IL 28/29	interleukin 28/29
IgG	immunoglobulin G
IgM	immunoglobulin M
IKKε	inhibitor of kappaB kinase
INF	interferon
IP10	interferon gamma-induced protein
IRAK	interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISG	interferon stimulated gene
ISGF3	interferon stimulated gene factor 3
ISRE	IFN-stimulated response element
JAK	janus kinase
kDA	kilodalton
LAM	lamivudine
LdT	telbivudine
LLoD	lower limit of detection
LTX	liver transplantation
MAVS	mitochondrial antiviral-signaling protein
MOI	multiplicity of infection

mRNA	messenger RNA
MVB	multivescular bodies
MxA	myxovirus resistance A
NF-κB	nuclear factor-κB
Myd88	Myeloid differentiation primary response gene 88
NK cells	natural killer cells
NLR	NOD-like receptor
NOD	(I) non-obese diabetic (II) nucleotide oligomerization domain
NTBC	2 (2-nitro-4-trifluormethybenzoyl)-cyclohexane-1,3-dione
NTCP	Na-taurocholate cotransporting polypeptide
NUCs	nucleoside/nucleotide analogues
OAS1	2´,5´-oligoadenylate synthetase 1
PAMPs	Pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	poly chain reaction
Peg-IFN	pegylated interferon
pgRNA	pregenomic RNA
PIAS	protein inhibitor of activated STAT
PMSF	phenylmethylsulfonyl fluoride
Prkdc	protein kinase, DNA-activated, catalytic polypeptide
PRR	pattern recognition receptors
Rag-2	recombinant activation gene-2
Rc DNA	relaxed circular DNA
Rv	reverse
Rig-I	retinoic acid-inducible gene
RIP	receptor-interacting protein
RNA	ribonucleic acid
RLR	Rig-I-like receptor
RPL30	ribosomal protein L30
RT	reverse transcriptase
RT-PCR	real time polymerase chain reaction
SCID	severe combined immunodeficiency
STAT	signal transducer and activator of transcription
SVP	subviral envelope particle

TAP1	transporter associated with antigen processing
TBK	TANK-binding kinase
TBS-T	Tris-buffered saline with 0.1% Tween 20
TDF	tenofovir
TGF-ß	transforming growth factor ß
TLR	toll-like-receptor
TRAF	tumor necrosis factor receptor-associated factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
uPA	urokinase plasminogen activator
USP18	ubiquitin-specific peptidase 18
WHV	woodchuck hepatitis virus
WHO	world health organization

Contents

A. Introduction

A.1. Hepatitis Delta virus

A.1.1 Epidemiology and genotypes

The hepatitis Delta virus (HDV) was discovered in 1977 by Marcus Rizzetto[1]. The outbreak, which started in Italy, has been brought under control in Europe and in industrialized countries during the past 20 years[2], [3]. Despite efficient vaccination, still more than 15 million people worldwide are chronically infected with HDV[4]. Especially in developing continents like Asia and Africa hepatitis Delta infections remain one of the major health problems[5]. But also in industrialized countries HDV infections are not eradicated completely. Due to its fast decline in Europe and North America, the attention to HDV diminished, virus diagnosis was often neglected, the virus re-emerged and infection rates are increasing slightly nowadays[6], [7]. The Hepnet, a German competence network for hepatitis, estimates that around 30,000 people are infected with HDV in Germany[8].

Eight major genotypes of HDV with highly variable sequences are reported in different parts of the world (see **figure A.1.1**). Genotype 1 is the most frequent one with an occurrence in Europe, North America, Middle East, North Africa, India and partly in South America and Asia. Genotype 2 and 4 are seen in the Far East, whereas genotype 3 is observed in the northern parts of South America. The genotypes 5, 6, 7 and 8 are exclusively found in Africa or African migrants[9].

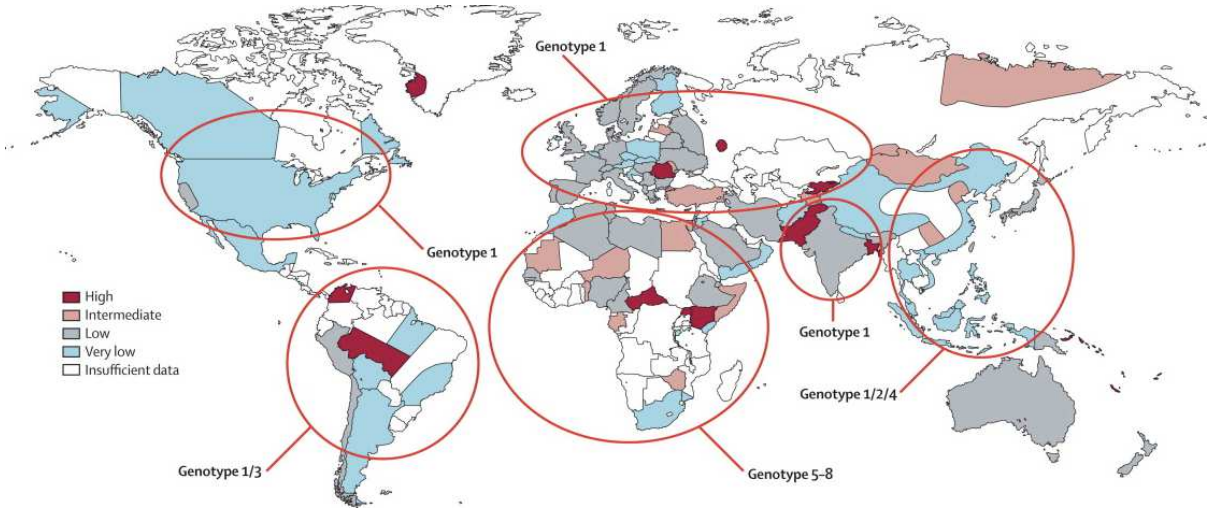


Figure A.1.1. Geographical distribution of the eight different HDV genotypes. *HDV genotype 1 is distributed in most parts of the world (Europe, Northern and Southern America, India, Middle East and Asia). Genotype 3 is predominant in Southern America, genotypes 2 and 4 in Asia and genotypes 5-8 are exclusively found in Africa**[10]**.*

A.1.2 Virus structure and replication cycle

The hepatitis Delta virion has a particle size of approximately 36 nm in diameter and is the smallest known RNA pathogen that can interact with a human host and cause substantial global morbidity and mortality. The inner nucleocapsid of the virus contains an only 1679 nucleotide long, circular RNA and around 200 molecules of hepatitis Delta antigen (HDAg), which is the only known protein encoded by the HDV RNA. Due to its simplicity in genome structure, HDV is a very unique animal virus with similarities to plant viroids[11], which are also small, single-stranded RNA pathogens, but which in contrast do not encode any protein[12].

HDV is a defective virus whose genome is surrounded by three hepatitis B virus (HBV) envelope proteins, termed small, medium and large hepatitis B surface antigen (HBsAg), and host lipids[13] (see **figure A.1.2**). HBV plays an important role as a helper virus for HDV and is stringently necessary for HDV transmission[14]. Therefore, HDV infections can only occur in patients co-infected with HBV or as a super-infection of an individual already infected with HBV.

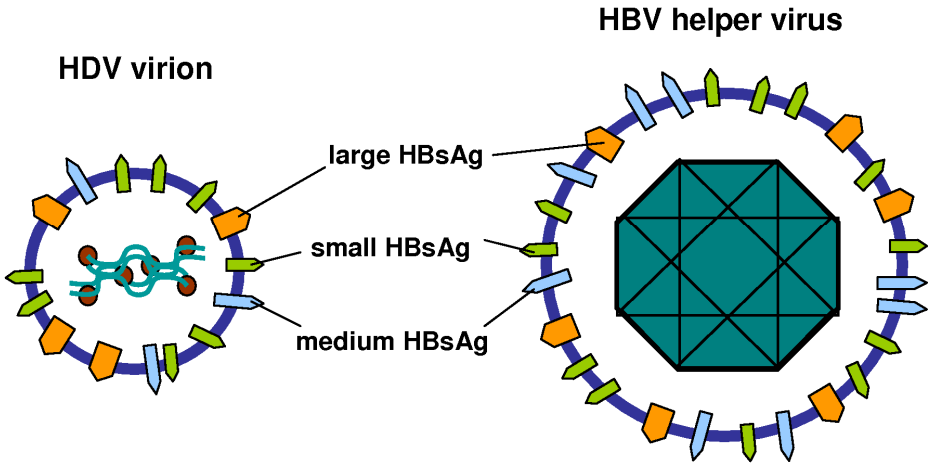


Figure A.1.2. HBV as a helper virus for HDV. *HBV shares its small, medium and large hepatitis B surface antigens (HBsAg) with HDV.*

The genome of hepatitis Delta virions is the circular, negative, single-stranded so called genomic RNA, which folds into an unbranched rod-like structure with many paired nucleotides[15]. However, as seen in **figure A.1.3 and A.1.4**, two other RNAs accumulate during virus replication, which takes place in hepatocytes: the antigenomic RNA as an exact complement of the genome and an 800 nucleotide mRNA with the same polarity as the antigenomic RNA. Approximately 100,000 to 300,000 copies of genomic, 7,000 to 65,000 copies of antigenomic and 600 copies of mRNA can be found in a replicating liver cell of woodchucks and chimpanzees[16]. In a double-rolling circle, host RNA polymerases I and II transform genomic and antigenomic RNA into each other without any DNA intermediates and therefore enable virus replication and assembly (see **figure A.1.3**).

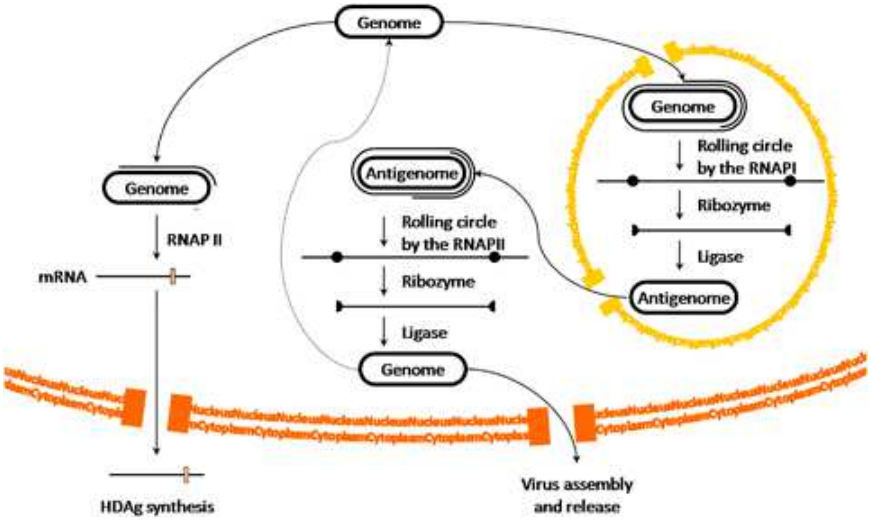


Figure A.1.3. Double rolling circle of replicating HDV. *The HDV genome replicates through the generation of genomic and antigenomic RNA with the help of the host RNA polymerase I and II but without DNA intermediates[17].*

A unique open reading frame on the antigenomic RNA leads via a complementary genomic RNA and mRNA to the synthesis of the HDAg, which occurs in two different forms: the small and large HDAg[18]. The small HDAg (24 kDa) is important for virus replication, whereas the large form (27 kDa) negatively influences replication and leads to virion assembly[19], [20]. Initially the small HDAg is synthesized. The large HDAg is generated through an ADAR-mediated RNA editing step at the antigenomic RNA, where an adenosine of a stop codon unit is switched into an ionosine[21], [22]. The single nucleotide change leads to a loss of the stop codon and produces the 3 kDa longer large HDAg (see **figure A.1.4**). The small HDAg is more abundant but both forms are essential for HDV productivity. The ratio of large to small HDAg is estimated to be about 0.1 in the infected liver, whereas in virions the ratio can increase up to 0.9[23].

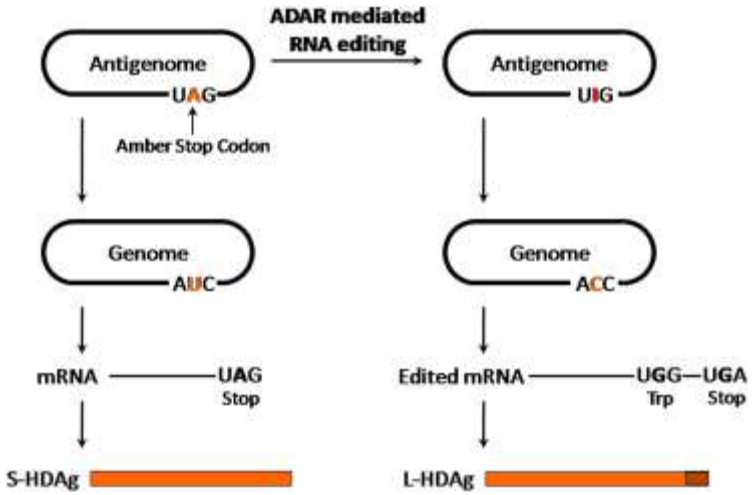


Figure A.1.4. Synthesis of small and large HDAg. *ADAR mediates a single nucleotide change from adenosine to ionosine, abolishing the formation of a stop codon and thus leading via a smaller and larger mRNA to the small and large hepatitis delta antigen (HDAg)[17].*

HDV shows a strict hepatotropism[24] and attaches to the hepatocytes of the host via a newly found receptor, the sodium taurocholate cotransporting polypeptide (NTCP), a transmembrane transporter for bile acids predominantly expressed in the liver[25]. After a reversible attachment of HDV to cell-surface-associated heparin sulfate proteoglycans[26], the large HBsAg (with its myristoylated N-terminus) irreversibly binds to NTCP[27] and HDV enters the hepatocyte via unknown mechanisms. Because HDV and HBV share the same envelope proteins, it is tempting to believe that the attachment and entry steps in the replication cycle of both viruses are similar. However, Sureau et al revealed that HBV looses its infectivity, when the virion is surrounded by small, medium and large HBV envelope proteins lacking the essential N-linked carbohydrates, whereas HDV remains infectious[28]. Although the detailed post entry mechanisms have not been elucidated completely, the basic replication cycle of HDV is known. After virion entry and uncoating[29], the HDV ribonucleoprotein is transported to the nucleus, where the genomic RNA is translated into its antigenomic form and the double-rolling replication circle starts[30]. HDV does not encode its own polymerase, therefore the virus hijacks the cellular host RNA polymerases to replicate its genome[31]. The mRNA is exported to the endoplasmic reticulum (ER) and transcribed into small and large HDAg, which then support virus replication and assembly at the nucleus. The balance between viral replication and assembly is conducted by the ratio of small and large HDAg and also by different post-

translational modifications of these proteins such as prenylation, phosphorylation, methylation and sumoylation[32]. In that way, newly synthesized ribonucleoproteins are released from the nucleus to the ER and associate with HBV envelope proteins to form new virus particles. HDV particles are exported from the hepatocytes via the Golgi complex and can infect other liver cells (see **figure A.1.5**).

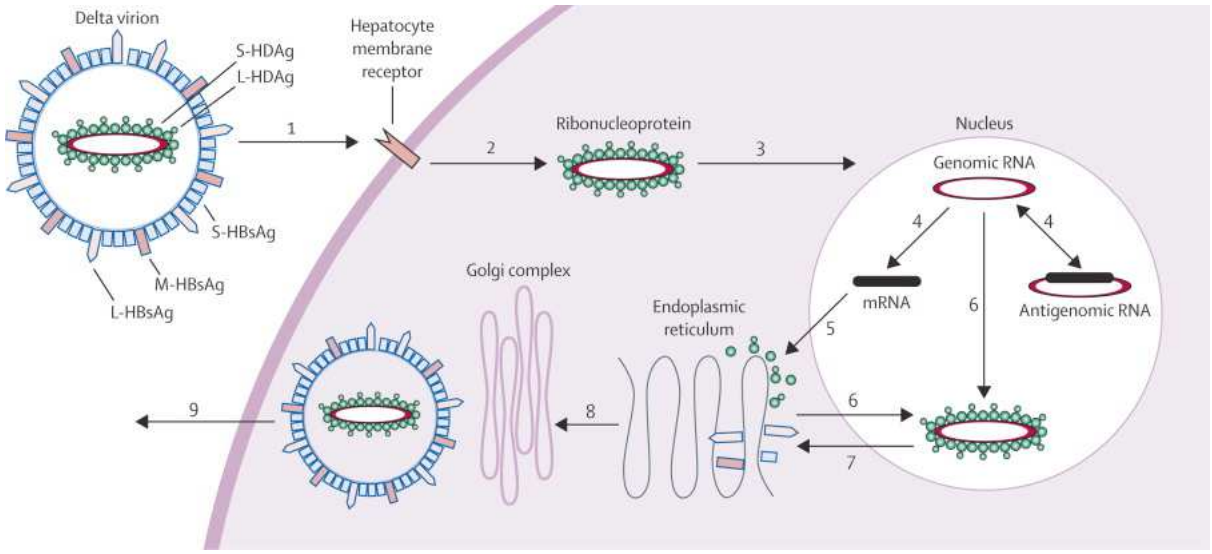


Figure A.1.5. HDV structure and replication cycle. *The hepatitis Delta virion attaches via its large HBsAg to the host hepatocyte (1). After entry and uncoating (2) the ribonucleoprotein is transported to the nucleus (3). The transformation of genomic and antigenomic RNA (4) enables the generation of HDAg at the ER (5). Small and large HDAg support virus replication at the nucleus (6) and virus assembly with HBsAg at the ER (7). New HDV particles are released from the hepatocyte via the Golgi complex (8, 9) to infect other cells[4].*

A.1.3 Diagnosis and treatment

HDV is transmitted parenterally through infected blood or body fluids. HBV/HDV co-infections often cause more severe symptoms than HBV mono-infections. An acute co-infection emerges after an incubation time of three to seven weeks and can either take an asymptomatic course or can show several non-specific symptoms like fatigue, lethargy, anorexia and nausea or result in acute liver failure[33]. In the setting of an HDV super-infection, up to 80% of the patients show a chronic course of disease, which is associated

with hepatocytes inflammation and fibrosis and may lead to fulminant liver cirrhosis over years. In contrast, an HBV/HDV co-infection mostly occurs as an acute, self-limited infection and leads in less than 5% to a chronic manifestation[34].

To determine an acute or chronic HBV/HDV co- or super-infection, HDV RNA, HDAg, IgM and IgG antibodies to HDAg are measured as specific markers. The first step towards establishing a diagnosis is to test for IgG antibodies to HDAg, which are universal in all infected patients and remain persistent during the chronic state of infection and even after seroconversion[35]. HDV RNA is an early, sensitive marker during the acute phase of infection with HBV/HDV and is present in most of the patients but becomes transient during the chronic state of co-infection. In a chronic super-infection, HDV RNA is persistent and usually remains detectable over time[36]. The determination of HDV RNA is not internationally standardized and does not correlate with disease activity or stage of liver fibrosis[37]. Another specific marker for HDV is HDAg, which is apparent early but disappears quickly in serum and may also become negative in hepatocytes during chronic infection[38]. Additionally testing for IgM antibodies to HDAg might be useful and to ascertain the extent of liver damage and failure, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) might be quantified as well.

Vaccination against HBV also protects efficiently against HDV infections. But in contrast to HBV infections and besides liver transplantation, therapy options for HDV are limited. Standard or pegylated interferon-α, which are licensed therapeutics in HBV infections, are also used as a treatment against HDV but with unsatisfied outcomes. When applying standard interferon-α, long term (12 months) and high dose administration (five million units daily or nine million units three times a week) seem to be the most effective condition. But despite a significant decrease of serum HDV RNA, viremia remain detectable until the end of treatment and HDV relapse is observed after treatment cessation in almost all patients [39]–[41]. Furthermore, complicating side effects like neutropenia, anaemia, fatigue and depression hamper the administration of interferon-α as a therapeutic against HDV[42].

Since pegylated interferon-α has a higher half-life and therefore an only weekly administration is required, standard interferon-α was mainly replaced by its pegylated form. However, different clinical trials reveal that better sustained virological responses cannot be

achieved. 57% of the patients treated with pegylated interferon-α for 12 months showed undetectable HDV RNA levels at the end of treatment, but only 43% at the end of a follow-up period of 6-42 months[43]. In three other clinical studies, pegylated interferon-α was administered for 48 or 52 weeks and sustained virological responses were achieved in 17%, 21% and 25% of patients at the end of follow up[44]–[46], demonstrating that recrudescence of HDV after treatment discontinuation remains a major problem.

Due to its simplicity in structure and the lack of producing its own polymerase, HDV offers much fewer targets than other viruses and common HBV or HCV therapeutics cannot directly target HDV infection. Nucleoside/nucleotide analogue as lamivudine[47], adefovir[48], [49] and entecavir[50] or the antiviral famciclovir[51] are potent reverse transcriptase inhibitors against HBV, but they do not show beneficial effects against HDV infections. Also ribavirin, which is a commonly used antiviral in chronic HCV infections and which was shown to block HDV genome replication in vitro[52], is ineffective for the treatment of HDV infected patients[41].

Since the knowledge about HDV is increasing (e.g. NTCP was discovered to be the entry receptor of HBV and HDV), novel therapies considering alternative antiviral targets emerge. Myrcludex-B, which is a myristoylated synthetic peptide binding NTCP and inhibiting viral attachment and entry, was shown to efficiently hinder the establishment of HDV infection in humanized mice[53]. Another preclinical study revealed that prenylation of the large HDAg is essential for virus assembly and release and that therefore prenylation inhibitors are able to decrease HDV RNA levels in vivo[54].

A.2. Hepatitis B virus

A.2.1 Epidemiology and genotypes

According to the WHO, about two billion people worldwide have been in contact with the hepatitis B virus (HBV) and 300 to 420 millions (five to seven percent of the human population) are chronically infected with HBV. Every year 600,000 people die due to the consequences of an HBV infection. The results of a German National Health survey in the year 1998 revealed that 7% of the German population had or have an acute HBV infection, while 0.7% are chronically infected[55]. Currently, the Hepnet estimates that approximately 300,000 to 650,000 people are carrier of the virus in Germany.

Eight main genotypes of HBV are distributed in different parts of the world. Genotype A is prevalent in Africa and Northwestern Europe, while genotype B and C are mainly found in Asia, Australia and New Zealand. Genotype D is predominant in Mediterranean countries, Middle East, Central Asia and India. Genotype E is restricted to West Africa, genotype F and H to Mexico and South America and genotype G to the United States and France. In the United States genotypes A, B, C, D and G are found[56] (**figure A.2.1**). The latest genotypes are genotype I, which was isolated in Vietnam[57] and Laos[58] in 2008, and genotype J, which was discovered in Japan in 2009[59].

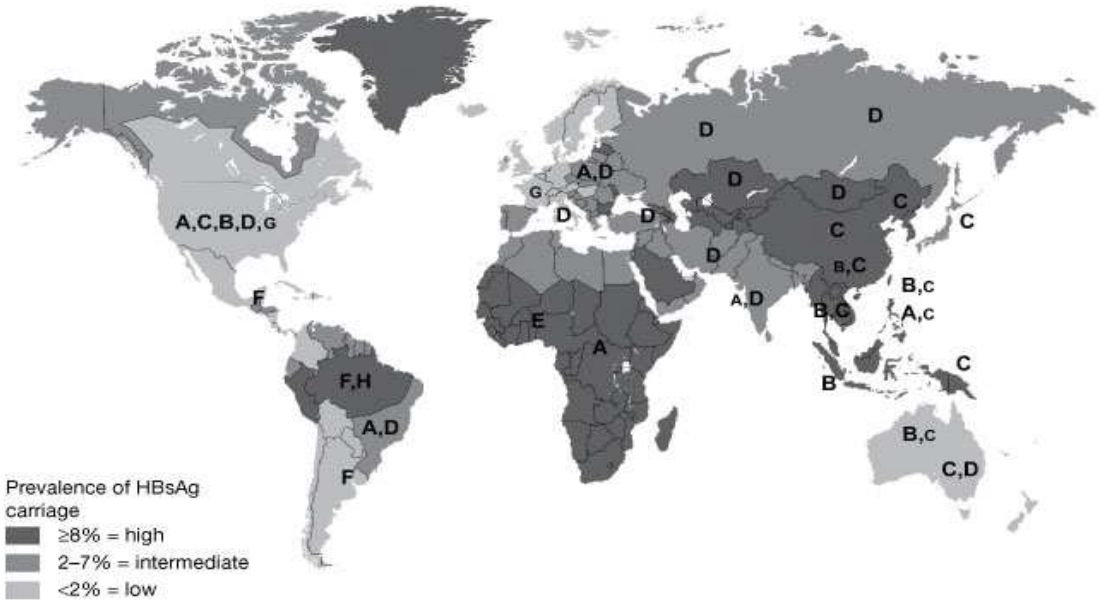


Figure A.2.1. Geographical distribution of HBV. *In Europe HBV genotypes A and D are predominant, while in Northern America genotypes A, B, C, D and G are found. Genotypes A, D and exclusively F and H are distributed in Middle and Southern America. In Africa genotypes A and E dominate, in Asia and the Middle East genotype D, while genotypes B, C and D are mainly found in Australia[60].*

A.2.2 Virus structure and replication cycle

The hepatitis B virus is a member of the hepadnaviridae family with a unique genome structure and replication cycle. HBV is a human-specific virus, which exclusively infects humans and chimpanzees[61], while HBV-related viruses can also infect mammals (woodchucks[62], squirrels[63]) and birds (peking duck[64], snow goose[65]).

The circular HBV genome is approximately 3,200 bp long, relaxed circular, partially double-stranded (rcDNA) and associated with the hepatitis B core protein (HBcAg), forming the nucleocapsid[66]. The viral envelope encloses the nucleocapsid and consists of a lipid membrane and the hepatitis B surface antigen (HBsAg), which exists in three different forms, the small, medium and large HBsAg[67]. As indicated in **figure A.2.2** the HBV genome contains four different genes: the S gene, which encodes for the three envelope proteins (HBsAg); the C gene, which encodes for the HBcAg and the hepatitis B e antigen (HBeAg); the P gene, which encodes for a multifunctional DNA polymerase and

the X gene, which encodes for the small regulatory x-protein. The three surface proteins are generated by different encoding RNAs, which all have the S domain in common. The medium and large form also contain the preS2 domain, whereas the large HBsAg is the longest of the three proteins and has an additional preS1 domain. The HBV polymerase is linked to the rcDNA, synthesizes DNA from a DNA or RNA template (polymerase and reverse transcriptase activity) and is involved in packing the pregenomic RNA into the nucleocapsid and is able to degrade RNA (nuclease activity)[68]. The HBeAg is produced from a small part of the C gene, termed preCore domain, during virus replication, is excreted from the virus into the blood and is used as an important serological marker for the status of a chronic HBV infection[69]. The HBeAg is believed to distract the immune system and to contribute to the persistence of an HBV infection[70]. The function of HBV X protein has not been uncovered completely, but it has been shown to play a regulatory role in virus replication and to be essential for cccDNA transcription in vivo[71].

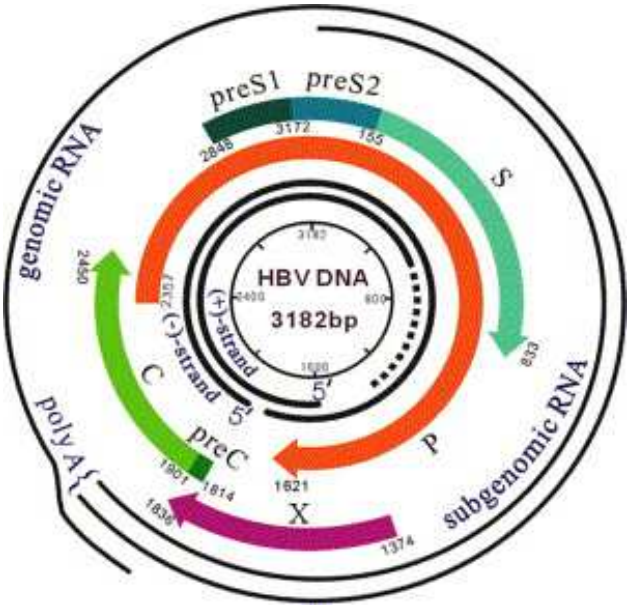


Figure A.2.2. Structure of HBV. *The HBV genome consists of an approximately 3,200bp long minus strand and an incomplete complementary plus strand. Four open reading frames (preS1/presS2/S-, P-, X-, preC/C-gene) encode for large, medium, small HBsAg, viral polymerase, small regulatory X-protein and the HBeAg or HBcAg[72].*

For a long time the entry receptor for HDV and HBV was unknown, but lately in 2012 sodium taurocholate cotransporting polypeptide (NTCP), a transmembrane transporter predominantly expressed in the liver, was identified as the essential receptor for virus entry.

After a reversible interaction of the virus with cell-surface-associated heparan sulfate proteoglycans, the preS1 domain of the large HBsAg specifically interacts with NTCP and enables virus entry into the host[25]. How exactly the virus enters liver cells remains to be elucidated. After HBV entry, the nucleocapsid with the relaxed circular DNA (rcDNA) and its covalently linked HBV polymerase is released into the cytoplasm and transported to the nucleus. The rcDNA consists of a complete minus strand and an incomplete plus strand, which is repaired by cellular DNA repair enzymes within the nucleus[73]. After the completion of the rcDNA a covalently closed circular DNA (cccDNA) is formed, which is structured as a minichromosome[74], appears chromatin-like and with histon and non-histon proteins. The cccDNA serves as a stable template for the transcription of all viral RNAs by cellular DNA polymerases and therefore contributes to the persistence of infection[75]. In the cytoplasm the pregenomic RNA (pgRNA) is translated into the HBcAg and the viral polymerase, while the subgenomic RNA encodes for the envelope proteins (HBsAg) and the HBx protein. The pgRNA, which self-assembles with the HBcAg and the polymerase to new, RNA-containing nucleocapsids, is reverse transcribed within the nucleocapsid and a complementary plus DNA strand is built[76]. Although HBV has a reverse transcriptase activity, the virus is not part of the retrovirus family, because in contrast to retroviruses HBV can replicate without an integration of its genome into the host genome[77]. However, these DNA-containing nucleocapsids can either re-enter the nucleus and form further cccDNA molecules or can be enveloped by HBsAg and secreted via multivescular bodies (MVB) and with the help of the cellular ESCRT machinery („endosomal sorting complex required for transport“) as infectious virions[78]. The release of enveloped nucleocapsids into the blood is exceeded 10^3 to 10^6 times by the secretion of subviral envelope particles (SVPs), which consist of sole envelope proteins without nucleocapsids and are not infectious. SVPs (and also HBeAg) are released via the ER-Golgi intermediate compartment (ERGIC) and seem to be able to bind neutralizing antibodies and therefore to increase the infectivity of HBV[79] (see **figure A.2.3**).

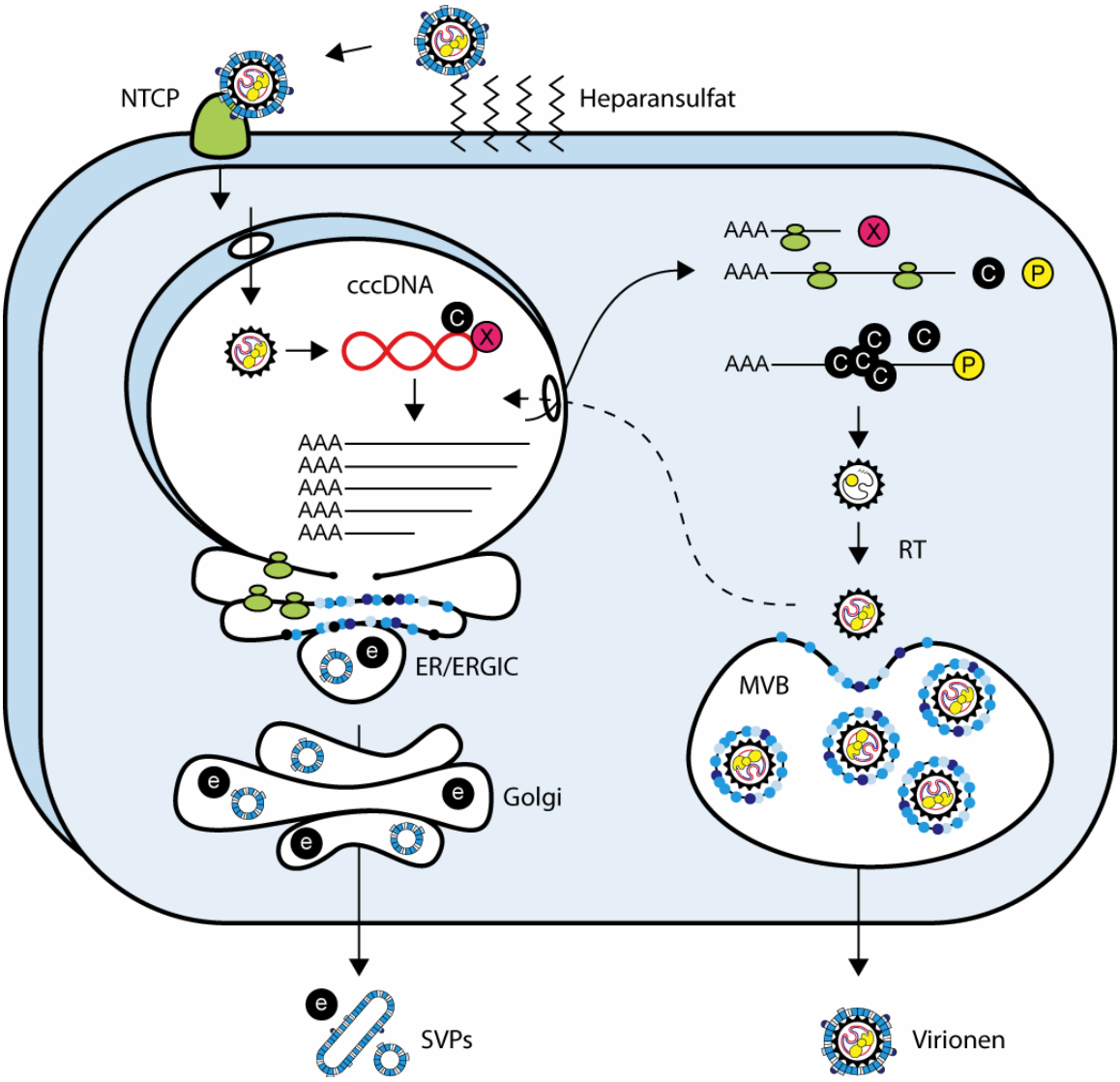


Figure A.2.3. Replication cycle of HBV. *After reversible attachment of HBV to cell-surface associated heparan sulfate proteoglycans follows an irreversible binding to the specific receptor, NTCP. HBV enters the hepatocyte via an unknown mechanism. The rcDNA-, HBcAg- and polymerase-containing nucleocapsid is released and transported to the nucleus. Within the nucleus the rcDNA is repaired and a stable cccDNA is formed. Viral proteins (C=HBcAg, X= HBx protein, P= viral polymerase) are produced via cccDNA transcription by cellular DNA polymerases. A new RNA-containing nucleocapsid is formed and matured via reverse transcription (RT) into a DNA-containing nucleocapsid, which then can either re-enter the nucleus where new cccDNA is amplified or it can be enveloped and secreted via multivesicular bodies (MVB) from the hepatocytes. Non-infectious subviral particles (SVP) consist of sole envelope proteins without nucleocapsids and are released with the HBeAg (e) via the ER-Golgi intermediate compartment (ERGIC)[80].*

A.2.3 Diagnosis and treatment

HBV transmission occurs parenterally through infected blood or body fluids. In high endemic areas HBV infection originates from transmission of an infected mother to her child during birth (perinatal transmission), while in low prevalence countries like Germany reason for HBV infections are mainly unprotected sexual contact and needle sharing among drug users[81]. After an incubation time of one to six months an acute HBV infection emerges without symptoms in 2/3 of the cases (asymptomatic course) and in only 1/3 of the patients with unspecific symptoms like fatigue, weight-loss, anorexia and nausea and during the progress of the disease with liver specific symptoms (jaundice, liver failure). 90% of acute infections in adults are resolved spontaneously with the development of long-lasting immunity[82], while approximately 10% develop a chronic HBV infection which either has an asymptomatic course or is associated with liver inflammation leading to cirrhosis over years and increases the incidence of hepatocellular carcinoma[83]. Interestingly, 90% of children infected before 1 year of age develop a chronic HBV infection[84]. To determine an acute or chronic HBV infection the occurrence of HBsAg and of antibodies against the core protein (anti-HBc) in the serum should be proved. Additionally markers as HBeAg, antibodies against HBeAg (anti-HBe) and serum HBV DNA can be measured[85]. The concentrations of serum transaminase enzymes ALT and AST are also quantified to estimate the extent of liver damage[86].

Treatment options are more promising for chronic HBV mono-infection than for chronic HBV/HDV co-infection. Besides an efficient vaccination against HBV, standard and pegylated interferon-α and nucleoside/nucleotide analogues (NUCs) are potent and approved candidates to decrease the morbidity and mortality of a chronic HBV infection. Although a seroconversion of HBeAg and serum HBV DNA levels below the lower limit of detection might be achieved with current therapeutics, a loss of HBsAg is rarely observed and a complete cure of the infection is not possible. Interferon-α acts both by modulating the immune system (by stimulating interferon stimulated genes and modulating natural killer cells)[87]–[89], as well as by inducing direct antiviral effects, including epigenetic suppression of cccDNA transcription[90], while NUCs inhibit the polymerase of the virus[91]. However, both antiviral compound classes are not able to target the cccDNA and therefore to decrease the persistence of the cccDNA and eradicate the virus completely[92].

Standard interferon-α, which shows cleared HBeAg and HBV DNA levels in around 1/3 of the treated patients, has to be administered three times a week and is mainly replaced by pegylated interferon-α, which has a prolonged half-life and is injected once a week subcutaneously[93]. An advantage of pegylated interferon-α administration is the finite duration of treatment and the highest rate of off-treatment sustained response, but on the other hand patients often suffer from severe side effects with influenza-like symptoms[94].

Five NUCs are currently approved in Europe for the treatment of chronic HBV infections: lamivudine, adefovir, entecavir, telbivudine and tenofovir. NUCs are administered orally and have fewer side effects than pegylated interferon-α, but due to decreased sustained virological responses during the follow-up phase a life-long treatment is required[91].

Several novel therapeutics are in development: The entry inhibitor Myrcludex-B, which has also promising antiviral effects against HDV infections[95], new nucleoside analogues such as clevudine, which is approved for HBV in South Korea and the Philippines[96] and emtricitabine, which is already licensed for the therapy of HIV infections[97].

A.3. Immune responses in viral infections

A.3.1 Innate immune responses

The innate immune system, as an immediate first line defence, protects the host against foreign pathogens in a general, non-specific manner. Especially the activation of the interferon system is known to play a fundamental role in counteracting virus infections.

Invading viruses can be recognized by host cells, which respond with an activation of the innate immune system. Pattern recognition receptors (PRRs) detect viral components (pathogen-associated molecular patterns, PAMPs) such as genomic DNA, single- and double stranded RNA and viral proteins and activate intracellular signaling cascades associated with antiviral immunity[98]. Three different types of PRRs are involved in the detection of viruses: Toll-like-receptors (TLRs) and retinoic acid-inducible gene (Rig-I)-like receptors (RLRs), which both are involved in the production of type I interferons (IFN), interferon stimulated genes (ISGs) and various cytokines[99]; and nucleotide oligomerization domain (NOD)-like receptors (NLRs) which are known for the regulation of interleukin-1ß maturation[100]. In detail, viral nuclei molecules are mainly sensed by the endosome located TLR 3, 7 and 9, which are primarily expressed on innate immune cells. TLR signaling involves the association of Myd88 to the receptors´ intracellular domains and the recruitment of different interleukin-1 receptor-associated kinases (IRAKs) and the interferon regulatory factor 7 (IRF7), as well as the generation of a TRAF6/RIP1 complex and an activation of NF-κB, promoting the expression of type I interferons. RLRs are ubiquitously expressed in the cytoplasm of all nucleated cells and RIG-I signaling is triggered when cells become infected. Activated RIG-I recruits MAVS through an interaction with its CARD domain and mediates the recruitment of TBK1, IKKε and IRF3, which similarly to IRF7 and NF-κB induce the expression of type I interferons (**figure A.3.1**)[101].

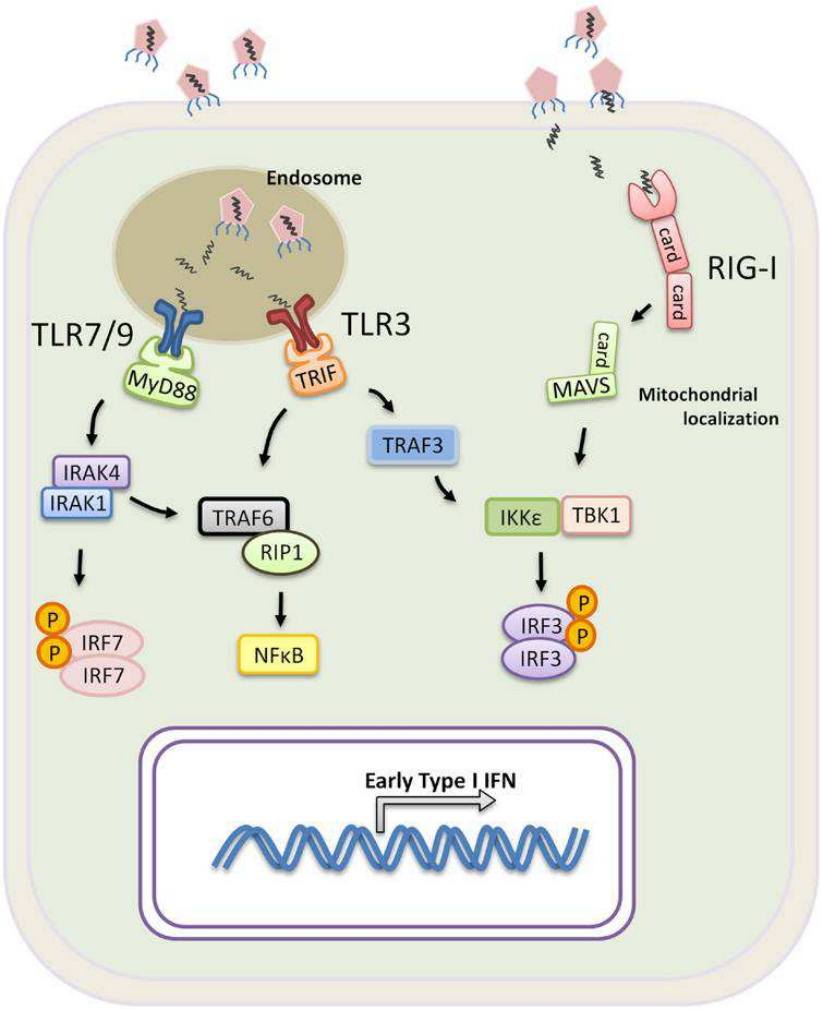


Figure A.3.1. Virus recognition and IFN production. *PAMPS are sensed by TLR3, 7, 9 and RIG-I, which upon their activation lead via complex pathways involving IRF 3 and 7 to the induction of type I interferons[101].*

Type I interferons (IFN-α and -ß) bind to their specific cell-surface receptors, interferon α/ß receptors 1/2 (IFNAR1/2), and induce the JAK/STAT pathway. STAT proteins are activated through a complex mechanism which involves their phosporylation mediated by JAK1 and Tyk2, their dimerization and the generation of the interferon stimulated gene factor 3 (ISGF3) complex, which consists of STAT1, STAT2 and the protein p48 or the IFN-regulatory factor 9 (IRF9). After the translocation of ISGF3 to the nucleus, the STAT containing complex binds IFN-stimulated response elements (ISRE) or IFN-γ-activated sequence (GAS) elements and leads to the induction of ISGs (e.g. OAS1, MxA, ISG20), which act as antiviral agents and play a fundamental role in counteracting viral infections (**figure A.3.2**)[102]–[104]. In detail, OAS1 (2´, 5´-oligoadenylate synthetase) activates an RNAse and degrades RNA, which is then able to induce interferon pathways via Rig-I.

MxA (myxovirus resistance A) is a GTPase, which forms oligomers and accumulates in the cytoplasm of infected cells. MxA complexes are able to trap and degrade nucleocapsids and other viral components. Other ISGs, such as ISG20 act as RNAses and inhibit virus replication by cleaving viral RNA[103].

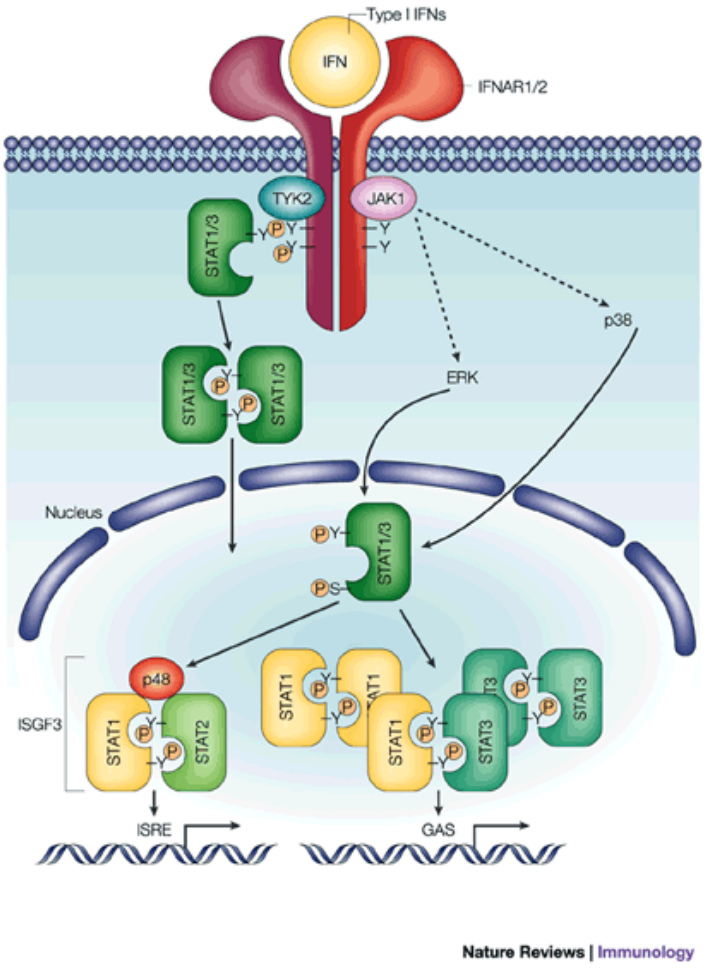


Figure A.3.2. Interferon signaling. *Type I interferons bind to the interferon-α/ß receptor 1/2 and lead via an activation of the JAK/STAT pathway and the generation of ISGF3 to the induction of antiviral ISGs[104].*

Another complex group of proteins playing an important regulatory role in innate immunity are cytokines, which can be divided into interferons (e.g. IFN-ß, IL28), interleukins, colony stimulating factors, tumor necrosis factors (e.g. TNF-α) and chemokines. Cytokines are secreted by many different immune cells and have various functions. They are able to mediate the communication between immune cells, enhance or

inhibit cell proliferation, differentiation or maturation or are involved in chemotaxis, phagocytosis or apoptosis and can therefore have pro- or anti-inflammatory, pro-fibrogenic, antiviral or antitumor effects.

Besides the induction of the interferon system, the activation of natural killer cells (NK cells) and of the complement system also plays an important role in innate immunity. Natural killer cells recognize infected host cells due to an alteration of the cell surface molecules (named MHC complex or HLA complex) and activate immune mechanisms and apoptosis of infected cells. The complement system consists of more than 30 plasma proteins, which induce inflammation, vasodilatation, proteolysis and cell lysis.

Clinical observations indicate that the liver damage associated with chronic HDV infections mainly seems to be immune-mediated. On the other hand, data from chimpanzees and specific clinical cases also suggest direct cytopathic effects of HDV on hepatocytes, but particularly in an acute hepatitis setting[10]. The small HDAg is thought to be responsible for this direct cytopathic effect of HDV, while the large HDAg is non-cytotoxic, promotes the persistence of HDV and makes hepatocytes susceptible to immune-mediated damage[105], [106]. However, detailed host immune responses in HDV infections are poorly investigated so far[105]. One very early study suggested that natural killer cells are activated upon interferon treatment in HDV infected individuals[107]. A more recent analysis in HDV infected patients revealed elevated levels of peripheral blood NK cells with a reduced functional capacity regarding their ability to respond to interferon alpha treatment compared to healthy donors. A high frequency of NK cells before and during interferon treatment is positively associated with treatment outcome[108]. Moreover, in vitro experiments showed that the large HDAg might be able to activate STAT3 and NF-κB signaling[109], [110] and that HDV interferes with interferon alpha signaling by blocking the activation and translocation of STAT proteins and thereby contributing to the persistence of HDV and impairing therapy outcomes[111]. However, further detailed studies are needed to describe innate immune responses in HDV infected human hepatocytes in vivo.

Adaptive immune responses in HDV infections generally seem to be weak[108]. Some investigations in chronic HDV infected patients suggest responses of helper T cells with a high frequency of secreting interleukin-10, which has immunomodulatory effects and

inhibits interferon pathways[112]. Also perforin-positive cytotoxic CD4+ T cells, which are associated with killing of infected cells, accumulate in chronically infected patients and might explain the more severe course of HDV associated liver diseases[113]. CD8+ T cell responses seem to be weaker and were only detected in patients with past, but not active HDV infections[114]. Impaired T cells responses observed in the setting of a chronic HBV/HDV co-infection occur possibly due to the presence of HBV. HBV persistence and high viral loads are associated with suppression, dysfunction and exhaustion of HBV-specific T-cells. The defective T cells responses in chronic HBV-infected patients are further characterized by a dysregulation of co-stimulatory pathways, an impairment of T cell receptor signaling and an enhanced T cell apoptosis[115].

A.4. HDV infection models

A.4.1 Early animal and in vitro models

Since HDV infections still play an important role as a difficult-to-treat disease, appropriate models to investigate the complete viral life cycle, virus host interactions and the efficacy of antiviral treatments are needed. The only animal species, which can be co-infected with human HDV and HBV are chimpanzees (pan troglodytes)[13] and tree shrews (tupaia belangeri sinensis), which are small non-rodent animals from Southeast Asia phylogenetically close to primates[116]. Woodchucks (marmota monax), ground and tree squirrels (spermophilus beecheyi) or pekin ducks (anas domesticus) can be infected with human HDV and HBV-related hepadnavirus[117] and can therefore be used as surrogate animal models for the study of HBV/HDV co-infections[118]. Experiments with chimpanzees[119], [120] and woodchucks[121], [122] revealed important information about the establishment of HDV infection and viral persistence, but their limited availability, high costs, ethical concerns and difficulties in the experimental performance restricted their use for further HDV infection studies.

Due to the narrow host range of HDV the idea of using transgenic mice as an HDV infection model came up. Guilhot et al developed transgenic mice producing the small and large form of HDAg in their hepatocytes. But during 18 months of observation no biological or histopathological evidence of liver disease was detectable[123]. In another attempt, transgenic mice were generated which expressed replication-competent genomic dimers of HDV RNA. Although these transgenic mice were able to replicate RNA transcripts in different tissues, intrahepatic genome replication occurred only in a very limited amount (less than 1%), RNA-editing to produce the large HDAg did not take place and mRNA species were not detected. Again, signs of liver cytotoxicity were missing completely, suggesting that transgenic mice cannot be used as a suitable model for chronic HDV infection studies[124].

In 1999, another model emerged which attempted to infect rodents with hepatotropic viruses. Liu et al described the hydrodynamics-based transfection model for the first time. By rapidly injecting DNA in a large volume into the tail vein of mice, organs, especially the liver, start to express transgenes, reaching a peak after some hours and decreasing thereafter[125]. Chang et al developed this method further and injected HDV cDNA or in

vitro transcribed HDV RNA into mice using the hydrodynamics-based transfection procedure. Five to nine days after inoculation an increasing accumulation of genomic HDV RNA was detected intrahepatically, which then decreased from day 15 to 30[126]. This model might help studying aspects of genome replication, RNA transcription and the role of host proteins in acute HBV/HDV infections, but certainly it has its limitations since host immune responses eliminate virus particles after a few days and the rapid injection of large volumes of fluids can cause liver damage so that chronic viral infections cannot be examined[127].

Primary cultures of hepatocytes support HDV replication in principle, but they are difficult to maintain, become non-permissive for HDV and HBV quickly after plating and revealed donor-dependant differences[128]. The hepatic cell line HepaRG[129] and, after the discovery of NTCP as HBV/HDV entry receptor in 2013, also the NTCP transfected hepatoma cell lines Huh7 and HepG2 are susceptible to HDV and HBV and are used for in-vitro studies[27].

A.4.2 Development of chimeric mouse models

Rodents cannot be infected with HDV and HBV, but during the past 15 years researches offered different strategies to transplant human hepatocytes into mice and developed novel mouse models for in vivo infections with HDV and HBV. It is well known that the liver is a highly regenerative organ and after liver injury or surgical removal of more than 2/3, liver mass can be restored completely without loosing hepatocyte structure and function. Therefore the idea of transplanting cells into the liver to restore liver mass emerged and investigations showed that primary isolated human hepatocytes are able to repopulate the liver after being transplanted into dorsal fat pads[130], spleen[131], peritoneal cavity[132] or under the renal capsule[133].

In 2000, Ohashi et al developed a novel in vivo model for the study of HBV mono- and HDV super-infections. Isolated human hepatocytes were engrafted via the kidney capsule into livers of non-obese diabetic (NOD) mice crossbred with severe combined immunodeficiency (SCID) mice. SCID mice lack T- and B-lymphocytes due to spontaneous mutation in the Prkdc gene and therefore do not have an adaptive immune

system. After human cells integrated in mice livers without loosing their characteristic morphology, inoculation with HBV and super-infection with HDV was performed successfully and human chimeric mice supported completion of the viral life cycle[134]. In the same year, Brown et al generated a primary human hepatocyte cell line which was stably transfected with a full-length HBV genome, expressed HBV genes and which supported replication of HBV. After intrasplenic transplantation of these HBV-producing cells into livers of combined immunodeficient Rag-2 mice, lacking mature B- and T-lymphocytes due to a Rag-2 deletion, long-term engraftment up to eight months and virion secretion could be observed[135].

These two models demonstrate that human hepatocytes can be engrafted successfully and survive in immunodeficient mice for months, but without showing high levels of host liver repopulation. However, under certain genetic or acquired conditions a better repopulation of human hepatocytes in murine livers can be obtained. Alb-uPA-transgenic mice, first developed in 1990 to study neonatal bleeding disorders[136], sustain hepatocytes which express the hepatotoxic urokinase-type plasminogen activator (uPA) transgene under the control of an albumin promoter causing murine hepatocytes death and thus providing a growth advantage for transplanted human hepatocytes. In 2001 Dandri et al transplanted human hepatocytes isolated from an adult human liver into uPA-transgenic mice that were crossbred with immunodeficient Rag-2 mice and therefore enabled hepatocytes repopulation and reconstitution of mouse livers up to 15%. Transplanted human hepatocytes remained permissive for human hepatotropic viruses, which was proved by the establishment of a productive HBV infection after inoculating uPA/Rag-2 mice with a human serum containing HBV DNA[137].

Another human-liver chimeric model where efficient liver repopulation can be achieved is the fumarylacetoacetate hydrolase (Fah) -deficient mouse. Fah is the last enzyme of the tyrosine catabolism cascade and its deletion in fah-deficient mice leads to an accumulation of hepatotoxic intermediates of tyrosine catabolism (e.g. fumarylacetoacetate) and therefore to injury of host hepatocytes[138]. Accumulation of fumarylacetoacetate can be prevented by the pharmacologic inhibitor of tyrosine catabolism 2 (2-nitro-4-trifluormethybenzoyl)-cyclohexane-1,3-dione (NTBC) and cyclic administration of NTBC allows control of liver failure in Fah null mice. In 2010, He et al crossed Fah-deficient mice with immunodeficient Rag-2 mice, titrated liver failure by the use of NTBC and transplanted human hepatocytes

via intrasplenic injection into mice livers. After inoculation of a HBV DNA positive human serum actively replicating HBV was found in livers of human chimeric mice[139].

A.4.3 UPA/SCID mouse model

Since generation of human chimeric mice by transplanting human hepatocytes into the liver seems to be the most suitable in vivo infection model and promises a better understanding of stable HBV/HDV infections, a further development of this animal model took place. In 2001, Mercer et al. engrafted hepatocytes from a human donor into livers of immunodeficient uPA/SCID mice and therefore generated the first murine model suitable for investigations of infections with human hepatitis C virus (HCV) in vivo. By using Alb-uPA homozygous instead of heterozygous mice repopulation rates could be increased from 15% [137] to more than 50% and therefore augmenting the chance of establishing an extensive virus infection[140]. Meuleman et al. characterized human chimeric livers of homozygous uPA/SCID mice and demonstrated a successful chronic mono-infection with HCV and HBV[141]. Seven years later, in 2012 our group infected human chimeric uPA/SCID mice, which lack an adaptive immune system, with a human HDV RNA positive serum for the first time and created an efficient mouse model for the study of HBV and HDV infections, virus-host interactions and preclinical drug evaluation[53].

All experiments in this doctoral thesis were performed using the human chimeric uPA/SCID model under following conditions (see **figure A.4.1**). Three to four week old uPA/SCID mice were engrafted with cryopreserved human hepatocytes by intrasplenic injection. Eight weeks later successful repopulation of human hepatocytes in the host liver was determined measuring human serum albumin, which is exclusively expressed by transplanted human hepatocytes. Mice with appropriate albumin levels were inoculated with a human or serial passaged murine serum positive for HDV RNA and HBV DNA. Co-infection was monitored collecting blood samples to determine viremia. At the end of the experiments, mice were sacrificed, livers were collected and intrahepatic measurements were performed.

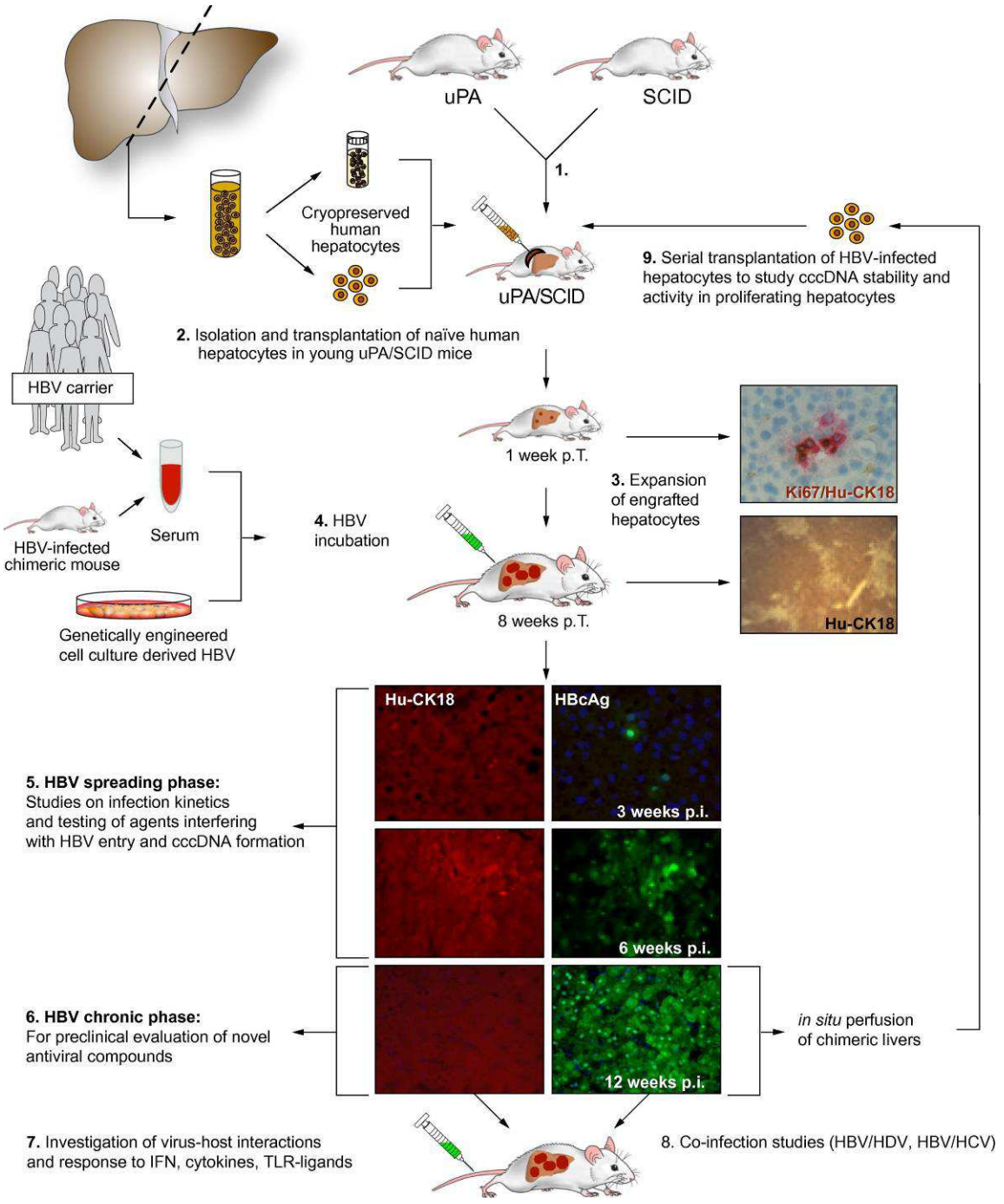


Figure A.4.1. UPA/SCID mouse model. *Human hepatocytes are engrafted into livers of uPA/SCID mice to generate human chimeric livers consisting of human and mouse hepatocytes. Inoculation with HBV and HDV enables establishment of chronic HBV mono- and HDV/HBV co-infections[142].*

A.5. Aim of the Work

Worldwide more than 15 million people are chronically infected with HDV, which leads to a hepatitis infection with the most severe clinical course of disease. The limited availability of HDV infection models has hindered studies of the interactions between HDV and the infected human hepatocytes. Therefore, the aim of the work was to describe different aspects of HDV infected human hepatocytes in the recently developed humanized uPA/SCID mouse model for stable HDV infections. Specific aims were to characterize virus host interactions in a chronic HBV/HDV co-infection, viral persistence and cell toxicity of an HDV mono-infection and changes of intrahepatic virus activity upon antiviral treatment.

The first aim was to investigate the antiviral state of the human hepatocytes in the setting of chronic HBV/HDV co-infection compared to HBV mono-infection using the uPA/SCID mouse model. The expression of human-specific interferon stimulated genes (ISGs), signaling genes and cytokines are determined on RNA and protein levels. The findings might contribute to a better understanding of the interplay between HDV and the innate immune system and might also help to explain the more severe course of HDV-associated liver disease.

To elucidate the persistence of HDV in human hepatocytes in the absence of HBV, the aim was to investigate whether primary human hepatocytes support a non-productive HDV replication in vivo and whether intracellular replicating HDV can be rescued upon HBV super-infection. Knowledge about HDV persistence and conversion of latent HDV infection to productive HBV/HDV co-infection might help to explain the recently described HDV activity in patients even with low HBV replication and re-occurrence of HDAg in the setting of liver transplantation.

For a more comprehensive understanding of the HDV replication cycle in humanized mouse livers, new quantification methods need to be established. Aim was to develop a new quantitative RT-PCR strategy to specifically distinguish between genomic and antigenomic HDV RNA in humanized mice and patient liver biopsies. As a proof of concept this new assay is used to investigate the antiviral effects induced by peg-IFN-α and the nucleotide reverse transcription inhibitor entecavir on intrahepatic HDV productivity in HBV/HDV co-infected humanized uPA/SCID mice. The findings of this doctoral thesis might contribute to a better understanding of HDV infection in human hepatocytes and might support the search for an appropriate antiviral therapy of HDV infected patients.

B. Material and Methods

B.1. Instruments

device	producer	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 Transfer cell	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)	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

Table B.1. Instruments.

B.2. Material

B.2.1 General reagents

Reagents	Producer	Country
ABI Fast 1-Step Virus Master	Applied Biosystems	USA
ABI Fast Advanced Master	Applied Biosystems	USA
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
Bovine serum albumin	PAA Laboratories GmbH	Austria
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
HRP Conjugated Goat anti-Human Albumin Detection Antibody A80-129P	Bethyl Laboratories	USA
Hepatocyte Wash Medium	Invitrogen™ (Life Technologies)	USA
Hoechst	Hoechst AG	Germany
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
Natrium chloride	Carl Roth GmbH & Co	Germany
Natrium chloride solution 0,9%	B. Braun Melsungen AG	Germany
Phosphate buffered saline	Invitrogen™ (Life Technologies)	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™ (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
Standard Quant-it	Invitrogen™ (Life Technologies)	USA
Sulfuric acid	Carl Roth GmbH & Co	Germany
Tissue Preparation Solution	Sigma	USA
T-Per Tissue Protein Extraction Reagent	Pierce	USA
Transcriptor Reverse Transcriptase	Hoffmann-La Roche	Switzerland
Transcriptor RT Reaction Buffer	Hoffmann-La Roche	Switzerland
Tris acetate EDTA buffer	Sigma	USA
Trizima base	Sigma-Aldrich	USA
Trizol	Invitrogen™ (Life Technologies)	USA
Tween 20	Sigma-Aldrich	USA
Uracil-DNA glycosylase	Hoffmann-La Roche	Switzerland

Table B.2. General reagents.

B.2.2 Kits

Kits	Producer	Country
ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit	Applied Biosystems	USA
Architect HBsAg assay	Abbott Ireland Diagnostics	Ireland
BCA Protein Assay Kit	Pierce	USA
Beta-globin Gene Kit	Hoffmann-La Roche	Switzerland
CXCL10/IP-10 Quantikine ELISA Kit	R&D	USA
DNA Gel Extraction Kit	Millipore	USA
Dynal Kilobase Binder Kit	Invitrogen™ (Life Technologies)	USA
Immunoprecipitation Kit - Dynabeads Protein G	Invitrogen™ (Life Technologies)	USA
In Situ Cell Death Detection Kit, Fluorescein (TUNEL)	Hoffmann-La Roche	Switzerland
MasterPure DNA Purification Kit	Epicentre	USA
MAXIscript kit	Ambion (Life Technologies)	USA
MEGAScript Kit SP6	Ambion (Life Technologies)	USA
MEGAScript Kit T7	Ambion (Life Technologies)	USA
MinElute PCR Purification Kit	Qiagen	Netherlands
QiAmp MinElute Virus Spin Kit	Qiagen	Netherlands
RNeasy RNA Mini Kit	Qiagen	Netherlands
Super Signal West Dura Chemiluminescent Substrate	Pierce	USA
Taqman Gene Expression Assays	Applied Biosystems	USA
TSA Fluorescein System	Perkin Elmer	Germany
Transcriptor First Strand cDNA Synthesis Kit	Hoffmann-La Roche	Switzerland

Table B.3. Kits.

B.3. Methods

B.3.1 Generation of humanized uPA/SCID mice

Humanized uPA/SCID mice were generated by crossing UPA transgenic mice (Jackson Laboratories, ME, USA) with SCID/beige mice (Taconic Farms, Denmark, EU) and by injecting 1 million viable thawed cryo-preserved human hepatocytes into the spleen of 3-week old homozygous uPA/SCID/beige (uPA/SCID) mice. Eight to ten weeks after transplantation repopulation of human hepatocytes into mice livers was completed and viral infections could be established by inoculation of humanized mice with an HBV- and HDV-positive patient derived serum[53].

Part 1 (innate immune responses): To evaluate the virus-dependent ISG and cytokine induction, groups of uninfected (n=13), HBV mono-infected (n=11) and HBV/HDV co-infected (n=19) mice, which received human hepatocytes from four different human donors (two with C/C and two with C/T IL28 locus), were analyzed. Human hepatocytes from these different donors were distributed equally among mice. For kinetic studies (t=0, 3, 8 and >12 weeks; n= 3 for each group) humanized mice were generated by transplantation of human hepatocytes obtained from a single human donor.

Part 2 (HDV persistence): For all HDV mono-infection (n=3 for each group and time point) and serial passaging (n=2) experiments, humanized uPA/SCID mice were generated by transplanting one million viable thawed cryo-preserved human hepatocytes from two different human donors (both with C/C locus) into homozygous uPA/SCID mice.

Part 3 (antiviral treatment): Humanized uPA/SCID mice, which were treated with pegylated interferon alpha (n=3) or entecavir (n=4) or left untreated (n=7) were generated by transplanting one million viable thawed cryo-preserved human hepatocytes obtained from two different human donors (both with C/T IL28 locus) into homozygous uPA/SCID mice.

Repopulation rates of all mice were estimated by determining human serum albumin (HSA) concentrations in mouse sera (Bethyl Laboratories, Biomol GmbH, Hamburg, Germany) and confirmed at sacrifice by determining human cell contents by histology (staining of the human marker CK18) and real-time PCR using the beta-globin gene kit (Roche DNA control Kit, Roche Diagnostics)[53]. Animals displaying levels of human chimerism ranging from 40 to 60% were used for the studies. All mice were sacrificed as indicated in the results and liver specimens were cryo-conserved in chilled isopentane and stored at -80°C for further histological and molecular analyses.

All mice were maintained 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

Part 1 (innate immune responses): HBV-positive patient serum (genotype D) serially passaged in mice was used to establish an HBV mono-infection. For HBV/HDV co-infection human chimeric mice received a single intraperitoneal injection of an HBV/HDV-positive, patient serum serially passaged in mice ($1x10^7$ HDV RNA copies/mouse, HDV genotype 1; $1x10^7$ HBV DNA copies/mouse, HBV genotype D). The inoculum corresponded to an MOI of approximately 0.33 for each virus by estimating an average of $3x10^7$ primary human hepatocytes per mouse liver[143].

Part 2 (HDV persistence): To establish an HDV mono-infection, humanized mice received a single peritoneal injection of either cell culture-derived HDV virions ($5x10^5$ HDV RNA copies/mouse) or patient-derived serum ($1x10^5$ HDV RNA copies/mouse). Cell culture-derived HDV particles were produced as previously described[144]. Briefly, two plasmids able to express the HBV envelope proteins (pSVB45H) and to initiate HDV replication (pSVL(D3)) respectively were transfected into Huh7 cells. Released virions were concentrated by precipitation with 10% polyethylene glycol, HDV loads were estimated by real-time PCR ($1x10^7$ genome copies/ml) and infectivity was verified in human hepatocyte cultures. Patient inoculum ($1x10^7$ HDV RNA copies/ml; HBV DNA

<LLoD 7 IU/ml) was obtained from a patient receiving entecavir treatment, under informed consent and during routine screening procedures in the outpatient department of the University Medical Centre Hamburg-Eppendorf, Hamburg. Both inocula corresponded to an MOI of approximately 0.016 and 0.003, by estimating an average of $3x10^7$ human hepatocytes per mouse liver[143]. HBV super-infection was performed using an HBV-positive patient serum ($1x10^8$ HBV DNA copies/mouse, genotype D; HDV negative; MOI≈3).

Part 3 (antiviral treatment): To establish an HBV/HDV co-infection, human chimeric uPA/SCID mice received a single peritoneal injection of an HBV- and HDV-positive, patient derived serum serially passaged in mice ($5x10^6$ HBV DNA copies/mouse, genotype D; $5x10^6$ HDV RNA copies/mouse, genotype 1). The inoculum corresponded to an MOI of approximately 0.2 by estimating an average of $3x10^7$ human hepatocytes per mouse liver.

B.3.3 Virological measurements and intrahepatic quantification

Viral DNA and RNA were extracted from serum samples (5 µl) using the QiAmp MinElute Virus Spin Kit (Qiagen, Venlo, Netherlands). To determine HDV viremia, cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). For the reverse transcription, 10 µl extracted RNA was mixed with random hexamer primers (60 µM) and denatured for 10 minutes at 65 °C. Denatured RNA was added to the reverse transcription mixture (total volume 20 µl) supplied by the manufacturer. The reaction was incubated for 10 minutes at 25 °C, followed by 30 minutes at 65 °C, and stopped by incubation at 85 °C for 5 minutes[53]. Real-time PCR was performed in a LightCycler 1.5 (Roche Applied Science, Basel, Switzerland) using 10 µl of cDNA with HDV- specific primers and TaqMan probes (**table B.4**) The reaction consisted of an initiating step of 10 minutes at 95 °C, followed by 45 cycles of amplification, with each cycle consisting of 10 seconds at 95 °C, 30 seconds at 62 °C, and 1 second at 72 °C[53].

Alternatively, HDV viremia was determined via reverse transcription and quantitative RT-PCR using the ABI Fast 1-Step Virus Master (Applied Biosystems, Carlsbad, USA), HDV specific primers and probes[145] on a ABI Viia7 (Applied Biosystems, Carlsbad,

USA). In detail, 5µl serum RNA was denatured at 95°C for 10 minutes, immediately cooled down at -20°C and reverse transcribed at 50°C for 5 minutes with HDV specific primers and probes[145]. After inactivation of the reverse transcriptase at 95°C for 20 seconds, amplification was performed under the following conditions: initial step 95°C 20 seconds, 40 cycles at 95°C for 3 seconds and 60 °C for 30 seconds[146].

Published HBV-specific primers and hybridization probes[147] were used to determine HBV DNA (**table B.4**). Known references of cloned HBV DNA were amplified in parallel to establish a standard curve for quantification. The plasmid pBluescript II SK(þ), containing one copy of the HDV genome (genotype 1), was used as a standard for HDV cDNA quantification[53].

DNA and RNA were extracted from mouse liver specimens using the MasterPure DNA Purification Kit (Epicentre, Madison, USA) and the RNeasy RNA Mini Kit (Qiagen, Venlo, Netherlands) according to manufacturers` instructions. Intrahepatic HBV DNA values were determined using HBV specific primers and fluorescence hybridization probes (**table B.4**) and were normalized for cellular DNA contents using the beta-globin gene kit (LightCycler VR Control Kit DNA; Roche Diagnostics), which specifically recognizes sequences of human origin[148]. Viral RNA was reverse-transcribed from 1 µg total RNA using oligo-dT primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) and quantified by using primers specific for total HBV RNA (**table B.4**). To carry out HDV intrahepatic measurements, 1 µg (for the 2-step analysis) or 1µl (for the 1-step analysis) of liver RNA was used for reverse transcription. HDV RNA loads were quantified using the same protocol described for the serological HDV RNA measurements. Steady-state levels of intrahepatic HDV and HBV RNA amounts were normalized using, as an internal control, human-specific GAPDH primers (QuantiTect Primer Assay; Qiagen). HBsAg quantification was performed using the Architect HBsAg assay (Abbott Ireland Diagnostics, Sligo, Ireland). Briefly, the mouse serum was diluted 1:150-1:500 in manual dilution serum (Abbott). HBsAg was quantified after assay calibration (HBsAg calibrator; Abbott), as recommended by the manufacture for the testing of reactive human serum.[53]

primer/probe	sequence
HBV Fw	CTCG TGGT GGAC TTCT CTC
HBV Rv	CAGC AGGA TGAA GAGG AA
HBV FL probe	CACT CACC AACC TCCT GTCC TCCA A
HBV LC probe	TGTC CTGG TTAT CGCT GGAT GTGT CT
HDV Fw1	TGGA CGTK CGTC CTCC T
HDV Fw2	TGGA CGTC TGTC CTCC TT
HDV Rv	TCTT CGGG TCGG CATG G
HDV probe	ATGC CCAG GTCG GAC
HDV1 Fw (genomic)	GCGC CGGC YGGG CAAC
HDV1 Rv (antigenomic)	TTCCTCTT CGGG TCGG CATG
HDV probe	CGCG GTCC GACC TGGG CATC CG

Table B.4. Virus specific primer and probes.

B.3.4 Quantification of genomic and antigenomic HDV RNA

10µl serum RNA or 1µg of intrahepatic RNA was denatured at 95°C for 10 minutes with 0.5µM of a biotinylated HDV specific forward primer for genomic HDV RNA quantification or a biotinylated HDV specific reverse primer for antigenomic HDV RNA quantification[145] (**table B.4**) and immediately cooled down at -20°C. After addition of the ABI Fast 1-Step Virus Master (Applied Biosystems, Carlsbad, USA) to a final volume of 20µl, reverse transcription was performed at 50°C for 5 minutes on an ABI Viia7 (Applied Biosystems, Carlsbad, USA) and enzymes were inactivated at 95°C for 20 seconds. Biotinylated cDNA was purified with the MinElute PCR Purification Kit (Qiagen, Venlo, Netherlands) and isolated with dynabeads specifically interacting with biotin (Dynal Kilobase Binder Kit, Invitrogen, Carlsbad, USA) following the manufacturer's instructions. In brief, 5µl dynabead solution was washed and re-suspended in Binding Buffer, incubated with 20µl biotinylated cDNA for 3 hours and washed twice with Washing Buffer and once with sterile, RNase-free water. For quantitative RT-PCR 1µl of purified biotinylated cDNA bound to dynabeads, HDV specific primers and probes[145] (**table B.4**) and an ABI Fast Advanced Master (Applied Biosystems, Carlsbad, USA) were used under the following conditions: Initial step 95°C 20 seconds; 40 cycles at 95°C for 3 seconds and 60 °C for 30 seconds. The quantification of genomic and antigenomic HDV RNA was performed in

triplicates, values are relative to human GAPDH and ratios were calculated using median ct values.

B.3.5 Preparation of genomic and antigenomic HDV RNA standard

For the genomic HDV RNA standard the HDV full genome plasmid (pBluescript + HDV) was digested with BamHI and in vitro transcribed with the T7 promoter, using the MAXIscript kit (Ambion, Carlsbad, USA). The antigenomic HDV RNA standard was generated after HindIII digestion of the HDV plasmid and transcription with the T3 promoter (**figure B.1.1**). Genomic and antigenomic HDV RNA standards were purified with isopropanol/trizol (Invitrogen, Carlsbad, USA) and the Master Pure Complete DNA and RNA purification kit (Epicentre, Madison, USA). For the quantification of genomic and antigenomic HDV RNA standard 10^7 copies were used and mixed with 1µg RNA of uninfected livers, imitating real measurement conditions in livers.

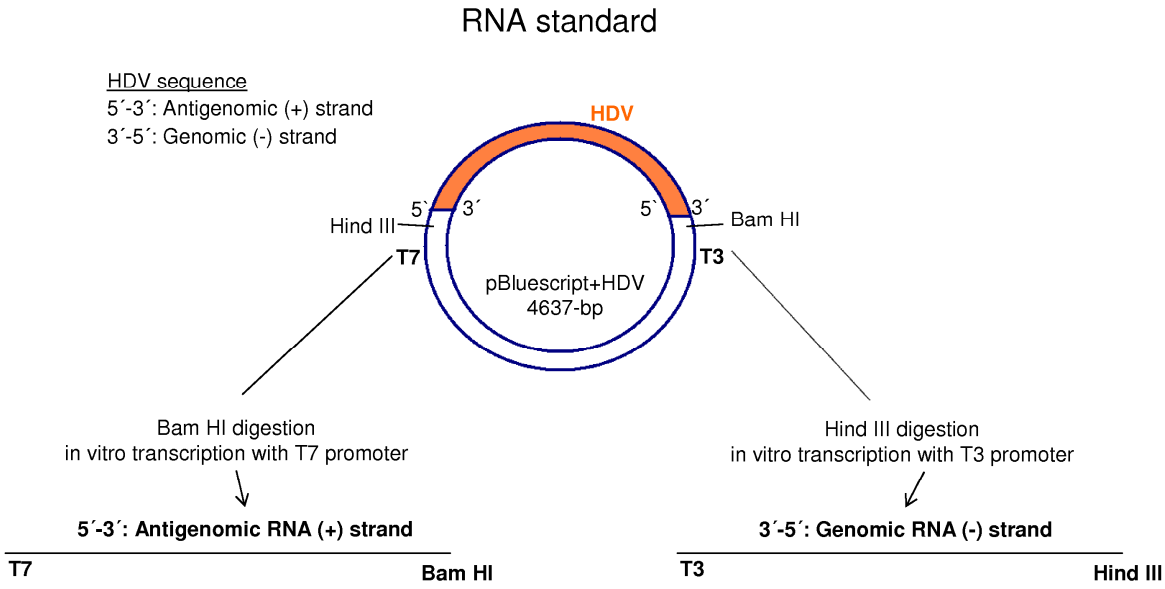


Figure B.1.1. Preparation of genomic and antigenomic HDV RNA standard.

B.3.6 ISG and cytokine expression

To determine expression levels of genes related to IFN signaling (ISGs and cytokines) in human hepatocytes repopulating the mouse liver, primers and probes specifically recognizing human transcripts and not cross-reacting with murine genes were used (Taqman Gene Expression Assays, Applied Biosystems, **table B.5**)[149]. For determining the ISG and cytokine expression, RNA was extracted as described above. CDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) using oligo-dT primer according to the manufacturer's instructions and qRT-PCR was performed with the ABI Fast Advanced Master (Applied Biosystems, Carlsbad, USA) in an ABI Viia7 (Applied Biosystems, Carlsbad, USA) under the following conditions: Initial step 95°C 20 sec; 40 cycles at 95°C for 1 sec and 60 °C for 20 sec. The mean of hGAPDH and hRPL30 was used for normalization to human specific genes.

gene	assay ID
hADAR	Hs01017595_g1
hAPOBEC3A	Hs00377444_m1
hAPOBEC3F	Hs01665324_m1
hAPOBEC3G	Hs00222415_m1
hAPOBEC3B	Hs00358981_m1
hAPOBEC3C	Hs00819353_m1
hCASP8	Hs01018151_m1
hGAPDH	Hs99999905_m1
hHLA-E	Hs03045171_m1
hHSPA6/ hHSP70B	Hs04187232_g1
hIFN-α	Hs00855471_g1
hIFN-ß	Hs00277188_s1
hIL28A+B / hIFNλ2/3	Hs04193049_gH
hIL29	Hs00601677_g1
hIP10 / hCXCL10	Hs00171042_m1
hISG15	Hs00192713_m1
hISG20	Hs00158122_m1
hMX1	Hs00895608_m1
hNTCP / hSLC10A1	Hs00914889_m1
hOAS1	Hs00973637_m1
hPIAS3	Hs00966031_m
hPIAS 4	Hs01071948_m1
hRig-I / hDDX58	Hs01061432_m1
hRPL30	Hs00265497_m1
hSTAT1	Hs01013989_m1
hSTAT2	Hs01013133_g1
hSTAT3	Hs01051722_s1
hTAP1	Hs00388675_m1
hTGF-ß	Hs00171257_m1
hTLR2	Hs01014511_m1
hTLR3	Hs00152933_m1
hTLR 4	Hs00152939_m1
hTLR7	Hs01933259_s1
hTLR8	Hs00152972_m1
hTLR9	Hs00370913_s1
hUSP18	Hs00276441_m1

Table B.5. Taqman Gene Expression Assays.

B.3.7 Immunofluorescence

For immunofluorescence staining 12µm cryostat sections of chimeric mouse livers were used. Sections were fixed with acetone or 4% paraformaldehyde, washed with PBS and incubated at 4 °C over night with mouse anti-CK18 (1:400; Dako, Glostrup, Denmark), human anti-delta (anti-HDAg-positive human serum 1:8000), rabbit anti-HBcAg (1:1000; Dako), mouse anti-STAT1 (1:200; BD Biosciences, New Jersey, USA), mouse anti-HLA-E (1:50; Leinco Technologies, St.Louis, USA), rabbit anti-ISG15 (1:100; Sigma, St.Louis, USA), or rabbit anti-Caspase 3 (1:400; BD Bioscience, New Jersey, USA) (**table B.6**)[53]. For the presence of apoptotic and/or necrotic human hepatocytes in mouse liver sections, frozen liver sections obtained from uPA recipients were analyzed using TUNEL labelling. Briefly, cryosections were fixed with 4% paraformaldehyde before TUNEL labelling was performed following the manufacturer's instructions (In situ Cell Death Detection Kit; Roche Applied Science). As positive control serial liver sections were incubated for 15 min at 37°C with 100 IU DNAse I (Epicentre®, Biozym), prior to TUNEL labelling[150]. Specific signals were visualized with Alexa 488-, 546- or 633-labeled secondary antibodies (Invitrogen, Carlsbad, USA) and the TSA Fluorescein System (Perkin Elmer, Jügesheim, Germany) to enhance HBcAg staining. Nuclear staining was achieved by Hoechst 33258 (1:20,000, Invitrogen, Darmstadt, Germany) [151]. Stained sections were then mounted with fluorescent mounting media (Dako) and analyzed by the fluorescence microscope BZ9000 (Keyence, Osaka, Japan) using the same settings for different experimental groups. By performing triple and quadruple staining (i.e. DAPI, Hu-CK-18, HDAg and HBcAg), the percentage of HBcAg-positive, HDAg-positive, or caspase 3-positive human hepatocytes was estimated as previously described[53] and by using 2 visual fields (displaying approximately 500 human hepatocytes each) from 3 different liver sections per mouse that was sacrificed.

antibody	dilution	producer	country
Alexa 488-labeled secondary antibody	1:400	Invitrogen	Germany
Alexa 546-labeled secondary antibody	1:800	Invitrogen	Germany
Alexa 633-labeled secondary antibody	1:200	Invitrogen	Germany
rabbit anti-Caspase3	1:400	BD Bioscience	USA
mouse anti-CK18	1:400	Dako	Denmark
rabbit anti-HBcAg	1:2000	Dako	Denmark
human anti-HDAg	1:8000	anti-HDAg positive human serum	
mouse anti-HLA-ABC	1:50	Leinco Technologies	USA
peroxidase-conjugated AffiniPure anti-rabbit secondary antibody	1:200	Jackson	USA
rabbit anti-ISG15	1:100	Sigma	USA
mouse anti-STAT1	1:200	BD Bioscience	USA

Table B.6. Primary and secondary antibodies.

B.3.8 Human serum IP10 ELISA

Human specific IP10 levels of uninfected, HBV mono-infected and HBV/HDV co-infected mice were determined with the human CXCL10/IP-10 Quantikine ELISA Kit (R&D, Minneapolis, USA) using 16 µl of mouse serum according to the manufacturer's instructions. Samples and standard dilutions were detected at 450nm with a wavelength correction at 570nm using the EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, USA).

B.3.9 Western blot and immunoprecipitation

Protein lysates were obtained by extracting liver tissue with T-Per Tissue Protein Extraction Reagent (Pierce, Rockford, USA) supplemented with the following protease and phosphatase inhibitors: NaF (10 mM), EDTA (2 mM), benzamidine (10 mM), PMSF (1 mM), leupeptin (1 µg/ml), Na3VO4 (2 mM) and aprotinin (1,5 µg/ml)[152]. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, USA).

For immunoprecipation the protein lysates (containing 1 mg protein) were first incubated with a highly diluted anti-HDAg serum (1:100) and the Immunoprecipitation Kit - Dynabeads Protein G (Invitrogen, Carlsbad, USA) was used following the manufacturer's instructions. 20 µg of protein (western blot) or 5 ml of HDAg enriched protein lysates (immunoprecipitation) were denatured at 95°C, separated on a 12% sodium dodecyl sulfate-polyacrylamide gel (nUView Precast gels, Peqlab, Erlangen, Germany) and blotted onto a nitrocellulose membrane (Hybond ECL Nitrocellulose Membrane, GE Healthcare, Buckinghamshire, UK). Small and large hepatitis Delta antigen (S-HDAg, L-HDAg) were detected with a high-diluted patient serum (1:2000), or a polyclonal rabbit anti-HDV antibody (1:2000). Normalization of HDAg was performed with an alpha-antitrypsin (AAT) antibody (1:1000, Biotrend, Cologne, Germany) detecting only human AAT. Horseradish-peroxidase conjugated secondary antibodies (Jackson Immunoresearch, Suffolk, UK) were used in a dilution of 1:10,000. Blocking reagent was TBS-T with 5% non-fat dry milk and washing steps were performed three times after first and second antibody incubation by using TBS-T. Signals were visualized with Super Signal West Dura Chemiluminescent Substrate (Pierce) and the Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, USA).

B.3.10 Sequencing

For viral sequencing the HDV genomic regions R1 (location: 305-1285, product size: 980bp, according to the numeration of Wang et al[15]) and R2 (location: 885-327, product size: 1121bp) were amplified by PCR using cDNA, respective primers (R1 fw 305-327: ccagaggacccccttcagcgaac, R1 rv 1285-1261: gaaggaaggccctcgagaacaaga, R2 fw 885-908: catgccgacccgaagaggaaag, R2 rv 327-306: gttcgctgaaggggtcctctg)[153] and a Red-Taq Polymerase (Sigma-Aldrich, St. Louis, USA) in a total volume of 50 µl. The reaction consisted of an initial 4 min at 94°C, 45 cycles of 1 min at 94°C, 1 min at 62°C and 2 min at 72°C. PCR product length was analyzed on a 0.8% agarose gel and DNA fragments were extracted with the DNA Gel Extraction Kit (Millipore, Billerica, USA) as recommended by the manufacturer. Both strands were sequenced on an ABI Prism 377 automated sequencer (PE Applied Biosystems, Foster City, USA) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

B.3.11 Treatment with pegylated interferon-α and entecavir

Stably HBV/HDV-co-infected (>10 weeks post virus inoculation) human-chimeric uPA-SCID mice were either treated with peg-INF-α or with ETV for 4 weeks. Peg-INF-α (25 ng/g body weight) or saline as control was injected subcutaneously twice a week. For ETV treatment, mice received drinking water supplemented with 1 µg/mL Baraclude Solution (Bristol-Myers Squibb, Munich, Germany). Administration was repeated daily for the following 4 weeks.

B.3.12 Statistics

Statistical analysis was performed with the GraphPad Prism 5 software. For group-wise comparisons the nonparametric Mann-Whitney U test or the nonparametric Kruskal-Wallis Test using Dunns test to compare all pairs as post-test were applied. P values < 0.05 were considered statistically significant.

C. Results

C.1. Hepatitis D virus infection and innate immune responses

C.1.1 Kinetics of HBV and HDV infection and ISG induction

The induction of interferon-stimulated genes (ISGs) is known to be the hallmark of antiviral immunity. While ISG activation plays a fundamental role in counteracting viral infections, elevated pre-treatment ISG levels have been associated with weaker responses to IFN-α treatment[154], [155] and may even support viral replication[156], [157]. The limited availability of HDV infection models has hindered studies of interactions between HDV and infected human hepatocytes. Therefore, the aim of this study was to investigate the antiviral state of the human hepatocytes in the setting of chronic HBV/HDV co-infection compared to HBV mono-infection using human liver chimeric mice.

To investigate the induction of innate immune responses during the course of HBV/HDV co-infection uPA/SCID mice repopulated with human hepatocytes obtained from one human donor (C/T IL28 locus) were used. In mice simultaneously inoculated with HBV and HDV both viruses could be detected in mouse serum samples after 3 weeks of infection, reached maximum viral loads (median titres of $4.5x10^7$ HDV RNA copies/ml and $6x10^6$ HBV DNA copies/ml) at week 8 of infection and remained enhanced thereafter (**figure C.1.1 A**). Development of viremia was accompanied by increased amounts of intrahepatic HDV RNA, showing median levels of 0.008, 0.224 and 0.191 HDV RNA relative to human GAPDH at 3, 8 and 16 weeks, respectively (**figure C.1.1 B**). Immunofluorescence staining demonstrated that a large number of human hepatocytes stained HDAg-positive in liver sections of 8-week HBV/HDV co-infected mice (**figure C.1.1 C**).

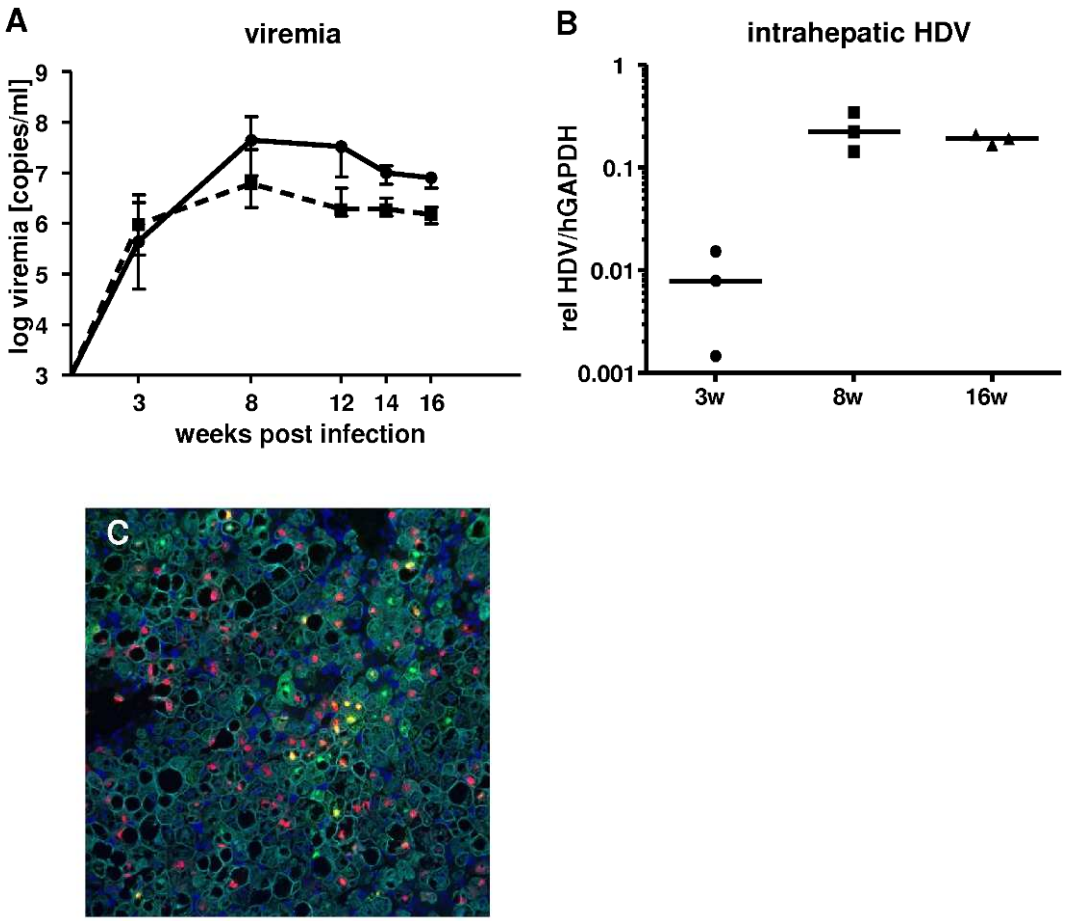


Figure C.1.1. Viremia and intrahepatic HDV parameters. A) *Median HBV (dashed line) and HDV (solid line) viremia development in HBV/HDV co-infected mice after 0, 3, 8, 12, 14 and 16 weeks of infection (n≥3 for each point).* ***B)*** *Intrahepatic HDV RNA levels relative to human GAPDH in 3, 8 and 16 weeks co-infected mice, reaching a maximum at 8 weeks of infection. Immunohistlogical staining (**C**) in a 8-week co-infected mice shows a large number of human hepatocytes (CK18; blue) positive for HDAg (red), while fewer human hepatocytes are HBcAg-positive (green) or co-infected (HDAg- and HBcAg-positive, yellow).*

In line with previous studies[53], HBV RNA transcript levels of HBV/HDV co-infected mice were low after 3 weeks of infection (median 0.0289), but reached high levels around week 8 post-infection (median 1.2) and remained in this range thereafter (median 0.6, week 16) (**figure C.1.2 A**). Also HBV viremia (**figure C.1.1 A**) reached its maximum at around week 8 post-infection, although viral titers in HBV/HDV co-infection generally remained lower than in the setting of HBV mono-infection (**figure C.1.2 B**).

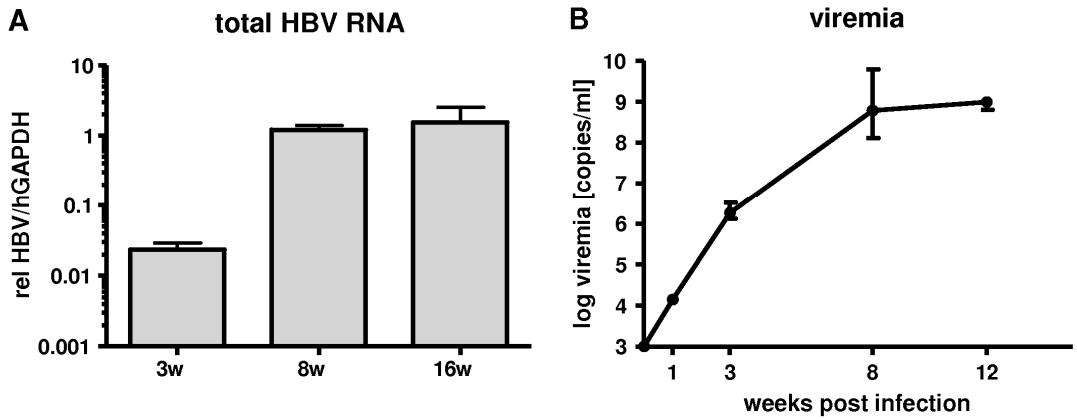


Figure C.1.2. HBV viremia. ***A****) Intrahepatic expression of total HBV RNA relative to human GAPDH in 3-, 8- and 16-week HBV/HDV co-infected mice.* ***B****) HBV viremia of HBV mono-infected humanized mice used for ISG kinetic analyses (0, 1, 3, 8 and 12 weeks of infection).*

Gene expression analysis in HBV/HDV co-infected mice revealed that viremia development was accompanied by a clear induction of human-specific ISGs, which was ascertained at RNA level by quantitative real-time PCR. The induction of classic human ISGs (2´, 5´-oligoadenylate synthetase, hOAS1; interferon stimulated gene 15, hISG15; ubiquitin-specific peptidase 18, hUSP18), of upstream IFN signaling (Signal Transducers and Activators of Transcription gene 1, hSTAT1) and of genes that are involved in the process of antigen presentation (HLA class I histocompatibility antigen, alpha chain, hHLA-E; transporter associated with antigen processing 1, hTAP1) were clearly increased during HDV spreading (3 weeks post infection). Induction of these genes reached the highest levels at the peak of infection (8 week) and remained enhanced in the following weeks (>12 weeks) (**figure C.1.3 A-F**). Interestingly, the induction of the same ISGs was also detected during the course of HBV mono-infection, although their enhancement was generally less pronounced than in the setting of HBV/HDV co-infection (**figure C.1.3 A-F**).

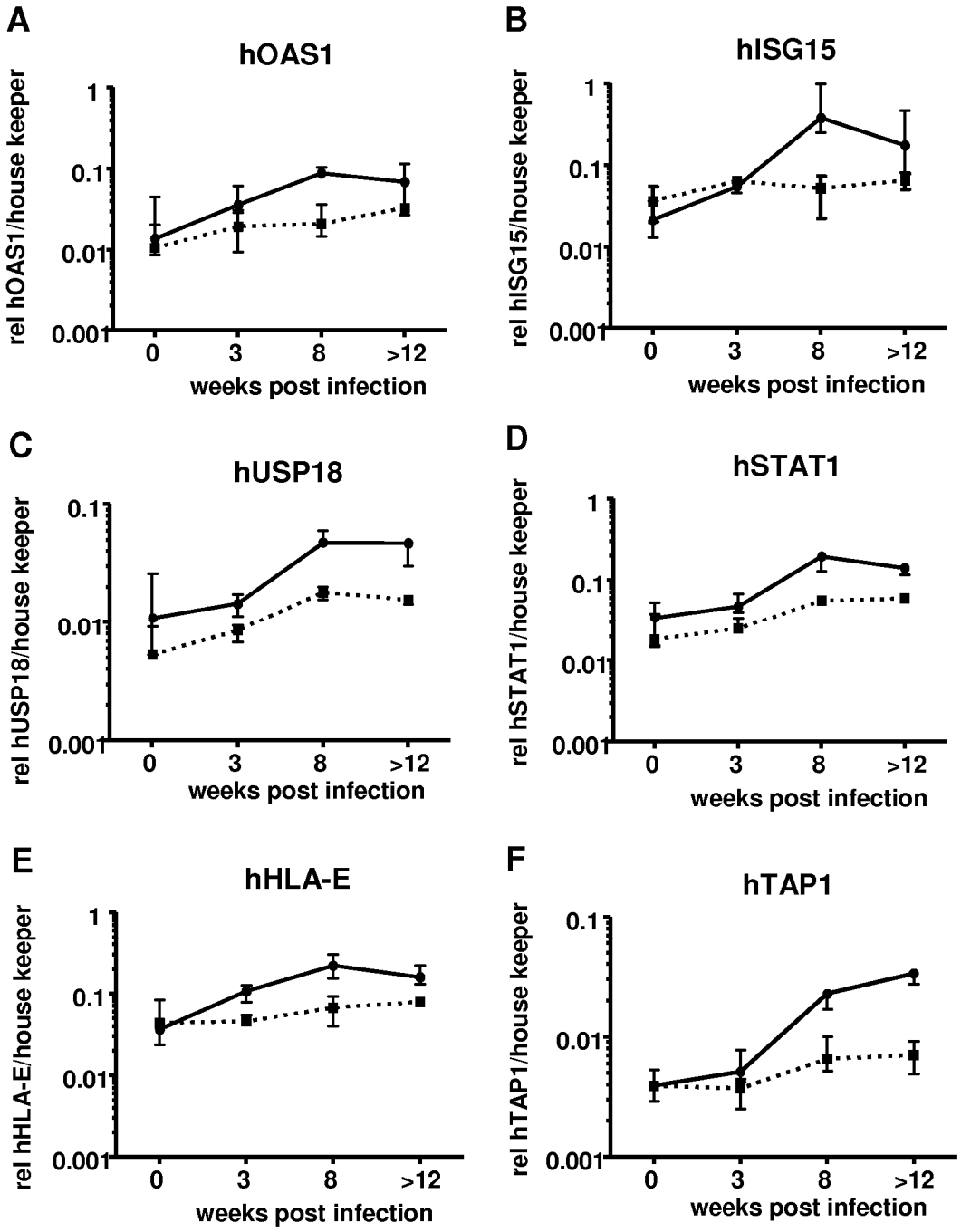


Figure C.1.3. ISG kinetic in HBV/HDV infected mice. A-F) *RNA expression of human-specific ISGs (hOAS1, hISG15, hUSP18), signaling genes (hSTAT1) and genes that are involved in antigen presentation (hHLA-E, hTAP1) relative to house keeping genes (mean of hGAPDH and hRPL30) during the course of HBV mono-infection (dashed line) and HBV/HDV co-infection (solid line) (0, 3, 8 and >12 weeks) in mice livers repopulated with human hepatocytes from a single human donor.*

C.1.2 Enhancement of innate immune responses in stably infected mice

To investigate whether innate immune responses observed during the kinetic of HBV/HDV co-infection remained also enhanced in the setting of chronic HDV infection and in a larger cohort of mice, the expression of human-specific ISGs and signaling genes were then investigated in the liver of mice that had been co-infected with HBV and HDV for at least 6 weeks and displayed titres >$1x10^6$ HDV RNA copies/ml (median $1.1x10^7$ HDV RNA copies/ml and $6.2x10^6$ HBV DNA copies/ml). These HBV/HDV co-infected mice were compared to chronic HBV mono-infected (median $2.6x10^8$ HBV DNA copies/ml) and uninfected humanized mice. To avoid possible donor-specific biases uPA/SCID mice were transplanted with four different human hepatocyte donors. All human donors were equally distributed among each group of mice (uninfected n=13, HBV n=11, HBV/HDV n=19).

As summarized in **table C.1** and shown in **figure C.1.4 A-D**, various human ISGs, including classic antiviral ISGs (e.g. hOAS1, hMxA), signaling genes (e.g. hSTAT1) and receptors (e.g. hRig-I), appeared only slightly increased in HBV mono-infected livers. However, the RNA expression levels of the same genes were significantly higher in the setting of HBV/HDV co-infection compared to uninfected control mice and even to HBV mono-infected animals (**table C.1**; fold differences HBV vs. HDV), therefore confirming the results obtained in the kinetic study using a single human donor.

gene	fold difference: HBV vs. uninf (median/median)	p-value	fold difference: HBV/HDV vs. uninf (median/median)	p-value	fold difference: HBV/HDV vs. HBV (median/median)	p-value
hMxA	1.0	ns	3.0	**<0.0001*** **	3.5	**0.0014****
hOAS1	2.2	**0.0031****	3.3	**<0.0001*** **	1.8	**0.0039****
hUSP18	1.5	**0.037***	3.0	**<0.0001*** **	2.5	**<0.0001*** **
hISG15	1.0	ns	4.3	**<0.0001*** **	3.5	**0.0006*** **
hISG20	1.4	ns	4.5	**0.0017****	2.9	**<0.0001*** **
hHLA-E	1.4	ns	3.2	**<0.0001*** **	2.0	**<0.0001*** **
hTAP1	2.0	**0.0045****	5.1	**<0.0001*** **	2.6	**<0.0001*** **
hADAR	1.3	ns	2.2	**<0.0001*** **	1.6	**0.0006*** **
hIP10	6.9	**0.0091****	40.2	**<0.0001*** **	5.3	**<0.0001*** **
hNTCP	1.2	ns	0.8	**0.0383***	0.7	**0.0179***
hSTAT1	2.1	**0.0054****	3.5	**0.0002*** **	1.8	**0.0226***
hSTAT2	1.7	**0.0277***	2.0	**<0.0001*** **	1.5	**0.0179***
hSTAT3	1.4	ns	2.9	**<0.0001*** **	2.0	**0.0076****
hPIAS3	1.4	**0.0236***	1.6	**0.0078****	1.0	ns
hPIAS4	1.4	**0.0205***	1.9	**0.0006*** **	1.3	**0.0111***
hRIG-I	1.6	**0.0426***	2.9	**<0.0001*** **	1.9	**0.0022****
hTLR2	1.7	ns	2.8	**0.0021****	1.6	**0.0238***
hTLR3	1.5	**0.0239***	1.3	**0.0193***	1.1	ns
hTLR4	3.7	**0.0370***	1.3	ns	0.5	ns
hTLR7	nd	ns	nd	ns	nd	ns
hTLR8	nd	ns	nd	ns	nd	ns
hTLR9	0.4	ns	1.2	ns	1.4	ns
hAPOBEC3A	nd	ns	nd	ns	nd	ns
hAPOBEC3B	3.5	**0.0006*** **	6.4	**<0.0001*** **	1.4	**0.0350***
hAPOBEC3C	nd	ns	nd	ns	nd	ns
hAPOBEC3F	1.9	**0.0021****	3.2	**0.004****	1.5	ns
hAPOBEC3G	2.3	**0.0012****	4.5	**0.0006*** **	2.2	**0.0111***
hCasp8	nd	ns	nd	ns	nd	ns
hHSPA6	1.9	**0.0175***	2.3	**<0.0001*** **	1.2	ns
hIFN-α	nd	ns	nd	ns	nd	ns
hIFN-ß	nd	ns	d	**0.0199***	d	**0.0066****
hTGF-ß	8.2	**0.0045****	22.7	**<0.0001*** **	1.8	ns
hIL28A+B	nd	ns	d	**0.0334***	d	**0.0036****
hIL29	nd	ns	d	**0.002****	d	**0.0004*** **

Table C.1. ISG induction. *The RNA expression of classic ISGs, signaling genes and cytokines was comparatively analyzed in stable HBV mono-infected, HBV/HDV co-infected and uninfected mice previously reconstituted with human hepatocytes derived from 4 different human donors. The last column indicates the relative expression differences determined in HBV/HDV co-infected mice compared to HBV mono-infected mice. Values are median fold inductions (median infected / median uninfected) and p-values are calculated with the nonparametric Mann-Whitney U test. Ns= not significant; nd= not detectable; d= detectable (i.e. when RNA expression in uninfected mice is below the lower limit of detection and fold inductions cannot be calculated).*

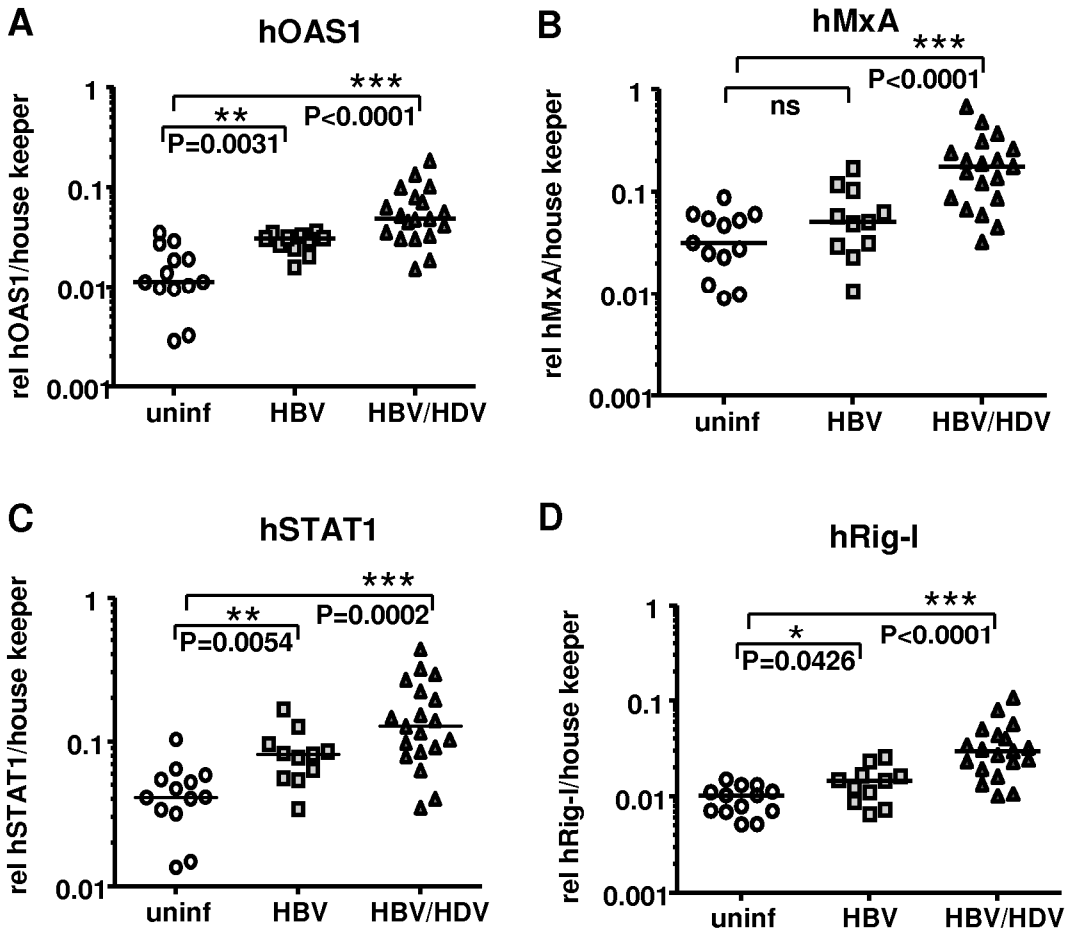


Figure C.1.4. ISG induction. A-D) *RNA expression of human-specific ISGs (hOAS1, hMxA) and signaling genes (hSTAT1, hRig-I) in mice which were uninfected (n=13), chronically HBV mono- (n=11) or HBV/HDV co-infected (n=19) and received human hepatocytes from 4 different human donors. All ISG expression levels are relative to housekeeping genes (mean of hGAPDH and hRPL30) and were determined with qRT-PCR using human-specific primers and probes which did not detect mouse signals.*

The human adenosine deaminase (hADAR), which was shown to convert the small into the large HDAg by an RNA-editing event[21], was not significantly induced in HBV mono-infected mice (**Figure C.1.5** and **table C.1**), but its expression was clearly increased in HBV/HDV co-infected livers ($p<0.0001$).

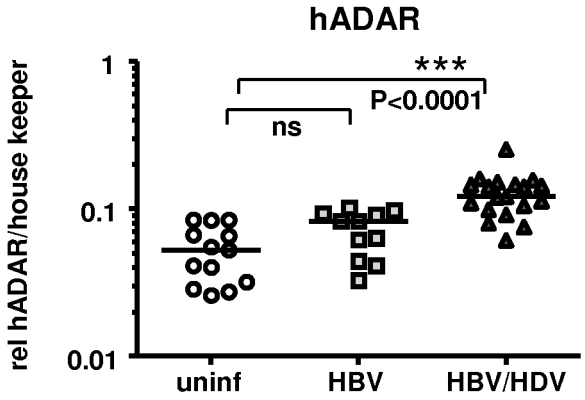


Figure C.1.5. hADAR induction. *RNA expression of hADAR in uninfected (n=13), chronically HBV mono- (n=11) or HBV/HDV co-infected (n=19) mice.*

Within the TLR family, hTLR2, appeared as the most enhanced gene in the setting of HBV/HDV co-infection ($p=0.0021$) (**Figure C.1.6 A**), while hTLR3 and hTLR4 were only slightly elevated already in HBV mono-infected livers and hTLR7, hTLR8 and hTLR9 were not induced or remained below the lower limit of detection in all groups. Furthermore, the expression levels of apolipoprotein B mRNA editing enzymes, catalytic polypeptide-like, such as hAPOBEC3B (**Figure C.1.6 B**), hAPOBEC3F and hAPOBEC3G, which are as cytidine deaminase enzymes also involved in antiviral innate immunity, appeared significantly induced not only in HBV/HDV co-infected animals, but also in HBV mono-infected mice. In contrast, the expression levels of human hAPOBEC3A and hAPOBEC3C remained below detection limits in human hepatocytes, regardless of the infection status (**table C.1**).

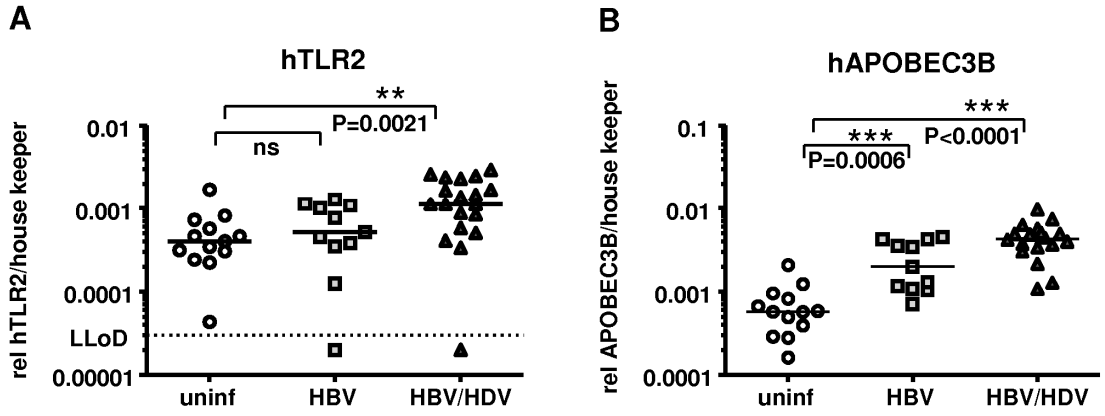


Figure C.1.6. hTLR2 and hAPOBEC3B induction. *RNA expression of hTLR2 (**A**) and hAPOBEC3B (**B**) in uninfected (n=13), chronically HBV mono- (n=11) or HBV/HDV co-infected (n=19) mice.*

The most remarkable expression change on RNA levels was observed with the interferon gamma-induced protein 10 (hIP10), a chemokine that is involved in the chemotaxis of macrophages, NK cells, dendritic cells and T cells. As shown in **figure C.1.7 A**, significant induction of hIP10 was already evident in HBV mono-infected animals (median 6.9-fold; p=0.0091) in comparison to uninfected humanized control mice. Nevertheless, even in this case, HBV/HDV co-infection provoked an even stronger induction of hIP10 (median 40.2-fold; p<0.0001). To confirm this strong RNA induction on protein levels, serum concentrations of human IP10 were determined also by ELISA in mice harbouring human hepatocytes obtained from the same human donor, which had been infected either with HBV or with HBV and HDV for 8 weeks. As indicated in **figure C.1.7 B**, the strongest increase of serum hIP10 was determined in HBV/HDV co-infected mice, while only a moderate increase was found in HBV mono-infected mice in comparison with the hIP10 concentrations determined in uninfected animals.

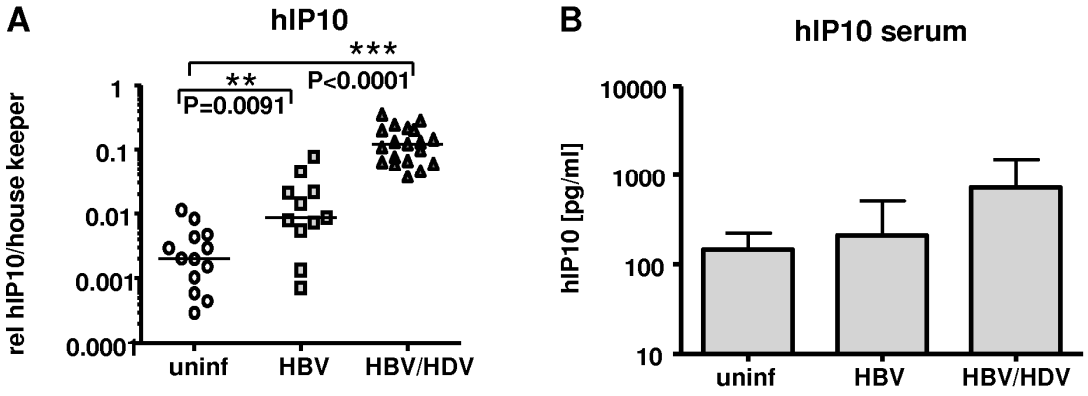


Figure C.1.7. hIP10 on RNA and protein levels. ***A****) RNA expression of hIP10 in uninfected (n=13), chronically HBV mono- (n=11) or HBV/HDV co-infected (n=19) mice.* ***B****) Serum hIP10 levels of uninfected, 8-week HBV mono-infected and 8-week HBV/HDV co-infected mice.*

Among the genes involved in antigen presentation, hTAP1 (**table C.1**) and hHLA-E (**figure C.1.8 A** and **table C.1**) appeared more strongly induced in HBV/HDV co-infected mice versus HBV mono-infected mice. Accordingly, immunohistological staining revealed an only moderate expression of hHLA-ABC proteins in the setting of HBV mono-infection, but a clearly stronger induction in HBV/HDV co-infected human hepatocytes compared to uninfected mice (**figure C.1.8 B-D**).

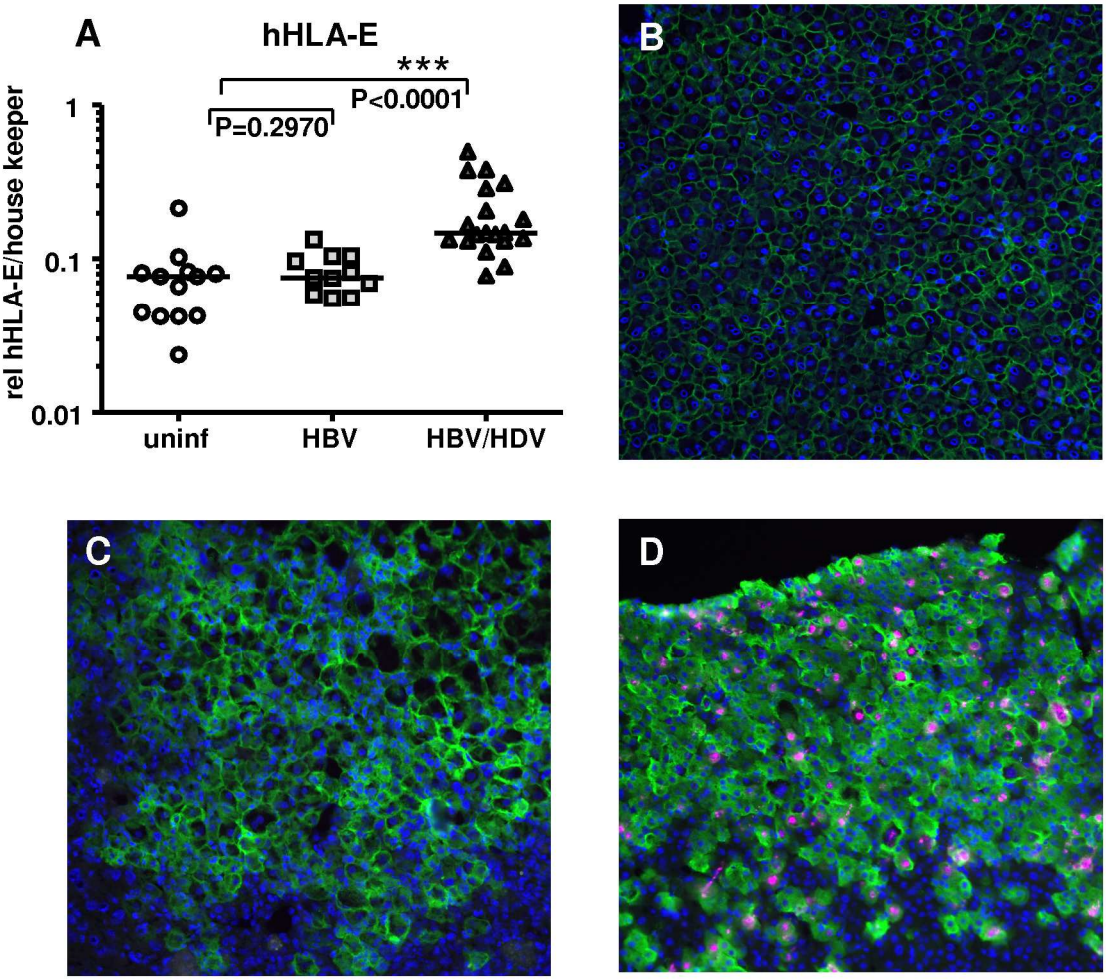


Figure C.1.8. hHLA-E expression. *The relative RNA expression of hHLA-E (**A**) and the protein expression of hHLA-ABC (green, immunohistological staining, **B-D**) was more induced in HBV/HDV co-infected mice (**D**) than in uninfected (**B**) and HBV mono-infected mice (**C**). HDAg-positive human hepatocytes are stained red (**D**).*

RNA expression measurements of human interferon stimulated gene 15 (hISG15) showed a significant increase (median 4.3-fold RNA; p<0.0001) in HBV/HDV co-infected mice, but no enhancement in HBV mono-infected animals (**figure C.1.9 A** and **table C.1**). In line with the RNA results, hISG15 protein amounts were clearly higher in human hepatocytes of HBV/HDV co-infected mice (**figure C.1.9 D**) than in uninfected (**figure C.1.9 B**) and HBV mono-infected mice (**figure C.1.9 C**).

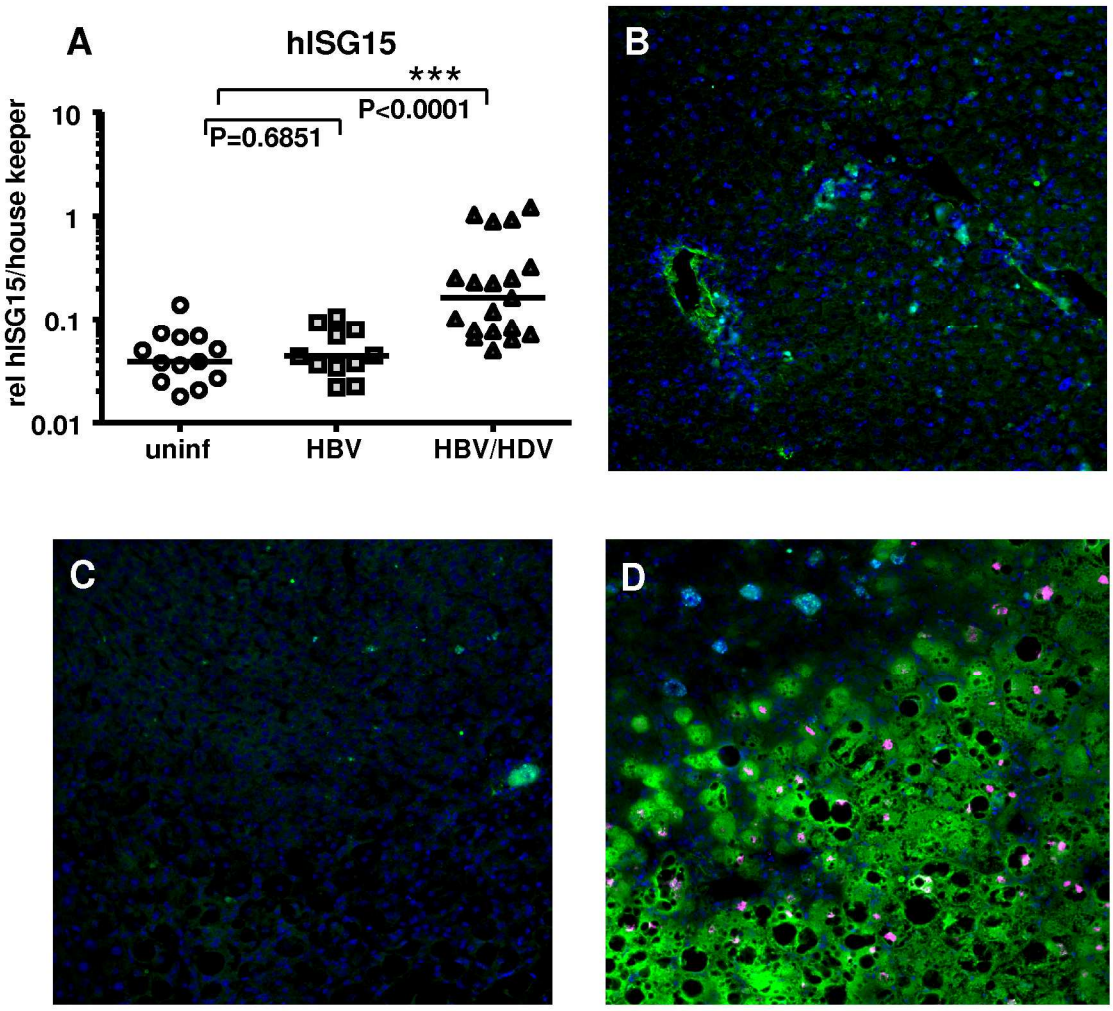


Figure C.1.9. hISG15 expression. ***A****) shows the relative RNA expression of hISG15 in uninfected, HBV mono-infected and HBD/HDV co-infected mice, which received human hepatocytes from 4 different human donors and which were stably infected (> 6 weeks of infection). In line, immunohistological staining showed that protein levels of hISG15 (green) are strongly expressed in HBV/HDV co-infected mice (****D****; HDAg=red) and remained lower in uninfected (****B****) and HBV mono-infected animals (****C****).*

The more pronounced enhancement of ISGs in HBV/HDV co-infected mice could be appreciated also when the four different groups of mice harbouring distinct human donor hepatocytes were analyzed separately. **Figure C.1.10** depicts the fold induction of hIP10 and hISG15 in HBV mono- and co-infected mice compared to uninfected baseline levels, subdivided into each mice group transplanted with the different human donors.

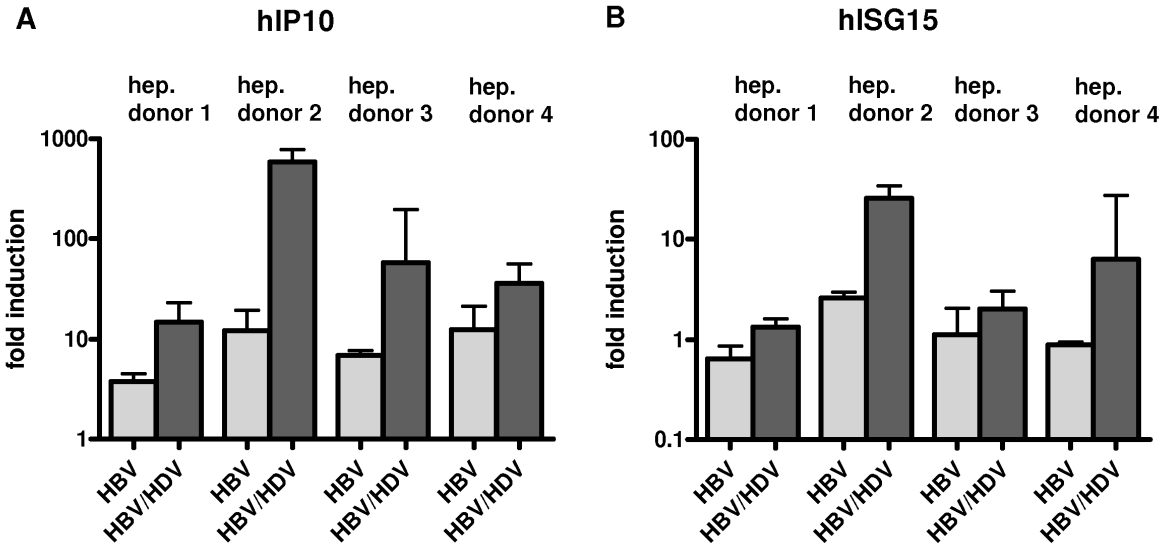


Figure C.1.10. hHLA-E and hISG15 induction of different human donors. *Fold induction of hIP10 (**A**) and hISG15 (**B**) in HBV mono- and co-infected mice compared to uninfected baseline levels, subdivided into each mice group transplanted with the different human hepatocytes donors (hep. donor 1-4).*

Human-specific cytokines were also determined by qRT-PCR in uPA/SCID mice, which received human hepatocytes from four different donors and were stably infected either with HBV or HBV/HDV for at least 6 weeks, or were transplanted in parallel but remained uninfected. Gene expression of hTGF-ß, hIFN-ß, hIL28A+B and hIL29 reveal a significant induction of these cytokines in the setting of HBV/HDV co-infection (e.g. fold inductions: 22.7x hTGF-ß, $p<0.0001$***) while levels remained lower (fold-induction: 8.2x hTGF-ß, $p=0.0045$**) or below detection (hIFN-ß, hIL28A+B, hIL29 > LLoD) in HBV-mono-infected mice as well as in uninfected control animals (**Figure C.1.11 A-D** and **table C.1**).

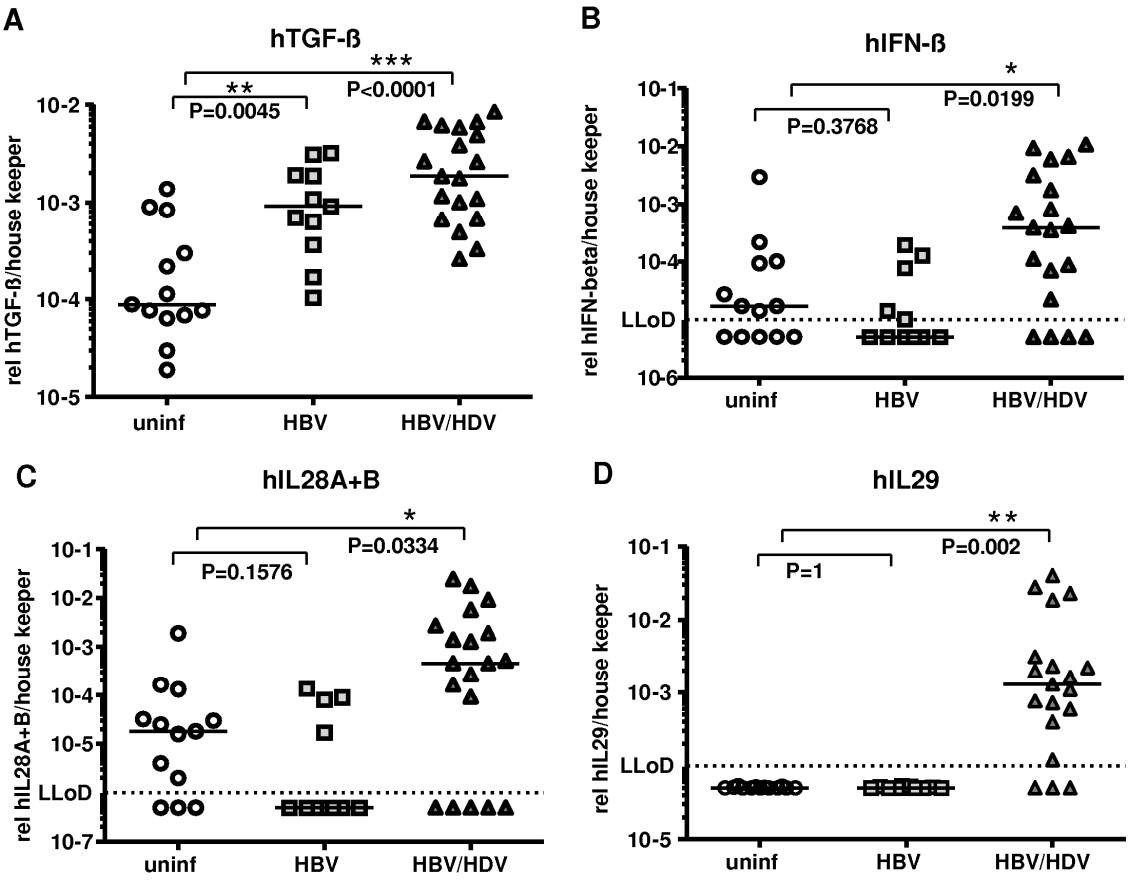


Figure C.1.11. Cytokine expression. *The relative RNA expression of human-specific cytokines (hTGF-ß, hIFN-ß, hIL28A+B, hIL29;* ***A-D****) is significantly enhanced in HBV/HDV co-infected mice and remains lower or below the lower limit of detection (LLoD) in uninfected and HBV mono-infected mice.*

C.1.3 Expression and cellular localization of STAT proteins

Since the RNA expression levels of hSTAT1 were strongly enhanced in HBV/HDV co-infected humanized mice both in comparison to uninfected mice and HBV mono-infected mice (**figure C.1.4 C** and **table C.1**), immunofluorescence staining was performed also to assess levels and cellular distribution of STAT proteins. As shown in **figure C.1.12 A**, a strong enhancement of hSTAT protein levels, as well as its accumulation in the nucleus of the human hepatocytes could be determined in HBV/HDV co-infected mice. In contrast, human hepatocytes in uninfected (**figure C.1.12 B**) and HBV mono-infected (**figure C.1.12 C**) mice did not show a similarly pronounced hSTAT staining. Notably, co-staining of hSTAT with HDAg indicated an inverse correlation between the amounts of these two proteins, since STAT accumulation was most prominent in the nuclei of cells that appeared uninfected or at least displayed undetectable or lower amounts of HDAg staining (**figure C.1.12 D-G**), thus suggesting that STAT nuclear accumulation was hindered in the infected human hepatocytes displaying high amounts of HDAg proteins. The decreased hSTAT accumulation and nuclear translocation in human hepatocytes with high amounts of HDAg may be facilitated by the expression of hPIAS3 and hPIAS4 (**table C.1**), which was significantly stronger induced in HBV/HDV co-infected mice livers and which are known to interact with JAK/STAT pathways by negatively regulating hSTAT transcription.

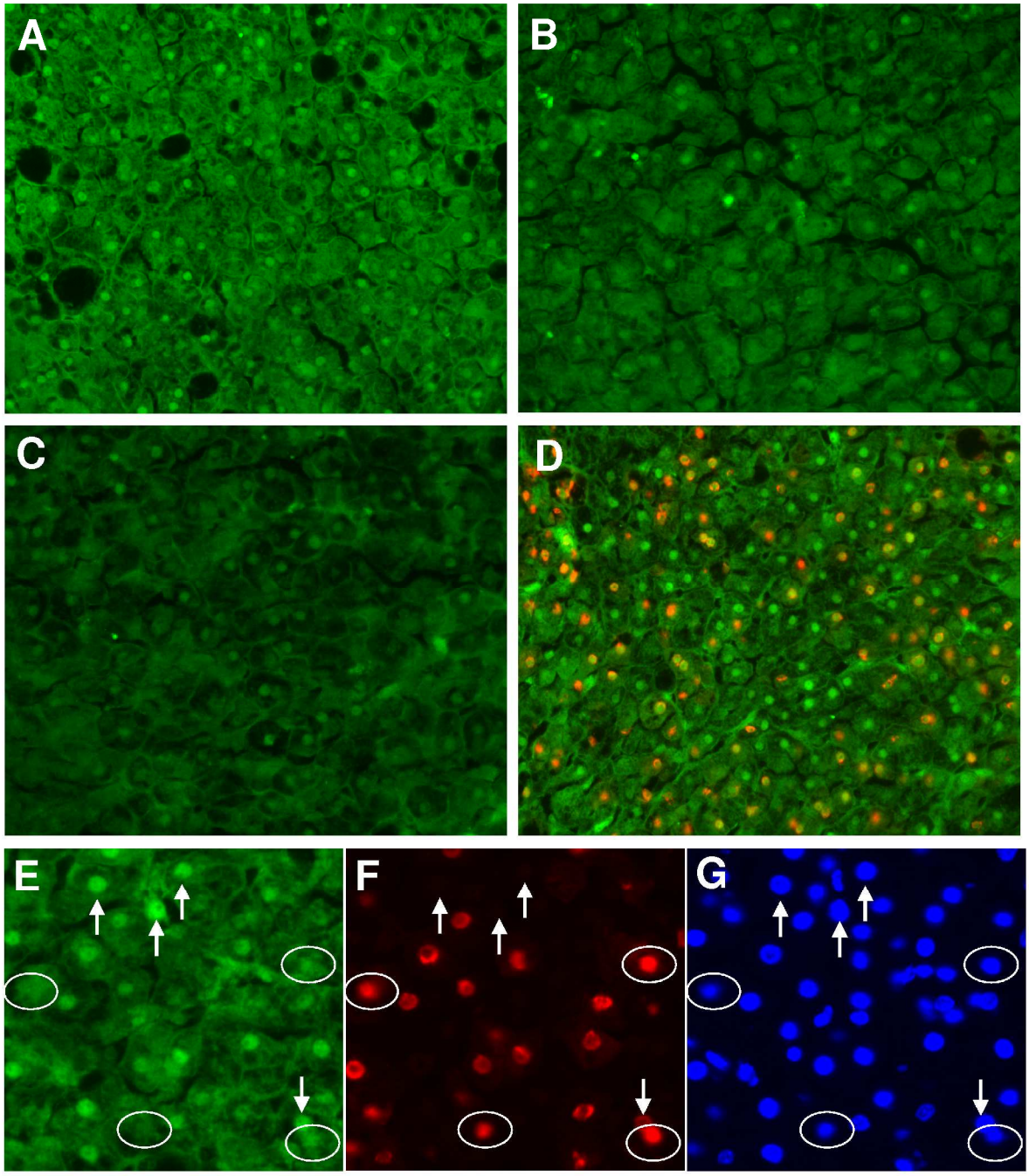


Figure C.1.12. hSTAT1 protein expression. *Immunofluorescence staining of hSTAT1 protein expression (green) in liver tissues from HBV/HDV co-infected (**A**), uninfected (**B**) and HBV mono-infected (**C**) mice. **D**) Co-staining of hSTAT1 (green) with HDAg (red) indicated an inverse correlation between the amounts of these two proteins. hSTAT accumulation was most prominent (arrows; **E**) in the nuclei of cells (DAPI staining; **G**) showing undetectable or lower amounts of HDAg staining (**F**), while cells that strongly express HDAg (circles, **F**) showed a reduced nuclear accumulation of hSTAT proteins (**E**).*

C.2. Persistence of hepatitis D virus mono-infection

All following results are already published in:

Persistent hepatitis D virus mono-infection in humanized mice is efficiently converted by hepatitis B virus to a productive co-infection
Giersch K, Helbig M, Volz T, Allweiss L, Mancke LV, Lohse AW, Polywka S, Pollok JM, Petersen J, Taylor J, Dandri M, Lütgehetmann M
J Hepatol. 2014 Mar;60(3):538-44. doi: 10.1016/j.jhep.2013.11.010. Epub 2013 Nov 23.

C.2.1 Establishment of HDV mono-infection in naïve humanized mice

Clinically an HDV infection occurs as a co-infection with HBV or as a super-infection of HBV infected patients. As a third and very rare form, HDV infection was also shown to persist for years in patients in the presence of low levels of HBV replication and HDAg positive cells have been observed up to 19 months after liver transplantation without detected evidence of HBV replication[158], [159]. These observations suggest that HDV might exist as a latent infection until rescued by HBV. An experimental study in woodchucks showed that HDV mono-infection might last at least one month[160], while studies in chimpanzees indicated HDV survival for only one week[161]. To investigate whether primary human hepatocytes support a non-productive HDV replication in vivo, the second aim of this thesis was to establish an HDV mono-infection in human hepatocytes of uPA/SCID mice and to assess the survival capacities of HDV in the absence of HBV, and whether an HDV mono-infection can be converted into a productive co-infection upon super-infection with HBV.

To establish an intrahepatic HDV mono-infection, naïve humanized uPA/SCID mice were inoculated with cell culture-derived HDV particles and sacrificed either after 2 (n=2), 3 (n=3) or 6 (n=3) weeks post-infection. To investigate the amount of HDV infected human hepatocytes in the absence of HBV immunofluorescence staining and quantitative RT-PCR measurements were performed. **Figure C.2.1 A-C** depicts the presence of HDAg-positive human hepatocytes (average 1.5%) in all HDV mono-infected livers and at each time point. In line, the relative intrahepatic expression of HDV RNA (**figure C.2.1 D)** was detectable at 2 weeks (0.07 HDV RNA copies/100 human GAPDH), 3 weeks (0.04 HDV RNA

copies/100 human GAPDH) and 6 weeks (0.03 HDV RNA copies/100 human GAPDH) post HDV mono-infection. Moreover, the amount of HDAg positive human hepatocytes and intrahepatic HDV RNA levels neither increased nor significantly diminished over time (p=0.58), since a median of 1.9% (range 1.0 to 5.3%), 1.2% (range 1.1 to 1.3%) and 1.6% (range 0.8 to 2.6%) HDAg-positive human hepatocytes were determined at week 2, 3 and 6 post HDV infection, respectively and thus demonstrating the successful establishment of intrahepatic HDV mono-infection[162].

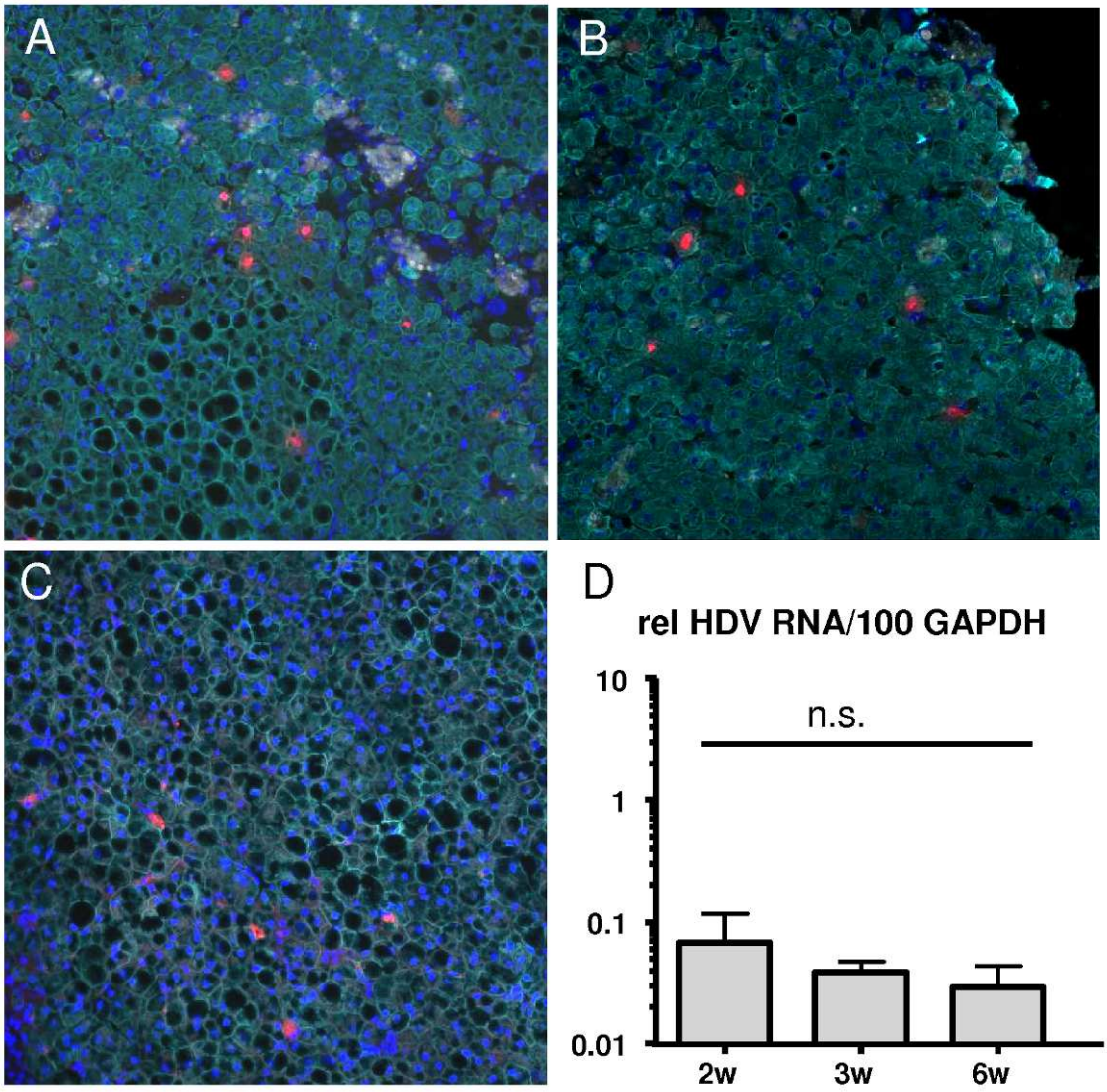


Figure C.2.1. Establishment of HDV mono-infection. *A small but comparable fraction (average 1.5%) of the human hepatocytes (blue) stained HDAg-positive (red) in mice that were sacrificed either after 2, 3 or 6 weeks of HDV mono-infection (**A, B, C**, respectively). **D**) Intrahepatic HDV RNA expression relative to 100 copies of GAPDH was detectable in all HDV mono-infected animals and remained unchanged among all time-points.*

Notably, similar levels of intrahepatic HDV mono-infection could also be established in all mice (n=5) that were inoculated with an HDV-positive serum derived from an entecavir treated patient displaying undetectable HBV viremia levels (**figure C.2.2**).

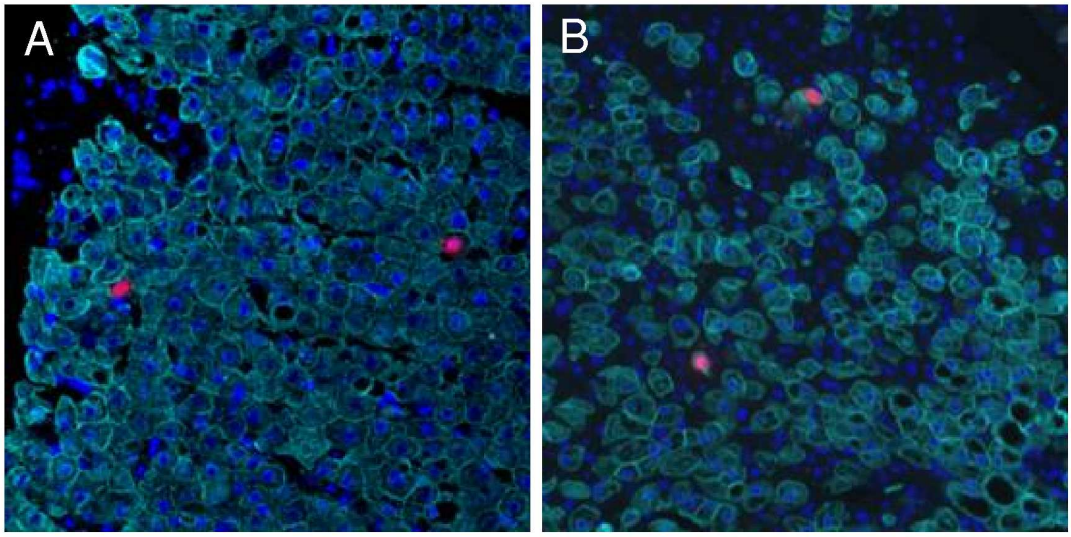


Figure C.2.2. Establishment of mono-infection. *HDAg (red) was detectable in a minority of the human hepatocytes (blue) 3 weeks after injection of HDV particles. Comparable amounts of HDAg-positive human hepatocytes were observed both by using cell culture derived HDV virions (3/3 animals)* ***(A)*** *and a patient-derived serum (HDV RNA positive and HBV DNA negative) in 5/5 animals* ***(B)****, demonstrating successful establishment of HDV mono-infection in human hepatocytes.*

To estimate whether HDV induced significant damage or apoptosis in mono-infected human hepatocytes, immunohistological assays for cell death markers (TUNEL and Caspase 3 staining) as well as quantitative measurements of human serum albumin (HSA) levels were performed. HSA levels, which fairly reflect the amount of human hepatocytes in human chimeric mouse livers, remained unchanged during the course of both HDV mono-infection experiments, indicating the absence of human hepatocyte death (**figure C.2.3**).

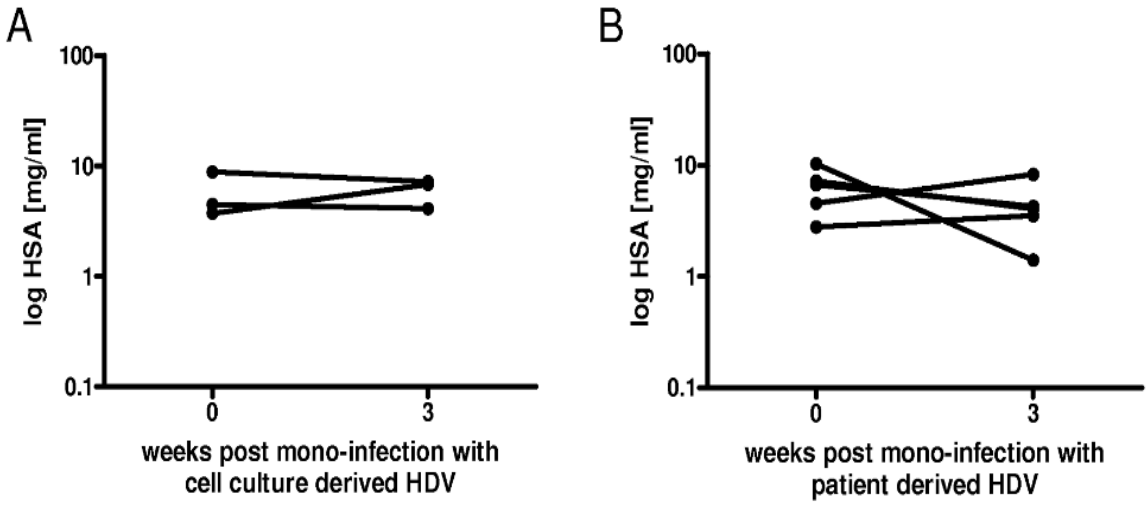


Figure C.2.3. Human serum albumin. *Concentrations of human serum albumin (HSA), quantified with ELISA measurements, remained stable in mice during HDV mono-infection both by using cell culture-derived supernatant (n=3) (**A**) or patient-derived serum (n=5) (**B**), indicating the absence of significant human hepatocyte loss during the course of HDV mono-infection.*

TUNEL staining, which detects the nuclei of apoptotic cells, appeared negative in all human hepatocytes of HDV mono-infected mice (**figure C.2.4 A**). The hepatotoxic uPA transgene in mouse hepatocytes causes murine hepatocytes death and therefore diseased hepatocytes of uPA-transgenic mice demonstrated a positive TUNEL staining, which was serving as positive control in this experimental setting (**figure C.2.4 B**).

Caspase 3, which plays a central role in cell apoptosis, was detected in some human hepatocytes of HDV mono-infected mice livers sacrificed either at week 2, 3 or 6 post-infection (mean 0.05%; 0.02%, 0.11%, respectively), although these values did not differ significantly ($p>0.05$) and similar human hepatocyte amounts were stained caspase 3-positive also in uninfected age-matched control mice (n=3; mean 0.02%). However, not a single human hepatocyte could be detected, which was co-stained positive for both HDAg and caspase 3 among all liver samples (n=13) that were screened (**figure C.2.4 C**)[162].

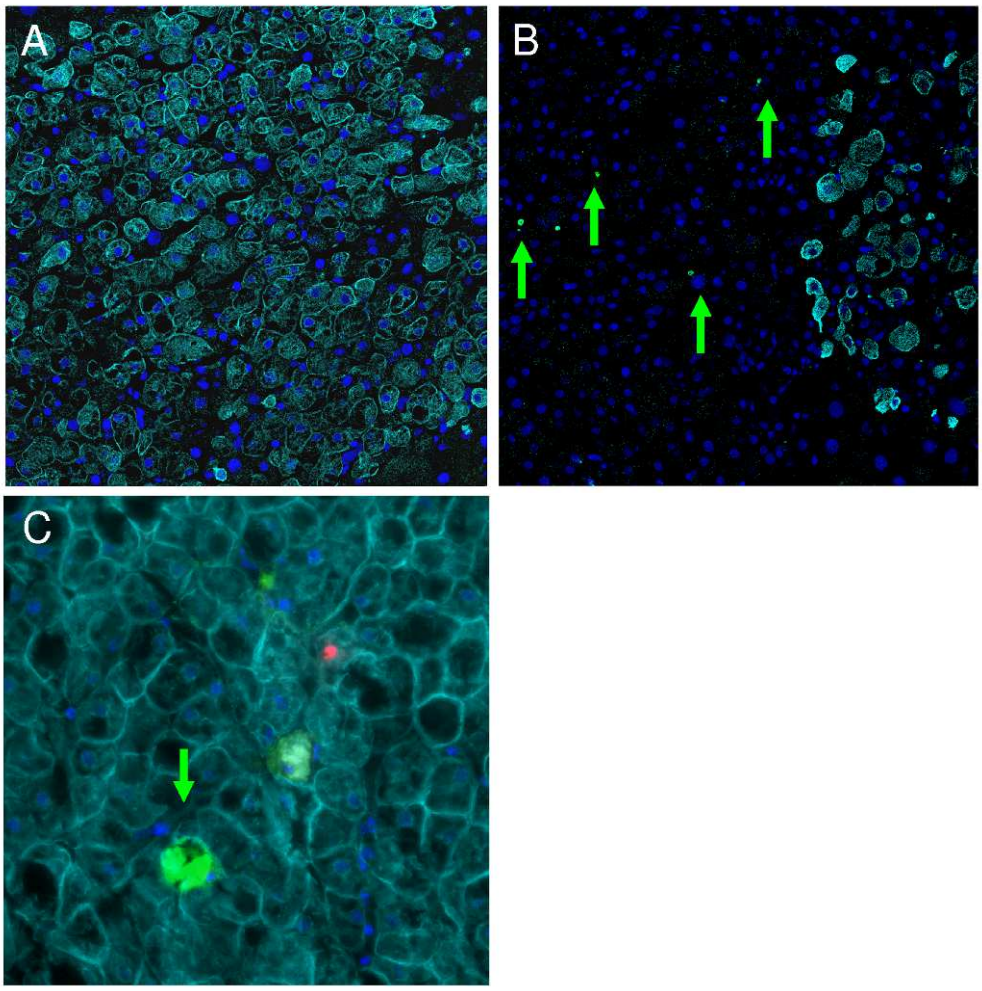


***Figure C.2.4**. **Cell death markers. A)** Cell death by TUNEL staining appeared negative in all human hepatocytes and was exclusively detectable (green arrows) in some liver cells of murine origin (positive control) **(B)**. **C)** Caspase 3 (green) was detected in very few human hepatocytes within the humanized mouse livers, but co-staining with HDAg (red) failed to identify caspase 3 expression in HDAg-positive cells.*

C.2.2 Rescue and infectivity of HDV virions upon HBV super-infection

To investigate whether persistent intrahepatic HDV particles of HDV mono-infected mice maintained their ability to replicate, assemble and spread as a productive virus, human chimeric mice that had been mono-infected with cell culture-derived HDV were super-infected with HBV ($1x10^8$ HBV DNA copies/mouse) either after three (n=3) or six weeks (n=3) post HDV inoculation.

As shown in **figures C.2.5 A** and **B**, mice that were inoculated with HBV three weeks after HDV mono-infection developed high viremia and reached median titers of $2.6x10^8$ HDV RNA copies/ml and $8.5x10^6$ HBV DNA copies/ml after 12 weeks of HBV super-infection. Notably, the rise of HBV and HDV viremia displayed comparable kinetics also in animals that had been super-infected with HBV six weeks after HDV mono-infection (**figures C.2.5 D** and **E**). In this case, median viremia was $2.6x10^8$ HDV RNA copies/ml and $2.4x10^7$ HBV DNA copies/ml nine weeks post HBV super-infection. In line with the HBV titers, increase of circulating HBsAg concentrations could be demonstrated in all HBV super-infected mice (**figures C.2.5 C** and **F**). These results indicate that a super-infection with HBV enables the assembly, release, and spread of intracellular persistent HDV particles and that HDV remains its ability of being a replicating virus also after 6 weeks of latency in human hepatocytes[162].

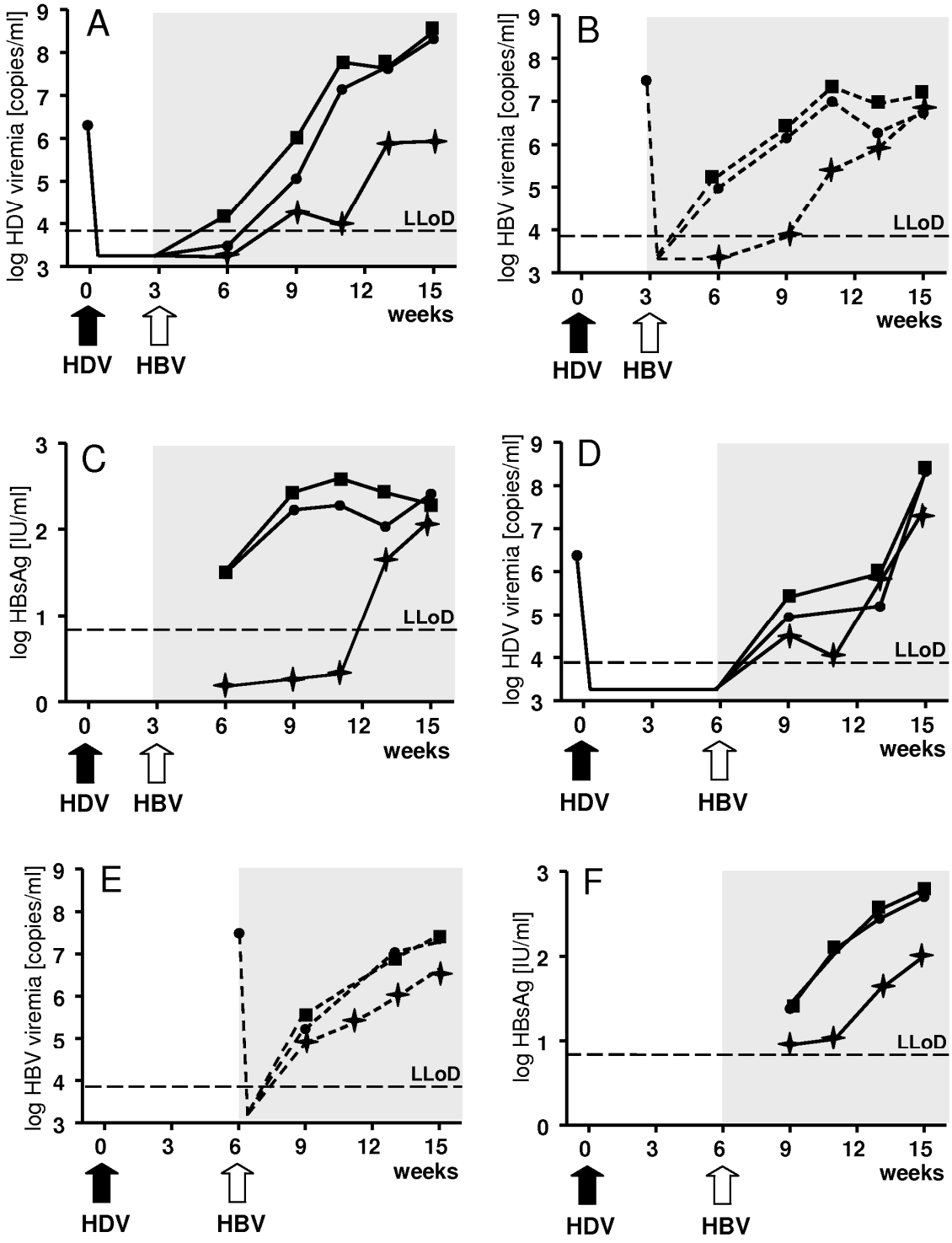


Figure C.2.5. Rescue of HDV virions upon HBV super-infection. *Serological viral loads of HDV RNA* ***(A, D)*** *and HBV DNA* ***(B, E)*** *were measured by quantitative real-time PCR. Development of HBV and HDV viremia in all mice (n=6) demonstrated that intracellular HDV could be rescued by HBV either three weeks* ***(A, B)*** *or six weeks* ***(D, E)*** *after injection of cell culture-derived HDV virions. In line with HBV titers, HBsAg levels increased in all animals after HBV super-infection* ***(C, F)****. Distinct serological measurements performed in the same mouse (****A*** *to* ***C****, or* ***D*** *to F) can be identified by the symbols used (square, circle, star).*

HSA levels, which represent the amount of human hepatocytes in humanized mice livers, remained unchanged in all mice that had been super-infected with HBV (**figures C.2.6 A** and **B**), indicating the absence of significant human hepatocyte loss during the course of HDV mono-infection and HDV rescue.

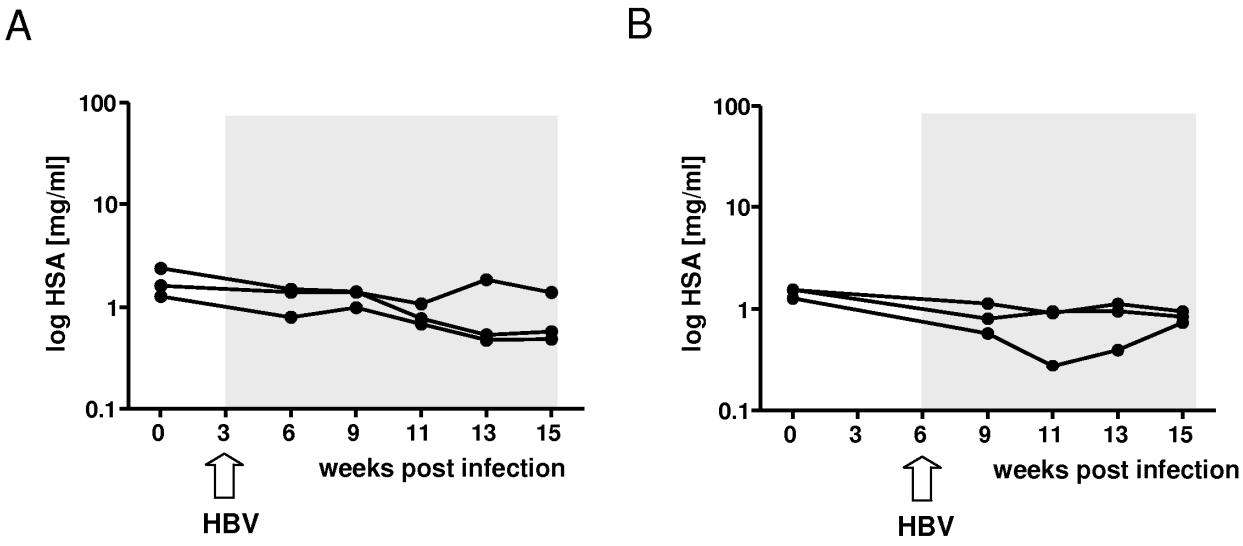


Figure C.2.6. Human serum albumin. *HSA levels remained also unchanged during the course of infection in mice which were HBV super-infected after 3 (**A**) or 6 weeks (**B**) of HDV mono-infection, indicating that human hepatocyte contents remained unchanged during HDV mono-infection and rescue experiments.*

To assess the extent of intrahepatic viral spreading after HBV super-infection, triple fluorescence staining of HDAg, HBcAg and the human-specific marker CK18 was performed on liver sections of HBV super-infected mice sacrificed after 15 weeks of HDV infection. **Figure C.2.7 A** depicts HDAg-positive staining in 53% (range 50.0 to 54.4%) of the human hepatocytes, thus indicating substantial spread of HDV infection among the human hepatocytes residing in the mouse livers.

Intrahepatic HDV RNA levels were determined by quantitative RT-PCR and mean HDV RNA expression levels relative to human housekeeping genes (100 GAPDH RNAs) demonstrated approximately 100-fold higher amounts of HDV RNA loads (7.13 HDV RNA copies/100 human GAPDH) in mice after 9 weeks of HBV super-infection (that is 15 weeks post HDV infection) than in HDV mono-infected mice (mean 0.04 HDV RNA

copies/100 human GAPDH) (**figure C.2.7 B**). These results again demonstrate that even after several weeks of a HDV mono-infection, HBV super-infection promptly led to the assembly, release and spread of infectious HDV, thus confirming that the latent HDV infection not only survived for at least six weeks before being rescued by HBV super-infection, but also maintained the ability to propagate within the chimeric mouse livers[162].

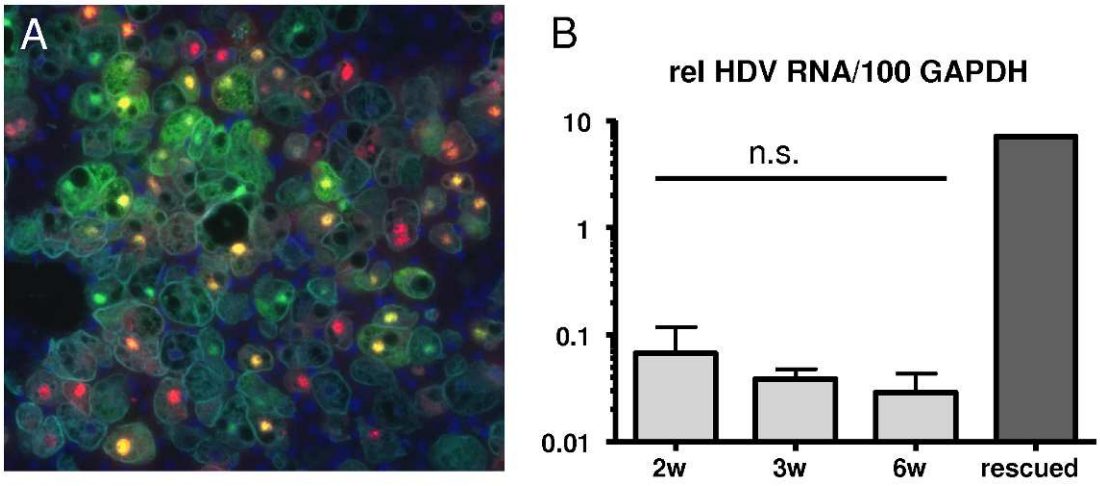


Figure C.2.7. Intrahepatic analyses of survival and spread of HDV virions. ***A****) Detection of HDAg (red) in the majority of the human hepatocytes (blue), as well as of HBcAg (green) and co-staining (HDAg+HBcAg; yellow) in mice sacrificed after 6 weeks of HDV mono-infection followed by 9 weeks of HBV super-infection, indicate successful spreading of HDV virions.* ***B****) Similar amounts of intrahepatic HDV RNAs were detected in the liver of animals sacrificed after 2 (n=2), 3 (n=8) or 6 weeks (n=3) of HDV mono-infection, which was achieved either with cell culture-derived (n=8) or patient derived (n=5) HDV particles. About 100-fold higher amounts of HDV RNA loads were determined in mouse livers after 9 weeks of HBV super-infection.*

To further test the infectivity of the HDV particles released upon HBV super-infection, two naïve, humanized mice were inoculated with 100µl of serum derived from one HBV super-infected mouse displaying high viremia levels ($2.7x10^8$ HDV RNA copies/ml, $2.4x10^7$ HBV DNA copies/ml). Development of HBV and HDV viremia was confirmed three weeks later in both animals. After six weeks, viral titers increased up to a median of $9x10^6$ HDV RNA copies/ml and $2x10^6$ HBV DNA copies/ml (**figure C.2.8 A**), demonstrating the ability of circulating HDV virions to establish an efficient de novo

infection even after six weeks of intrahepatic latency and to promote the development of a manifest HDV infection in newly infected mice. Histological investigation performed in liver sections of the two mice sacrificed 6 weeks post HBV/HDV co-infection indicated that a median of 58% (range 41 to 81%) of the human hepatocytes stained HDAg-positive, whereas only a minority of the cells (1.5%, range 0.5 to 5.4%) stained HBcAg-positive and 0.4% (range 0 to 1.5%) displayed both viral markers after 6 weeks of co-infection **(figure C.2.8 B)**[162]. In agreement with our previous study[53], the higher number of HDAg-positive human hepatocytes suggests that by injecting higher amounts of delta virions compared to HBV, HDV can infect hepatocytes and propagate with faster kinetics than HBV, provided that HBV/HDV co-infection is established at least in some hepatocytes.

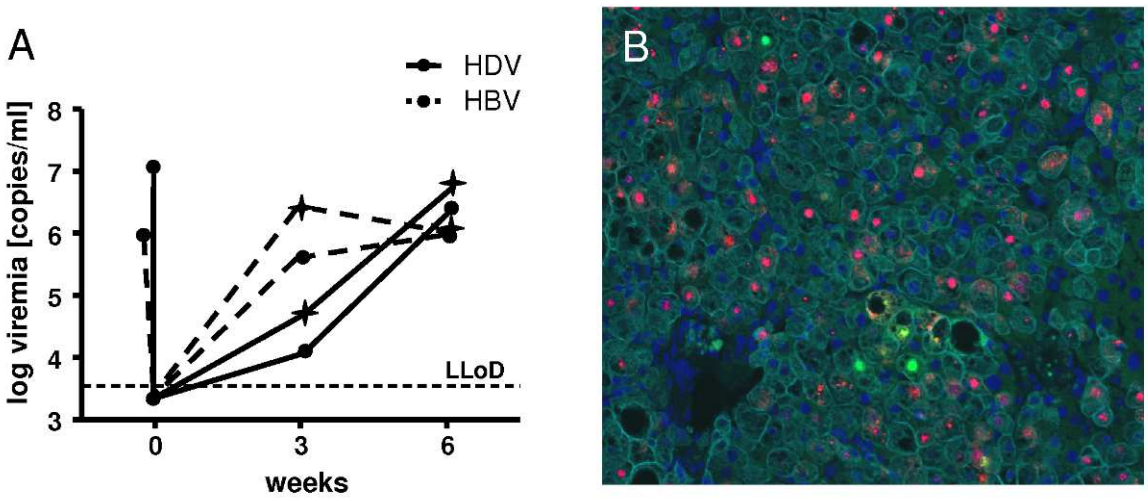


Figure C.2.8. Intrahepatic analyses of infectivity of HDV virions. A) *Three and six weeks after infection of mice with HDV particles rescued by HBV super-infection, development of HDV and HBV viremia was confirmed. Measurements performed in the same mouse can be identified by the symbols used (circle, star).* ***B)*** *In these mice a remarkable amount of HDAg-positive (red) human hepatocytes (blue) was detected by immunofluorescence, while only a minority of cells stained HBcAg-positive (green) after six weeks of HBV/HDV co-infection.*

C.2.3 Maintenance of dominant HDV quasi-species after latency and serial passage in mice

HDV is a virus known for its high genome variability and its ability to mutate in a short period of time. To determine whether mutations altering intrahepatic HDV replication may have occurred, sequencing of the RNA genome by using specific primers R1 and R2 was performed and analyzed with a special focus on the nucleotide sequences around the RNA-editing site responsible for the production of the large HDAg. **Figure C.2.9 A** displays examples of sequences derived from the input virus, from HDV genomes obtained from the liver of HDV mono-infected mice (2 and 6 weeks post-infection), as well as from the blood of one HBV super-infected mouse (after 6 weeks of HDV latency and 9 weeks of super-infection) and of one serial passaged HDV. This analysis revealed the coexistence of two predominant sequences, with only a detectable difference at the specific RNA-editing site (nucleotide 1023, according to numeration of Wang et al[15]). No accumulation of other mutations was detected by comparing sequences derived from input virions, intracellular HDV RNA, circulating rescued and serial passaged HDV RNA. As shown in the flow diagrams (**figure C.2.9 B**), approximately equal amounts of the unedited site ATC (UAG on mRNA) and of the noninfectious edited site ACC (UGG on mRNA) were found in all circulating HDV RNA forms (cell culture-derived, rescued after in vivo mono-infection and passaged virions). The thymine in this region is required to build a stop codon to generate the small HDAg, whereas RNA editing to cytosine leads to the longer HDV RNA sequence encoding for large HDAg. The co-existence of both HDV RNA quasi-species in this case is not surprising, since the generation of small and large HDAg is essential for virus replication and assembly and therefore leads to a productive HDV infection. In contrast, the ratio of these two HDV RNA sequences appeared slightly altered in the liver of HDV mono-infected mice, indicating a shift towards the edited HDV genome, which encodes for the large HDAg.

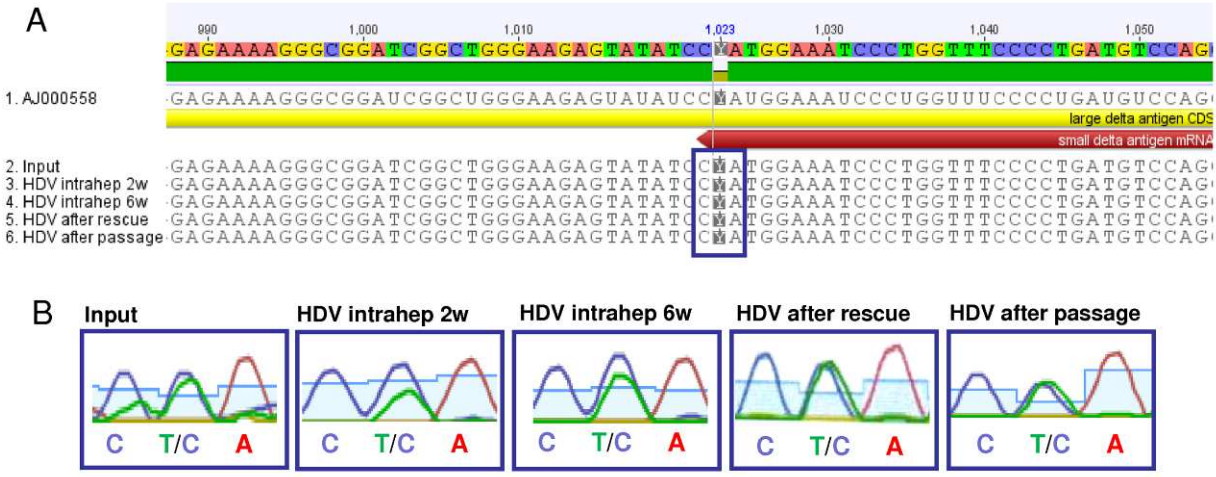


Figure C.2.9. Genome sequencing. *A) HDV RNA sequencing around the RNA-editing site responsible for the production of large HDAg protein was determined in the inoculum, in 2 and 6 weeks mono-infected mice (intrahepatic), in HDV particles rescued after 6 weeks (serum) and in serial passaged HDV (serum). The full genome sequence AJ000558 was used for comparison.* ***B****) The flow diagrams show a base pair mixture of cytosine and thymine in the stop codon position of small delta antigen, which demonstrates RNA-editing from ATC (UAG on mRNA, stop codon) to ACC (UGG on mRNA, no stop codon). The ratio of HDV RNA quasi-species was approximate 1:1 in input, rescued and passaged HDV RNA, while a cytosine-predominance was found in the livers of HDV mono-infected mice.*

To assess whether the relative intracellular increase of the edited HDV RNA species led to a detectable shift at the protein level, extracts obtained from liver of the same mice were analyzed by immunoprecipitation and western blot using anti-HDAg antibodies. In agreement with the nucleotide sequencing results, the ratio of the two HDAg isoforms was not significantly altered in circulating virions (rescued and in vivo passaged; **figure C.2.10 A**), but a decrease of the small HDAg protein could be detected after 6 weeks of intrahepatic HDV mono-infection (**figure C.2.10 B**). Nevertheless, the large HDAg predominance was apparently not sufficient to abrogate HDV propagation since HBV super-infection led to prompt development of HDV viremia both after three and six weeks of latent mono-infection[162].

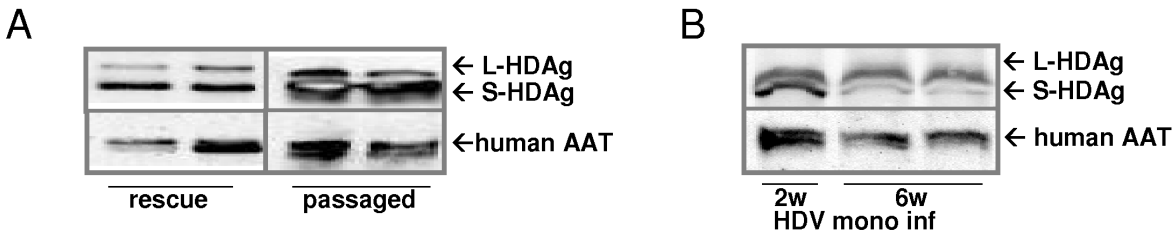


Figure C.2.10. HDAg analyses. ***A****) Immunoprecipitation and immunoblot analyses indicate that intrahepatic production of small and large HDAg is detected in all HDV/HBV replicating animals sacrificed either after 3 (lane 1) or 6 weeks (lane 2) of HBV super-infection, as well as after passaging of the rescued HDV (n=2) (lane 3, 4). A decrease of small HDAg relative to large HDAg was observed between week 2 (lane 1) and 6 (lane 2 and 3) in livers of HDV mono-infected mice (****B****). Human α-antitrypsin was employed to normalize values for the amount of human hepatocytes present in selected liver specimen.*

C.3. Quantification of genomic and antigenomic HDV RNA

Genomic and antigenomic HDV RNAs are essential for a productive HDV replication and distinguishing between both HDV RNA forms enables more detailed analysis of the intrahepatic HDV activities in human hepatocytes. Since genomic and antigenomic HDV RNA are exact complements of each other and lead to self-priming and self-annealing and therefore to the occurrence of many unspecific transcription products, their specific detection is challenging and to date limited to northern blot analyses. The aim was to develop a PCR based method to quantify genomic and antigenomic HDV RNA in human hepatocytes of uPA/SCID mice and in patient liver biopsies.

To establish a quantitative RT-PCR assay for the strand-specific determination of genomic and antigenomic HDV RNAs, artificial genomic and antigenomic HDV RNA standards were generated as described in **Material & Methods, B.3.5**. HDV RNA standards were first incubated either with a genomic HDV or antigenomic HDV biotinylated strand-specific primer[145]. After reverse transcription, specific cDNA was separated from unspecifically transcribed products via magnetic beads, which specifically bind the biotinylated cDNA products. In a next step, qRT-PCR was performed with primers and probes recognizing all known HDV genotypes[145] (**figure C.3.1**).

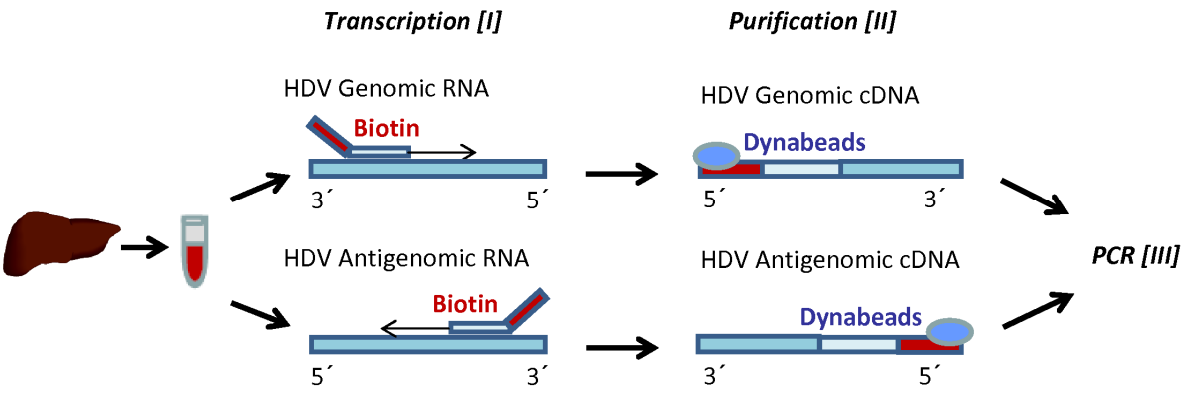


Figure C.3.1. Establishment of strand-specific genomic/antigenomic HDV RNA assay. *HDV RNAs were separately reverse transcribed using biotinylated HDV specific genomic or antigenomic primers, purified with magnetic beads and then quantified by performing RT-PCR.*

By performing this assay, specific RT-PCR signals of the genomic and antigenomic HDV RNA standard could be detected, while controls without reverse transcriptase or without reverse transcription primer did not yield a product in the PCR and therefore excluded self-priming and self-annealing of HDV RNA in this assay. Artificial full length genomic and antigenomic HDV RNAs were diluted and used as standard demonstrating that this new assay can detect genomic and antigenomic RNA from 10^9 down to 10^5 copies (**figure C.3.2 A-D**).

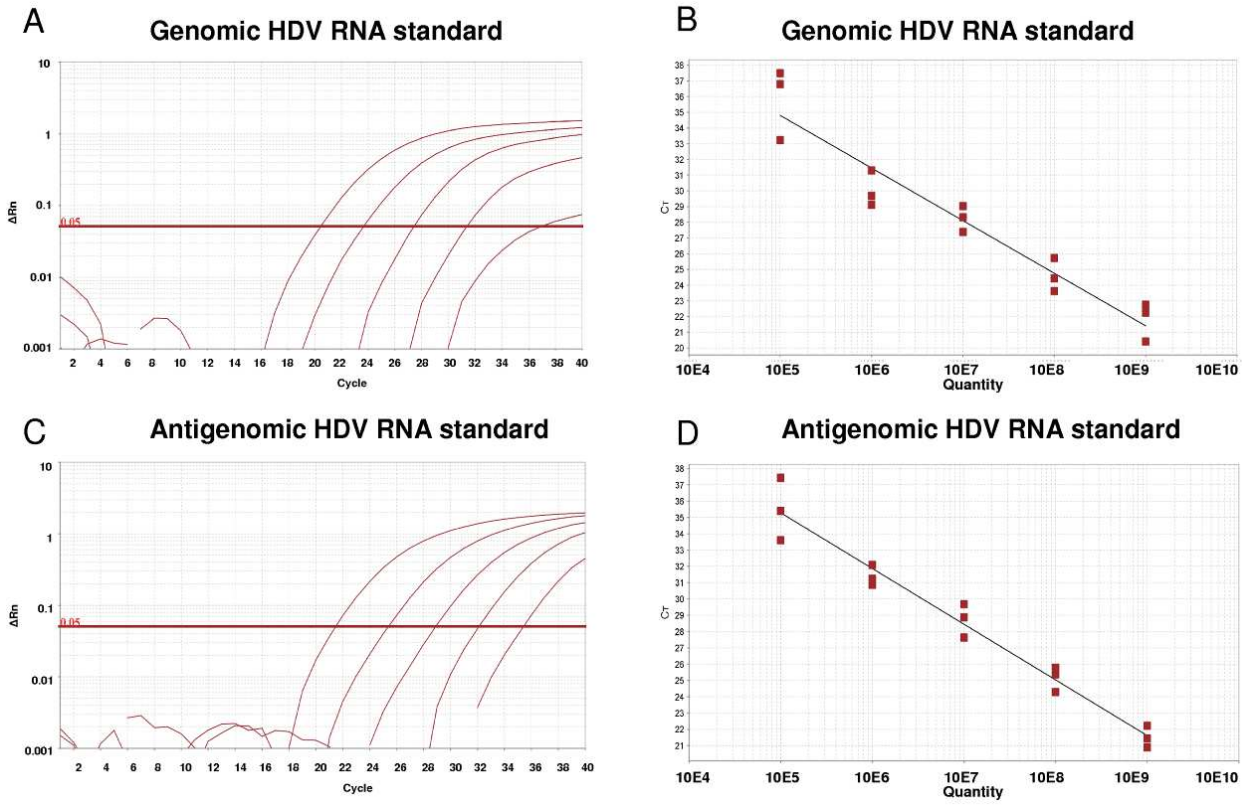
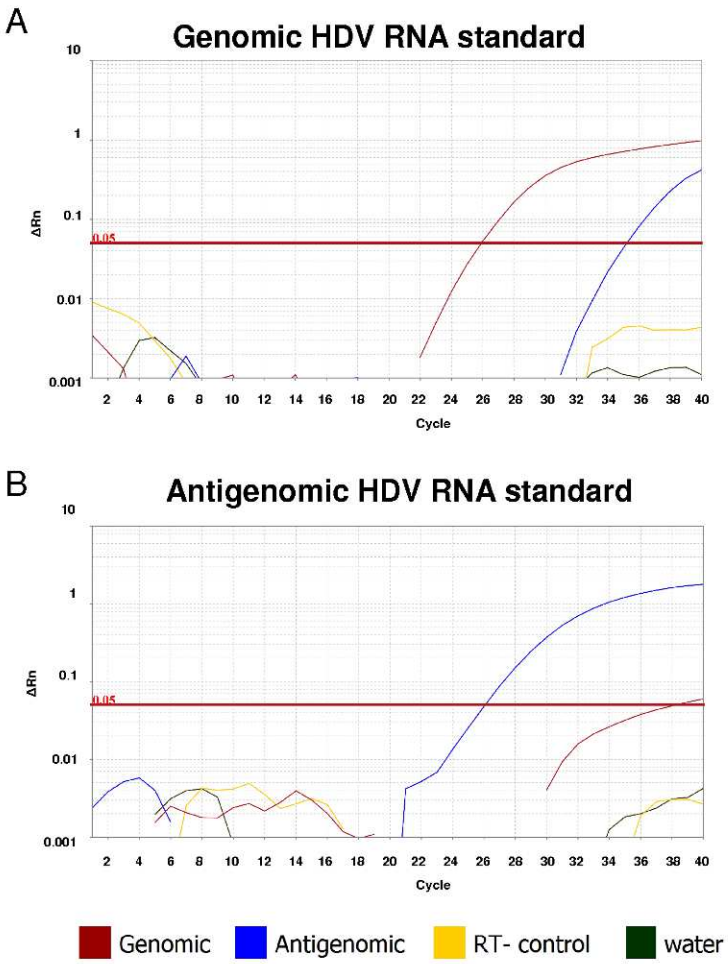


Figure C.3.2. Standard curves. *Standard curves of genomic HDV RNA with a slope of 3.351 and an efficiency of 98.8%* ***(A)*** *and antigenomic HDV RNA with a slope of 3.417 and an efficiency of 96.2%* ***(C)****. Amplification curves of 10^9, 10^8, 10^7, 10^6 and 10^5 copies of genomic* ***(B)*** *and antigenomic* ***(D)*** *HDV RNA.*

The specificity of this assay was investigated by the determination of artificial genomic and antigenomic RNA with the correct primer and the respective opposite primer (genomic primer on antigenomic HDV RNA standard and vice versa) (**figure C.3.3 A, B**). Unspecific reverse transcription with the opposite primer occurred, though in a very limited extent and

without relevance for the natural ratio of genomic and antigenomic RNA in livers. Ratios of genomic to antigenomic RNA can be quantified up to 1:100 without crosstalk (**figure C.3.3 C**), which was revealed by quantifying artificial mixtures of $5x10^8$ copies genomic HDV RNA with $5x10^8$ (ratio 1:1), $5x10^7$ (ratio 1:10) and $5x10^6$ (ratio 1:100) copies of antigenomic HDV RNA. These solutions were transcribed with the antigenomic primer and amplification curves showed that the ct value is 31 for $5x10^6$ copies antigenomic HDV RNA (in a mixture with $5x10^8$ copies of genomic HDV RNA; 1:100 ratio) and in accordance with the unspecific amplification signal for $5x10^8$ copies of pure genomic HDV RNA (also ct value of 31) transcribed with the antigenomic primer, indicating that the antigenomic HDV RNA standard in a mixture with genomic HDV RNA can only be detected until a dilution of 1:100 copies (genomic: antigenomic). As another control experiment, genomic and antigenomic HDV RNA levels in the serum of a chronically HBV/HDV infected uPA/SCID mouse ($2.4x10^5$ copies/ml) were quantified. During the replication cycle of HDV genomic HDV RNA is exclusively used for the assembly and release of new HDV virions. Therefore HDV particles containing antigenomic HDV RNA forms should not be detected in serum. As assumed, **figure C.3.3 D** depicts detectable genomic HDV RNA (ct value = 31) and undetectable antigenomic HDV RNA in mouse serum.


A
Genomic HDV RNA standard
10
1
0.1
0.01
0.001
ΔRn
0.05
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
B
Antigenomic HDV RNA standard
10
1
0.1
0.01
0.001
ΔRn
0.05
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
Genomic
Antigenomic
RT- control
water

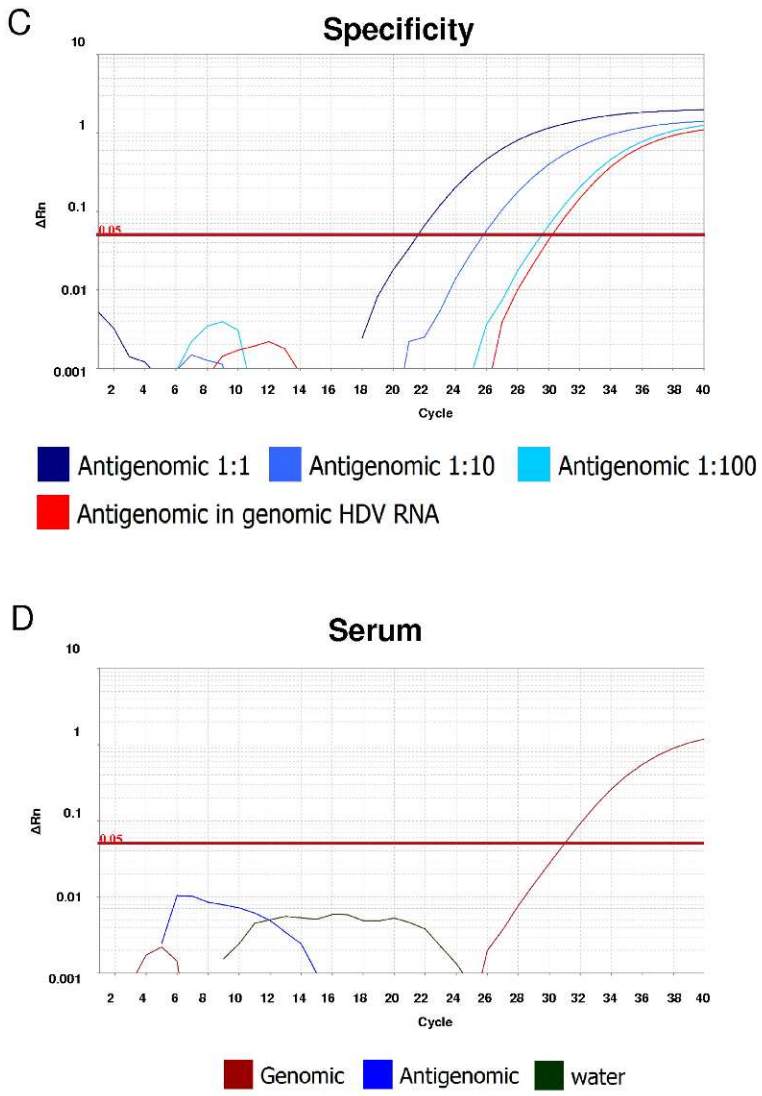


Figure C.3.3. Establishment of a specific genomic/antigenomic assay. A) *Amplicifcation curves of 10^7 copies genomic HDV RNA standard transcribed with the biotinylated HDV genomic (ct=26.0, red curve) or antigenomic (ct=34.5, blue curve) primer.* ***B)*** *Amplicifcation curves of 10^7 copies antigenomic HDV RNA standard transcribed with the biotinylated HDV antigenomic (ct=26.0, blue curve) or genomic (ct=38, red curve) primer.* ***C)*** *Amplification curves of $5x10^8$ copies genomic HDV RNA mixed with $5x10^8$ (1:1), $5x10^7$ (1:10) and $5x10^6$ (1:100) copies of antigenomic HDV RNA and transcribed with the antigenomic primer. The ct value for $5x10^6$ copies antigenomic HDV RNA (red curve) is 31 and the same as the unspecific ct value for $5x10^8$ copies genomic HDV RNA also transcribed with the antigenomic primer, indicating that the detection of antigenomic HDV RNA in a mixture with genomic HDV RNA can only be detected down to a dilution of 1:100 (genomic: antigenomic).* ***D)*** *Serum control from a chronically HBV/HDV-infected uPA/SCID mouse.*

HDV infected human chimeric mice (n=3, median viremia of $6.7x10^7$ HDV RNA copies/ml) show median genomic HDV RNA levels of 0.824 and median antigenomic HDV RNA levels of 0.021 relative to the housekeeping gene hGAPDH (**figure C.3.4 A**). The ratio of genomic to antigenomic HDV RNA ranged between 19.4 and 73.0 to 1(**figure C.3.4 B**). Interestingly, similar levels of genomic (median relative 1.791 HDV/hGAPDH) and antigenomic RNAs (median relative 0.043 HDV/hGAPDH; median ratio: 2.9-41.3) were observed in human liver biopsies from patients with chronic HDV/HBV infection (**figure C.3.4 A, B**), indicating that this novel assay is suitable for intrahepatic measurements in both humanized mouse livers and liver patient biopsies.

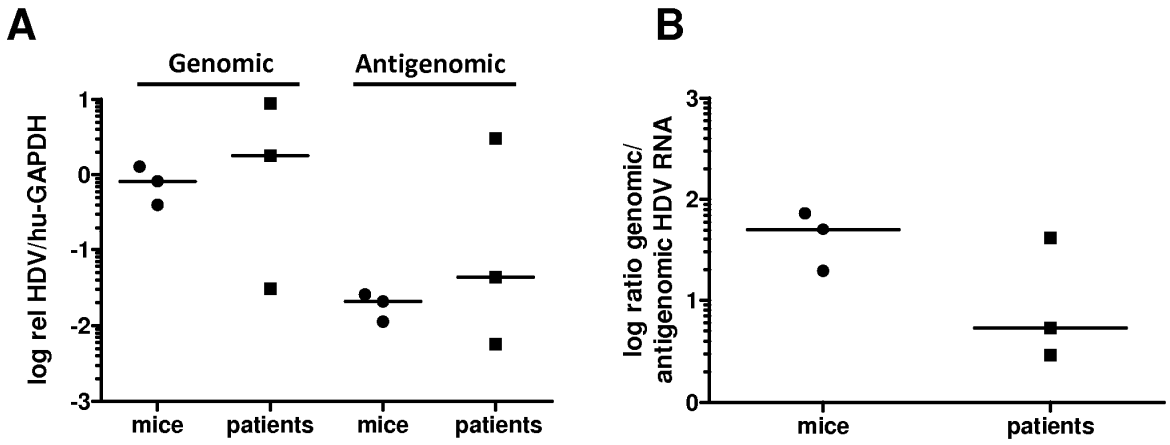


Figure C.3.4. Genomic/antigenomic HDV RNA in mice and patients. *Amounts of genomic and antigenomic HDV RNA relative to human GAPDH (**A**) and ratios of genomic to antigenomic HDV RNA (**B**) obtained from this new assay are comparable between cryo-conserved livers of 8-weeks HBV/HDV co-infected, humanized mice (n=3) and liver biopsies of chronic HBV/HDV co-infected patients (n=3).*

C.4. Antiviral effects of pegylated interferon-α and entecavir in HBV/HDV co-infected UPA/SCID mice

C.4.1 Reduction of HDV viremia

Nucleoside/nucleotide analogues as lamivudine, adefovir and tenofovir, which are potent reverse transcriptase inhibitors for the therapy of HBV infections, do not show beneficial effects in most chronically HBV/HDV co-infected patients. Treatment options for HDV infections are therefore limited to regular or pegylated interferon-α (peg-IFN-α)[4]. However, the knowledge of intrahepatic virological changes induced by the different antiviral treatments available in HBV/HDV co-infected patients or in animal models is scant. Therefore the aim was to study the intrahepatic changes of HDV replication, especially of genomic and antigenomic HDV RNA, during a 4-week antiviral therapy with peg-IFN-α or the HBV-polymerase inhibitor entecavir (ETV) in human-chimeric uPA/SCID mice.

Chronic HBV/HDV infected human chimeric mice (median viremia at baseline: $3.2x10^6$ HBV DNA copies/ml and $3.5x10^7$ HDV RNA copies/ml) were treated with peg-INF-α (n=3) twice a week for 4 weeks or remained untreated as controls (n=3). As depicted in **figure C.4.1 A, B**, the antiviral treatment induced a reduction of 2.0-log and 2.2-log in HBV and HDV viremia, respectively. HBsAg levels were decreased by 1.1-log in peg-INF-α treated animals and remained stable in untreated animals (**figure C.4.1 C**). A second group of human chimeric uPA/SCID mice (median viremia at baseline: $3.4x10^6$ HBV DNA copies/ml and median $4.8x10^6$ HDV RNA copies/ml) were treated with ETV (n=4) daily for 4 weeks or remained untreated as controls (n=3). ETV treated mice showed a 3.4-log decrease of HBV viremia, but no significant decrease of HDV viremia (**figure C.4.1 D, E**) or HBsAg levels (**figure C.4.1 F**). Again untreated control mice remained stable in all three parameters (**figure C.4.1 D-F**).

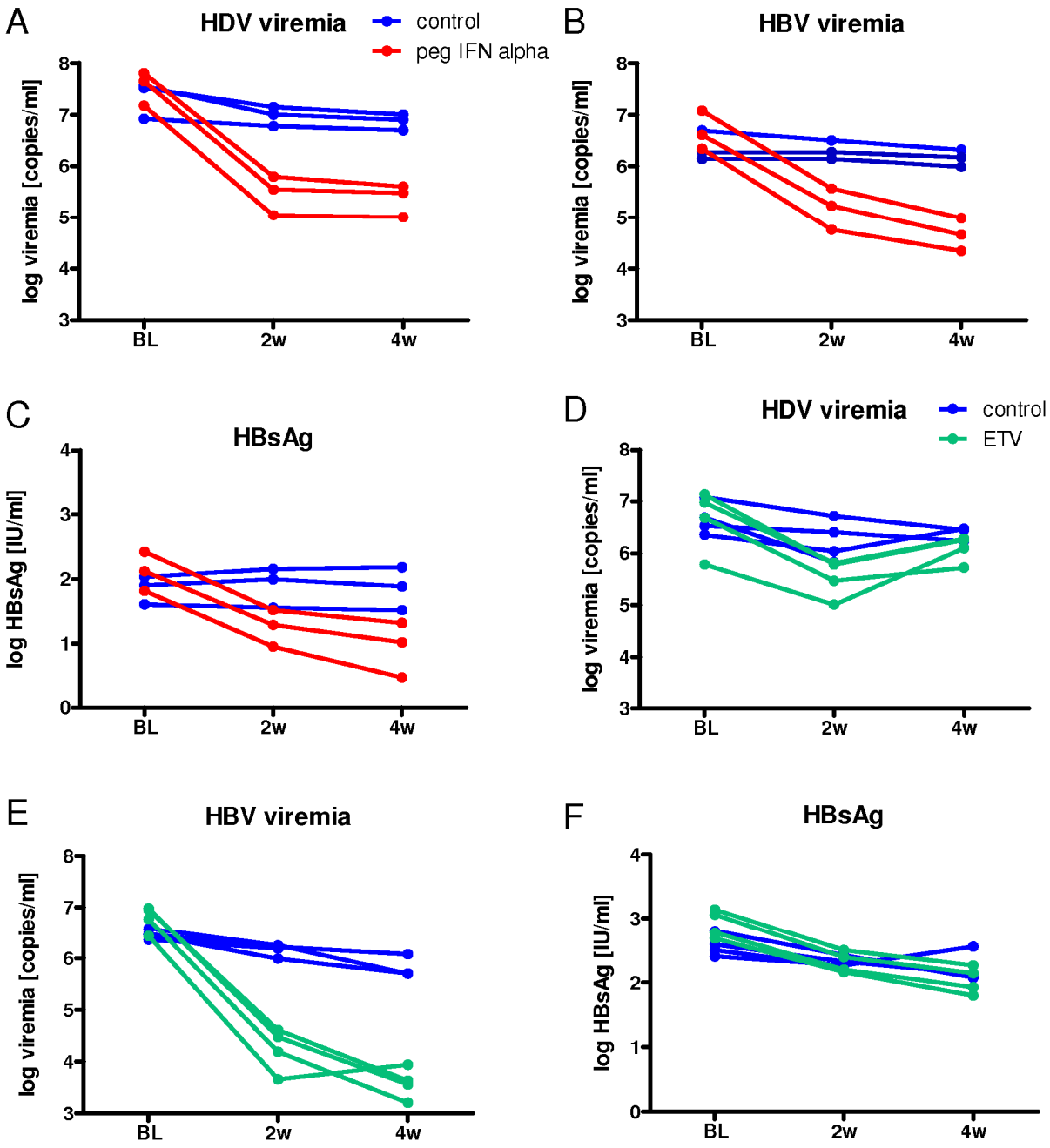


Figure C.4.1. Serological measurements. *4 weeks of peg-INF-α treatment (twice a week) in chronic HBV/HDV co-infected mice led to a reduction of HDV viremia by 2.2-log (**A**), HBV viremia by 2.0-log (**B**) and HBsAg levels by 1.1–log (**C**). In contrast, daily ETV administration for 4 weeks decreased HBV viremia by 3.4-log (**E**), while HDV viremia (**D**) and HBsAg levels (**F**) remained unchanged.*

C.4.2 Changes of genomic and antigenomic HDV RNA

To evaluate intrahepatic activities of HBV and HDV, some treated (peg-INF-α: n=3; ETV: n=2) and untreated (n=5) human chimeric mice were sacrificed at the end of the treatment period. First, amounts of genomic and antigenomic HDV RNAs were determined to elucidate whether treatment with these compounds has specific effects on the replication of the virus. **Figure C.4.2 A** shows that peg-IFN-α but not ETV reduced both genomic and antigenomic HDV RNA relative to human GAPDH clearly by 6.5-fold and 6.0-fold, respectively, compared to untreated control mice. Ratios of genomic to antigenomic HDV RNA remain constant in all groups (median control: 17.7; peg-IFN-α: 20.3; ETV: 18.0) (**figure C.4.2 B**), indicating that peg-INF-α alters genomic and antigenomic HDV RNA levels similarly.

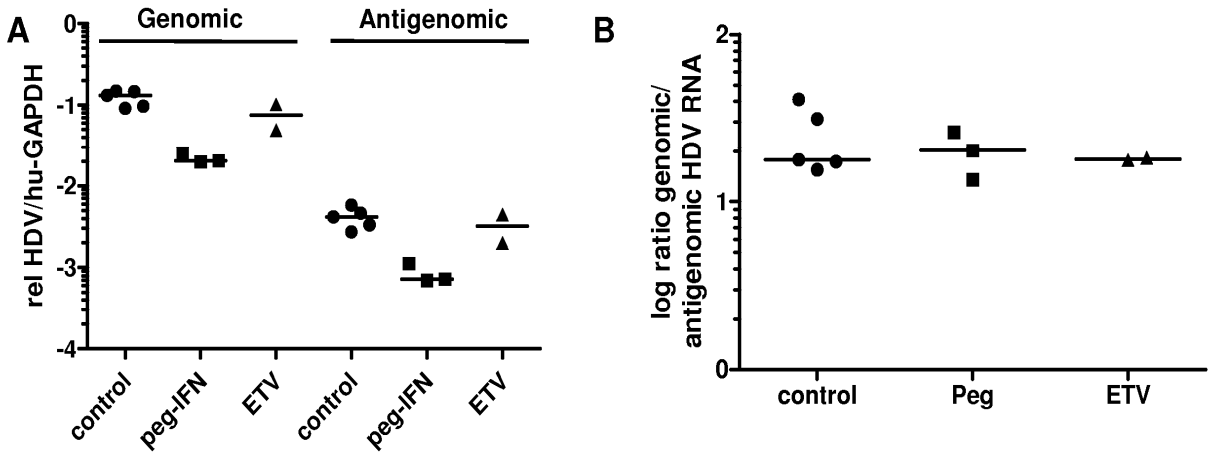


Figure C.4.2. Intrahepatic HDV measurements. *Peg-INF-α treatment clearly reduced amounts of genomic and antigenomic HDV RNA relative to human GAPDH, while ETV did not show a significant reduction of HDV RNA levels compared to untreated control mice (**A**). Ratios of genomic to antigenomic HDV RNA remained constant in all three groups (**B**)*

Results of this new quantitative RT-PCR assay were confirmed by northern blot analyses (**figure C.4.3),** which were performed by the group of T. Pollicino (within a cooperation project) at the Department of Paediatric, Gynaecologic, Microbiological, and Biomedical Sciences, University of Messina in Messina, Italy. In line with qRT-PCR measurements, densitometric quantification of northern blot bands revealed that peg-IFN-α but not ETV is able to decrease amounts of both genomic and antigenomic HDV RNA.

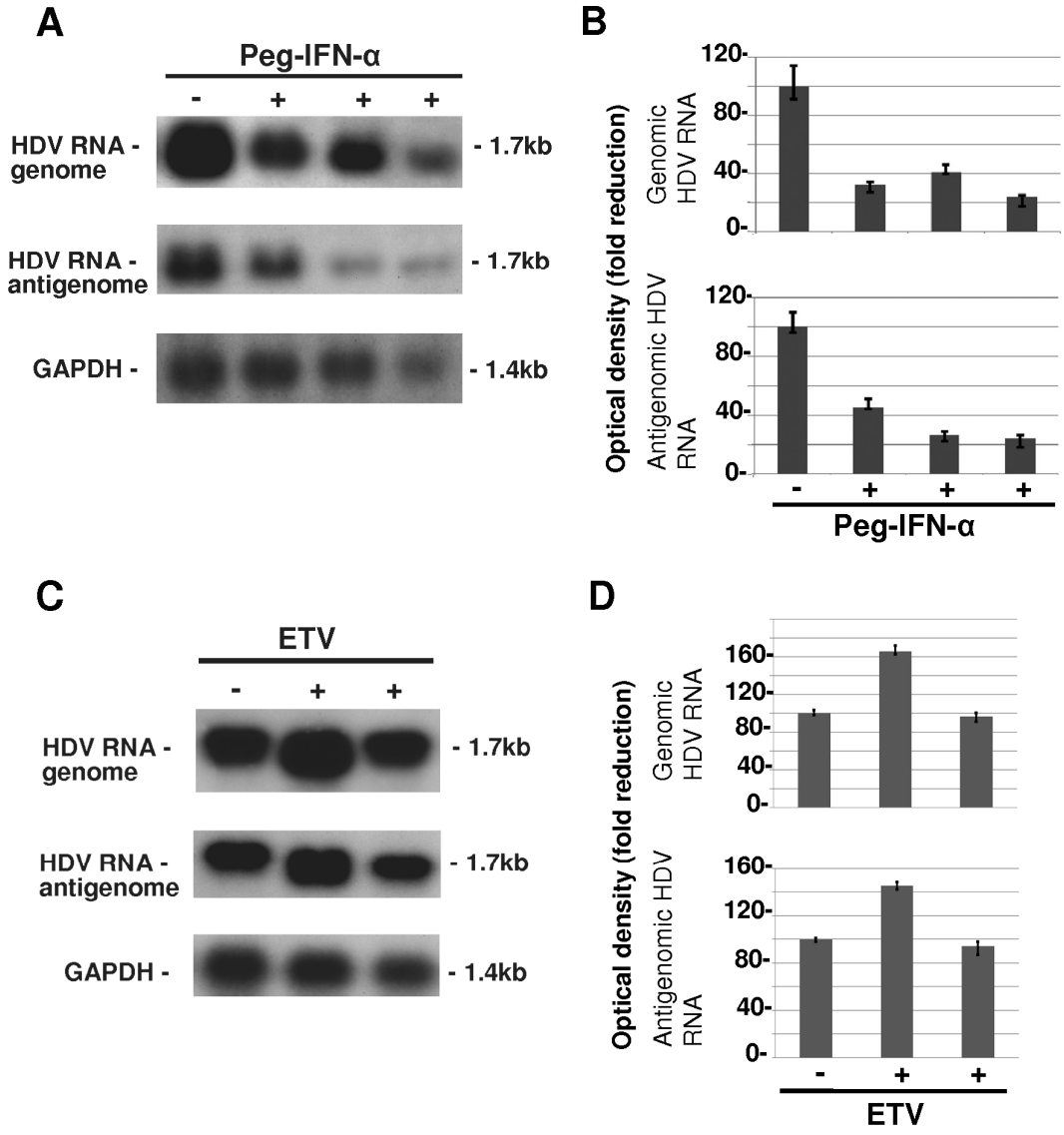


Figure C.4.3. Northern blot analyses. *Representative northern blot analyses (**A, C**) and the densitometric quantification (**B, D**) of signal bands for genomic (1.7kb) and antigenomic HDV RNA (1.7kb) from untreated (-) and peg-IFN-α (**A, B**) or ETV treated (+) mice (**C, D**).*

Moreover, **figure C.4.4** demonstrates that both peg-IFN-α and ETV were able to decrease total HBV DNA copies per human cell compared to untreated control animals by 6.9-fold and a 15.9-fold, respectively, confirming that HBV productivity, in contrast to HDV, is clearly reduced upon treatment with both drugs.

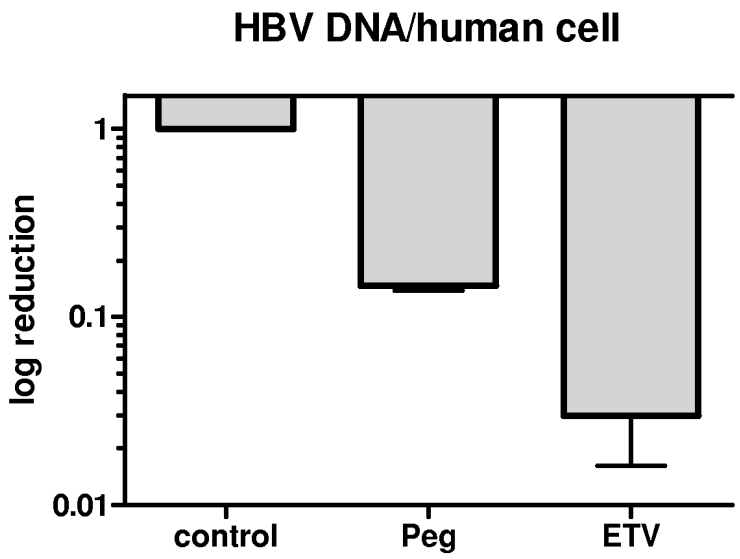


Figure C.4.4. HBV DNA levels. *Peg-INF-α and ETV treatment decreased intrahepatic HBV DNA/human cell (median reduction with range) compared to untreated control mice.*

C.4.3 Reduction of intrahepatic HDAg

Immunofluorescence stainings of liver specimens revealed that the number of HDAg-positive human hepatocytes is significantly reduced in peg-IFN-α treated mice. 60.2% (range=51.0-76.4%) of human hepatocytes in control animals are infected with HDV (**figure C.4.5 A**), while only 9.6% (range=4.7-18.3%) of IFN-α treated mice demonstrate HDAg-positive human hepatocytes (**figure C.4.5 B**). In contrast, in ETV-treated animals the number of HDAg-positive human hepatocytes remained comparable to levels obtained in untreated humanized mice (51.2%, range=41.3-63.6%; **figure C.4.5 C**).

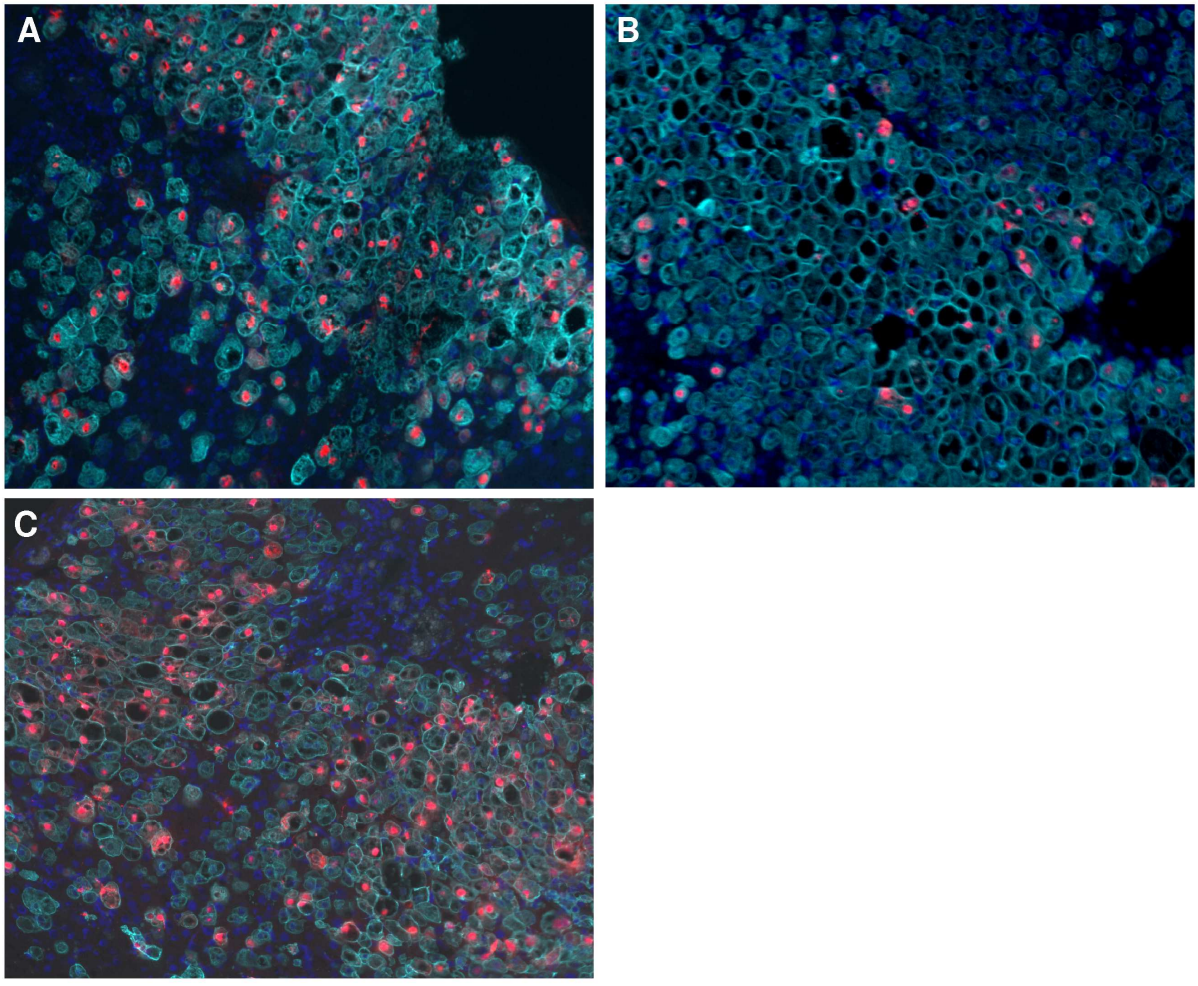


Figure C.4.5. Immunofluorescence stainings. *Peg-INF-α (**B**), but not ETV (**C**) treatment decreased the expression of intrahepatic HDAg (red) in human hepatocytes (blue) compared to untreated control livers (**A**).*

D. Discussion

The limited availability of HDV infection models has hindered investigation of the interactions occurring between HDV and infected human hepatocytes. Our group recently developed a small animal model for stable HDV infections using humanized uPA/SCID mice[53]. By using this in vivo model different aspects of HDV infection could be questioned and successfully answered within this doctoral thesis. This study characterizes the interplay of HDV and the innate immune system in human hepatocytes and describes the intrahepatic persistence of HDV in the absence of HBV and its conversion to a productive co-infection upon HBV super-infection. Moreover, a novel RT-PCR assay was established to specifically quantify genomic and antigenomic HDV RNA, which are both essential for virus replication and assembly. As a proof of concept, this assay was used to investigate intrahepatic antiviral effects on HDV productivity upon treatment with peg-INF-α and the HBV polymerase inhibitor entecavir.

D.1. Hepatitis D virus infection and innate immune responses

The innate immune system is the first-line defense against foreign pathogens and the induction of interferon pathways is considered the hallmark of antiviral immunity. Although the activation of ISGs is known to play a fundamental role in counteracting viral infections, knowledge of intrahepatic innate immune processes during chronic HDV infection is very limited. Therefore, in this study the humanized uPA/SCID mouse system, which lacks functional B, T and NK cells, was used to investigate the interplay of HDV and innate immune responses in human hepatocytes. In HBV/HDV co-infected humanized mice the development of viral parameters, which reached a maximum at week 8 of infection, was accompanied by a strong induction of the antiviral state of the human hepatocytes during the course of co-infection. These co-infected mice were repopulated with human hepatocytes isolated from the same human donor (to avoid possible host-related bias) and demonstrated that the intrahepatic expression of human specific ISGs (e.g. hOAS1, hMxA, hISG15) and signaling genes (e.g. hSTAT1) was increased during the spreading phase of infection (week 3), reached its highest level at the peak of infection (8 week), when HDV spreading appeared to be accomplished, and remained enhanced in the following weeks of

stable infection (>12 weeks). In contrast, and in line with previous studies highlighting the ability of HBV to avoid the induction of the innate signaling pathways in infected cells[151], [163]–[165], establishment of HBV mono-infection provoked a less pronounced induction of human ISGs.

While HBV is regarded as a virus that under most conditions is not directly cytopathic to the infected hepatocytes, data from chimpanzees and specific clinical cases suggest direct cytopathic effects of HDV on hepatocytes, but particularly in an acute hepatitis setting[10]. However, HDV infected patients advance more rapidly in their disease than patients infected with HBV or HCV[4] and clinical observations indicate that the liver damage associated with chronic HDV infections mainly seems to be immune-mediated. Although both innate and adaptive immune responses are believed to contribute to the pathogenesis of HDV infection, the mechanisms responsible for the more severe course of the disease remain largely unknown. Adaptive immune responses to HDV are generally weak in untreated patients[48] and a previous study suggested an elevated frequency of peripheral NK cells in chronically HDV-infected individuals compared to healthy donors, while the differentiation status of these NK cells appeared to remain unaltered[108]. It seems plausible that HDV exerts most of its effects in the liver, where the infection can induce the antiviral state of the human hepatocytes and virus-mediated cellular changes can initiate inflammatory pathways. Therefore, the innate immune responses of infected human hepatocytes were also investigated in a larger cohort of humanized mice, whose livers had been reconstituted with human hepatocytes derived from four distinct donors and which had been stably infected either with HBV or were co-infected with HBV and HDV (>6 weeks of infection). Compared to an HBV mono-infection, establishment of stable HBV/HDV co-infection provoked a significant and sustained enhancement of the innate defense mechanisms. Classic human ISGs (e.g. hOAS1, hMxA, hISG15) and broadly acting effectors of the innate antiviral responses such as hRig-I, as well as hSTAT transcription factors, which are key signaling molecules that can be activated through direct viral actions[166], were found significantly higher in the setting of a chronic HDV infection. Also the expression of genes involved in antigen presentation and in recognition of infected cells by natural killer cells (e.g. hHLA-E, hTAP1) was significantly increased compared to uninfected and HBV mono-infected mice. Furthermore, the expression levels of apolipoprotein B mRNA editing enzymes, catalytic polypeptide-like, such as hAPOBEC3B, hAPOBEC3F and hAPOBEC3G, which are cytidine deaminase enzymes

also involved in antiviral innate immunity, appeared significantly induced not only in HBV/HDV co-infected animals, but also in HBV mono-infected mice. Also when mouse livers repopulated with distinct human donors were investigated separately, the expression of these human ISG and signaling genes was generally higher in HBV/HDV co-infected than in HBV mono-infected mice, although baseline expression levels of ISGs and the extent of their induction differed among these different human donors.

In line with these RNA measurements, hSTAT1 demonstrated not only a clear induction on protein levels but also a more pronounced nuclear accumulation in human hepatocytes of HBV/HDV infected mice, indicating that an HDV infection could trigger the JAK/STAT signaling cascade. Nevertheless, previous in vitro studies reported an HDV-mediated inhibition of STAT nuclear translocation in response to interferon alpha[111]. In humanized mice hSTAT nuclear accumulation also was most evident in human hepatocytes displaying undetectable or lower amounts of HDAg, thus indicating that intracellular interference mechanisms may be active in the presence of high viral protein amounts. Human protein inhibitors of activated STAT, such as hPIAS3 and hPIAS4, interact with JAK/STAT pathways by negatively regulating hSTAT transcription. The finding that these factors were also significantly enhanced in HBV/HDV co-infected mice, suggests that the decreased hSTAT accumulation and nuclear translocation in human hepatocytes with high amounts of HDAg may be facilitated by the increased induction of hPIAS.

The expression of the RNA-editing enzyme hADAR, which not only functions as a molecular trigger of innate responses to destabilize foreign double-stranded RNA structures but is also important for the generation of the large HDAg[21], was more enhanced in HBV/HDV infected livers than in HBV mono-infected mice and may indicate that the enhancement of specific innate pathways may even support viral infection. Another noticeable ISG is the ubiquitin homologue hISG15 which is strongly induced in HCV infected patients associated with a poor response to interferon alpha and ribavirin treatment[154]. Although hISG15 is known for its antiviral function in various viral infections (such as herpes simplex virus, influenza virus, sindbis virus infections), recent in vitro studies showed that hISG15 might also play a pro-viral role in HCV infections by supporting the virus replication directly and by inhibiting interferon pathways and ISG expressions[156, p. 15], [157]. Interestingly, hISG15 was strongly enhanced both at RNA and protein level in HBV/HDV infected human hepatocytes. Future studies will be needed

to assess whether hISG15 plays a major role in HDV infection and whether its induction or suppression might affect HDV infection (pro-viral effect), as shown for HCV[157].

In this study, the most remarkable expression change was observed with hIP10 both on RNA and serum protein levels in HBV/HDV co-infected mice. From recent studies in HCV infected patients it is known that elevated pre-treatment ISG levels, especially hIP10, correlate not only with high viral loads but also with suboptimal IFN responses[155]. It has also been shown that viral infections can activate various ISGs even when type I interferons are minimally induced at mRNA levels, while the induction of type III interferons can be the dominant type of interferons produced in hepatocytes during HCV infection[167]. This study revealed a significant induction of hTGF-ß, which is considered a major pro-fibrogenic cytokine, as well as of hIFN-ß and hIFN-λ in the setting of HBV/HDV co-infection, while the expression levels of these cytokines remained low or below the detection limit in the livers of HBV mono-infected and uninfected animals. The enhanced production of hIFN-β, which is known to cause hepatic injury by mediating immune responses and antiviral activity, is also in line with previous studies indicating that pathogen-mediated nucleic acid sensing can induce hIFN-β production in hepatocytes[166]. Of note, within the toll like receptor family, which plays a key role in innate immunity and is able to activate pro-inflammatory cytokines, hTLR2, appeared as the most enhanced gene in the setting of HBV/HDV co-infection, while other hTLRs were only slightly induced in HBV and HBV/HDV infected livers or remained unchanged compared to uninfected controls. Observations in chronic HBV infected patients suggest that an impaired activation of toll like receptor signaling molecules may lead to weak immune responses against HBV infection[168], [169].

Furthermore, observations in HBV/HDV co-infected patients and humanized mice recently showed that the activity of HBV is slightly suppressed in an HBV/HDV co-infection compared to an HBV mono-infection[53]. The strong antiviral state caused by HDV observed in this study could affect HBV replication in co-infected livers and hence may in part explain the lower levels of HBV infection.

In conclusion, the results of this study indicate that an HBV/HDV co-infection provokes a stronger enhancement of the innate responses of the human hepatocytes in comparison to HBV in human liver chimeric mice. These findings suggest that in HDV infections high basal ISG and increased amounts of inflammatory and pro-fibrogenic cytokine levels may directly contribute to liver damage and inflammation, providing a rationale for the more severe course of HDV-associated liver disease.

D.2. Persistence of hepatitis D virus mono-infection

An HDV infection always needs the presence of HBV as a satellite virus to build productive HDV particles, which consist of HDV RNA, HDAg and the surface proteins of HBV (HBsAg)[170]. Clinically, a productive HDV infection occurs as a super- or co-infection with HBV. However, another, very rare form of HDV infection was first observed by Samuel et al[158] and later confirmed by the recent study of Mederacke and colleagues[159], where the presence of HDAg-positive cells have been observed up to 19 months after liver transplantation (LTX) without detected evidence of HBV replication. Despite the administration of HBV immunoglobulins (HBIG) and HBV-polymerase inhibitors, low amounts of HBV and HDV virions are still present in the blood circulation in the days following liver transplantation and HDV infection of individual hepatocytes within the transplanted organ may occur [159]. The treatment with nucleos(t)ide analogues, which show efficient antiviral effects on HBV, is not able to directly suppress HDV replication and therefore may also lead to an HDV infection which persists for years in patients in the presence of low levels of HBV replication[171]. Nevertheless, these studies did not assess whether HDV mono-infection can persist in human hepatocytes without causing significant hepatocyte death and whether a prolonged latency leads to the development of non-infectious delta virions with restricted or no capability to propagate upon HBV super-infection. Therefore, the second aim of this doctoral thesis was to elucidate whether and how long HDV can survive as a latent mono-infection in humanized uPA/SCID mice and if a persistent mono-infection can efficiently converted into a productive HDV infection upon super-infection with HBV.

An HDV mono-infection in humanized uPA/SCID mice was established and characterized by using either a patient derived serum, which was HDV positive, but HBV

negative (due to entecavir treatment) or a cell-culture derived supernatant consisting of HDV particles. After 2, 3 and 6 weeks of HDV mono-infection HDAg-positive human hepatocytes (average 1.5%) and HDV RNA transcripts could be detected in both experimental settings and thus proving that HDV mono-infection can be efficiently established in primary human hepatocytes in the absence of HBV. Although only a small fraction of the human hepatocytes could initially be infected with HDV, the intrahepatic amounts of HDAg and HDV RNA appeared to persist between week 2 and 6, since no significant decrease of intracellular HDV markers could be detected. Events of possible cell death during HDV mono-infection were investigated by performing TUNEL and Caspase 3 immunofluorescence staining. TUNEL staining remained undetectable in all human cells, while caspase 3-positive staining could be determined in some human hepatocytes (up to 0.1% in mice sacrificed after 6 weeks of HDV mono-infection), but exclusively in human hepatocytes that were HDAg negative. Since a similar extent of caspase 3-positive signals was also found in uninfected humanized livers, it currently cannot be proven or disproven whether some events of cell death/apoptosis may have been associated with HDV mono-infection. These first experiments showed that HDV is able to persist as a mono-infection in the absence of HBV in human hepatocytes and without causing significant intrahepatic cell death[162].

It was also investigated whether persistent HDV particles can be rescued and converted into a productive co-infection upon super-infection with HBV. Therefore, HDV mono-infected humanized uPA/SCID mice were super-infected with an HBV-positive serum after 3 and 6 weeks of HDV mono-infection. In both mice groups the development of HBV and HDV viremia as well as circulating HBsAg could be detected in the serum after 3 weeks of super-infection and was increasing constantly in the following weeks. After the complete establishment of virus spreading (9 weeks after HBV super-infection, which is 15 weeks post HDV infection), the majority of human hepatocytes could be stained positive for HDAg and HBcAg. These results demonstrate that HDV particles not only survive as a persistent mono-infection in human hepatocytes for at least 6 weeks, but can also be rescued upon HBV super-infection leading to a productive HBV/HDV co-infection. The HDV virions derived from these HDV mono-infected mice also remained infectious after their rescue, which was demonstrated by propagating the serum derived from a mouse that was at first HDV mono-infected and then HBV super-infected after 6 weeks, into 2 naïve humanized uPA/SCID mice. These 2 co-infected mice developed HBV and HDV viremia

after 3 weeks of infection and also showed HDAg- and HBcAg-positive human hepatocytes after 6 weeks post infection[162].

It is known from in vitro studies that HDV requires new rounds of infections to build HDV virions and to remain productive during the course of disease. In cell culture experiments HDV replication leads to increased RNA editing events and to the accumulation of the large HDAg, which inhibits HDV replication and prevents the initiation of new rounds of infection[172]. Sequencing results of this study performed on input virus, intrahepatic HDV RNA genomes, as well as rescued and serial passaged HDV virions, revealed the co-existence and maintenance of two predominant quasi-species, with the unique difference at the specific RNA-editing site and without the emergence of major amounts of additional gene alterations or mutations in other regions of the genome. During HDV replication many altered genomes are usually known to arise, which essentially contribute to viral adaptability. The maintenance of the wild-type HDV form in this study indicates that the pressure to select viral quasi-species largely depends on the environment in which replication takes place. The absence of viral inhibitors and adaptive immune responses in humanized mice of this study may explain the maintenance of the dominant wild-type virus. However, in the livers of long-term HDV mono-infected mice (6 weeks) intrahepatic analyses revealed the occurrence of a shift in the ratio of RNA quasi-species to an increased number of edited HDV RNA species (which encode for the large HDAg) as well as a decreased amount of small HDAg. Although the large HDAg was predominant a significant amount of small HDAg and virions harboring the unedited genome was shown to persist during the 6 weeks of HDV mono-infection and therefore enabling rescue of HDV particles and conversion into a productive co-infection. Following HBV super-infection experiments demonstrated that HDV particles rescued after 3 or 6 weeks of latency maintained similar cell-to-cell spreading capacities in vivo and the conversion of HDV mono-infection to a productive HBV/HDV co-infection occurred with comparable kinetics[162]. These observations are in line with previous in vitro studies demonstrating that the small HDAg is essential for a productive HDV replication, since an accumulation of the edited genomes inhibits HDV replication in cell culture[173], [174] and that HDV replication could persist in vitro for at least one year only when limited amounts of the small HDAg were provided from a separate DNA source (all in the absence of large HDAg)[172].

Beside the observations that HDV can persist in patients with low replication of HBV[171] and that HDAg remained detectable in transplanted livers[158],[159], controversial data obtained from animal experiments have been reported in the past about the capability of HDV to survive in the liver. A study in woodchucks showed that an HDV mono-infection survives for up to 33 days after being super-infected with the HBV-related woodchuck hepatitis virus (WHV)[160]. In contrast, previous experimental studies in chimpanzees indicated that HDV cannot persist as a latent infection for longer than seven days, since a subsequent HBV infection failed to rescue HDV[161].

In this doctoral thesis HDV latency was investigated in human hepatocytes for the first time and findings provide direct evidence that an HDV-mono-infection can persist in the absence of an HBV infection in human hepatocytes for at least 6 weeks, thus giving an explanation for previous observations in liver-transplanted patients[158], [159], [175], [176]. Also the observation that an HDV mono-infection can not only survive but promptly be converted into a productive co-infection after super-infection with HBV and that these rescued HDV particles still remain infectious may explain why HDV clearance is rarely achieved in HBV/HDV co-infected patients despite successful treatment of HBV with HBV polymerase inhibitors. These results also emphasize the importance to develop antivirals directly targeting HDV replication and blocking re-infection of the liver[53].

D.3. Quantification of genomic and antigenomic HDV RNA

Both genomic and antigenomic HDV RNAs are essential for a productive HDV replication and distinguishing between both HDV RNA forms enables more detailed analyses of intrahepatic HDV activities in human hepatocytes. However, due to the lack of suitable assays and HDV infection models knowledge of intrahepatic virological changes induced by different antiviral treatments in HBV/HDV co-infected patients is scant. Therefore, a new strand-specific quantitative RT-PCR assay to determine genomic and antigenomic HDV RNA was established and employed in HBV/HDV co-infected patient liver biopsies as well as in humanized livers of HBV/HDV co-infected uPA/SCID mice. In the past a differentiation between genomic and antigenomic HDV RNA was performed with northern blot analyses[16] and in 2008 an approach from Tseng et al emerged quantifying genomic and antigenomic HDV RNA by performing reverse transcription with

nucleotide tagged HDV strand-specific primers followed by RT-PCR[177]. Semi-quantitative northern blot analyses are time-consuming, involve the use of radioactivity and do not enable a simple absolute quantification. Since genomic and antigenomic HDV RNA are exact complements of each other and lead to self-priming and self-annealing, quantitative RT-PCR procedures without an additional purification step have its limitations and may generate false positive PCR products. By using a full-length genomic and antigenomic HDV RNA standard, a novel quantitative RT-PCR assay was developed within this doctoral thesis, which specifically distinguishes between genomic and antigenomic HDV RNA. This novel assay is strand-specific and circumvents self-priming and self-annealing of genomic and antigenomic HDV RNA. The use of biotin tagged strand-specific primers for reverse transcription and a magnetic bead separation strategy for purification leads to highly specific cDNA products without false positive signals. This assay is reproducible and shows a linear trend from 10^9 to 10^5 copies. A very early study of 1986 estimated that a replicating liver cell of woodchucks and chimpanzees contains around 100,000 to 300,000 copies of genomic and 7,000 to 65,000 copies of antigenomic HDV RNA per liver cell[16]. Furthermore, this assay detects all genotypes by using HDV-specific primers and probes that bind in a highly conserved ribozyme region of the HDV genome[145]. The quantification of artificial mixtures of genomic and antigenomic HDV RNA showed that this novel assay enables the detection of both RNAs up to a ratio of 1:100 (genomic: antigenomic HDV RNA). Since early studies in woodchucks and chimpanzees demonstrated that the genomic RNA is 5-22 times more abundant than the antigenomic strand[16], this obtained ratio seems to lie within the biological range of both RNAs. The suitability of the genomic and antigenomic HDV RNA quantification assay was demonstrated not only by using cryo-conserved mice livers but also in patient liver biopsies. Quantitative amounts of genomic and antigenomic HDV RNA in patients infected with HDV are not known and can to date only be estimated from data that exist from woodchuck and chimpanzee experiments. This new assay enables the detection of genomic and antigenomic HDV RNA in patients and analyses in three different patient liver biopsies reveal ratios of 2.9, 5.4 and 41.3 for the first time. Such distinct measurements of the two viral genome forms may also support future studies aiming at determining the impact of new therapeutic agents on HDV replication and possibly on the distinct HDV RNA strains.

D.4. Antiviral effects of pegylated interferon-α and entecavir in HBV/HDV co-infected UPA/SCID mice

A chronic HDV infection remains the most progressive form of chronic viral hepatitis and as such its successful treatment is important. However, in contrast to chronic hepatitis B and C virus infections, no new drugs for the treatment of HDV infected patients have been introduced recently[178]. To date HDV treatment is restricted to interferon alpha, which shows low efficacies, high relapse rates after treatment cessation and severe side effects[39]–[41]. In HBV infections interferon alpha is known to exert its antiviral effects directly by inhibiting HBV replication through epigenetic repression of HBV cccDNA transcriptional activities[90] and indirectly through JAK/STAT signaling and the induction of interferon stimulated genes[179]. However, due to the limited availability of HDV infection models and patient liver biopsies detailed changes of intrahepatic HDV RNA activity upon interferon treatment have not been investigated. HBV replication can successfully be suppressed with nucleoside/nucleotide analogs such as lamivudine, adefovir and entecavir, but the persistence of cccDNA hampers complete viral clearance[91]. Although HDV occurs only in a co- or super-infection with HBV and HDV release is dependant on the presence of HBsAg[14], nucleoside/nucleotide analogs do not seem to be beneficial in HDV infections[47], [49], [50], [178].

The development of the uPA/SCID mouse model for HDV infections and the novel quantitative RT-PCR assay to specifically determine genomic and antigenomic RNA now provide new possibilities to investigate the intrahepatic life cycle of the virus and the impact of different antiviral compounds on the replication of HDV. Therefore – and as a proof of concept – the aim of this study was to analyze humanized uPA/SCID mice treated either with peg-IFN-α or with ETV for four weeks and determine intrahepatic parameters reflecting HDV productivity, e.g. amounts of genomic and antigenomic HDV RNA. Treatment of humanized uPA/SCID mice showed that peg-IFN-α efficiently suppressed HBV and HDV productivity by lowering HBV and HDV viremia, circulating HBsAg and amounts of HDAg positive human hepatocytes. Peg-IFN-α led to a clear decrease of total intrahepatic HDV RNA and specifically also to a suppression of both genomic and antigenomic HDV RNA levels. The two different HDV RNA forms seem to be affected similarly since ratios of genomic to antigenomic HDV RNA remained constant between control and treated mice groups. These findings demonstrate that peg-IFN-α is able to

affect virus replication by directly targeting HDV RNAs and might in part also lead to viral clearance in humanized mice. However, in HDV infected patients peg-IFN-α treatment fails to clear HDV, suggesting that HDV similar to HBV[151] and other viruses[180] may have developed strategies to evade effects of peg-IFN-α or that different host environments may somehow favor viral survival[111]. In contrast, the strong suppression of HBV replication mediated by a 4-week treatment with ETV (also shown in a recent study in HBV mono-infected mice[179]) did not significantly affect HBsAg levels and HDV productivity, as documented by the unchanged levels of HDV viremia and intrahepatic HDV levels. These observations are in line with clinical studies in patients[50] and not surprising because ETV suppresses HBV replication without affecting the transcriptional activity of HBV cccDNA which encodes for all HBV RNAs as well as HBsAg. Since HBsAg production can still be maintained upon ETV treatment HDV nucleocapsids remain able to assemble into infectious virions[127]. In line, ETV did neither affect genomic nor antigenomic HDV RNA levels, demonstrating that ETV fails to directly target HDV virus replication as well as virus assembly or release.

In summary, a novel quantitative RT-PCR assay was developed which specifically distinguishes between genomic and antigenomic HDV RNA in patient liver biopsies and in humanized uPA/SCID mice. By using this new assay and the uPA/SCID mice model, which lacks adaptive immune responses, it could be demonstrated that peg-IFN-α efficiently suppresses HBV and HDV activity and as a first proof of concept also intrahepatic genomic and antigenomic HDV RNA. In contrast the nucleoside analogue ETV had no influence on serological and intrahepatic HDV activity. Using the example of interferon alpha it was demonstrated that by targeting HDV replication intrahepatic viral clearance might be achievable in humanized mice. At the same time these findings underline that due to its simplicity in structure HDV offers only very limited targets to eradicate the virus and that new therapeutic approaches are urgently needed.

D.5. Final conclusion

Overall, findings of this doctoral thesis demonstrate that an HDV infection in humanized uPA/SCID mice is accompanied by a significant induction of the intrinsic innate immune responses of the human hepatocytes, suggesting that both the enhancement of ISGs and

higher levels of pro-inflammatory cytokines might directly contribute to inflammation and the more severe liver damage that is frequently observed in HBV/HDV co-infected patients. We also demonstrated that HDV is able to persist intrahepatically in the absence of HBV for at least six weeks before being rescued by HBV super-infection, at which time persistent HDV mono-infection could be converted into a productive HBV/HDV co-infection. Moreover, the establishment of a novel assay to distinguish between genomic and antigenomic HDV RNA provided new possibilities to investigate intrahepatic changes on HDV productivity induced by different antiviral treatments. Pegylated interferon alpha, but not ETV, not only reduced HDV viremia but also showed a decrease of both genomic and antigenomic HDV RNA levels, suggesting that by targeting HDV replication intrahepatic clearance could be achievable.

The observations of this doctoral thesis reveal new aspects of the HDV life cycle and its interaction with the host and underline that HDV as a deceivingly simple virus has acquired unique biological features[181] turning HDV infections into a difficult-to-treat disease with a severe clinical course. Moreover, a novel assay to investigate intrahepatic replication of HDV was established, which might support preclinical drug analyses and therefore contribute to the search of new antiviral treatments against HDV infections.

E. Abstract

Abstract

About 15 million people worldwide are chronically infected with the hepatitis D virus (HDV). A chronic HDV infection shows the most severe clinical course of all hepatitis infections leading to liver cirrhosis in up to 70 % and to the development of hepatocellular carcinoma. Virus replication requires the generation of genomic and antigenomic HDV RNAs which are generated in a double rolling circle, while the envelope proteins of the hepatitis B virus (HBV) are needed for assembly and release of infectious virions. The limited availability of HDV infection models has hindered investigations of HDV pathogenesis and interactions between HDV and infected human hepatocytes. Currently an HDV specific therapy does not exist. Our group recently developed an HDV in vivo infection model using humanized uPA/SCID mice, which after transplantation of primary human hepatocytes into mice livers can stably be infected with HBV and HDV. Aims of this study were to characterize interactions of HDV and the innate immune system, to investigate the persistence and cell toxicity of an HDV mono-infection and to determine effects of different antiviral drugs on HDV productivity by establishing and using a novel assay to quantify genomic and antigenomic HDV RNA. By using the uPA/SCID mouse model it could be shown that the development of HDV viremia was accompanied by a clear induction of human-specific interferon stimulated genes (ISGs), which play an important role in the defense of viruses. While an HBV mono-infection only showed moderate inductions of classic ISGs (e.g. hMxA, hOAS1, hHLA-E, hIP10) in human hepatocytes, the expression of these ISGs was significantly stronger in an HBV/HDV co-infection. Also levels of human specific cytokines (hTGF-ß, hIFN-ß, hIFN-λ) were clearly increased in HBV/HDV co-infected mice, while their expression was lower or even below the lower limit of detection in HBV mono- and uninfected mice. This strong activation of the innate immune system might directly lead to liver damage and inflammation providing a rationale for the more severe course of HDV-associated liver disease. Another investigation within this thesis showed that an HDV mono-infection in humanized mice can persist for at least 6 weeks in the absence of HBV. Moreover persistent HDV particles could be rescued upon HBV super-infection and serially passaged into naïve human chimeric mice proofing the maintenance of HDV infectivity after a 6-weeks-phase of intracellular latency. These results explain observations in patients showing detectable intrahepatic HDV markers

despite very low HBV activity or even after liver transplantation and emphasize the risk for these patients to develop a productive HBV/HDV infection after re-infection with HBV. Furthermore, a novel, quantitative RT-PCR assay was established, which specifically detects genomic and antigenomic HDV RNA in human chimeric mice livers and human liver patient biopsies. This assay now provides new possibilities to investigate the intrahepatic life cycle of HDV and the impact of different antivirals on HDV productivity in a more detailed way. E.g. a 4-week administration of pegylated interferon alpha not only reduced HDV viremia but also showed a decrease of both intrahepatic genomic and antigenomic HDV RNA levels. In contrast, a therapy with the nucleotide analogue entecavir efficiently suppressed HBV viremia but had no influence on HDV replication. Our interferon alpha studies also indicated that intrahepatic viral clearance could be achievable by suppressing HDV replication. At the same time these findings underline that due to its simplicity in structure HDV offers only very limited targets to eradicate the virus and that new therapeutic approaches are urgently needed.

Zusammenfassung

Circa 15 Millionen Menschen sind weltweit chronisch mit dem Hepatitis-D-Virus (HDV) infiziert. Die chronische HDV-Infektion zeigt den schwersten klinischen Verlauf aller viralen Hepatitiden und führt in bis zu 70% der Fälle zu Leberzirrhose und zur Entstehung des hepatozellulären Karzinoms. Für die Replikation des Virus ist das Vorhandensein von genomischer und antigenomischer HDV RNA essentiell, während es für die Bildung und Sekretion von infektiösen Viruspartikeln die Hüllproteine von HBV benötigt. Da geeignete HDV-Infektionsmodelle fehlen, ist bisher wenig über die Pathogenese einer HDV-Infektion und Virus-Wirts-Wechselwirkungen in infizierten humanen Hepatozyten bekannt. Eine HDV-spezifische Therapie existiert derzeit nicht. Kürzlich haben wir ein HDV-Infektionsmodell entwickelt, bei dem uPA-SCID-Mäuse mit primären, humanen Hepatozyten repopuliert und mit HBV und HDV infiziert werden können. Die Ziele dieser Studie waren, Interaktionen zwischen HDV und dem angeborenen Immunsystem zu charakterisieren, die Persistenz und Zellschädigung des Virus in einer HDV-Monoinfektion zu untersuchen und mit Hilfe einer neuen Methode zur Quantifizierung genomischer und antigenomischer HDV, antivirale Effekte verschiedener Virustatika zu bestimmen. Unter Verwendung des uPA/SCID-Mausmodells konnte gezeigt werden, dass die Entwicklung der HDV-Virämie von einer klaren Induktion humaner Interferon-stimulierter Gene (ISGs), die eine wichtige Rolle bei der Abwehr Viren spielen, begleitet wurde. Während eine HBV-Monoinfektion im Vergleich mit uninfizierten Kontrollmäusen nur zu einem moderaten Anstieg der klassischen ISGs (z.B. hMxA, hOAS1, hHLA-E, hIP10) in humanen Hepatozyten führte, war die Induktion der ISGs während einer HBV/HDV-Koinfektion signifikant stärker ausgeprägt. Humane Zytokine (hTGF-ß, hIFN-ß, hIFN-λ) waren in einer HBV/HDV-Koinfektion ebenfalls deutlich induziert, während die Expression dieser Zytokine in HBV-monoinfizierten und uninfizierten Mäusen niedriger oder unterhalb der Messgrenze war. Diese ausgeprägte Aktivierung des angeborenen Immunsystems könnte direkt zu Leberschädigung und Leberentzündung führen und somit den schweren klinischen Verlauf einer HDV-Infektion erklären. Zusätzlich wurde beobachtet, dass eine HDV-Monoinfektion im uPA/SCID-Mausmodell intrahepatisch in der Abwesenheit von HBV für mindestens 6 Wochen persistieren kann und dass persistierende HDV-Partikel durch eine HBV-Superinfektion „gerettet“ werden können. Darüber hinaus bewies eine serielle Übertragung dieser HDV-Partikel in naive, human-chimäre Mäuse, dass deren Infektiosität bestehen bleibt. Diese

Ergebnisse erklären Beobachtungen in Patienten, bei denen HDV auch bei sehr niedriger HBV-Replikation und sogar nach vollständiger Transplantation der Leber nachweisbar ist und unterstreichen das Risiko, dass nach erneuter Infektion mit HBV sofort eine aktive HBV/HDV-Koinfektion vorliegen kann. Weiterhin wurde eine neue, quantitative RT-PCR-Methode entwickelt, welche genomische und antigenomische HDV-RNA spezifisch in human chimären Mauslebern und Patientenbiopsien detektiert. Dieses Nachweisverfahren liefert neue Möglichkeiten, den Replikationszyklus von HDV und den Einfluss verschiedener Wirkstoffe detailliert zu untersuchen. So zeigte eine 4-wöchige Behandlung mit pegyliertem Interferon alpha nicht nur eine Erniedrigung der HDV-Virämie, sondern auch eine Reduktion der genomischen und antigenomischen RNA in humanen Hepatozyten, während eine Therapie mit Entecavir keinen Einfluss auf die Aktivität von HDV hatte. Am Beispiel von pegyliertem Interferon alpha ist zu sehen, dass durch erfolgreiche Hemmung der HDV-Replikation eine Viruselimination erreicht werden könnte. Gleichzeitig wird aber auch deutlich, dass HDV wegen seines einfachen Aufbaus sehr wenig Angriffspunkte für eine antivirale Therapie bietet und dringend neue Therapieansätze benötigt werden.

F. Bibliography

[1] M. Rizzetto, M. G. Canese, S. Aricò, O. Crivelli, C. Trepo, F. Bonino, and G. Verme, "Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and in serum of HBsAg carriers," *Gut*, vol. 18, no. 12, pp. 997–1003, Dec. 1977.

[2] C. A. Navascués, M. Rodríguez, N. G. Sotorrío, P. Sala, A. Linares, A. Suárez, and L. Rodrigo, "Epidemiology of hepatitis D virus infection: changes in the last 14 years," *Am. J. Gastroenterol.*, vol. 90, no. 11, pp. 1981–1984, Nov. 1995.

[3] G. B. Gaeta, T. Stroffolini, M. Chiaramonte, T. Ascione, G. Stornaiuolo, S. Lobello, E. Sagnelli, M. R. Brunetto, and M. Rizzetto, "Chronic hepatitis D: a vanishing Disease? An Italian multicenter study," *Hepatology*, vol. 32, no. 4 Pt 1, pp. 824–827, Oct. 2000.

[4] S. A. Hughes, H. Wedemeyer, and P. M. Harrison, "Hepatitis delta virus," *Lancet*, vol. 378, no. 9785, pp. 73–85, Jul. 2011.

[5] A. Ciancio and M. Rizzetto, "Chronic hepatitis D at a standstill: where do we go from here?," *Nat Rev Gastroenterol Hepatol*, Sep. 2013.

[6] H. Wedemeyer, B. Heidrich, and M. P. Manns, "Hepatitis D virus infection--not a vanishing disease in Europe!," *Hepatology*, vol. 45, no. 5, pp. 1331–1332; author reply 1332–1333, May 2007.

[7] T. J. S. Cross, P. Rizzi, M. Horner, A. Jolly, M. J. Hussain, H. M. Smith, D. Vergani, and P. M. Harrison, "The increasing prevalence of hepatitis delta virus (HDV) infection in South London," *J. Med. Virol.*, vol. 80, no. 2, pp. 277–282, Feb. 2008.

[8] C. Reinheimer, H. W. Doerr, and A. Berger, "Hepatitis delta: on soft paws across Germany," *Infection*, vol. 40, no. 6, pp. 621–625, Dec. 2012.

[9] P. Dény, "Hepatitis delta virus genetic variability: from genotypes I, II, III to eight major clades?," *Curr. Top. Microbiol. Immunol.*, vol. 307, pp. 151–171, 2006.

[10] H. Wedemeyer and M. P. Manns, "Epidemiology, pathogenesis and management of hepatitis D: update and challenges ahead," *Nat Rev Gastroenterol Hepatol*, vol. 7, no. 1, pp. 31–40, Jan. 2010.

[11] J. M. Taylor, "Replication of human hepatitis delta virus: influence of studies on subviral plant pathogens," *Adv. Virus Res.*, vol. 54, pp. 45–60, 1999.

[12] R. Flores, S. Delgado, M.-E. Gas, A. Carbonell, D. Molina, S. Gago, and M. De la Peña, "Viroids: the minimal non-coding RNAs with autonomous replication," *FEBS Lett.*, vol. 567, no. 1, pp. 42–48, Jun. 2004.

[13] M. Rizzetto, B. Hoyer, M. G. Canese, J. W. Shih, R. H. Purcell, and J. L. Gerin, "delta Agent: association of delta antigen with hepatitis B surface antigen and RNA in serum of delta-infected chimpanzees," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 77, no. 10, pp. 6124–6128, Oct. 1980.

[14] M. Rizzetto, M. G. Canese, J. L. Gerin, W. T. London, D. L. Sly, and R. H. Purcell, "Transmission of the hepatitis B virus-associated delta antigen to chimpanzees," *J. Infect. Dis.*, vol. 141, no. 5, pp. 590–602, May 1980.

[15] K. S. Wang, Q. L. Choo, A. J. Weiner, J. H. Ou, R. C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Denniston, J. L. Gerin, and M. Houghton, "Structure, sequence and expression of the hepatitis delta (delta) viral genome," *Nature*, vol. 323, no. 6088, pp. 508–514, Oct. 1986.

[16] P. J. Chen, G. Kalpana, J. Goldberg, W. Mason, B. Werner, J. Gerin, and J. Taylor, "Structure and replication of the genome of the hepatitis delta virus," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 83, no. 22, pp. 8774–8778, Nov. 1986.

[17] S. Pascarella and F. Negro, "Hepatitis D virus: an update," *Liver Int.*, vol. 31, no. 1, pp. 7–21, Jan. 2011.

[18] A. J. Weiner, Q. L. Choo, K. S. Wang, S. Govindarajan, A. G. Redeker, J. L. Gerin, and M. Houghton, "A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides p24 delta and p27 delta," *J. Virol.*, vol. 62, no. 2, pp. 594–599, Feb. 1988.

[19] P. J. Chen, F. L. Chang, C. J. Wang, C. J. Lin, S. Y. Sung, and D. S. Chen, "Functional study of hepatitis delta virus large antigen in packaging and replication inhibition: role of the amino-terminal leucine zipper," *J. Virol.*, vol. 66, no. 5, pp. 2853–2859, May 1992.

[20] H. W. Wang, P. J. Chen, C. Z. Lee, H. L. Wu, and D. S. Chen, "Packaging of hepatitis delta virus RNA via the RNA-binding domain of hepatitis delta antigens: different roles for the small and large delta antigens," *J. Virol.*, vol. 68, no. 10, pp. 6363–6371, Oct. 1994.

[21] S. K. Wong and D. W. Lazinski, "Replicating hepatitis delta virus RNA is edited in the nucleus by the small form of ADAR1," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 99, no. 23, pp. 15118–15123, Nov. 2002.

[22] A. G. Polson, B. L. Bass, and J. L. Casey, "RNA editing of hepatitis delta virus antigenome by dsRNA-adenosine deaminase," *Nature*, vol. 380, no. 6573, pp. 454–456, Apr. 1996.

[23] F. L. Chang, P. J. Chen, S. J. Tu, C. J. Wang, and D. S. Chen, "The large form of hepatitis delta antigen is crucial for assembly of hepatitis delta virus," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 88, no. 19, pp. 8490–8494, Oct. 1991.

[24] S. Dourakis, P. Karayiannis, R. Goldin, M. Taylor, J. Monjardino, and H. C. Thomas, "An in situ hybridization, molecular biological and immunohistochemical study of hepatitis delta virus in woodchucks," *Hepatology*, vol. 14, no. 3, pp. 534–539, Sep. 1991.

[25] H. Yan, G. Zhong, G. Xu, W. He, Z. Jing, Z. Gao, Y. Huang, Y. Qi, B. Peng, H. Wang, L. Fu, M. Song, P. Chen, W. Gao, B. Ren, Y. Sun, T. Cai, X. Feng, J. Sui, and W. Li, "Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus," *elife*, vol. 1, p. e00049, 2012.

[26] O. Lamas Longarela, T. T. Schmidt, K. Schöneweis, R. Romeo, H. Wedemeyer, S. Urban, and A. Schulze, "Proteoglycans act as cellular hepatitis delta virus attachment receptors," *PLoS ONE*, vol. 8, no. 3, p. e58340, 2013.

[27] Y. Ni, F. A. Lempp, S. Mehrle, S. Nkongolo, C. Kaufman, M. Fälth, J. Stindt, C. Königer, M. Nassal, R. Kubitz, H. Sültmann, and S. Urban, "Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes," *Gastroenterology*, vol. 146, no. 4, pp. 1070–1083, Apr. 2014.

[28] C. Sureau, C. Fournier-Wirth, and P. Maurel, "Role of N glycosylation of hepatitis B virus envelope proteins in morphogenesis and infectivity of hepatitis delta virus," *J. Virol.*, vol. 77, no. 9, pp. 5519–5523, May 2003.

[29] Y. P. Xia, C. T. Yeh, J. H. Ou, and M. M. Lai, "Characterization of nuclear targeting signal of hepatitis delta antigen: nuclear transport as a protein complex," *J. Virol.*, vol. 66, no. 2, pp. 914–921, Feb. 1992.

[30] M. M. C. Lai, "RNA replication without RNA-dependent RNA polymerase: surprises from hepatitis delta virus," *J. Virol.*, vol. 79, no. 13, pp. 7951–7958, Jul. 2005.

[31] J. Monjardino, "Replication of hepatitis delta virus," *J. Viral Hepat.*, vol. 3, no. 4, pp. 163–166, Jul. 1996.

[32] W. H. Huang, C. W. Chen, H. L. Wu, and P. J. Chen, "Post-translational modification of delta antigen of hepatitis D virus," *Curr. Top. Microbiol. Immunol.*, vol. 307, pp. 91–112, 2006.

[33] S. J. Hadziyannis, C. Papaioannou, and A. Alexopoulou, “The role of the hepatitis delta virus in acute hepatitis and in chronic liver disease in Greece,” *Prog. Clin. Biol. Res.*, vol. 364, pp. 51–62, 1991.

[34] F. Bonino, F. Negro, M. Baldi, M. R. Brunetto, E. Chiaberge, M. Capalbo, E. Maran, C. Lavarini, N. Rocca, and G. Rocca, “The natural history of chronic delta hepatitis,” *Prog. Clin. Biol. Res.*, vol. 234, pp. 145–152, 1987.

[35] A. Olivero and A. Smedile, “Hepatitis delta virus diagnosis,” *Semin. Liver Dis.*, vol. 32, no. 3, pp. 220–227, Aug. 2012.

[36] M. Buti, R. Esteban, M. Roggendorf, J. Fernandez, R. Jardi, R. Rashofer, H. Allende, J. Genesca, J. I. Esteban, and J. Guardia, “Hepatitis D virus RNA in acute delta infection: serological profile and correlation with other markers of hepatitis D virus infection,” *Hepatology*, vol. 8, no. 5, pp. 1125–1129, Oct. 1988.

[37] K. Zachou, C. Yurdaydin, U. Drebber, G. N. Dalekos, A. Erhardt, Y. Cakaloglu, H. Degertekin, S. Gurel, S. Zeuzem, H. Bozkaya, V. Schlaphoff, H. P. Dienes, T. C. Bock, M. P. Manns, and H. Wedemeyer, “Quantitative HBsAg and HDV-RNA levels in chronic delta hepatitis,” *Liver Int.*, vol. 30, no. 3, pp. 430–437, Mar. 2010.

[38] E. Sagnelli, F. M. Felaco, P. Filippini, G. Pasquale, P. Peinetti, E. Buonagurio, L. Aprea, C. Pulella, F. Piccinino, and G. Giusti, “Influence of HDV infection on clinical, biochemical and histological presentation of HBsAg positive chronic hepatitis,” *Liver*, vol. 9, no. 4, pp. 229–234, Aug. 1989.

[39] P. Farci, T. Roskams, L. Chessa, G. Peddis, A. P. Mazzoleni, R. Scioscia, G. Serra, M. E. Lai, M. Loy, L. Caruso, V. Desmet, R. H. Purcell, and A. Balestrieri, “Long-term benefit of interferon alpha therapy of chronic hepatitis D: regression of advanced hepatic fibrosis,” *Gastroenterology*, vol. 126, no. 7, pp. 1740–1749, Jun. 2004.

[40] F. Rosina, C. Pintus, C. Meschievitz, and M. Rizzetto, “A randomized controlled trial of a 12-month course of recombinant human interferon-alpha in chronic delta (type D) hepatitis: a multicenter Italian study,” *Hepatology*, vol. 13, no. 6, pp. 1052–1056, Jun. 1991.

[41] F. Gunsar, U. S. Akarca, G. Ersoz, A. C. Kobak, Z. Karasu, G. Yuce, T. Ilter, and Y. Batur, “Two-year interferon therapy with or without ribavirin in chronic delta hepatitis,” *Antivir. Ther. (Lond.)*, vol. 10, no. 6, pp. 721–726, 2005.

[42] J. L. Gaudin, P. Faure, H. Godinot, F. Gerard, and C. Trepo, “The French experience of treatment of chronic type D hepatitis with a 12-month course of interferon alpha-2B. Results of a randomized controlled trial,” *Liver*, vol. 15, no. 1, pp. 45–52, Feb. 1995.

[43] C. Castelnau, F. Le Gal, M.-P. Ripault, E. Gordien, M. Martinot-Peignoux, N. Boyer, B.-N. Pham, S. Maylin, P. Bedossa, P. Dény, P. Marcellin, and E. Gault, “Efficacy of peginterferon alpha-2b in chronic hepatitis delta: relevance of quantitative RT-PCR for follow-up,” *Hepatology*, vol. 44, no. 3, pp. 728–735, Sep. 2006.

[44] A. Erhardt, W. Gerlich, C. Starke, U. Wend, A. Donner, A. Sagir, T. Heintges, and D. Häussinger, “Treatment of chronic hepatitis delta with pegylated interferon-alpha2b,” *Liver Int.*, vol. 26, no. 7, pp. 805–810, Sep. 2006.

[45] G. A. Niro, A. Ciancio, G. B. Gaeta, A. Smedile, A. Marrone, A. Olivero, M. Stanzione, E. David, G. Brancaccio, R. Fontana, F. Perri, A. Andriulli, and M. Rizzetto, “Pegylated interferon alpha-2b as monotherapy or in combination with ribavirin in chronic hepatitis delta,” *Hepatology*, vol. 44, no. 3, pp. 713–720, Sep. 2006.

[46] L. Gheorghe, S. Iacob, I. Simionov, R. Vadan, I. Constantinescu, F. Caruntu, I. Sporea, and M. Grigorescu, “Weight-based dosing regimen of peg-interferon α-2b

for chronic hepatitis delta: a multicenter Romanian trial," *J Gastrointestin Liver Dis*, vol. 20, no. 4, pp. 377–382, Dec. 2011.

[47] G. A. Niro, A. Ciancio, H. L. Tillman, M. Lagget, A. Olivero, F. Perri, R. Fontana, N. Little, F. Campbell, A. Smedile, M. P. Manns, A. Andriulli, and M. Rizzetto, "Lamivudine therapy in chronic delta hepatitis: a multicentre randomized-controlled pilot study," *Aliment. Pharmacol. Ther.*, vol. 22, no. 3, pp. 227–232, Aug. 2005.

[48] J. Grabowski, C. Yurdaydìn, K. Zachou, P. Buggisch, W. P. Hofmann, J. Jaroszewicz, V. Schlaphoff, M. P. Manns, M. Cornberg, and H. Wedemeyer, "Hepatitis D virus-specific cytokine responses in patients with chronic hepatitis delta before and during interferon alfa-treatment," *Liver Int.*, vol. 31, no. 9, pp. 1395–1405, Oct. 2011.

[49] H. Wedemeyer, C. Yurdaydìn, G. N. Dalekos, A. Erhardt, Y. Çakaloğlu, H. Değertekin, S. Gürel, S. Zeuzem, K. Zachou, H. Bozkaya, A. Koch, T. Bock, H. P. Dienes, and M. P. Manns, "Peginterferon plus adefovir versus either drug alone for hepatitis delta," *N. Engl. J. Med.*, vol. 364, no. 4, pp. 322–331, Jan. 2011.

[50] G. Kabaçam, F. O. Onder, M. Yakut, G. Seven, S. C. Karatayli, E. Karatayli, B. Savas, R. Idilman, A. M. Bozdayi, and C. Yurdaydin, "Entecavir treatment of chronic hepatitis D," *Clin. Infect. Dis.*, vol. 55, no. 5, pp. 645–650, Sep. 2012.

[51] C. Yurdaydin, H. Bozkaya, S. Gürel, H. L. Tillmann, N. Aslan, A. Okçu-Heper, E. Erden, K. Yalçin, N. Iliman, O. Uzunalimoglu, M. P. Manns, and A. M. Bozdayi, "Famciclovir treatment of chronic delta hepatitis," *J. Hepatol.*, vol. 37, no. 2, pp. 266–271, Aug. 2002.

[52] S. S. Choi, R. Rasshofer, and M. Roggendorf, "Inhibition of hepatitis delta virus RNA replication in primary woodchuck hepatocytes," *Antiviral Res.*, vol. 12, no. 4, pp. 213–222, Nov. 1989.

[53] M. Lütgehetmann, L. V. Mancke, T. Volz, M. Helbig, L. Allweiss, T. Bornscheuer, J. M. Pollok, A. W. Lohse, J. Petersen, S. Urban, and M. Dandri, "Humanized chimeric uPA mouse model for the study of hepatitis B and D virus interactions and preclinical drug evaluation," *Hepatology*, vol. 55, no. 3, pp. 685–694, Mar. 2012.

[54] B. B. Bordier, J. Ohkanda, P. Liu, S.-Y. Lee, F. H. Salazar, P. L. Marion, K. Ohashi, L. Meuse, M. A. Kay, J. L. Casey, S. M. Sebti, A. D. Hamilton, and J. S. Glenn, "In vivo antiviral efficacy of prenylation inhibitors against hepatitis delta virus," *J. Clin. Invest.*, vol. 112, no. 3, pp. 407–414, Aug. 2003.

[55] W. Thierfelder, W. Hellenbrand, H. Meisel, E. Schreier, and R. Dortschy, "Prevalence of markers for hepatitis A, B and C in the German population. Results of the German National Health Interview and Examination Survey 1998," *Eur. J. Epidemiol.*, vol. 17, no. 5, pp. 429–435, 2001.

[56] J.-H. Kao, "Hepatitis B viral genotypes: clinical relevance and molecular characteristics," *J. Gastroenterol. Hepatol.*, vol. 17, no. 6, pp. 643–650, Jun. 2002.

[57] T. T. H. Tran, T. N. Trinh, and K. Abe, "New complex recombinant genotype of hepatitis B virus identified in Vietnam," *J. Virol.*, vol. 82, no. 11, pp. 5657–5663, Jun. 2008.

[58] C. M. Olinger, P. Jutavijittum, J. M. Hübschen, A. Yousukh, B. Samountry, T. Thammavong, K. Toriyama, and C. P. Muller, "Possible new hepatitis B virus genotype, southeast Asia," *Emerging Infect. Dis.*, vol. 14, no. 11, pp. 1777–1780, Nov. 2008.

[59] K. Tatematsu, Y. Tanaka, F. Kurbanov, F. Sugauchi, S. Mano, T. Maeshiro, T. Nakayoshi, M. Wakuta, Y. Miyakawa, and M. Mizokami, "A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J," *J. Virol.*, vol. 83, no. 20, pp. 10538–10547, Oct. 2009.

[60] Y.-F. Liaw, M. R. Brunetto, and S. Hadziyannis, “The natural history of chronic HBV infection and geographical differences,” *Antivir. Ther. (Lond.)*, vol. 15 Suppl 3, pp. 25–33, 2010.

[61] S. N. Thung, M. A. Gerber, R. H. Purcell, W. T. London, K. B. Mihalik, and H. Popper, “Animal model of human disease. Chimpanzee carriers of hepatitis B virus. Chimpanzee hepatitis B carriers,” *Am. J. Pathol.*, vol. 105, no. 3, pp. 328–332, Dec. 1981.

[62] F. Galibert, T. N. Chen, and E. Mandart, “Nucleotide sequence of a cloned woodchuck hepatitis virus genome: comparison with the hepatitis B virus sequence,” *J. Virol.*, vol. 41, no. 1, pp. 51–65, Jan. 1982.

[63] C. Seeger, D. Ganem, and H. E. Varmus, “Nucleotide sequence of an infectious molecularly cloned genome of ground squirrel hepatitis virus,” *J. Virol.*, vol. 51, no. 2, pp. 367–375, Aug. 1984.

[64] E. Mandart, A. Kay, and F. Galibert, “Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences,” *J. Virol.*, vol. 49, no. 3, pp. 782–792, Mar. 1984.

[65] S. F. Chang, H. J. Netter, M. Bruns, R. Schneider, K. Frölich, and H. Will, “A new avian hepadnavirus infecting snow geese (Anser caerulescens) produces a significant fraction of virions containing single-stranded DNA,” *Virology*, vol. 262, no. 1, pp. 39–54, Sep. 1999.

[66] H. Delius, N. M. Gough, C. H. Cameron, and K. Murray, “Structure of the hepatitis B virus genome,” *J. Virol.*, vol. 47, no. 2, pp. 337–343, Aug. 1983.

[67] V. Bruss, “Envelopment of the hepatitis B virus nucleocapsid,” *Virus Res.*, vol. 106, no. 2, pp. 199–209, Dec. 2004.

[68] X. Wei and D. L. Peterson, “Expression, purification, and characterization of an active RNase H domain of the hepatitis B viral polymerase,” *J. Biol. Chem.*, vol. 271, no. 51, pp. 32617–32622, Dec. 1996.

[69] Y.-F. Liaw, G. K. K. Lau, J.-H. Kao, and E. Gane, “Hepatitis B e antigen seroconversion: a critical event in chronic hepatitis B virus infection,” *Dig. Dis. Sci.*, vol. 55, no. 10, pp. 2727–2734, Oct. 2010.

[70] K. Visvanathan and S. R. Lewin, “Immunopathogenesis: role of innate and adaptive immune responses,” *Semin. Liver Dis.*, vol. 26, no. 2, pp. 104–115, May 2006.

[71] J. Lucifora, S. Arzberger, D. Durantel, L. Belloni, M. Strubin, M. Levrero, F. Zoulim, O. Hantz, and U. Protzer, “Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection,” *J. Hepatol.*, vol. 55, no. 5, pp. 996–1003, Nov. 2011.

[72] X. Zhang, H. Zhang, and L. Ye, “Effects of hepatitis B virus X protein on the development of liver cancer,” *J. Lab. Clin. Med.*, vol. 147, no. 2, pp. 58–66, Feb. 2006.

[73] H. Guo, D. Jiang, T. Zhou, A. Cuconati, T. M. Block, and J.-T. Guo, “Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation,” *J. Virol.*, vol. 81, no. 22, pp. 12472–12484, Nov. 2007.

[74] C. T. Bock, S. Schwinn, S. Locarnini, J. Fyfe, M. P. Manns, C. Trautwein, and H. Zentgraf, “Structural organization of the hepatitis B virus minichromosome,” *J. Mol. Biol.*, vol. 307, no. 1, pp. 183–196, Mar. 2001.

[75] M. Levrero, T. Pollicino, J. Petersen, L. Belloni, G. Raimondo, and M. Dandri, “Control of cccDNA function in hepatitis B virus infection,” *J. Hepatol.*, vol. 51, no. 3, pp. 581–592, Sep. 2009.

[76] J. Summers and W. S. Mason, “Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate,” *Cell*, vol. 29, no. 2, pp. 403–415, Jun. 1982.
[77] W. S. Robinson, R. H. Miller, and P. L. Marion, “Hepadnaviruses and retroviruses share genome homology and features of replication,” *Hepatology*, vol. 7, no. 1 Suppl, p. 64S–73S, Feb. 1987.
[78] R. Prange, “Host factors involved in hepatitis B virus maturation, assembly, and egress,” *Med. Microbiol. Immunol.*, vol. 201, no. 4, pp. 449–461, Nov. 2012.
[79] N. Chai, H. E. Chang, E. Nicolas, Z. Han, M. Jarnik, and J. Taylor, “Properties of subviral particles of hepatitis B virus,” *J. Virol.*, vol. 82, no. 16, pp. 7812–7817, Aug. 2008.
[80] S. Urban, A. Schulze, M. Dandri, and J. Petersen, “The replication cycle of hepatitis B virus,” *J. Hepatol.*, vol. 52, no. 2, pp. 282–284, Feb. 2010.
[81] C. W. Shepard, E. P. Simard, L. Finelli, A. E. Fiore, and B. P. Bell, “Hepatitis B virus infection: epidemiology and vaccination,” *Epidemiol Rev*, vol. 28, pp. 112–125, 2006.
[82] M. L. Shiffman, “Management of acute hepatitis B,” *Clin Liver Dis*, vol. 14, no. 1, pp. 75–91; viii–ix, Feb. 2010.
[83] A. Bertoletti and A. Gehring, “Immune response and tolerance during chronic hepatitis B virus infection,” *Hepatol. Res.*, vol. 37 Suppl 3, pp. S331–338, Oct. 2007.
[84] M.-H. Chang, “Natural history and clinical management of chronic hepatitis B virus infection in children,” *Hepatol Int*, vol. 2, no. Suppl 1, pp. 28–36, May 2008.
[85] S. Assar, M. K. Arababadi, B. N. Ahmadabadi, M. Salehi, and D. Kennedy, “Occult hepatitis B virus (HBV) infection: a global challenge for medicine,” *Clin. Lab.*, vol. 58, no. 11–12, pp. 1225–1230, 2012.
[86] M. Cornberg, U. Protzer, J. Petersen, H. Wedemeyer, T. Berg, W. Jilg, A. Erhardt, S. Wirth, C. Sarrazin, M. M. Dollinger, P. Schirmacher, K. Dathe, I. B. Kopp, S. Zeuzem, W. H. Gerlich, and M. P. Manns, “[Prophylaxis, diagnosis and therapy of hepatitis B virus infection - the German guideline],” *Z Gastroenterol*, vol. 49, no. 7, pp. 871–930, Jul. 2011.
[87] R. E. Randall and S. Goodbourn, “Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures,” *J. Gen. Virol.*, vol. 89, no. Pt 1, pp. 1–47, Jan. 2008.
[88] K. Kakimi, L. G. Guidotti, Y. Koezuka, and F. V. Chisari, “Natural killer T cell activation inhibits hepatitis B virus replication in vivo,” *J. Exp. Med.*, vol. 192, no. 7, pp. 921–930, Oct. 2000.
[89] K. A. Stegmann, N. K. Björkström, H. Veber, S. Ciesek, P. Riese, J. Wiegand, J. Hadem, P. V. Suneetha, J. Jaroszewicz, C. Wang, V. Schlaphoff, P. Fytili, M. Cornberg, M. P. Manns, R. Geffers, T. Pietschmann, C. A. Guzmán, H.-G. Ljunggren, and H. Wedemeyer, “Interferon-alpha-induced TRAIL on natural killer cells is associated with control of hepatitis C virus infection,” *Gastroenterology*, vol. 138, no. 5, pp. 1885–1897, May 2010.
[90] L. Belloni, L. Allweiss, F. Guerrieri, N. Pediconi, T. Volz, T. Pollicino, J. Petersen, G. Raimondo, M. Dandri, and M. Levrero, “IFN-α 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.*, vol. 122, no. 2, pp. 529–537, Feb. 2012.
[91] D. Grimm, R. Thimme, and H. E. Blum, “HBV life cycle and novel drug targets,” *Hepatol Int*, vol. 5, no. 2, pp. 644–653, Jun. 2011.
[92] R. P. Perrillo, “Therapy of hepatitis B -- viral suppression or eradication?,” *Hepatology*, vol. 43, no. 2 Suppl 1, pp. S182–193, Feb. 2006.

[93] J. L. Dienstag, “Hepatitis B virus infection,” *N. Engl. J. Med.*, vol. 359, no. 14, pp. 1486–1500, Oct. 2008.
[94] M. J. Sonneveld and H. L. A. Janssen, “Chronic hepatitis B: peginterferon or nucleos(t)ide analogues?,” *Liver Int.*, vol. 31 Suppl 1, pp. 78–84, Jan. 2011.
[95] T. Volz, L. Allweiss, M. B. M Barek, M. Warlich, A. W. Lohse, J. M. Pollok, A. Alexandrov, S. Urban, J. Petersen, M. Lütgehetmann, and M. Dandri, “The entry inhibitor Myrcludex-B efficiently blocks intrahepatic virus spreading in humanized mice previously infected with Hepatitis B Virus,” *J. Hepatol.*, Dec. 2012.
[96] J. S. Lee, E. T. Park, S. S. Kang, E. S. Gu, J. S. Kim, D. S. Jang, K. S. Lee, J.-S. Lee, N. H. Park, C. H. Bae, S. K. Baik, B. J. Yu, S. H. Lee, E. J. Lee, S. I. Park, M. Bae, J. W. Shin, J. H. Choi, C. Gu, S. K. Moon, G. J. Chun, J. H. Kim, H. S. Kim, and S.-K. Choi, “Clevudine demonstrates potent antiviral activity in naïve chronic hepatitis B patients,” *Intervirology*, vol. 53, no. 2, pp. 83–86, 2010.
[97] S. G. Lim, T. M. Ng, N. Kung, Z. Krastev, M. Volfova, P. Husa, S. S. Lee, S. Chan, M. L. Shiffman, M. K. Washington, A. Rigney, J. Anderson, E. Mondou, A. Snow, J. Sorbel, R. Guan, and F. Rousseau, “A double-blind placebo-controlled study of emtricitabine in chronic hepatitis B,” *Arch. Intern. Med.*, vol. 166, no. 1, pp. 49–56, Jan. 2006.
[98] O. Takeuchi and S. Akira, “Innate immunity to virus infection,” *Immunol. Rev.*, vol. 227, no. 1, pp. 75–86, Jan. 2009.
[99] V. Pétrilli, C. Dostert, D. A. Muruve, and J. Tschopp, “The inflammasome: a danger sensing complex triggering innate immunity,” *Curr. Opin. Immunol.*, vol. 19, no. 6, pp. 615–622, Dec. 2007.
[100] T.-D. Kanneganti, M. Lamkanfi, and G. Núñez, “Intracellular NOD-like receptors in host defense and disease,” *Immunity*, vol. 27, no. 4, pp. 549–559, Oct. 2007.
[101] D. Ng and J. L. Gommerman, “The Regulation of Immune Responses by DC Derived Type I IFN,” *Front Immunol*, vol. 4, p. 94, 2013.
[102] M. S. Kunzi and P. M. Pitha, “Interferon targeted genes in host defense,” *Autoimmunity*, vol. 36, no. 8, pp. 457–461, Dec. 2003.
[103] J. W. Schoggins and C. M. Rice, “Interferon-stimulated genes and their antiviral effector functions,” *Curr Opin Virol*, vol. 1, no. 6, pp. 519–525, Dec. 2011.
[104] M. G. Katze, Y. He, and M. Gale Jr, “Viruses and interferon: a fight for supremacy,” *Nat. Rev. Immunol.*, vol. 2, no. 9, pp. 675–687, Sep. 2002.
[105] Z. Abbas and R. Afzal, “Life cycle and pathogenesis of hepatitis D virus: A review,” *World J Hepatol*, vol. 5, no. 12, pp. 666–675, Dec. 2013.
[106] S. M. Cole, E. J. Gowans, T. B. Macnaughton, P. D. Hall, and C. J. Burrell, “Direct evidence for cytotoxicity associated with expression of hepatitis delta virus antigen,” *Hepatology*, vol. 13, no. 5, pp. 845–851, May 1991.
[107] G. C. Actis, E. Maran, F. Rosina, G. Saracco, G. Rocca, M. Rizzetto, F. Bonino, and G. Verme, “Natural killer response to exogenous interferon in delta hepatitis: boost or depression defined within the first week of therapy,” *Digestion*, vol. 37, no. 1, pp. 51–58, 1987.
[108] S. Lunemann, D. F. G. Malone, J. Grabowski, K. Port, V. Béziat, B. Bremer, K.-J. Malmberg, M. P. Manns, J. K. Sandberg, M. Cornberg, H.-G. Ljunggren, H. Wedemeyer, and N. K. Björkström, “Effects of HDV infection and pegylated interferon α treatment on the natural killer cell compartment in chronically infected individuals,” *Gut*, Apr. 2014.
[109] V. Williams, S. Brichler, E. Khan, M. Chami, P. Dény, D. Kremsdorf, and E. Gordien, “Large hepatitis delta antigen activates STAT-3 and NF-κB via oxidative stress,” *J. Viral Hepat.*, vol. 19, no. 10, pp. 744–753, Oct. 2012.

[110] C.-Y. Park, S.-H. Oh, S. M. Kang, Y.-S. Lim, and S. B. Hwang, "Hepatitis delta virus large antigen sensitizes to TNF-alpha-induced NF-kappaB signaling," *Mol. Cells*, vol. 28, no. 1, pp. 49–55, Jul. 2009.
[111] P. Pugnale, V. Pazienza, K. Guilloux, and F. Negro, "Hepatitis delta virus inhibits alpha interferon signaling," *Hepatology*, vol. 49, no. 2, pp. 398–406, Feb. 2009.
[112] N. Aslan, C. Yurdaydin, H. Bozkaya, P. Baglan, A. M. Bozdayi, H. L. Tillmann, M. P. Manns, and H. Wedemeyer, "Analysis and function of delta-hepatitis virus-specific cellular immune responses," *Journal of Hepatology*, vol. 38, pp. 15–16, Apr. 2003.
[113] N. Aslan, C. Yurdaydin, J. Wiegand, T. Greten, A. Ciner, M. F. Meyer, H. Heiken, B. Kuhlmann, T. Kaiser, H. Bozkaya, H. L. Tillmann, A. M. Bozdayi, M. P. Manns, and H. Wedemeyer, "Cytotoxic CD4 T cells in viral hepatitis," *J. Viral Hepat.*, vol. 13, no. 8, pp. 505–514, Aug. 2006.
[114] Y.-H. Huang, M.-H. Tao, C. Hu, W.-J. Syu, and J.-C. Wu, "Identification of novel HLA-A*0201-restricted CD8+ T-cell epitopes on hepatitis delta virus," *J. Gen. Virol.*, vol. 85, no. Pt 10, pp. 3089–3098, Oct. 2004.
[115] A. Bertoletti and C. Ferrari, "Innate and adaptive immune responses in chronic hepatitis B virus infections: towards restoration of immune control of viral infection," *Gut*, vol. 61, no. 12, pp. 1754–1764, Dec. 2012.
[116] Q. Li, M. Ding, and H. Wang, "[The infection of hepatitis D virus in adult tupaia]," *Zhonghua Yi Xue Za Zhi*, vol. 75, no. 10, pp. 611–613, 639–640, Oct. 1995.
[117] W. H. Caselmann, "HBV and HDV replication in experimental models: effect of interferon," *Antiviral Res.*, vol. 24, no. 2–3, pp. 121–129, Jul. 1994.
[118] A. Ponzetto, P. J. Cote, H. Popper, B. H. Hoyer, W. T. London, E. C. Ford, F. Bonino, R. H. Purcell, and J. L. Gerin, "Transmission of the hepatitis B virus-associated delta agent to the eastern woodchuck," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 81, no. 7, pp. 2208–2212, Apr. 1984.
[119] F. Negro, K. F. Bergmann, B. M. Baroudy, W. C. Satterfield, H. Popper, R. H. Purcell, and J. L. Gerin, "Chronic hepatitis D virus (HDV) infection in hepatitis B virus carrier chimpanzees experimentally superinfected with HDV," *J. Infect. Dis.*, vol. 158, no. 1, pp. 151–159, Jul. 1988.
[120] F. Negro, M. Shapiro, W. C. Satterfield, J. L. Gerin, and R. H. Purcell, "Reappearance of hepatitis D virus (HDV) replication in chronic hepatitis B virus carrier chimpanzees rechallenged with HDV," *J. Infect. Dis.*, vol. 160, no. 4, pp. 567–571, Oct. 1989.
[121] U. Schlipköter, A. Ponzetto, K. Fuchs, R. Rasshofer, S. S. Choi, S. Roos, M. Rapicetta, and M. Roggendorf, "Different outcomes of chronic hepatitis delta virus infection in woodchucks," *Liver*, vol. 10, no. 5, pp. 291–301, Oct. 1990.
[122] A. R. Ciccaglione, M. Rapicetta, A. Fabiano, C. Argentini, M. Silvestro, R. Giuseppetti, F. Varano, N. D'Urso, L. Dinolfo, and A. Morgando, "Chronic infection in woodchucks infected by a cloned hepatitis delta virus," *Arch. Virol. Suppl.*, vol. 8, pp. 15–21, 1993.
[123] S. Guilhot, S. N. Huang, Y. P. Xia, N. La Monica, M. M. Lai, and F. V. Chisari, "Expression of the hepatitis delta virus large and small antigens in transgenic mice," *J. Virol.*, vol. 68, no. 2, pp. 1052–1058, Feb. 1994.
[124] J. M. Polo, K. S. Jeng, B. Lim, S. Govindarajan, F. Hofman, F. Sangiorgi, and M. M. Lai, "Transgenic mice support replication of hepatitis delta virus RNA in multiple tissues, particularly in skeletal muscle," *J. Virol.*, vol. 69, no. 8, pp. 4880–4887, Aug. 1995.

[125] F. Liu, Y. Song, and D. Liu, “Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA,” *Gene Ther.*, vol. 6, no. 7, pp. 1258–1266, Jul. 1999.
[126] J. Chang, L. J. Sigal, A. Lerro, and J. Taylor, “Replication of the human hepatitis delta virus genome Is initiated in mouse hepatocytes following intravenous injection of naked DNA or RNA sequences,” *J. Virol.*, vol. 75, no. 7, pp. 3469–3473, Apr. 2001.
[127] M. Dandri and M. Lütgehetmann, “Mouse models of hepatitis B and delta virus infection,” *J. Immunol. Methods*, Mar. 2014.
[128] J. Taylor, W. Mason, J. Summers, J. Goldberg, C. Aldrich, L. Coates, J. Gerin, and E. Gowans, “Replication of human hepatitis delta virus in primary cultures of woodchuck hepatocytes,” *J. Virol.*, vol. 61, no. 9, pp. 2891–2895, Sep. 1987.
[129] M. Engelke, K. Mills, S. Seitz, P. Simon, P. Gripon, M. Schnölzer, and S. Urban, “Characterization of a hepatitis B and hepatitis delta virus receptor binding site,” *Hepatology*, vol. 43, no. 4, pp. 750–760, Apr. 2006.
[130] R. L. Jirtle, C. Biles, and G. Michalopoulos, “Morphologic and histochemical analysis of hepatocytes transplanted into syngeneic hosts,” *Am. J. Pathol.*, vol. 101, no. 1, pp. 115–126, Oct. 1980.
[131] M. Kusano and M. Mito, “Observations on the fine structure of long-survived isolated hepatocytes inoculated into rat spleen,” *Gastroenterology*, vol. 82, no. 4, pp. 616–628, Apr. 1982.
[132] A. A. Demetriou, S. M. Levenson, P. M. Novikoff, A. B. Novikoff, N. R. Chowdhury, J. Whiting, A. Reisner, and J. R. Chowdhury, “Survival, organization, and function of microcarrier-attached hepatocytes transplanted in rats,” *Proc. Natl. Acad. Sci. U.S.A.*, vol. 83, no. 19, pp. 7475–7479, Oct. 1986.
[133] C. Ricordi, P. E. Lacy, M. P. Callery, P. W. Park, and M. W. Flye, “Trophic factors from pancreatic islets in combined hepatocyte-islet allografts enhance hepatocellular survival,” *Surgery*, vol. 105, no. 2 Pt 1, pp. 218–223, Feb. 1989.
[134] K. Ohashi, P. L. Marion, H. Nakai, L. Meuse, J. M. Cullen, B. B. Bordier, R. Schwall, H. B. Greenberg, J. S. Glenn, and M. A. Kay, “Sustained survival of human hepatocytes in mice: A model for in vivo infection with human hepatitis B and hepatitis delta viruses,” *Nat. Med.*, vol. 6, no. 3, pp. 327–331, Mar. 2000.
[135] J. J. Brown, B. Parashar, H. Moshage, K. E. Tanaka, D. Engelhardt, E. Rabbani, N. Roy-Chowdhury, and J. Roy-Chowdhury, “A long-term hepatitis B viremia model generated by transplanting nontumorigenic immortalized human hepatocytes in Rag-2-deficient mice,” *Hepatology*, vol. 31, no. 1, pp. 173–181, Jan. 2000.
[136] J. L. Heckel, E. P. Sandgren, J. L. Degen, R. D. Palmiter, and R. L. Brinster, “Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator,” *Cell*, vol. 62, no. 3, pp. 447–456, Aug. 1990.
[137] M. Dandri, M. R. Burda, E. Török, J. M. Pollok, A. Iwanska, G. Sommer, X. Rogiers, C. E. Rogler, S. Gupta, H. Will, H. Greten, and J. Petersen, “Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus,” *Hepatology*, vol. 33, no. 4, pp. 981–988, Apr. 2001.
[138] G. Kelsey, S. Ruppert, A. Schedl, E. Schmid, E. Thies, and G. Schütz, “Multiple effects on liver-specific gene expression in albino lethal mice caused by deficiency of an enzyme in tyrosine metabolism,” *J. Cell Sci. Suppl.*, vol. 16, pp. 117–122, 1992.
[139] Z. He, H. Zhang, X. Zhang, D. Xie, Y. Chen, K. J. Wangensteen, S. C. Ekker, M. Firpo, C. Liu, D. Xiang, X. Zi, L. Hui, G. Yang, X. Ding, Y. Hu, and X. Wang, “Liver xeno-repopulation with human hepatocytes in Fah-/-Rag2-/- mice after

pharmacological immunosuppression," *Am. J. Pathol.*, vol. 177, no. 3, pp. 1311–1319, Sep. 2010.

[140] D. F. Mercer, D. E. Schiller, J. F. Elliott, D. N. Douglas, C. Hao, A. Rinfret, W. R. Addison, K. P. Fischer, T. A. Churchill, J. R. Lakey, D. L. Tyrrell, and N. M. Kneteman, "Hepatitis C virus replication in mice with chimeric human livers," *Nat. Med.*, vol. 7, no. 8, pp. 927–933, Aug. 2001.

[141] P. Meuleman, L. Libbrecht, R. De Vos, B. de Hemptinne, K. Gevaert, J. Vandekerckhove, T. Roskams, and G. Leroux-Roels, "Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera," *Hepatology*, vol. 41, no. 4, pp. 847–856, Apr. 2005.

[142] M. Dandri and J. Petersen, "Chimeric mouse model of hepatitis B virus infection," *J. Hepatol.*, vol. 56, no. 2, pp. 493–495, Feb. 2012.

[143] M. Dandri, J. M. Murray, M. Lutgehetmann, T. Volz, A. W. Lohse, and J. Petersen, "Virion half-life in chronic hepatitis B infection is strongly correlated with levels of viremia," *Hepatology*, vol. 48, no. 4, pp. 1079–1086, Oct. 2008.

[144] S. Gudima, Y. He, A. Meier, J. Chang, R. Chen, M. Jarnik, E. Nicolas, V. Bruss, and J. Taylor, "Assembly of hepatitis delta virus: particle characterization, including the ability to infect primary human hepatocytes," *J. Virol.*, vol. 81, no. 7, pp. 3608–3617, Apr. 2007.

[145] R. B. Ferns, E. Nastouli, and J. A. Garson, "Quantitation of hepatitis delta virus using a single-step internally controlled real-time RT-qPCR and a full-length genomic RNA calibration standard," *J. Virol. Methods*, vol. 179, no. 1, pp. 189–194, Jan. 2012.

[146] M. Homs, K. Giersch, M. Blasi, M. Lütgehetmann, M. Buti, R. Esteban, M. Dandri, and F. Rodriguez-Frias, "Relevance of a full-length genomic RNA standard and thermal shock step for optimal hepatitis delta virus quantification," *J. Clin. Microbiol.*, Jul. 2014.

[147] K. R. Loeb, K. R. Jerome, J. Goddard, M. Huang, A. Cent, and L. Corey, "High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system," *Hepatology*, vol. 32, no. 3, pp. 626–629, Sep. 2000.

[148] T. Volz, M. Lutgehetmann, P. Wachtler, A. Jacob, A. Quaas, J. M. Murray, M. Dandri, and J. Petersen, "Impaired intrahepatic hepatitis B virus productivity contributes to low viremia in most HBeAg-negative patients," *Gastroenterology*, vol. 133, no. 3, pp. 843–852, Sep. 2007.

[149] N. Oehler, T. Volz, O. D. Bhadra, J. Kah, L. Allweiss, K. Giersch, J. Bierwolf, K. Riecken, J. M. Pollok, A. W. Lohse, B. Fehse, J. Petersen, S. Urban, M. Lütgehetmann, J. Heeren, and M. Dandri, "Binding of hepatitis B virus to its cellular receptor alters the expression profile of genes of bile acid metabolism," *Hepatology*, Apr. 2014.

[150] T. Volz, M. Lütgehetmann, L. Allweiss, M. Warlich, J. Bierwolf, J. M. Pollok, J. Petersen, E. Matthes, and M. Dandri, "Strong antiviral activity of the new l-hydroxycytidine derivative, l-Hyd4FC, in HBV-infected human chimeric uPA/SCID mice," *Antivir. Ther. (Lond.)*, vol. 17, no. 4, pp. 623–631, 2012.

[151] M. Lütgehetmann, T. Bornscheuer, T. Volz, L. Allweiss, J.-H. Bockmann, J. M. Pollok, A. W. Lohse, J. Petersen, and M. Dandri, "Hepatitis B virus limits response of human hepatocytes to interferon-α in chimeric mice," *Gastroenterology*, vol. 140, no. 7, pp. 2074–2083, 2083.e1–2, Jun. 2011.

[152] S. Huber, J. Schrader, G. Fritz, K. Presser, S. Schmitt, A. Waisman, S. Lüth, M. Blessing, J. Herkel, and C. Schramm, "P38 MAP kinase signaling is required for the

conversion of CD4+CD25- T cells into iTreg," *PLoS ONE*, vol. 3, no. 10, p. e3302, 2008.

[153] V. Ivaniushina, N. Radjef, M. Alexeeva, E. Gault, S. Semenov, M. Salhi, O. Kiselev, and P. Dény, "Hepatitis delta virus genotypes I and II cocirculate in an endemic area of Yakutia, Russia," *J. Gen. Virol.*, vol. 82, no. Pt 11, pp. 2709–2718, Nov. 2001.

[154] L. Chen, I. Borozan, J. Feld, J. Sun, L.-L. Tannis, C. Coltescu, J. Heathcote, A. M. Edwards, and I. D. McGilvray, "Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection," *Gastroenterology*, vol. 128, no. 5, pp. 1437–1444, May 2005.

[155] M. Lagging, A. I. Romero, J. Westin, G. Norkrans, A. P. Dhillon, J.-M. Pawlotsky, S. Zeuzem, M. von Wagner, F. Negro, S. W. Schalm, B. L. Haagmans, C. Ferrari, G. Missale, A. U. Neumann, E. Verheij-Hart, K. Hellstrand, and DITTO-HCV Study Group, "IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection," *Hepatology*, vol. 44, no. 6, pp. 1617–1625, Dec. 2006.

[156] L. Chen, J. Sun, L. Meng, J. Heathcote, A. M. Edwards, and I. D. McGilvray, "ISG15, a ubiquitin-like interferon-stimulated gene, promotes hepatitis C virus production in vitro: implications for chronic infection and response to treatment," *J. Gen. Virol.*, vol. 91, no. Pt 2, pp. 382–388, Feb. 2010.

[157] R. Broering, X. Zhang, S. Kottilil, M. Trippler, M. Jiang, M. Lu, G. Gerken, and J. F. Schlaak, "The interferon stimulated gene 15 functions as a proviral factor for the hepatitis C virus and as a regulator of the IFN response," *Gut*, vol. 59, no. 8, pp. 1111–1119, Aug. 2010.

[158] D. Samuel, A. L. Zignego, M. Reynes, C. Feray, J. L. Arulnaden, M. F. David, M. Gigou, A. Bismuth, D. Mathieu, and P. Gentilini, "Long-term clinical and virological outcome after liver transplantation for cirrhosis caused by chronic delta hepatitis," *Hepatology*, vol. 21, no. 2, pp. 333–339, Feb. 1995.

[159] I. Mederacke, N. Filmann, C. Yurdaydin, B. Bremer, F. Puls, B. J. Zacher, B. Heidrich, H. L. Tillmann, J. Rosenau, C.-T. Bock, B. Savas, F. Helfritz, F. Lehner, C. P. Strassburg, J. Klempnauer, K. Wursthorn, U. Lehmann, M. P. Manns, E. Herrmann, and H. Wedemeyer, "Rapid early HDV RNA decline in the peripheral blood but prolonged intrahepatic hepatitis delta antigen persistence after liver transplantation," *J. Hepatol.*, vol. 56, no. 1, pp. 115–122, Jan. 2012.

[160] H. J. Netter, J. L. Gerin, B. C. Tennant, and J. M. Taylor, "Apparent helper-independent infection of woodchucks by hepatitis delta virus and subsequent rescue with woodchuck hepatitis virus," *J. Virol.*, vol. 68, no. 9, pp. 5344–5350, Sep. 1994.

[161] A. Smedile, J. L. Casey, P. J. Cote, M. Durazzo, B. Lavezzo, R. H. Purcell, M. Rizzetto, and J. L. Gerin, "Hepatitis D viremia following orthotopic liver transplantation involves a typical HDV virion with a hepatitis B surface antigen envelope," *Hepatology*, vol. 27, no. 6, pp. 1723–1729, Jun. 1998.

[162] K. Giersch, M. Helbig, T. Volz, L. Allweiss, L. V. Mancke, A. W. Lohse, S. Polywka, J. M. Pollok, J. Petersen, J. Taylor, M. Dandri, and M. Lütgehetmann, "Persistent hepatitis D virus mono-infection in humanized mice is efficiently converted by hepatitis B virus to a productive co-infection," *J. Hepatol.*, vol. 60, no. 3, pp. 538–544, Mar. 2014.

[163] M. Dandri and S. Locarnini, "New insight in the pathobiology of hepatitis B virus infection," *Gut*, vol. 61 Suppl 1, pp. i6–17, May 2012.

[164] T. Lang, C. Lo, N. Skinner, S. Locarnini, K. Visvanathan, and A. Mansell, "The hepatitis B e antigen (HBeAg) targets and suppresses activation of the toll-like receptor signaling pathway," *J. Hepatol.*, vol. 55, no. 4, pp. 762–769, Oct. 2011.

[165] S. F. Wieland and F. V. Chisari, “Stealth and cunning: hepatitis B and hepatitis C viruses,” *J. Virol.*, vol. 79, no. 15, pp. 9369–9380, Aug. 2005.
[166] D. Hare and K. L. Mossman, “Novel paradigms of innate immune sensing of viral infections,” *Cytokine*, vol. 63, no. 3, pp. 219–224, Sep. 2013.
[167] H. Park, E. Serti, O. Eke, B. Muchmore, L. Prokunina-Olsson, S. Capone, A. Folgori, and B. Rehermann, “IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection,” *Hepatology*, vol. 56, no. 6, pp. 2060–2070, Dec. 2012.
[168] M. Momeni, N. Zainodini, R. Bidaki, G. Hassanshahi, H. Daneshvar, M. Khaleghinia, M. Ebrahim, M. Karimi-Googheri, A. Askari, M. K. Arababadi, and D. Kennedy, “Decreased expression of toll like receptor signaling molecules in chronic HBV infected patients,” *Hum. Immunol.*, vol. 75, no. 1, pp. 15–19, Jan. 2014.
[169] M. Jiang, R. Broering, M. Trippler, L. Poggenpohl, M. Fiedler, G. Gerken, M. Lu, and J. F. Schlaak, “Toll-like receptor-mediated immune responses are attenuated in the presence of high levels of hepatitis B virus surface antigen,” *J. Viral Hepat.*, Feb. 2014.
[170] C. Sureau, “The role of the HBV envelope proteins in the HDV replication cycle,” *Curr. Top. Microbiol. Immunol.*, vol. 307, pp. 113–131, 2006.
[171] M. Rizzetto, “Current management of delta hepatitis,” *Liver Int.*, vol. 33 Suppl 1, pp. 195–197, Feb. 2013.
[172] J. Chang, S. O. Gudima, and J. M. Taylor, “Evolution of hepatitis delta virus RNA genome following long-term replication in cell culture,” *J. Virol.*, vol. 79, no. 21, pp. 13310–13316, Nov. 2005.
[173] Y. C. Chao, M. F. Chang, I. Gust, and M. M. Lai, “Sequence conservation and divergence of hepatitis delta virus RNA,” *Virology*, vol. 178, no. 2, pp. 384–392, Oct. 1990.
[174] J. M. Taylor, “Replication of human hepatitis delta virus: recent developments,” *Trends Microbiol.*, vol. 11, no. 4, pp. 185–190, Apr. 2003.
[175] T. Pollicino, G. Raffa, T. Santantonio, G. B. Gaeta, G. Iannello, A. Alibrandi, G. Squadrito, I. Cacciola, C. Calvi, G. Colucci, M. Levrero, and G. Raimondo, “Replicative and transcriptional activities of hepatitis B virus in patients coinfected with hepatitis B and hepatitis delta viruses,” *J. Virol.*, vol. 85, no. 1, pp. 432–439, Jan. 2011.
[176] M. Schaper, F. Rodriguez-Frias, R. Jardi, D. Tabernero, M. Homs, G. Ruiz, J. Quer, R. Esteban, and M. Buti, “Quantitative longitudinal evaluations of hepatitis delta virus RNA and hepatitis B virus DNA shows a dynamic, complex replicative profile in chronic hepatitis B and D,” *J. Hepatol.*, vol. 52, no. 5, pp. 658–664, May 2010.
[177] C.-H. Tseng, K.-S. Jeng, and M. M. C. Lai, “Transcription of subgenomic mRNA of hepatitis delta virus requires a modified hepatitis delta antigen that is distinct from antigenomic RNA synthesis,” *J. Virol.*, vol. 82, no. 19, pp. 9409–9416, Oct. 2008.
[178] C. Yurdaydin, “Treatment of chronic delta hepatitis,” *Semin. Liver Dis.*, vol. 32, no. 3, pp. 237–244, Aug. 2012.
[179] L. Allweiss, T. Volz, M. Lütgehetmann, K. Giersch, T. Bornscheuer, A. W. Lohse, J. Petersen, H. Ma, K. Klumpp, S. P. Fletcher, and M. Dandri, “Immune cell responses are not required to induce substantial hepatitis B virus antigen decline during pegylated interferon-alpha administration,” *J. Hepatol.*, vol. 60, no. 3, pp. 500–507, Mar. 2014.
[180] C. M. Cebulla, D. M. Miller, and D. D. Sedmak, “Viral inhibition of interferon signal transduction,” *Intervirology*, vol. 42, no. 5–6, pp. 325–330, 1999.
[181] C. Alves, C. Branco, and C. Cunha, “Hepatitis delta virus: a peculiar virus,” *Adv Virol*, vol. 2013, p. 560105, 2013.

G. Publication list

Publications

Relevance of a full-length genomic RNA standard and thermal shock step for optimal hepatitis delta virus quantification.
Homs M, Giersch K, Blasi M, Lütgehetmann M, Buti M, Esteban R, Dandri M, Rodriguez-Frias F.
J Clin Microbiol. 2014 Jul 2. pii: JCM.00940-14. Epub 2014 Jul 2.

Binding of hepatitis B virus to its cellular receptor alters the expression profile of genes of bile acid metabolism.
Oehler N, Volz T, Bhadra OD, Kah J, Allweiss L, Giersch K, Bierwolf J, Riecken K, Pollok JM, Lohse AW, Fehse B, Petersen J, Urban S, Lütgehetmann M, Heeren J, Dandri M.
Hepatology. 2014 Apr 8. doi: 10.1002/hep.27159. [Epub ahead of print]

Persistent hepatitis D virus mono-infection in humanized mice is efficiently converted by hepatitis B virus to a productive co-infection
Giersch K, Helbig M, Volz T, Allweiss L, Mancke LV, Lohse AW, Polywka S, Pollok JM, Petersen J, Taylor J, Dandri M, Lütgehetmann M
J Hepatol. 2014 Mar;60(3):538-44. doi: 10.1016/j.jhep.2013.11.010. Epub 2013 Nov 23.

Immune cell responses are not required to induce substantial hepatitis B virus antigen decline during pegylated interferon-alpha administration
Allweiss L, Volz T, Lütgehetmann M, Giersch K, Bornscheuer T, Lohse AW, Petersen J, Ma H, Klumpp K, Fletcher SP, Dandri M
J Hepatol. 2014 Mar;60(3):500-7. doi: 10.1016/j.jhep.2013.10.021. Epub 2013 Oct 26.

Congress Presentations

03-06/09/2014 Los Angeles, USA	**2014 International Meeting on Molecular Biology of Hepatitis B Virus** poster presentation **Interferon Alpha and Lambda can inhibit HDV replication but differ in their capacity to suppress HBV in humanized mice despite the induction of interferon stimulated genes UPA/SCID-mice** *Giersch K, Allweiss L, Volz T, Kah J, Bhadra OD, Lohse AW, Petersen J, Lütgehetmann M, Dandri M*
09-13/04/2014 London, UK	**2014 EASL The International Liver Congress** oral and poster presentation **Interferon Alpha and Lambda inhibit HBV replication with distinct efficacy and kinetics of gene regulation in humanized mice** *Giersch K, Allweiss L, Volz T, Lohse AW, Petersen J, Lütgehetmann M, Dandri M*

24-25/01/2014 Tubingen, Germany	**30th annual Meeting of the GASL** poster presentation **Different antiviral effects induced by interferon or entecavir in HDV infected human-chimeric uPA/SCID mice determined by new intrahepatic qRT-PCR assay** *Giersch K, Homs M, Allweiss L, Volz T, Lohse AW, Buti M, Petersen J, Dandri M, Lütgehetmann M*
29-30/12/2013 Lyon, France	**EASL Monothematic Conference Translational Research in Viral Hepatitis** poster presentation **Pegylated Interferon Lambda efficiently suppresses HDV productivity and shows comparable ability to induce ISGs as peg-IFNα in HBV/HDV co-infected humanized mice** *Giersch K, Lütgehetmann M, Volz T, Allweiss L, Lohse AW, Petersen J, Dandri M*
20-23/10/2013 Shanghai, China	**2013 International Meeting on Molecular Biology of Hepatitis B Virus** oral presentation **Pegylated Interferon Lambda efficiently suppresses HDV productivity in HBV/HDV co-infected humanized UPA/SCID-mice** *Giersch K, Lütgehetmann M, Volz T, Allweiss L, Oehler N, Lohse AW, Petersen J, Dandri M*
11-14/09/2013 Nurnberg, Germany	**68. Annual Meeting of the DGVS** oral presentation **Eine Hepatitis-Delta-Koinfektion führt im Vergleich zu einer Hepatits-B-Monoinfektion in humanisierten Mäusen zu einer verstärkten Antwort des angeborenen Immunsystems und zur ausgeprägteren Induktion humanspezifischer Zytokine** *Giersch K, Volz T, Allweiss L, Braunschmidt JL, Lohse AW, Petersen J, Dandri M, Lütgehetmann M*
25-28/04/2013 Amsterdam, Netherlands	**2013 EASL The International Liver Congress** oral presentation **Hepatitis Delta infection in humanized UPA/SCID-mice leads to pronounced Induction of human-specific Innate Immune Responses and Cytokine expression in comparison to HBV mono-infection** *Giersch K, Lütgehetmann M, Volz T, Allweiss L, Braunschmidt JL, Lohse AW, Petersen J, Dandri M*
22-25/09/2012 Oxford, UK	**2012 International Meeting on Molecular Biology of Hepatitis B Virus** oral presentation **Induction of Innate Immune Responses in Hepatocytes is more pronounced in HBV/HDV co-infection than in HBV mono-infection in humanized UPA/SCID-mice** *Giersch K, Lütgehetmann M, Volz T, Allweiss L, Braunschmidt JL, Lohse AW, Petersen J, Dandri M*

H. Appendix

H.1. List of Figures

H.2. List of Tables