Technische Universität München
TUM School of Medicine and Health

Dissecting the roles of HBV surface antigen locally expressed in the liver and secreted antigen in serum in mediating HBV immune tolerance

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Vollständiger Abdruck der von der TUM School of Medicine and Health der Technischen Universität München zur Erlangung eines Doktors der Medizin (Dr. med.) genehmigten Dissertation.

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Prüfende der Dissertation:

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Die Dissertation wurde am 04.10.2023 bei der Technischen Universität München eingereicht und durch die TUM School of Medicine and Health am 14.03.2024 angenommen.

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1 Abstract

Hepatitis B virus (HBV) is a viral thread with about 350 million chronically infected people worldwide, which causes up to 800 000 deaths annually. Currently, available therapies are limited to interferons and nucleos(t)ide analogues (NUC), which cannot cure the infection in most cases. The consequences are a lifelong treatment with poor compliance, costs and remaining risk of liver damage making new therapies urgently needed. Chronification inversely correlates with the strength and quality of the virus-specific immune response, and CD8 T cells have been identified as central mediators of cure. Several clinical trials investigated if virus-specific immunity could be induced in chronically infected patients using immunotherapies, but approaches such as TLR-agonists or therapeutic vaccination so far failed to induce curative

immunity. It is believed that HBV inhibits virus-specific immune responses, and high levels of viral antigens have been suggested as a causative agent. A previous study reported that the suppression of viral antigen expression using small interfering ribonucleic acid (siRNA) indeed restored the responsiveness of high-level HBV carrier mice towards therapeutic vaccination. These results sparked multiple efforts to develop a combinatorial therapy, in which first viral antigens are suppressed by a direct antiviral therapy followed by immunotherapy aimed at restoring antiviral immunity. Besides using siRNAs, the antiviral therapies include antisense oligonucleotides (ASOs), anti-HBs antibodies or HBs-secretion inhibitors. One key question in this regard is if suppression of high viral antigen levels in serum – achieved by anti-HBs antibodies or secretion inhibitors – or additional suppression of antigens in hepatocytes that is only achieved by siRNAs or ASOs, is needed to break HBV immunotolerance. The presented work aimed to answer this question and thereby contribute to the development of curative HBV therapies. Therefore, mice were infected with an adeno associated (AAV) vector that expressed either wildtype HBV (AAV-WT) or an HBs protein secretion defective HBV mutant (AAV-C65S). After establishing an infection with low HBsAg and HBV e (HBe) antigen titers, half of the mice were treated with TherVacB, and the immune response was measured in all individuals.

Mice without circulating antigens did not spontaneously clear the infection if they did not receive a therapeutic vaccine. Moreover, TherVacB treatment achieved similar results of viral reduction in all treated mice, indicating that clearance of HBsAg from serum alone did not break HBV-Immunotolerance. Afterwards, immunocompetent mice were infected with higher doses of the same HBV vectors to induce high-level HBV replication. Mice were either treated with HBV specific siRNAs or with control siRNAs, and afterwards all of them were immune stimulated with TherVacB. Again, a missing of circulating HBsAg did not lead to spontaneous seroconversion.

Furthermore, the pretreatment with HBV specific siRNAs compared to control siRNAs led to a more robust immune stimulation via TherVacB in all mice. The absence of circulating HBsAg did not equally contribute to the reestablishment of the immune response. The mice treated with siRNAs and TherVacB reestablished a robust immune response with a rise of alanine aminotransferase (ALT) levels, a better performance of HBV specific CD8+ T cells in the fluorescence activated cell sorting (FACS) assay and a reduction of the viral load in the liver. There was no significant difference detected between AAV-C65S mice and AAV-WT mice. The results support the thesis that clearing circulating HBs antigens from the serum of infected individuals does not suffice to restore HBV-specific immunity. It highlights the need for a siRNA

or ASO treatment to suppress protein expression in hepatocytes, allowing immune induction by subsequent immunotherapy.

2 Abbreviations

µg	microgram
µl	microliter
µm	micrometer
aa	aminoacid
AAV	adeno-associated virus
AAV-C65S	AAV vector carrying HBV C65S
AAV-HBV	AAV vector carrying HBV
AAV-WT	AAV vector carrying HBV WT
ALT	alanine amino transferase
APC	antigen-presenting cell
ASO	antisense oligonucleotide
BFA	brefeldin-A
bp	base pair
BSA	bovine serum albumin
C65S	cytosin 65 to serin mutation
cccDNA	covalently closed circular DNA
cDNA	complementary DNA
CMIA	Chemiluminescence-Microparticle-Immunoessay
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DMEM	dulbecoo´s Modified Eagle Medium
DMSO	dimethylsolfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting

FCS	fetal calf serum
GrzmB	granzyme B
HBc protein	HBV core protein
HBcAg	HBV core antigen
HBe protein	HBV e protein
HBeAg	HBV e antigen
HBs protein	HBV s protein
HBsAg	HBV surface antigen
HBV	hepatitis B virus
HBV-C65S	HBV C65S genome
HBV-WT	HBV WT genome
HBx protein	HBV x protein
HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
i.m.	intramuscularly
i.v.	intravenously
ICS	intracellular cytokine staining
IFNγ	interferon γ
IFN	interferon
L-HBs	large HBs
IL	Interleukin
kb	kilobase
LAL	liver associated lymphocytes
M-HBs	medium HBs
mg	milligram
MHC	major histocompatibility complex
min	minutes
ml	millilitre
mRNA	messenger RNA

MVA	modified vaccina virus Ankara
NK cell	natural killer cell
nm	nanometer
NOD	nucleotide-binding oligomerization domain
nt	nucleotide
NTCP	sodium taurocholate co-transporting polypeptide
NUC	Nucleos(t)ide analogues
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pcRNA	precore RNA
PD-1	programmed cell death protein 1
PEIU	Paul-Ehrlich-Institute-unit
Pen/Strep	penicillin/streptomycin
PRR	pattern recognition receptor
qPRC	quantitative PCR
rcDNA	relaxed circular DNA
RNA	ribonucleic acid
RNase	ribonuclease
rmp	rounds per minute
RPMI	Roswell Park Memorial Institute Medium
s.c.	subcutaneous
S-HBs	small HBs protein
SEM	standard error of the mean
SVP	subviral particle
T	temperature
t	time
TCR	T cell receptor
TherVacB	therapeutic vaccination against HBV

Tim-3	T cell immunoglobulin and mucin-domain containing-3
TLR	toll-like receptor
TNFα	tumor necrosis factor α
vg	viral genomes
WT	wildtype

3 Introduction

3.1 Hepatitis B

Hepatitis B is an infectious disease of the liver caused by HBV. Chronic hepatitis B is defined as seropositivity of HBsAg for more than 6 months (Lok and McMahon 2007). Although a safe and effective prophylactic vaccine against HBV infection is available, chronic HBV infection is one of the most common infectious diseases worldwide, affecting about 350 million people, corresponding to about 5% of the world's population. Since chronic HBV infection accounts for approximately 50% of all HCC cases and almost all HCC cases in children, it is a major threat to global health, leading to an estimated 887,000 deaths in 2015 (El-Serag 2012, WHO 2019).

HBV is transmitted almost exclusively parenterally by contact with infectious fluids such as blood, semen or breast milk (Komatsu, Inui et al. 2012). HBV is substantially more infectious than the human immunodeficiency virus (HIV), whereas the risk of infection is highest for perinatal transduction (Alter 2006).

The course of HBV infection is crucially determined by the cellular immune response mounted against the virus. Here, especially $CD8^+$ and $CD4^+$ T cells play an essential role to control the virus after infection. Contact with the virus can either cause a strong and polyclonal T cell response with symptomatic acute hepatitis leading to a self-limiting infection and induction of anti-HBs antibodies, or a weak or absent Immune response in patients who develop chronic infection (Böcher, Herzog-Hauff et al. 1999, Thimme, Wieland et al. 2003). In the group of chronically infected patients, 15 - 40% develop long-term damages during their lifetimes, such as liver cirrhosis, liver failure, or HCC (Lok and McMahon 2007).

The risk of becoming a chronic carrier after contact with HBV differs widely between the patients' groups. Approximately 90% of infants perinatally infected by their mother become

chronic carriers, whereas children between the age of one and four have a 30% risk of developing a chronic infection, and the risk shrinks to 5% in adults. When infected with HBV, patients either develop immunity (approximately 87-90%) and clear the infection or become chronic carriers. A smaller percentage of these patients suffer from chronically active hepatitis, being at risk to develop long-time effects like liver cirrhosis or HCC (Ott, Stevens et al. 2012). Of the 887,220 deaths resulting from HBV infection in 2015, an estimated 337,454 were caused by HCC, 462,690 were caused by cirrhosis and 87,076 were caused by acute hepatitis (McGlynn, Petrick et al. 2015, WHO 2019).

3.2 Structure of HBV virions and subviral particles

HBV is a small deoxyribonucleic acid (DNA) virus containing a nucleocapsid and an envelope and, as such, belongs to the HepaDNAvidirae (Dane, Cameron et al. 1970). Its nucleocapsid contains a small circular 3.2 kilobase (kb) double stranded DNA genome, viral polymerase and core protein. Its envelope is built of viral surface proteins and a lipid cell membrane provided by the host cell (Lu and Block 2004) (Figure 1 A). In the blood of infected patients, three different morphologic virus-like particles were detected. The subviral particles (SVPs), appearing in filamentous and spheric conformation and the virions, called Dane particles after their discoverer (Dane, Cameron et al. 1970, Hu and Liu 2017).

There are nine different genotypes of HBV described. They consist of A to I, which are dominant in different regions and thus provide epidemiological information on the virus's origin. In addition, the genotypes differ in their clinical manifestation and response to antiviral treatment (Kramvis 2014).

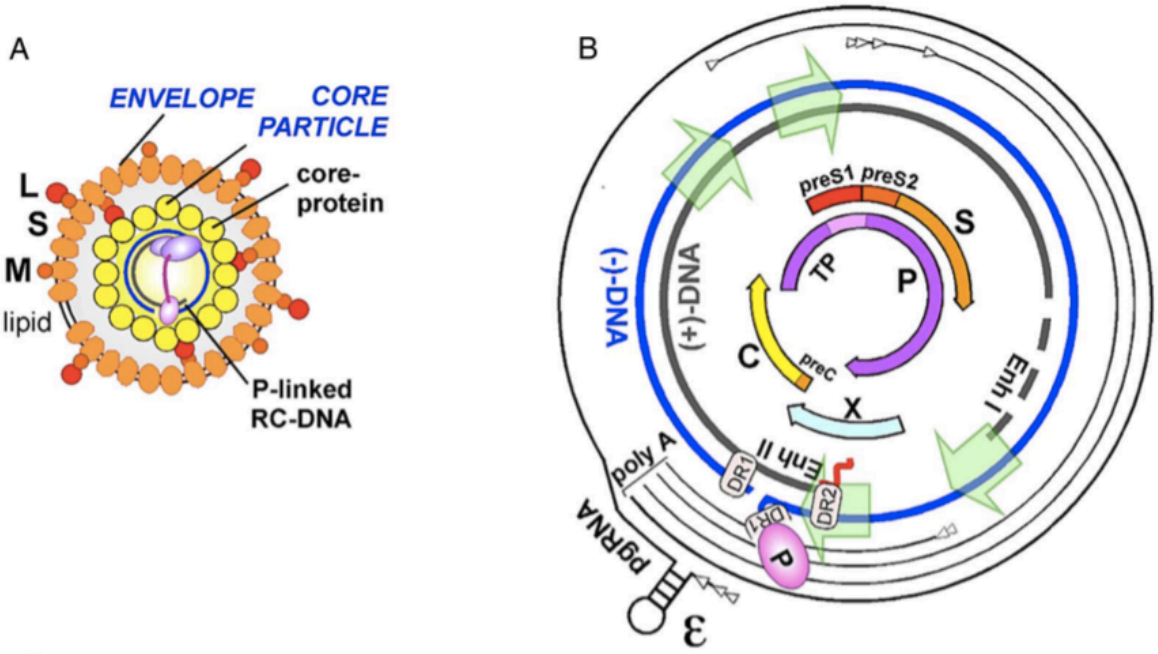

Figure 1 Structure of the HBV virion and organisation of the HBV genome
(A) Model of an HBV virion consisting of an envelope composed of the lipid embedded HBV small, medium and large protein (S-, M-, L- Protein). The icosahedral nucleocapsid is built by the core protein and contains the virus DNA in a relaxed circular form (rcDNA) linked to the polymerase (P) protein. (B) Genome organisation of HBV with the rcDNA and the blue, full length minus (-) strand and the incomplete plus (+) strand. The outer thin lines represent the mRNA transcripts with the green arrowheads at transcription starting points. In the centre, the translated proteins are represented: HBV envelope proteins (s-, m-, l- protein), polymerase (P) protein, X-protein, core (C) and pre-core (preC) protein (Graphic adapted from Nassal 2015).

3.2.1 Structure of subviral particles

Apart from virions, the HBs proteins (S-, M-, L-HBs protein) are also secreted into the bloodstream from post-endoplasmic reticulum (ER) pre-Golgi membranes without enveloping a nucleocapsid, appearing as spherical or filamentous SVPs (Huovila 1992). They are non-infectious lipoprotein particles with 22 nanometers (nm) diameter, containing the envelope proteins and are stabilised by disulphide bonds (Gilbert, Beales et al. 2005). The SVPs consist of 100 small (S-)HBs proteins, representing the majority, with only a lower amount of middle (M-) and large (L-)HBs proteins. In contrast, L-HBs protein is far more present in the envelope of Dane particles. Subviral particles are found in a 10,000-fold higher concentration in the serum of patients compared to the Dane particles, though the Dane particles are infecting the host (Alberti, Diana et al. 1978).

It is a current component of scientific discussions whether the massive overproduction of surface antigen and secretion via SVPs into the bloodstream has an immunomodulatory effect on the host, leading to an insufficient immune response (Gilbert, Beales et al. 2005, Bruss

2007). These immunomodulatory effects are further discussed under 3.5.2 *Adaptive immune response on HBV infection*.

3.2.2 Structure of Dane particles

In contrast to the SVPs, the Dane particles are the infectious virions of HBV (Alberti, Diana et al. 1978). The particle is an icosahedral structure with an inner nucleocapsid coated by an outer envelope. The nucleocapsid is formed by 180 or 240 copies of the HB core (HBc) protein and contains the viral polymerase and the relaxed circular (rc)DNA. The envelope is formed by the HBs proteins (S-, L-HBs proteins) and lipids of the cell membrane (Gilbert, Beales et al. 2005, Gallucci and Kann 2017). The small and large envelope proteins are crucial for forming the envelope of virions, whereas M-HBs protein is dispensable (Chua, Wang et al. 2005).

3.3 HBV proteins

There are four open reading frames (ORF) on the viral DNA, those of the surface (preS/S), precore-core, polymerase and X genes, encoding seven proteins. These proteins are called HBe protein, HBc protein, HBs proteins (S-, M- and L-HBs protein), polymerase and HBV x (HBx) protein (Karayiannis 2017) (Figure 1 B).

3.3.1 Core protein

The precore/core ORF on the viral DNA encodes for two different proteins, the precore-core and the core protein, translated from two different ribonucleic acids (RNAs) (Milich 2003). The core protein, consisting of 183 to 185 amino acids (aa) (depending on the HBV genotype), builds the shell of the viral nucleocapsid (Böttcher, Wynne et al. 1997). It can be divided into two parts. One part is an N-terminal end, responsible for self-assembly of the nucleocapsid and therefore called assembly domain. The other part is a C-terminal end which is involved in packaging the pregenome/reverse transcriptase complex (Bruss 2007). HBV capsid contains the rcDNA and the viral polymerase (Gallucci and Kann 2017). After the protein is folded correctly, it forms a dimer located in the capsid membrane like an upside-down "T", with the horizontal bar integrated into the capsids surface and the vertical bar forming a spike pointing to the outside of the capsid. At the spike, the main HBc antigen (HBcAg) domain is located (Bruss 2007). HBcAg is a very immunogenic antigen, that is why IgM and IgG antibodies

against HBc are established in almost all infected patients. Furthermore, studies showed that high levels of anti-HBc IgM antibodies correlated with fatality in acute liver failure (Chen, Diaz et al. 2018).

Using another 5'initiation site on the DNA, the RNA for the precore-core protein is transcribed. The precore-core proteins contain a hydrophobic portion that leads the protein to the ER and guides its uptake into the rough ER. There the C-terminal end is detached and the secreted HBe antigen (HBeAg) is formed (Milich 2003). The role of HBeAg is not fully understood yet, but studies showed that it is neither necessary for replication nor for infection in vivo (Chang, Enders et al. 1987). However, it is used alongside other parameters such as HBsAg as a marker for hepatitis B virus replication, infectivity, the severity of the disease and therapy response (Salfeld, Pfaff et al. 1989).

3.3.2 Viral polymerase

The longest viral RNA is the pregenomic (pg)RNA, which also serves as a template for translation of the viral polymerase. After the polymerase binds to a specific structure on the pgRNA, called ε, core protein encapsidates the pgRNA/polymerase complex and builds the nucleocapsid of the virus. Then, the reverse transcription of pgRNA into the partially double stranded rcDNA is started by the polymerase with a simultaneous degradation of pgRNA (Gallucci and Kann 2017).

3.3.3 X-Protein

The ORF of X-protein is 465 base pairs (bp) long and is translated to the 154aa long HBx protein, which is the smallest HBV protein. HBx protein is essential for HBV infectivity and establishing infection in vivo (Seeger 2000). The HBx protein achieves this by capturing the cellular DDB1-containing E3-ubiquitin ligase to selectively degrade the structural maintenance of chromosomes (Smc) complex Smc5/6 (Decorsière, Mueller et al. 2016). This mechanism makes it an important player in HBV replication. Furthermore, HBx is suspected of playing an important role in the carcinogenesis of HBV and thus leading to HCC in the long term (Geng 2015). First, studies showed that HBx expression correlates with the presence of 8-hydroxy-2 deoxyguanosine (8-OHdG), leading to oxidative stress in the cell and therefore DNA damage. Secondly, a high level of HBx suppressed the activity of glycosylase α, a DNA repair protein, also leading to DNA damage and mutagenesis on the long run (Cheng, Zheng et al. 2010).

3.3.4 Envelope proteins

The viral glycoproteins incorporated in the HBV envelope are encoded in a single ORF region and are translated by using different start codons to form the S-, M-, and L-HBs protein. While all three proteins contain a surface domain (S-domain), the M-HBs protein contains the 55 aa long pre-S2 domain, and additionally, the L-HBs protein contains the 108aa pre-S1 domain (Barrera, Guerra et al. 2005). In virions, the distribution of S, M and L-HBs proteins is approximately 4:1:1, whereas spherical SVPs contain S-HBs proteins predominantly and a few M-HBs proteins and filamentous SVPs mainly contain L-HBs proteins (Persing, Varmus et al. 1987, Siegler and Bruss 2013). Like all membrane proteins, the HBV envelope proteins are synthesised at the rough ER (Bruss 2007). When secreted into the bloodstream, the HBs proteins form the HBsAg. HBsAg levels over six month in the serum of patients defines a chronic HBV infection (Mangold, Unckell et al. 1995, Bruss 2007, Krebs, Böttinger et al. 2013). This antigen is currently under investigation in the academic setting, and the role of HBsAg in the induction of immune tolerance is not yet completely clarified. This project tries to contribute an important step to understanding the role of HBsAg in the induction of HBV immune tolerance.

3.3.4.1 S-Protein

The S-HBs Protein is the smallest envelope protein containing 226aa, is translated at the rough ER and insertion is guided by an N-terminal sequence (Siegler and Bruss 2013). The integration of S-HBs into the membrane is a complex biological mechanism. S-HBs consist of four transmembrane domains (TM1-TM4) integrated into the double lipid layer (Bruss 2007). TM1 (aa 8 – aa 22) is placed in the membrane and directs the N-terminal end of S-HBs into the ER lumen. The C-terminal end of TM1, therefore, lies in the cytosol. The following TM2 (aa 80 – aa 98) guides the C-terminal end of the transmembrane domain to the ER lumen again, building a cytosolic loop between TM1 and TM2. There are only theories about their location for the other two transmembrane domains. Since the region between aa 196 and aa 201 is suggested to be inside the cytosol, and the C-terminal end of S-HBs protein is located in the ER lumen, two more transmembrane domains exist between aa 170 and aa 226 (Suffner, Gerstenberg et al. 2018). Between these domains, the Luminal loop is formed (Figure 2), which is the major epitope of HBsAg (Mangold and Streeck 1993, Mangold, Unckell et al. 1995).

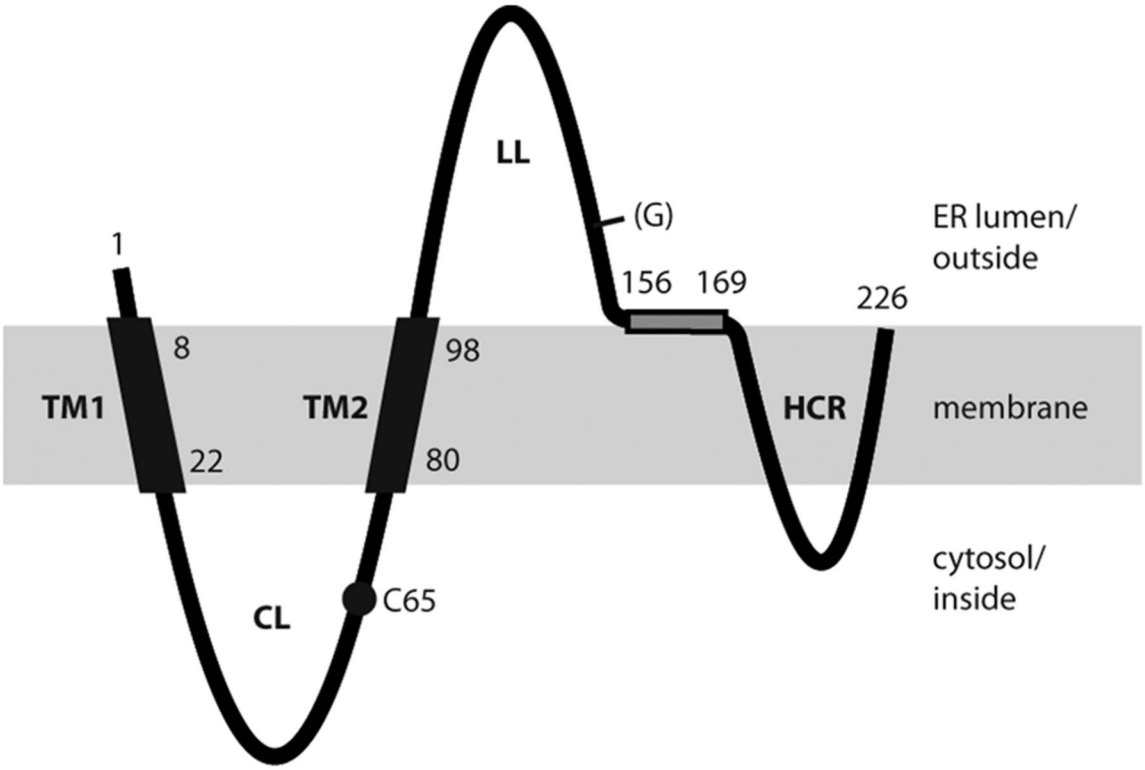

Figure 2 transmembrane structure of HBs protein
TM1 and TM2 are the transmembrane domains 1 and 2 with a cytosolic loop (CL) in between. They are followed by a luminal loop (LL) and a hydrophobic C terminal region (HCR). Numbers represent the aa position such as C65. Domains in the ER lumen are located at the outside of subviral particles, domains at the cytosol point to the inside of subviral particles (Suffner, Gerstenberg et al. 2018).

3.3.4.2 M-Protein

The M-HBs protein is transcribed using a different start-codon on the same RNA as the S-HBs protein. The M-HBs protein consists of the S-HBs domain at the C-terminal end and the additional preS2-domain at the N-terminal end. The transmembrane structure of M-HBs protein is identical to S-HBs protein locating the preS2 part in the cytosolic compartment after translation. At a so far unknown step, a translocation of the preS-parts across the membrane to the outside occurs (Urban, Schulze et al. 2010). It is additionally N-glycosylated at Asparagine 4 and O-glycosylated at Threonine 37. (Schmitt, Glebe et al. 1999, Bruss 2007).

3.3.4.3 L-Protein

The longest envelope protein, the L-HBs protein, is 389 to 400aa long and contains a preS1 domain additionally to the S-HBs part and the preS2 part. PreS1 is 108 or 119 aa long located at the N-terminal end of the protein next to the preS2 domain, also in the ER (Bruss 2007). The L-HBs protein contains a sequence detected by an N-myristoyl- Transferase at the N-

terminus, myristylating the Glycin on position two posttranslational (Resh 1999). This part of the L-HBs protein was identified as essential for HBV infectivity since it binds the sodium taurocholate co-transporting polypeptide (NTCP) receptor expressed on the basolateral membrane of hepatocytes and therefore leads to viral uptake into the host cell (Urban, Schulze et al. 2010, Meier, Mehrle et al. 2013, Yan, Peng et al. 2013).

3.3.5 C65S Mutant of HBV

For research purposes, an HBV mutant was designed, leading to infection without the secretion of HBsAg. This was based on the previous finding by Mangold and Streeck et al., being that the Cysteine number 65 (C65) is crucial for the secretion of subviral particles. After mutation of this cysteine to a serin (C65S), the subviral particles were not secreted but remained in the hepatocytes (Mangold and Streeck 1993). Regarding the protein structure of HBs, the C65 is located in the cytosolic loop (Figure 2).

3.4 Viral life cycle

The hepatitis B virus has unique genomic organisation and replication, and its infection is highly specific for species and tissue (Figure 3). It only infects primate hepatocytes and uses them as host cells for replication (Dandri and Petersen 2016). The replication steps are described in the following.

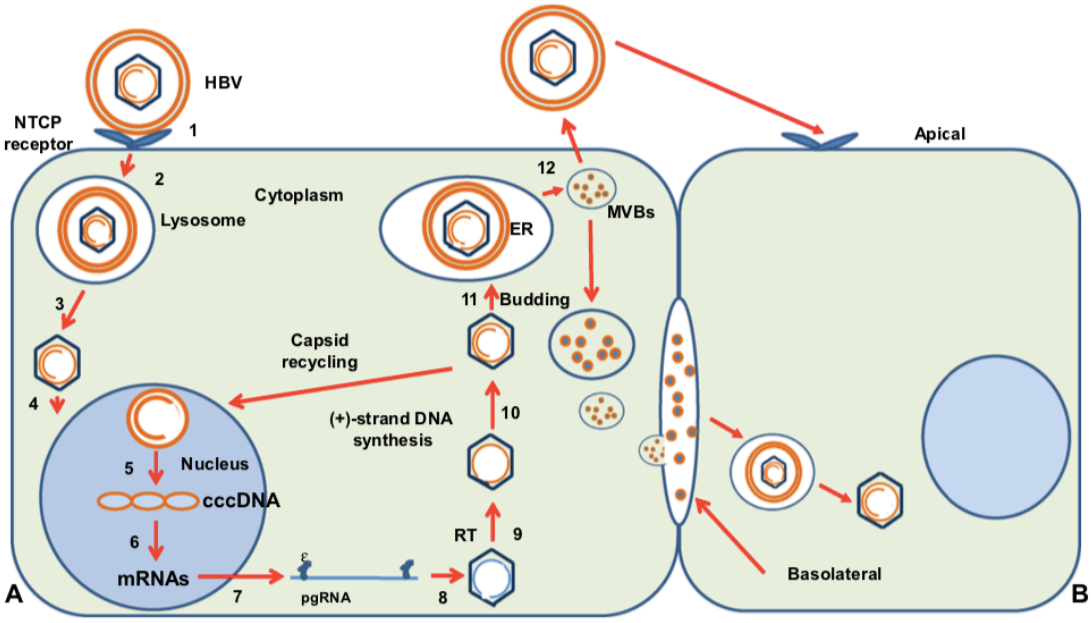

Figure 3 infection of a hepatocyte with HBV, schematic representation
(A) Stages of viral life cycle; (1) attachment, (2) endocytosis, (3) capsid release, (4) rcDNA entry into the nucleus, (5) cccDNA synthesis, (6) transcription, (7) mRNA transfer to the cytoplasm, (8) encapsidation, (9) (-)-DNA strand synthesis by reverse transcription, (10) (+)-DNA strand synthesis, (11) budding of virions into the ER lumen, (12) viral release. (B) basolateral cell to cell spread of the virus (Karayiannis 2017).

3.4.1 Cell uptake

The exact molecular mechanisms are still largely unclear, thus the knowledge about the path of infection is limited. However, it is known that numerous factors of the host play an essential role (Dandri and Petersen 2016). After entering the patient's bloodstream, the virus is transported to the liver of the host organism, where the N-terminal end of the L-HBs protein binds the NTCP receptor expressed on the apical membrane of hepatocytes. Also, heparan sulfate is crucial for the initial binding process (Karayiannis 2017). The following uptake into the host cell happens either via endocytosis and the release of viral DNA from endocytic vesicles or by fusion of the envelope part with the host cell's plasma membrane. After cellular uptake, the viral nucleocapsid is transported along the cellular microtubule to the nuclear pore complexes of the host cell. Then the viral rcDNA is released into the cellular nucleocapsid (Urban, Schulze et al. 2010). Within the nucleus, the rcDNA is converted into a covalently closed circular (ccc)DNA which is plasmid-like attached to the host genome (Figure 3). There it remains and serves as a matrix for the production of viral RNA and as a consequence for new virions (Nassal 2015). As none of the current therapies for chronic hepatitis B target cccDNA, they do not achieve a real cure (Lucifora and Protzer 2016).

3.4.2 Genome replication

Using the cccDNA as a template, Polymerase II, the cellular polymerase essential for cellular RNA synthesis, transcribes all viral RNAs (Beck 2007). This includes the sub genomic RNAs coding for the HBs proteins and the HBx protein and the precore (pc)RNA and pgRNA, which are all necessary for viral replication (Nassal 2015).

Packed in the nucleocapsid, the C-terminal end of HBc protein forms the polymerase/pgRNA complex where pgRNA is reverse transcribed into rcDNA (Figure 3) (Gallina, Bonelli et al. 1989, Seeger 2000, Hu and Liu 2017)

3.5 Pathogenesis and immune response to HBV infection

The immunological course of infection is divided into two phases. In the early phase of infection, HBV does not efficiently replicate, and HBV-DNA and HBV antigens are not detectable in serum or liver (Thimme, Wieland et al. 2003). In the second phase, HBV replicates logarithmically, and HBV-DNA can be detected (Bertoletti and Gehring 2006). The immune response following the second phase of HBV infection determines the course of the disease. Either acute hepatitis or chronic persistence of the virus are possible outcomes. For virus control, innate and adaptive immune responses are crucial. When these pathways are dysfunctional, chronic infection is established with a permanent persistence of the virus and a constant release of virions and subviral particles from infected hepatocytes into the bloodstream (Bertoletti and Gehring 2006). Via this mechanism, a large fraction of hepatocytes are infected, leading to a constant infiltration of leucocytes into the liver, causing destruction and continuous inflammation with the risk of developing liver fibrosis and cirrhosis (Seeger 2000, Ferrari 2015). Both acute (fulminant) or chronic decompensation of liver disease caused by chronic hepatitis B can lead to acute liver failure, defined as hepatic encephalopathy and coagulopathy (Lee 1993), often ending fatally by multiorgan failure (Chen, Diaz et al. 2018). A chronic HBV infection is established primarily after vertical or perinatal transmission to newborns. HBV is believed to exploit the immaturity of the neonatal immune system to establish a chronic infection due to a weak virus-specific response. A high HBV replication rate and a low incidence of liver inflammation is measured (Bertoletti and Kennedy 2015).

The liver is an organ with vast immunogenic activity. As parts of the innate immune system, antigen presenting cells (APCs) like Kupffer cells, natural killer (NK) cells and others are present in the liver interstitium. Also, the key players of the adaptive immune system, the B-

and T cells migrate to the liver and develop their immunogenic potential (Thomson and Knolle 2010).

The key contributors of the innate and the adaptive immune response and their dysfunctionality during chronic HBV infection are described in the following.

3.5.1 Innate immune response on HBV infection

The innate immune system is the first defence system against external pathogens. It acts immediately and leads to inflammation and activation of the adaptive immune system. It comprises epithelial barriers, cytokines, the complement system, the cell autonomous defence and cells like macrophages, granulocytes, natural killer cells and dendritic cells (Akira, Uematsu et al. 2006). These immune cells are activated by direct recognition of pattern recognition receptors (PRR) such as toll like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors and nucleotide-binding oligomerisation domain (NOD) like receptors. Following the activation of these cells, secretion of pro-inflammatory proteins like tumour necrosis factor (TNF), interleukin (IL) 1 and IL-6 and other interferons (IFN) and chemokines happens. They induce cell death and degradation of infected cells, increase the vascular endothelial permeability, recruit blood cells and induce the production of acute-phase-proteins (Takeuchi and Akira 2010).

The activation of the innate immune system in the early phase after viral infection is mainly characterised by the production of type 1 IFN and the activation of NK cells. While the production of Type 1 IFN is triggered by the recognition of viral DNA or RNA, the activation of NK cells is mainly driven by the recognition of stress induced molecules presented on major histocompatibility complex (MHC) I molecules on the surface of infected cells (Alexopoulou, Holt et al. 2001, Moretta, Bottino et al. 2005). In the early phase of HBV infection with low replication rates, the virus avoids recognition by the innate immune system (Ferrari 2015). It is discussed that the cccDNA, as a transcriptional template for genome replication, can hide within the nucleus and thereby escape recognition by PRRs (Wieland and Chisari 2005). It was shown that HBV is a “stealth” virus in terms of IFN response. While some authors found at least some IFN response following HBV infection (Wang, Li et al. 2010, Kumar, Jung et al. 2011), a recent study could not confirm these findings (Mutz, Metz et al. 2018). Moreover, secreted HBV proteins (HBsAg, HBeAg) suppress TLR expression and inhibit TLR-induced immune responses (Visvanathan, Skinner et al. 2007, Wu, Meng et al. 2009). To restore the innate immune system is a future therapy option further investigated under 3.7.3.1 Innate immunotherapies.

3.5.2 Adaptive immune response on HBV infection

In contrast to the innate immune system, the adaptive immune system responds slower but more specific. The concept of the adaptive immune system is the antigen-specific immune response. When contracting an external antigen, the adaptive immune system is induced, and an antigen specific humoral and cellular response is launched. The key players of the cellular response are T cells, which recognise antigens presented on the MHC I or II using T cell receptors (TCR). They are divided into CD4+ T cells, which recognise the antigens presented on MHC II and lead to an activation of other immune cells like macrophages and B cells. The other T cells are called CD8+ T cells or cytotoxic T cells, recognising antigens presented on MHC I and leading to the destruction of the infected cells. (Guermonprez, Valladeau et al. 2002).

B cells are another player of the cellular adaptive immune system and are responsible for the humoral immune response by secreting antigen specific antibodies. After activation by a T cell, the B cell further develops into an antibody-secreting plasma cell or a memory cell. The B cells undergo differentiation and proliferation, resulting in massive secretion of antigen-specific antibodies released into the bloodstream. The antibodies develop their effects in three different ways. The first is to inactivate or block binding sites of the pathogen. By the second, they opsonise the pathogen and promote phagocytosis. The third is triggering the activation of the complement system as a part of the innate immune system. Thereby the pathogens can be directly killed (Klaus, Pepys et al. 1979, French, Laskov et al. 1989).

For HBV control, especially the CD8+ T cell response is crucial. The viral load in infected hosts can only be diminished when CD8+ T cells appear in the liver (Thimme, Wieland et al. 2003). They can either directly induce apoptosis of HBV-infected hepatocytes or activate antiviral responses in infected cells via cytokine secretion (Guidotti, Rochford et al. 1999, Xia and Protzer 2017). By IFNs, CD8+ T cells clear the infected hepatocytes and destroy viral DNA. This mechanism is also possible without degradation of the cells but via intrahepatic cccDNA degradation with IFNα (Lucifora, Xia et al. 2014). A correlation between high frequencies of intrahepatic CD8+ T cells and HBV control was detected. Also, patients that controlled the virus after long term treatment with NUCs showed comparable HBV specific T cell responses to patients resolving acute HBV infection (Boni, Laccabue et al. 2012).

Apart from the essential immune response of CD8+ T cells, the CD4+ T cell response and other mechanisms of the adaptive immune response contribute to the control of the infection. For example, CD4+ helper T cells activate B cells leading to antibody secretion such as anti-

HBs and anti HBe antibodies (Gehring and Protzer 2019). Furthermore, HBV-specific CD4+ and CD8+ T cells and NK and NK-T cells can inhibit the replication of infected hepatocytes, which is mainly mediated via INFy (Guidotti, Ando et al. 1994).

Patients who develop acute hepatitis mostly have a self-limiting infection with a strong and polyclonal CD4+ and CD8+ T cell response. Viremia drops rapidly, followed by an elevation of alanine aminotransferase (ALT) levels (Guidotti, Rochford et al. 1999). In chronic hepatitis B, however, a dysfunctional adaptive immune system cannot clear the infection. There are different mechanisms of how HBV induces this immunotolerance:

During the development of T- and B cells, they undergo a natural selection to remove incorrectly primed cells and prevent a false immune response. Otherwise, autoimmune responses of B- and T cells primed on antigens of the host would occur frequently (Gallegos and Bevan 2006, Mathis and Benoist 2010). Still, some self-reacting T cells can migrate to the periphery. By a mechanism called peripheral tolerance, the immune system prevents destructive damage of these T cells to organs of the host, limiting false immune reactions. Therefore, it is an essential mechanism for the host organism and is described in more detail in the following (Protzer and Knolle 2016).

In the liver, CD4+ and Foxp3+ regulatory T cells (Treg) and APCs inhibit the activation of effector T cells. Tregs do so by suppressing the proliferation, cytokine secretion and cytotoxic activity of effector T cells (Collison and Workman 2008). Moreover, they can inhibit APCs and effector T cells by expressing molecules like Transforming Growth Factor (TGF)β, cytotoxic T-lymphocyte-associated protein 4 (CTLA4), TLR-2, -8 and other molecules (Miyara and Sakaguchi 2007).

Another protein important for immune tolerance is the surface protein programmed death protein 1 (PD-1) and its ligands PD-L1 and PD-L2. PD-1 is present in the liver and is expressed on all activated T-, B- and NKT cells. PD-L1 is, among others, expressed on nonparenchymal cells of the liver. In T cells, the activation of PD-1 by its ligands PD-L1 and PD-L2 leads to a downregulation of IL-2 and IFNy and, therefore, inhibits T cells' immunogenic effect (Fife and Pauken 2011).

The third mechanism of immune tolerance is provided by the APCs. Without inflammation, APCs express lower amounts of co-stimulatory molecules for T cells. Therefore, when T cells detect an antigen without being activated by co-stimulatory molecules from APCs, they are going in an irreversible state of anergy (Hamilton-Williams, Lang et al. 2005, Schietinger, Delrow et al. 2012).

However, in chronically HBV infected patients, these immunomodulatory effects lead to dysregulation and prevent the adaptive immune system from clearing the viral infection (Dembek, Protzer et al. 2018). This effect is also observed in other viral infections such as HIV or cytomegalovirus, whilst it is particularly strong in the chronic phase of HBV infection (Protzer and Knolle 2016).

In chronic HBV infected patients, exhausted HBV-specific T cells are found, characterised by the expression of inhibitory ligands like PD-1, T cell immunoglobulin and mucin-domain containing-3 (Tim3) and CTLA4 and the upregulation of their corresponding ligands like PD-L1 and PD-L2 (Boni, Fisicaro et al. 2007, Gehring and Protzer 2019). However, it is still discussed which mechanism leads to such a solid peripheral immune tolerance. One possible explanation is high levels of HBsAg and HBeAg in chronically infected patients with frequent TCR engagement via MHC molecules. This engagement is expected to mainly lead to a high expression of inhibitory ligands such as PD1, TIM3 and CTLA4, marking the exhaustion of antigen specific T cells (Utzschneider, Alfei et al. 2016).

After understanding the mechanisms of the adaptive immune system, it is evident that its reactivation could lead to a cure of HBV or at least provide promising new therapeutic possibilities (Gehring and Protzer 2019). These possibilities are discussed further under 3.7.3.2 *Adaptive immunotherapies*.

3.6 Standard of care and its limitations

Treatment of HBV aims to prevent the development of liver cirrhosis, HCC, acute liver failure and death related to HBV infection (Lok, Zoulim et al. 2017). For clinical treatment of HBV infection, different markers in the serum of infected patients are particularly important, including the HBsAg and anti-HBs antibodies, HBcAg and HBeAg, and the HBV DNA (Dienstag 2008). Using these markers, different grades of cure are defined and set as an aim for therapy success. A sterilising cure is defined as the eradication of intrahepatic cccDNA and integrated HBV DNA. When HBV antigens and HBV DNA are not found in the serum of infected patients over a longer period, and the liver injury is regressive, functional cure is achieved. Partially cured patients show detectable HBsAg but undetectable HBV DNA in the serum after treatment (Lok, Zoulim et al. 2017, Gehring and Protzer 2019). In current therapy strategies, cccDNA and the integrated HBV DNA are the major problems when trying to achieve a sterilising cure. Even in persons who recovered from acute HBV infection, cccDNA is found in the liver, leading to reactivation of the virus after immunosuppression. Therefore, developing therapy options for a functional cure is the primary goal of the scientific community at the

moment (Lok, Zoulim et al. 2017, Xia and Protzer 2017). These are further described under 3.7 Novel therapeutic strategies for Hepatitis B.

For the treatment of HBV, there are two standard therapies available, IFNα and NUCs (Xia and Protzer 2017).

NUCs such as lamivudine, entecavir and tenofovir have become the standard of care therapy for chronic hepatitis B since they can be administered orally, show very few side effects and have a potent antiviral activity (Yapali, Talaat et al. 2014). Because of the excellent safety profile of NUCs, long term treatment is possible (Durantel and Zoulim 2016). Attacking the viral reverse transcriptase interferes with the synthesis of genomes of progeny viruses (rcDNA) from pgRNA templates, therefore leading to viral suppression. The long term treatment with NUCs reduces liver damage, such as necroinflammatory activity, fibrosis and the development of HCC (Liaw, Sung et al. 2004, Wang, Zou et al. 2014). However, NUCs neither eliminate cccDNA nor integrate HBV DNA by targeting the viral reverse transcriptase. Furthermore, HBsAg and HBeAg secretion persist, and less than 1% of HBV infected patients are cured per year (Trépo, Chan et al. 2014).

In contrast, interferons are injected subcutaneously and often involve a broader spectrum of side effects, particularly influenza-like symptoms, bone marrow suppression, depression, and exacerbation of autoimmune illnesses (Trépo, Chan et al. 2014). Interferon has an immunomodulatory and antiviral effect in the host organism, aiming for HBsAg and HBeAg seroconversion and HBV DNA reduction (Yapali, Talaat et al. 2014). It has been demonstrated in clinical studies that a one-year treatment with pegylated interferon in HBeAg-positive patients resulted in 29% to 32% HBeAg seroconversion and 3% to 7% HBsAg loss 24 weeks after completing the treatment (Lau, Piratvisuth et al. 2005).

The current therapeutic options require either a lifelong treatment with NUCs and a permanent risk of a rebound after ending the therapy or a wide spectrum of side effects during treatment with interferons. Therefore, developing a finite therapy that enables long-term virus control or eventually a cure is the major goal aspired by academia and the pharmaceutical industry (Lok, Zoulim et al. 2017, Lazarus, Block et al. 2018, Gehring and Protzer 2019).

3.7 Novel therapeutic strategies for Hepatitis B

Novel therapeutic strategies are urgently needed since the present therapeutics cannot cure the infection in most cases. Therefore, to bring the virus under control and lower the deaths by

acute hepatitis, liver fibrosis, liver cirrhosis and HCC, the scientific community aims to develop new strategies to achieve functional cure (Durantel and Zoulim 2016, Lok, Zoulim et al. 2017).

Different therapeutical setups are under investigation and further explored via in vivo experiments and clinical trials. HBs secretion inhibitors, TLR agonists, specific antibody therapy, adoptive T cell therapy, therapeutic vaccination and siRNA therapy are among the most promising approaches. A few of these are further described in the following.

3.7.1 Targeting viral RNAs

RNA interference (RNAi) was discovered in 1998, and a novel price was given for the discovery in 2006 (Kelleher, Cortez-Jugo et al. 2020). In 2018, the US Food and Drug Administration approved the first RNAi therapeutical, and more have followed since then. The immense potential of RNAi therapeutics is also highlighted by the many drugs currently undergoing clinical trials (Kelleher, Cortez-Jugo et al. 2020).

RNAi is a mechanism of posttranscriptional gene regulation and is physiological in human cells. The endogenous RNAi is mediated by micro (mi)RNAs, which are relatively small RNAs that do not encode for proteins but interfere with mRNA expression (Bushati and Cohen 2007). The synthesis of miRNAs starts with the transcription from nuclear genes by polymerase II. They are called primary (pri)-miRNA thereafter. These pri-miRNAs are cleaved either by the microprocessor complex of Drosha and DiGeorge syndrome critical region gene 8 or by the spliceosome into hairpin shaped pre-miRNA. After the transport from the nucleus to the cytoplasm, the pre-miRNA is cleaved by the RNase III enzyme called Dicer and TBRP. Helicases separate the now originated imperfect miRNA duplex, and a single stranded miRNA is formed. This miRNA is incorporated into the RNA-induced silencing complex and targets mRNAs, leading to translational repression or mRNA degradation (Mizuguchi, Takizawa et al. 2016).

RNAi may also be used to treat certain diseases such as HBV and is currently undergoing clinical trials. Targeting and degrading the viral RNAs prevents the translation of viral proteins. HBV appears particularly suitable for this therapy approach, as all HBV transcripts share a common sequence in the 3'region (X ORF), making it possible to suppress all viral transcripts and proteins with a single therapeutic molecule (Michler, Große et al. 2016). The main approaches which are being pursued are antisense oligonucleotides (ASOs) or RNAi based methods. The latter has gained the most momentum in recent years (Mak, Seto et al. 2019). Apart from miRNAs, short hairpin (sh)RNAs and siRNAs are currently being evaluated for clinical use (McCaffrey, Nakai et al. 2003, Zhang, Li et al. 2010).

SiRNAs are small (approximately 19-21 nucleotides (nt) long) synthetic double stranded RNAs containing two complementary RNA strands that enter the RNAi pathway downstream of Drosha. In shRNAs, in contrast, the two strands are connected by a small loop, making it possible to transcribe this single RNA in vivo from an externally applied transgene. Both target mRNAs in the host cell bind it and initiate its degradation (Fire, Xu et al. 1998, McCaffrey, Meuse et al. 2002). Already few years after first reports that RNAi is also active in mammalian cells, it was found that siRNAs can also be used to inhibit HBV (Klein, Bock et al. 2003).

An important step for the clinical translation of siRNAs for HBV therapy was made by linking the modified RNA to a synthetic triantennary N-acetylgalactosamine (GalNAc). It allows siRNA uptake via the asialoglycoprotein receptor (ASGPR), a hepatocellular transmembrane glycoprotein highly expressed on hepatocytes. Highly efficient delivery of siRNAs to hepatocytes is even achieved following subcutaneous (s.c.) injection (Matsuda, Keiser et al. 2015). Furthermore, the conjugation of siRNAs to GalNac does not suppress the inhibitory effect of the siRNA on HBV replication (Foster, Brown et al. 2018).

Over the past years, progress has also been made by identifying chemical modifications that increase siRNAs' stability. Typical modifications are 2'-O-methyl and 2'-deoxy-2'-fluoro ribosugar modifications with terminal phosphorothioate linkages of both strands of the siRNA. The modifications enhance the stability of siRNAs against 3’ and 5’ enucleases, the most common nucleases in humans and prevent their degradation in the blood and other body fluids. Moreover, the recognition of the siRNAs by innate immune receptors such as TLRs and thereby a degradation is diminished. These modifications have a vast impact on the performance of GalNac-siRNA conjugates and make long term gene suppression possible (Wittrup and Lieberman 2015, Nair, Attarwala et al. 2017, Foster, Brown et al. 2018).

Several siRNAs for chronic hepatitis B treatment are currently in phase II clinical studies and aim to become therapy modalities in the following years. All of them have an impressive suppression of HBsAg and HBeAg in common up to a decline of >1 log10 from the baseline, exceeding the standard of care (NUCs) (Mak, Seto et al. 2019).

3.7.2 HBsAg secretion inhibitors

Treating HBV with nucleic acid polymers (NAPs) is a new therapeutic approach in the HBV field. NAPs aim to clear HBsAg in the bloodstream by suppressing the secretion of subviral particles of chronically infected patients. The exact mechanism is still largely unclear, but especially the NAP REP 2139 is currently undergoing clinical trials to prove its function (Vaillant 2019). In contrast to siRNA treatment, NAPs are only suppressing the secretion of HBsAg and

not the translation in the cytosol (Al-Mahtab, Bazinet et al. 2016). In vivo data showed the ability of NAPs to reduce HBsAg and even HBV DNA in the serum and establish a functional control of the chronic infection in Pekin ducks (Quinet, Jamard et al. 2018). Clinical trials in patients were already performed and showed a reduction of HBsAg in the serum of infected patients (Bazinet, Pântea et al. 2017).

3.7.3 Immunotherapeutic approaches

Immunotherapeutic approaches offer a wide spectrum of possible therapy options for the future. However, reactivating the suppressed immune response specifically for HBV is a challenging task with different approaches. A few of them are described in the following.

3.7.3.1 Innate immunotherapies

As described under 3.5.1 Innate immune response on HBV infection, the innate immune system is the first and fastest defence mechanism against viral infections. To trigger the innate immune system on HBV bears the potential to attack the viral infection in an early stage of its replication. Therefore, Immune modulators of the innate immune system are intensely investigated. Various TLR agonists, RIG-1 agonists and NOD-2 agonists achieve good results in viral control in pre-clinical models (Lucifora, Bonnin et al. 2018, Mak, Seto et al. 2019).

3.7.3.2 Adaptive immunotherapies

Targeting the adaptive immune response offers a wide range of possible therapy options. Antibody therapy, adoptive T cell therapy and therapeutic vaccination are the most promising approaches.

Injecting monoclonal antibodies that target HBsAg reduces the host's HBsAg load and even leads to seroclearance in phase I studies. This approach is currently undergoing phase II studies (Mak, Seto et al. 2019).

T cell therapy is also currently in phase I and II trials, aiming to restore the HBV specific T cell response. Since HBV suppresses the T cell response differently, it is difficult to restore the response via one pathway. Blocking the inhibitory signals transmitted by PD1, Tim3 and CTLA4 and the induction of an environment that facilitates T cell expansion or complete bypass of T cell dysfunction by adoptive T cell therapy are investigated approaches. By adoptive T cell therapy, genetically modified T cells are transferred to the host. A strong T cell response is

promised. It is aimed to produce genetically modified T cells in vitro with redirected specificity against HBV and infuse them into the host organism. Different targets for adoptive T cell therapy are currently investigated and tested in clinical trials (Boni, Laccabue et al. 2012). These concepts have been incorporated into therapeutic strategies and are further developed (Boni, Laccabue et al. 2012, Boni, Barili et al. 2019, Gehring and Protzer 2019).

Therapeutic vaccination as a promising approach of adaptive immunotherapies is described further in the following.

3.7.3.3 Therapeutic vaccination

Another promising immunotherapeutic approach is therapeutic vaccination. In contrast to the prophylactic vaccination, with therapeutic vaccination, control or even eradication of the virus in chronically infected patients is aimed. By activating the patients' immune response, certain therapeutic vaccinations can induce a multi-specific and multifunctional T cell response against viral antigens (Kutscher, Bauer et al. 2012). Since the treatment of chronically infected patients is a lifelong therapy that is costly and has side effects, the need for a therapeutic vaccination is obvious (Cornberg, Protzer et al. 2011). One promising approach of therapeutic vaccination is a combination of a protein prime with a modified vaccina virus Ankara (MVA) boost. It has been shown in mouse models that the protein prime composed of HBsAg and HBcAg induced an HBV specific neutralising antibody secretion, complexing the circulating antigens and therefore lowering the effect of the HBV antigens on T cell tolerance. Adding an adjuvant to the protein prime allows a balanced HBV specific T cell response. Cyclic-di-AMP as an adjuvant induces neutralising antibodies and priming of HBV specific CD4+ and CD8+ T cells. The adjuvant is crucial for therapy success, and cyclic-di-AMP achieved the best results in recent studies (Parvatiyar, Zhang et al. 2012). The subsequent MVA boost expresses HBsAg and HBcAg, expanding the HBV specific immune response, including HBs-seroconversion and an effective CD8+ and CD4+ T cell response (Figure 4 C). This therapy scheme is used in the present study and is called TherVacB hereafter.

Different pre-treatments are discussed before treating with therapeutic vaccination, including siRNA therapy and NUCs. By lowering the viraemia and antigenemia with a pre-treatment (Figure 4 A), a facilitated immune response and a diminished inflammatory response after therapeutic vaccination is aimed. It was shown in AAV-HBV mouse models that in individuals with low viremia and low levels of HBV antigens, HBV immune tolerance can be broken with TherVacB. HBs seroconversion is induced, and a robust CD8+ T cell response is established. However, when treating high HBV titer mice, TherVacB therapy itself could not control HBV

infection. It was suggested that high levels of viral antigens inhibited vaccine-induced T cell responses. Indeed, suppression of viral antigens in the liver using siRNAs restored the responsiveness of high titer HBV-carrier mice towards TherVacB immunisation. Animals in which siRNAs reduced antigen levels before vaccination, strong HBV-specific CD8+ T cell responses were developed, which cured HBV (Michler, Kosinska et al. 2020). While it is not clear which HBV proteins cause the strong HBV immune tolerance, one often suspected candidate is HBsAg. SiRNA therapy reduces HBV antigen levels in the infected hepatocytes and the serum of animals. It is unclear yet, whether the suppression of antigens in the hepatocytes or the absence of antigens in the serum leads to a better therapy outcome with TherVacB. This, however, is an important question, as several therapeutic approaches aim to reduce serum antigen levels (neutralising antibodies, HBs-secretion inhibitors). None of them affect local antigen expression in the liver, though, and it is unclear if such approaches could also restore the responsiveness towards therapeutic vaccination.

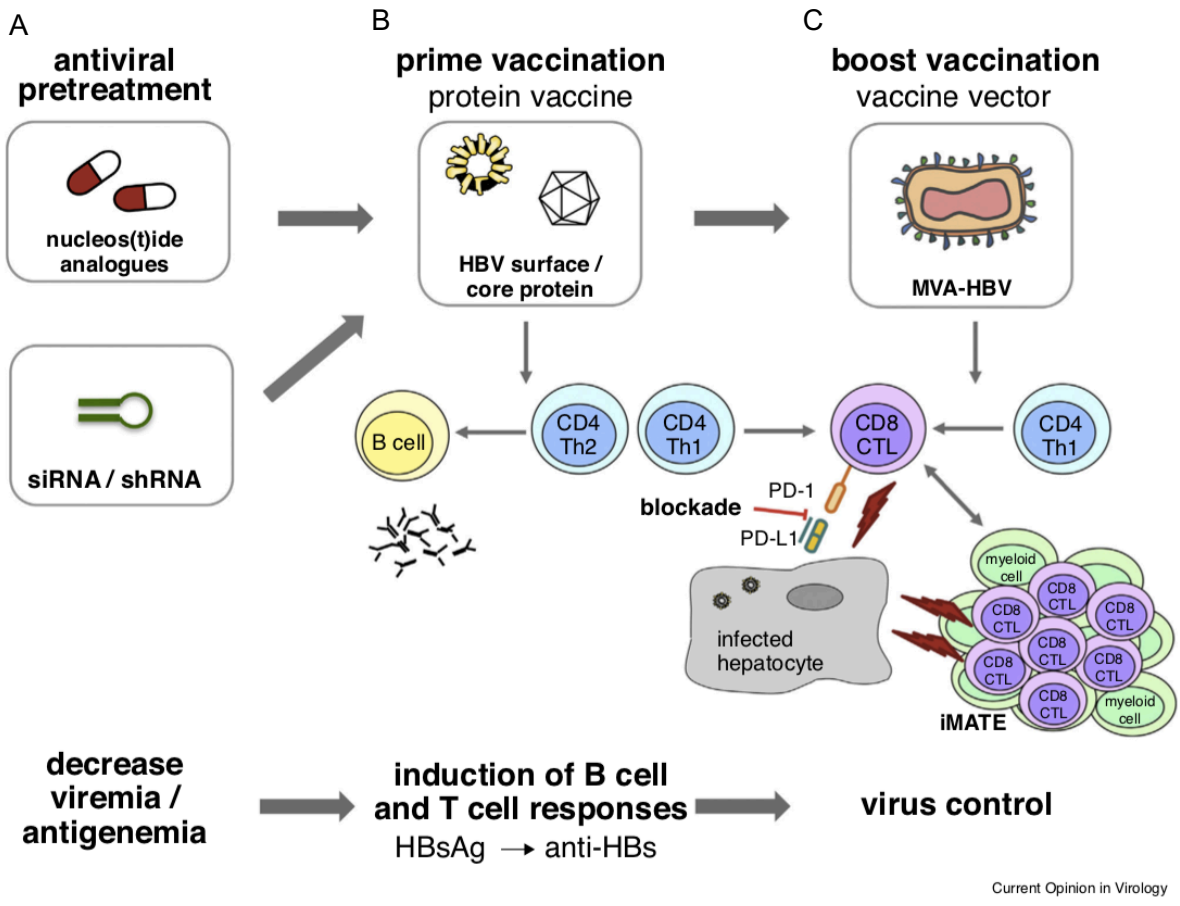

Figure 4 TherVacB strategy for chronic HBV infection
Several steps of therapeutic vaccination are required to achieve a promising result in HBV control. (A) Pretreating with either NUCs or siRNAs lowers the viraemia intending to facilitate the induction of virus-specific immune responses and diminish the inflammatory reaction. (B) Protein prime induces HBV specific antibody secretion and a balanced T cell response. (C) Boosting with a recombinant viral vector vaccine (e.g. MVA), a stable and robust CD8+ T cell response shall be induced, leading to a permanent virus control (Kosinska, Bauer et al. 2017).

3.8 In vivo model systems to study chronic HBV infection

For studies evaluating the effect of novel therapies on HBV-specific immunity and antiviral efficacy, in vivo models are irreplaceable. This poses a challenge, as HBV infection is strictly restricted to the animal family Hominidae, which besides humans, include other great apes such as Chimpanzees. These are not accessible for experimental studies.

A commonly used alternative in scientific research is to use different mammalian hepatitis B viruses, including, for example, the duck hepatitis virus (DHBV) and the woodchuck hepatitis virus (WHV). In contrast, no related hepatitis virus infecting mice has been found yet (Nassal 2015). For in vivo experiments in mice, it is possible to deliver the genome of human HBV into livers of mice using a viral vector such as Adeno-Associated Virus (AAV). It was shown that these mice established a permanent HBV infection with HBsAg and HBeAg positivity and HBV DNA in the serum. Furthermore, immunohistochemical staining for core showed a correlation between the dosage of AAV injection and core positive cells (Yang, Liu et al. 2014). The AAV infected mice are a reliable setup for research on HBV cure. Infected mice establish an immune tolerance that is not broken by conventional HBV vaccines. Also, the mice did neither undergo an anti-HBs or anti-HBe seroconversion nor develop a strong endogenous T cell response (Michler, Kosinska et al. 2020).

4 Results

For immunotolerance of chronic HBV infection, different causes are discussed. The viral HBe and HBs Antigens are proposed as key mediators of T cell dysfunction, but it is unclear if the secreted forms or local expression in hepatocytes are responsible for their immunomodulatory effects. To determine the role of HBs antigen locally expressed in the liver and secreted HBs antigen, the C65S mutant was used. For infection of cells and mice, the C65S mutation was inserted into the HBV expression plasmid 345 pAAV-HBV-C65S by Fuwang Chen and packed into an AAV-vector by Julia Hasreiter. To determine the role of secreted HBs Antigen for immune tolerance in chronically HBV infected patients, in vitro and in vivo experiments were performed using the C65S mutant.

4.1 In vitro experiments to confirm C65S mutant

To confirm that the C65S mutant is indeed secretion defective, in vitro experiments using Huh 7 cells were performed. Therefore, the 345 pAAV-HBV-C65S plasmid was transfected and is called HBV-C65S plasmid hereafter. One experimental setup was performed with dimethylsulfoxide (DMSO), and one was performed without DMSO. Also, an AAV vector (produced by Julia Hasreiter), carrying the HBV-overlength genome D, called AAV-HBV hereafter, with inserted mutation, was transduced into the cells. 345 pAAV-HBV1.3WT plasmid/AAV vectors carrying the wildtype (WT) HBV genome served as controls.

The supernatant of the plasmid transfected cells was taken after 1, 3, 4, and 6 days and further analysed. HBe and HBs Antigen was measured. The two experimental setups with and without DMSO were compared regarding the secretion of antigens. The cells incubating in DMSO medium showed a higher secretion of HBe antigen but a lower secretion of HBs antigen during the length of the experiment. In both experimental setups, the same trend was seen, however. Between the HBV wildtype group and the HBV C65S group, no significant difference of HBe antigen secretion after 3 and 6 days was found (Figure 5 A, B). In contrast, only the wildtype group secreted HBsAg, whereas no HBsAg was found in the supernatant of the C65S group (Figure 5 D, E). Overall, the addition of DMSO did not improve the outcome of the experimental setup, so it was not added during the AAV-HBV transduction.

AAV vectors were transduced into Huh 7 cells to deliver the HBV transgene with or without C65S mutation, and the supernatant was collected on days 3 and 6. In this experiment, the same effect was seen in the previous experiment: HBe antigen was equally secreted during the experiment (Figure 5 C), whereas HBs antigen was only found in the supernatant of the cells infected with the vector expressing WT HBV (Figure 5 F).

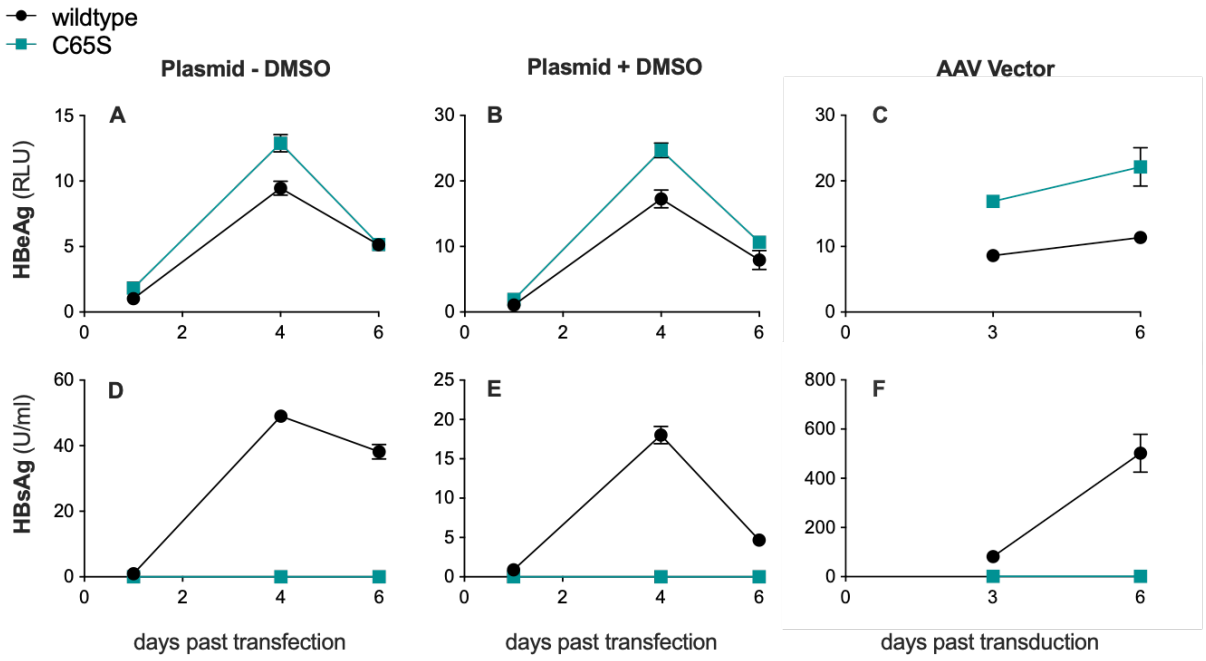

Figure 5 Effect of C65S mutation on the secretion of HBV antigens in vitro
Huh 7 cells transfected with the HBV-C65S and HBV-WT plasmid incubated in the supernatant with and without DMSO. Huh 7 cells transduced with AAV-WT and AAV-C65S. The supernatant was taken and analysed for (A, B, C) HBeAg and (D, E, F) HBsAg.

Fuwang Chen already investigated the intracellular production of HBs proteins in cells transfected with the C65S plasmid. He performed a western blot of Huh7 cells after transfecting them with a C65S genome and a wildtype HBV genome. In both cell lines, intracellular production of HBs proteins was detected (Figure 6). The results confirmed that the C65S mutation indeed prevented the secretion of HBsAg.

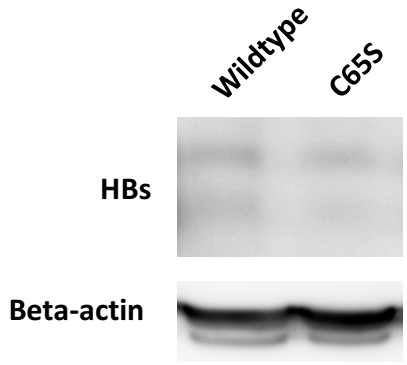

Figure 6 Western Blot detecting HBs proteins after plasmid transfection with C65S and WT HBV genome

4.2 Confirming C65S mutant in vivo

To evaluate the role of secreted HBs antigen in the immune tolerance of chronic HBV infection, we perceived a mouse model as indispensable. Therefore, after confirming the C65S mutant in vitro, in vivo testing in mice was performed. The mice were infected with AAV-C65S and AAV-WT. Immunocompetent C57Bl/6J mice were infected intravenously (i.v.) with $1x10^9$ viral genomes (vg) of AAV-HBV wildtype vector and the same amount of C65S mutated vector.

Eight weeks after injection of AAV-HBV, therapeutic immunization following the TherVacB scheme was started. First, immunization with a protein prime was performed four and six weeks after infection. It consisted of HBsAg and HBeAg combined with a cyclic-di-AMP as an adjuvant. Then, two weeks after the second priming, a boost immunization was performed with a modified Vaccinia virus Ankara (MVA) viral boost, expressing HBsAg and HBcAg, inducing a boost of the HBV specific Immune response (Figure 7).

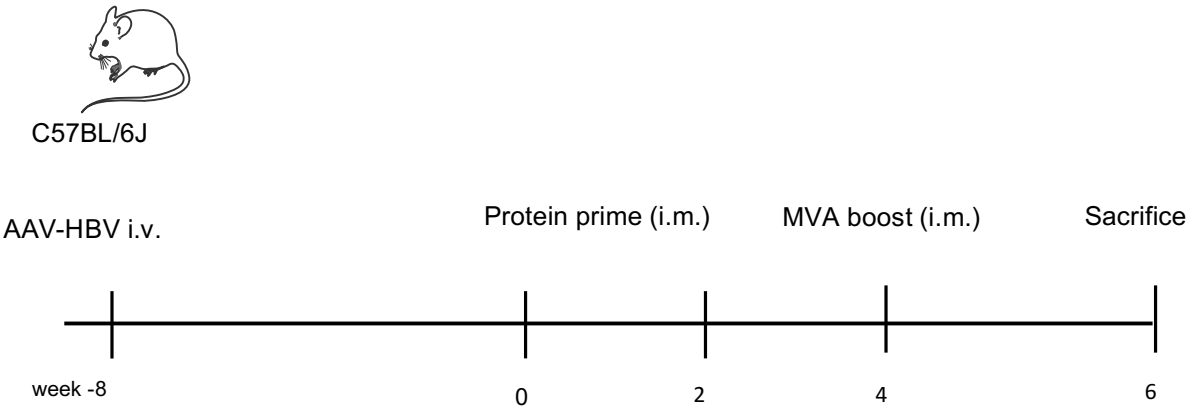

Figure 7. Experimental setup to evaluate the C65S mutation in vivo.
C57BL/6J mice were intravenously injected with AAV-HBV 8 weeks before starting TherVacB immunization scheme. Vaccination consists of two intramuscularly (i.m.) injected protein primes at week 0 and 2 (10µg HBcAg + 10µg HBsAg + 10µg cyclic-di-AMP) and one intramuscularly injected (i.m.) MVA boost at week 4. The mice were sacrificed 6 weeks after the first protein prime

Serum HBe and HBs Antigen levels were monitored over ten weeks after vector injection. Furthermore, the body weight and ALT levels were measured (Figure 8). Serum HBs Antigen in the C65S mutated group was not detected over the whole period of the experiment. The HBsAg levels of the wildtype mice were comparably high between the individuals. After

TherVacB immunisation, the HBs antigen levels of the treated wildtype group dropped under the threshold (Figure 8 A). HBe antigen was detected in all animals of both groups. Four weeks after infection, different serum HBe antigen levels between the groups were found. The HBe antigen titer of the C65S group had a mean value of 20,48 PEIU/ml, whereas serum HBeAg levels of the wildtype group had a mean value of 9,36 PEIU/ml (Figure 8 B). Eight weeks after infection, the TherVacB treatment was started with the first i.m. injection of protein prime. Four weeks after the first protein prime, blood was taken, and a drop of serum HBeAg titers in both treated groups was seen. When the mice were sacrificed six weeks after the first protein prime, HBeAg titers in both groups fell below the threshold level (Figure 8 B).

Before therapeutic vaccination, all four groups showed an ALT level in the physiological range for mice. The mutant groups did not show a significantly higher ALT level before therapeutic immunisation than the wildtype group. After TherVacB treatment, the two treated groups showed a significant increase in ALT levels up to 60 U/l in the wildtype group and 45 U/l in the C65S group. There was no significant difference between the two immunised groups (Figure 8 C). The mice constantly gained weight during the experiment, but the C65S mutant groups showed a trend towards lower weight than the wildtype groups (Figure 8 D).

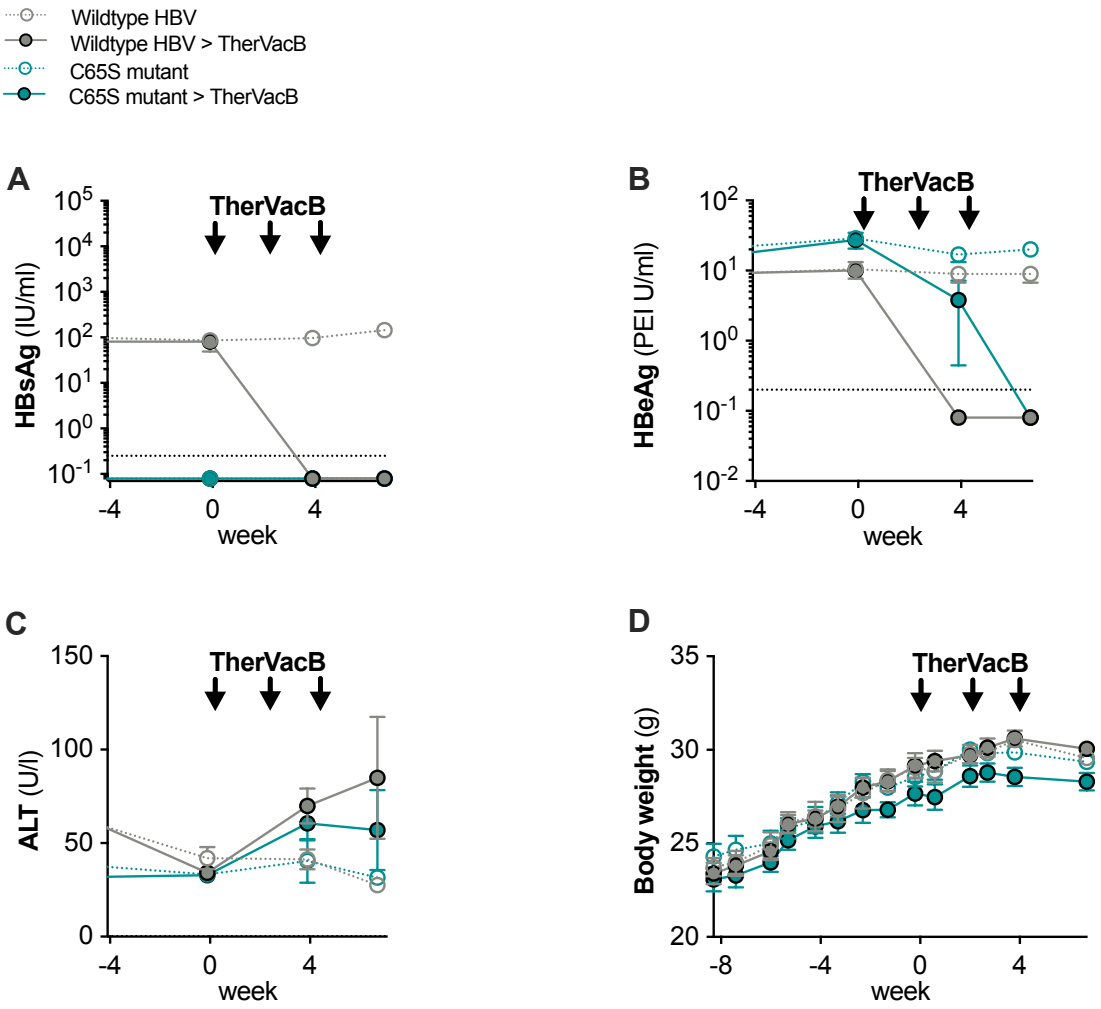

Figure 8 Effect of C65S mutant on HBV antigen levels in serum and response to therapeutic vaccination. Blood was taken four weeks before the first protein prime and on weeks 0, 4 and 6. It was analysed for (A) serum HBsAg (B) serum HBeAg and (C) ALT levels. (D) Bodyweight was measured over the whole time of the experiment.

Simultaneously to the decline of HBsAg and HBeAg serum antigen levels (Figure 8 A, B), the HBV specific anti-HBs and anti-HBe antibodies started to rise continuously in the TherVacB treated mice. In this group, the C65S group showed a trend towards higher final anti-HBs antibody titers compared to the wildtype group. In the C65S group without TherVacB treatment, one mouse secreted anti-HBs antibodies, rising during the experiment. After immunisation, the anti-HBe titers of the treated groups rose equally (Figure 9 B).

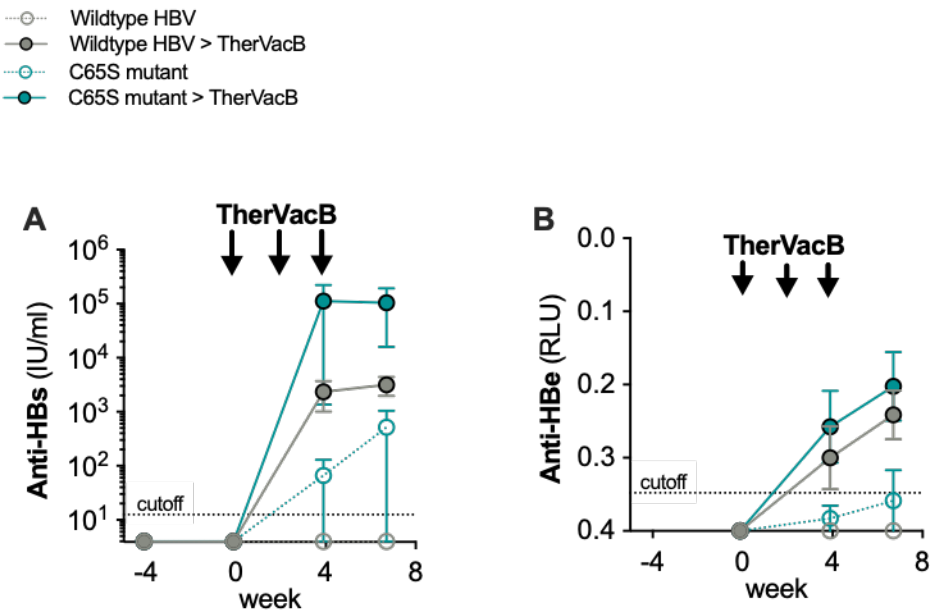

Figure 9 Effect of C65S mutant on HBV-specific antibody responses.
(A) anti-HBs and (B) anti-HBe Antibodies of the same experiment, as shown in Figure 6, were measured at weeks 0, 4 and 6. The mean and positive and negative standard error of the mean (SEM) is shown.

Two weeks after injecting the MVA boost, the mice were sacrificed. Liver and spleen were taken, and the liver associated lymphocytes and the spleen associated lymphocytes were isolated. The number and co-inhibitory receptor expression of HBV-specific CD8+ T cells were determined by flow cytometry following staining using HBV- or MVA-specific MHC I - multimers. In parallel, immune cells were ex vivo stimulated with HBV- or MVA-specific peptides and responsiveness evaluated by intracellular cytokine staining and flow cytometry. Furthermore, liver slices were immunohistochemically stained for HBc proteins, and PCRs from liver lysate were performed.

In the analysis of C93 specific CD8+ T cells, each group of vaccinated animals showed four individuals with sufficient T cells for further analyses of co-inhibitory receptor expression found. The unvaccinated mice showed no or only low amounts of C93 specific CD8+ T cells and were not analysed further (Figure 10 A). Co-inhibitory receptor expression (PD1, $PD1^{high}$, Tim3) and cytokine positivity (Granzyme B, TNFα, IFNγ) were analysed. The percentage of C_{93} specific CD8+ T cells expressing PD1 was significantly higher in the C65S group than the wildtype group, whereas no significant difference was observed in the percentage of $PD1^{high}$ expressing cells (Figure 10 B). Also, the percentage of PD1 and Tim3 double-positive cells was significantly higher in the C65S group (Figure 10 B). After peptide restimulation, the CD8+ T cells showed a different positivity for cytokines between the groups. For the C_{93} specific CD8+

T cells of mice infected with the C65S vector, the percentage of Granzyme B (Figure 10 E) and TNFα (Figure 10 C) after peptide restimulation was significantly lower. In summary, more C_{93} specific CD8+ T cells in the C65S group were PD1+, which coincided with reduced functionality.

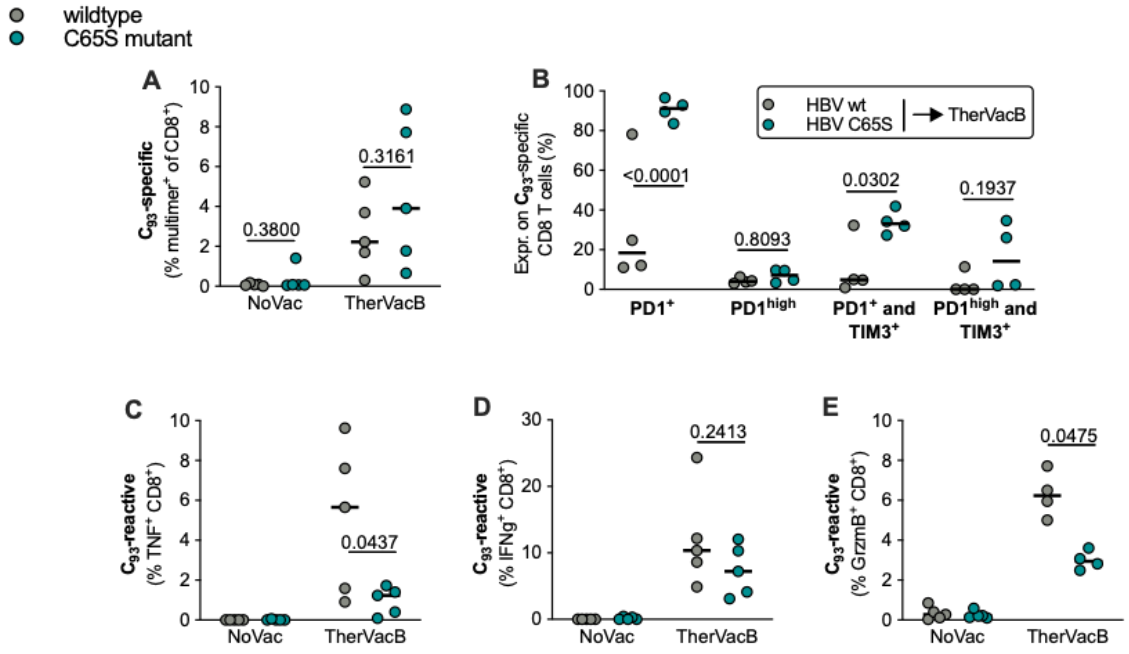

Figure 10 Effect of C65S mutation on vaccine-induced C_{93}-specific CD8+ T cell response in liver
The same experiment is shown in Figures 6 and 7. After sacrificing mice and isolating liver associated lymphocytes, the CD8+ T cells were stained with (A) C_{93}-specific MHCI-multimers as well as (B) PD1 and Tim3 specific antibodies and analysed by flow cytometry. (C-E) After peptide restimulation, T cells were intracellularly stained with (C) TNF (D) IFNγ and (E) Granzyme B specific antibodies. For statistical analysis, the median is displayed, and significance is measured by multiple t-test.

To further analyse how the C65S mutation would affect the antiviral effect of TherVacB, PCRs from liver lysate were performed, and HBV pgRNA, as well as total HBV RNA expression relative to cyclophilin A, was measured. Between non-vaccinated groups, a significant difference in total HBV RNA expression and HBV pgRNA expression was seen. The C65S mutant showed a significantly higher expression of total HBV RNA (C) and a significantly higher pgRNA expression (D). The same trend was seen in IHC stainings of liver slices for HBc (B). Furthermore, in the non-vaccinated group, the mutant showed a mean of 180 core positive cells per mm^3, whereas in the wildtype group, only a mean of 20 core positive cells per mm^3 were counted (A). Taken together, mice transduced with the C65S mutant AAV-HBV replicated higher levels of HBV. Considering that we did not observe such an effect in our in

vitro studies, we assumed that the two AAV preparations contained slightly varying numbers of functional AAV (Figure 8 B).

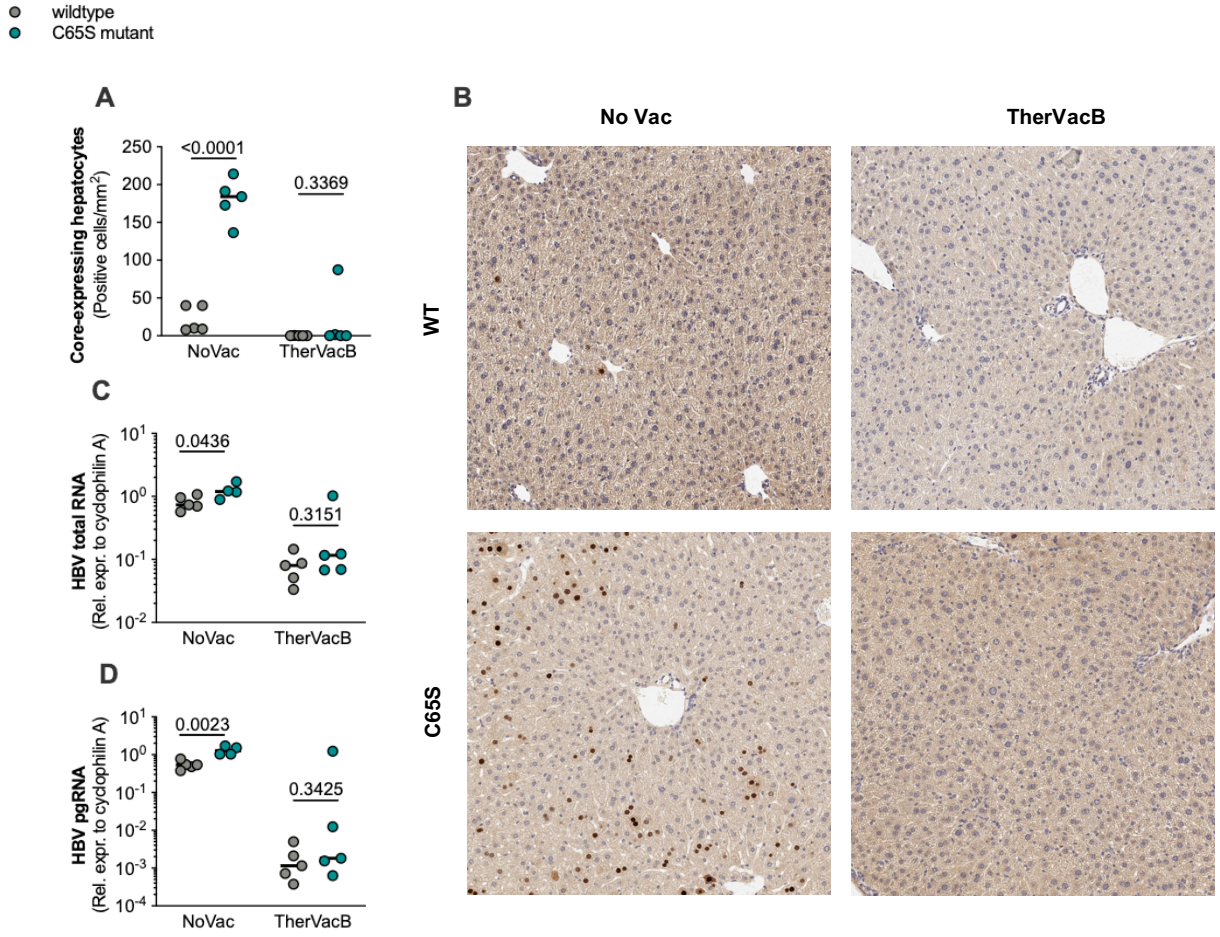

Figure 11 Antiviral efficacy of therapeutic vaccination in mice replicating wildtype or C65S HBV
Liver slices from the mice were made and (B) Immunohistochemically stained for core. (A) Core positive hepatocytes per mm^3 were counted and compared between the groups. Furthermore, liver lysates were analysed by RT-PCRs for expression of (C) HBV totalRNA and (D) HBV pgRNA relatively to cyclophilin A expression. For statistical analysis, the median is displayed, and significance is measured by multiple t-test.

The S_{190} specific T cells were analysed equally as the C_{93} specific CD8+ T cells. For the staining of PD1 and Tim3 expression, the mice with a low number of S_{190} specific T cells were excluded. Therefore, five mice of the vaccinated C65S group and three of the vaccinated wildtype group remained for this analysis (Figure 12 A).

The percentage of PD1 and $PD1^{high}$ expression on S_{190} specific CD8+ T cells was equally high in both groups, but the C65S group showed a significantly higher percentage of PD1 and Tim3 double-positive CD8+ T cells (Figure 12 B). Furthermore, the Granzyme B+ cells after peptide

restimulation were significantly lower in the C65S group than the WT group (Figure 12 E). However, in the amount of TNF+ (Figure 12 C) and IFNγ+ cells (Figure 12 D), both groups showed a similar level after restimulation. In summary, CD8+ T cells showed a tendency towards higher reactivities against S_{190} if isolated from livers of mice that had received the C65S mutant HBV.

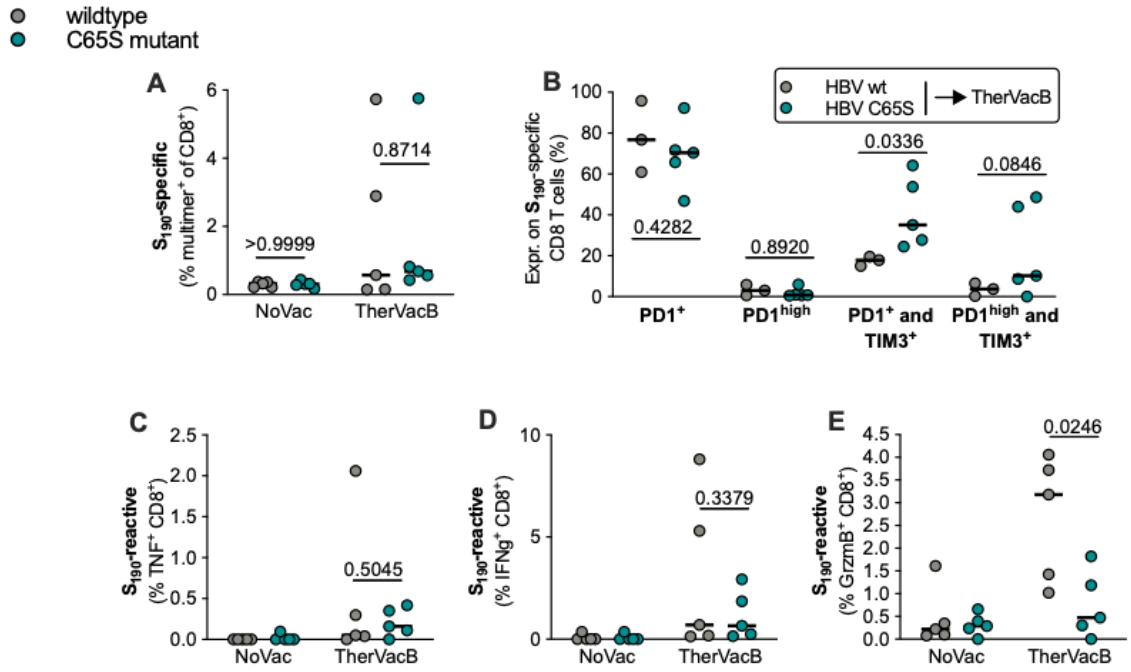

Figure 12 Effect of C65S mutation on S_{190}-specific CD8+ T cell response in liver
After sacrificing mice and isolating liver associated lymphocytes, the CD8+ T cells were stained using (A) S_{190}-specific MHCI-multimers and afterwards for (B) co-inhibitory receptor expression like PD1, $PD1^{high}$ and Tim3 as well as their combinations. After restimulation with HBV-specific peptides, (C) TNFα (D) IFNγ and (E) Granzyme B production was measured by ICS and FACS assay. For statistical analysis, the median is displayed, and significance is measured by multiple t-test.

In the analysation of functionality, not only S_{190} specific CD8+ T cells were investigated, but also S_{208} specific CD8+ T cells. When stimulated with the S_{190} peptide, CD8+ cells from mice treated with the C65S mutant were stained significantly less frequently positive for Granzyme B (Figure 13 C), correlating to the Co-inhibitory receptor expression of S_{190} specific CD8+ T cells. On the contrary, in the S_{208} specific CD8+ T cells of the C65S group, significantly more TNFα positive (Figure 13 D) and INFγ positive (Figure 13 E) cells were detected after peptide

restimulation. In summary, the CD8+ T cell response developed contrary between the S_{190} and S_{209} specific CD8+ T cells.

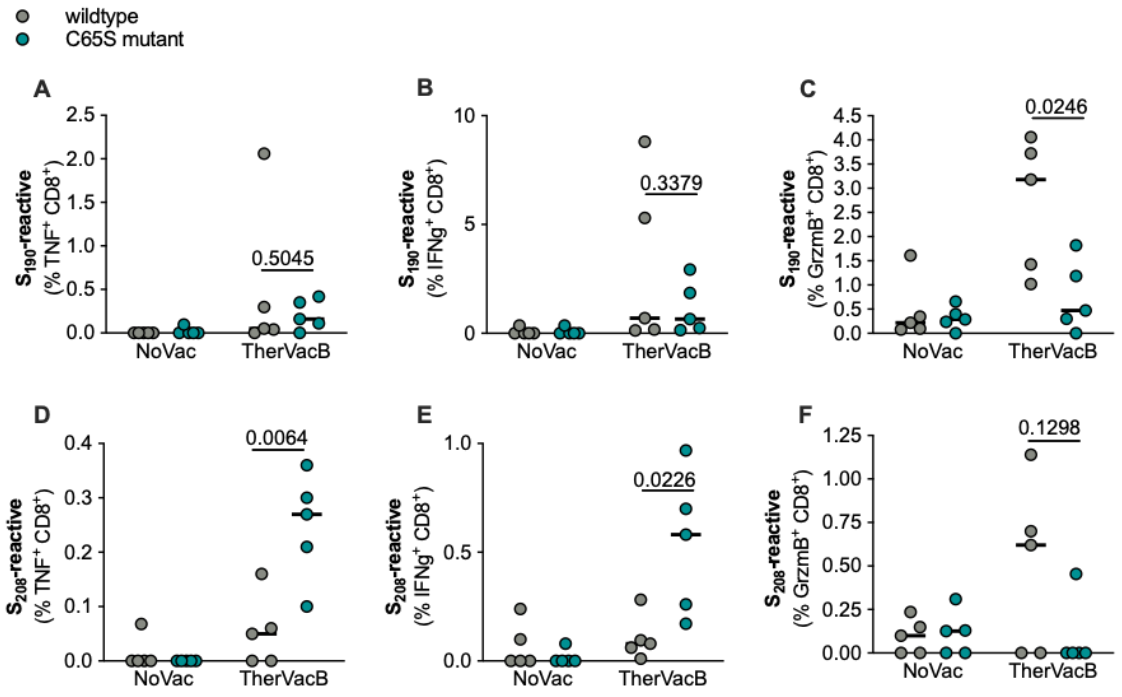

Figure 13 Effect of C65S mutation on S_{190} and S_{208} specific CD8+ T cell response in liver
Comparing the (A) TNFα, (B) IFNγ and (C) Granzyme B secretion of S_{190} specific CD8+ T cells with (D) TNFα, (E) IFNγ and (F) Granzyme B secretion of S_{209} specific CD8+ T cells after peptide restimulation. Liver associated lymphocytes (LALs) were restimulated with HBV-specific peptides, and cytokine production was measured by ICS and flow cytometry. For statistical analysis, the median is displayed, and significance is measured by multiple t-test.

To confirm that TherVacB immunisation, in general, worked equally well in all animals, we measured T cell responses against an MVA epitope (MVA_{B8R}). MVA-specific CD8+ T cells from immunised mice showed a low percentage of PD1+ and $PD1^{high}$ expression on CD8+ T cells and a low percentage of PD1+ and Tim3+ double expression on CD8+ T cells (Figure 14 B), in line with the notion that they had not encountered the MVA_{B8R} antigen following immunisation. After peptide restimulation, a TNF production of MVA_{B8R} specific CD8+ T cells was detected in both groups treated with TherVacB (Figure 14 C) as well as an IFNγ production (Figure 14 D). Also, Granzyme B was produced in all mice (Figure 14 E), leading to the conclusion that the secretion of HBsAg in mice did not affect MVA-specific T cell responses.

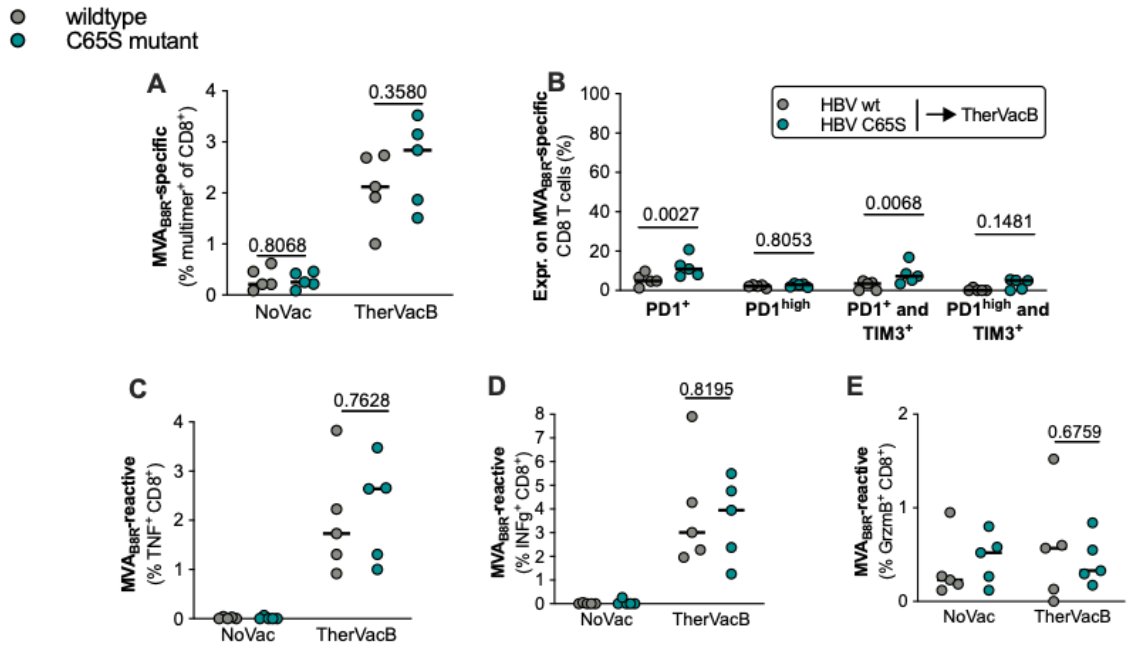

Figure 14 MVA$_{B8R}$ specific CD8+ T cell response in liver
After sacrificing mice and isolating liver associated lymphocytes, the CD8+ T cells were stained using (A) MVA$_{B8R}$-specific MHCI-multimers and afterwards stained for (B) co-inhibitory receptor expression like PD1, PD1^{high} and Tim3 as well as their combinations. After restimulation with HBV-specific peptides, (C) TNFα (D) IFNγ and (E) Granzyme B production was measured by ICS and FACS assay. For statistical analysis, the median is displayed, and significance is measured by multiple t-test.

4.3 Effect of siRNA-mediated HBV antigen suppression on vaccine-induced immune responses in mice replicating wildtype or C65S mutant HBV

Another mouse experiment was designed and conducted to discriminate further the roles of a high HBV antigen load in serum or liver on HBV-specific immune response. In this experiment, mice were again infected with AAV-HBV1.3 vectors, encoding for the WT HBV genome or the C65S genome. Two groups with ten mice each were infected either with AAV-WT or AAV-C65S. We injected a higher AAV-HBV dose to achieve high viral replication where TherVacB could not break immune tolerance alone without siRNA pretreatment. Based on our previous experiment (Figure 8), we adjusted doses to receive equal levels of HBV replication for both AAV-HBV vectors. Eight weeks after the infection with HBV vectors, siRNA treatment was started, and four groups were discriminated. Five mice of the AAV-HBV and AAV-C65S groups were treated with HBV siRNA, and five mice of AAV-HBV and AAV-C65S were treated with Ctrl siRNA. The treatment was completed with the injection of the same siRNA twelve and sixteen weeks after HBV infection. TherVacB immunisation was started for all mice with the

first protein prime simultaneously with the last siRNA treatment to induce the immune response. Mice were sacrificed 5 weeks after the MVA-boost (Figure 15).

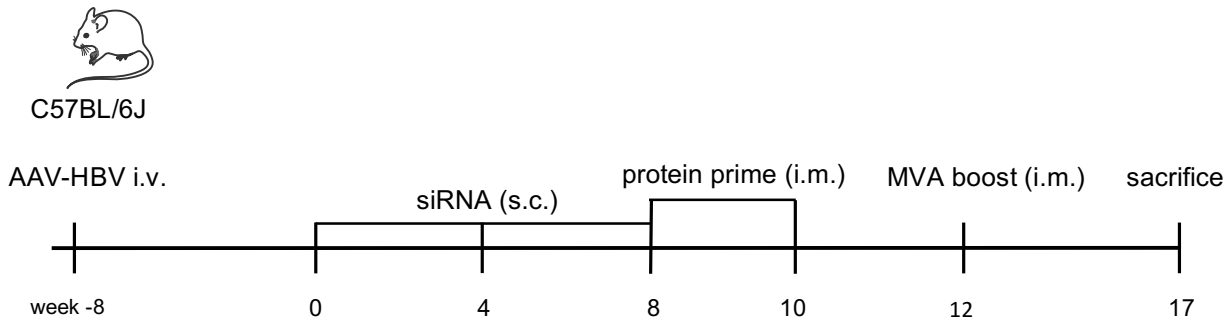

Figure 15 Experimental setup to examine if antigen suppression in the liver is necessary to restore responsiveness of HBV-replicating mice to therapeutic vaccination
C57BL/6J mice were intravenously injected with an AAV8-vector with a 1.3 overlength genome encoding either for HBV WT genome or HBV C65S genome. Eight weeks after infection, the siRNA treatment was started, where one group of each mouse population was treated with siHBV siRNA and one group was treated with siCtrl siRNA. Then, with the third dose of subcutaneously injected siRNA, the TherVacB therapy scheme was started with the first protein prime and conducted following the TherVacB therapy scheme. Seventeen weeks after the first siRNA treatment, the mice were sacrificed.

Over the whole time of the experiment, body weight, serum ALT levels and serum HBsAg and HBeAg levels were measured (Figure 16). Mice continuously gained body weight throughout the experiment. While the siRNA-treatment did not affect body weight, animals expressing the C65S mutant showed a tendency towards decreased body weights compared to animals expressing wildtype HBV (Figure 16 F). This trend was also shown in the first mouse experiment (Figure 8 D) and could indicate that inhibition of HBsAg secretion may have adverse effects on mice. HBs serum antigen was not detected in the AAV-C65S mice, whereas the titer in AAV-WT mice was ten times higher in this experiment than the experiment described before due to the larger number of viral genomes injected. HBs serum antigen levels in the AAV-WT groups were similar until the siRNA treatment was started, and the HBsAg titer of the siHBV group dropped directly after the first injection of siRNA until it was not detectable anymore after the third siRNA injection. One mouse of the AAV-C65S group showed an HBsAg elevation in the serum at weeks four and eight having a peak of 3,9 IU/ml at week four (Figure 14 A), which was possibly released from dying hepatocytes.

Ten times higher HBeAg serum levels than in the first experiment were measured, equally high in all four groups. When siRNA injection was started, the HBeAg titer of both groups treated with siHBV dropped equally to a tenth of the initial value until it stabilised at about 10 PEIU/ml until the end of the experiment. There was no difference in the drop of HBe antigen comparing

the AAV-WT and AAV-C65S group after siRNA treatment and after TherVacB immunisation (Figure 16 B). The drop of serum antigen titers was followed by an elevation of ALT serum levels after TherVacB immunisation in all four groups, indicating destruction of infected hepatocytes by HBV-specific CD8+ T cells (Figure 16 C).

Anti HBs antibodies of the AAV-C65S group were detectable during the experiment and rose equally after TherVacB treatment. Before TherVacB treatment, the anti-HBs antibodies in the WT groups were not detectable but also rose equally after therapeutic vaccination (Figure 16 D). The anti HBe antibodies of most mice remained negative, and only two mice developed a detectable titer (Figure 16 E). The HBeAg levels of the same individuals dropped under the threshold before anti-HBe titers rose. This effect is probably attributable to immunocomplexes built between antibodies and antigens (Figure 16 B).

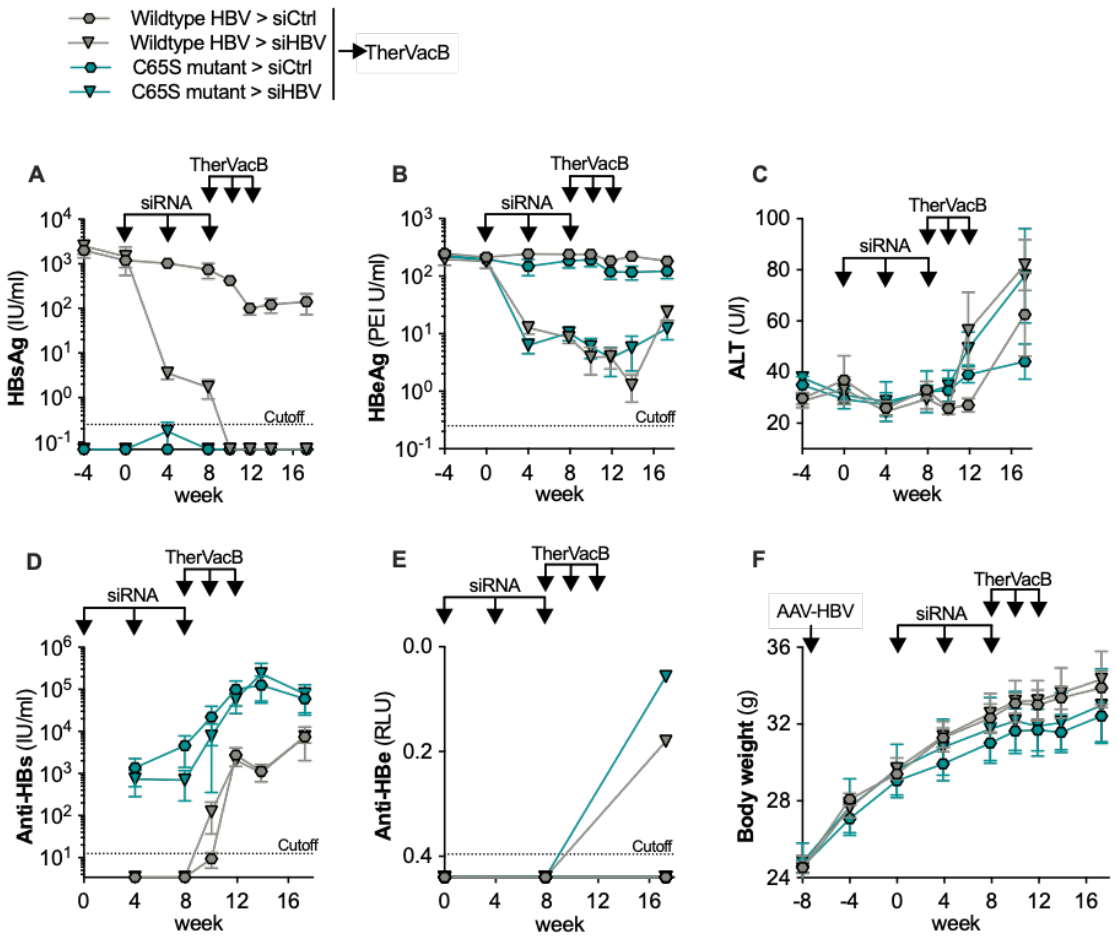

Figure 16 Effect of C65S mutation on efficacy of combinatorial siRNA and vaccination therapy
Four weeks before the first siRNA treatment, blood was taken the first time from mice. In the following time before treatment, blood was retaken, and testing was performed. (A) serum HBsAg levels as well as (B) serum HBeAg levels were tested by ELISA. (C) ALT levels were tested on a Reflotron® Revlovet Plus (Roche diagnostics). At specific time points before and after TherVacB treatment (D) anti-HBs and (E) anti-HBe antibodies were tested.

The anti-HBe antibody graph shows individual mice. (F) Bodyweight was monitored over the whole time of the experiment. For statistical analysis, the mean and positive and negative standard error of the mean (SEM) is displayed.

After terminating the experiment 17 weeks after the first siRNA dose, liver associated lymphocytes were isolated and stained with multimers, specific for C_{93}, S_{190} and MVA_{B8R}. The multimer positive T cells were further analysed, detecting the co-inhibitory receptor expression of PD1, Tim3 and CTLA4. Also, the cytokine positivity after peptide restimulation was measured, detecting the TNFα, IFNγ and Granzyme B production of the HBV specific T lymphocytes.

The multimer staining showed sufficient amounts of C_{93} specific CD8+ T cells to analyse subgroups further. Their quantity was comparable between the AAV-C65S and the AAV-WT groups (Figure 17 A). The percentage of PD1 expression on the surface of the T cells showed a significantly lower signal on the AAV-WT + siCtrl group compared with the other groups. However, mice treated with AAV-C65S + siCtrl expressed PD1 in a similar percentage as the two groups treated with siHBV (Figure 17 C). The same trend was seen in the mean PD1 expression of C_{93} specific T cells, where the mean of the AAV-C65S + siCtrl group was on an equal level as the mean of the siHBV groups (Figure 17 B). Furthermore, the AAV-C65S + siCtrl showed a similar Tim3 expression as the siHBV groups, all significantly lower than the AAV-WT + siCtrl group (Figure 17 C). In summary, more CD8+ T cells of the siHBV groups were PD1+ and Tim3+ coinciding with a preserved functionality. The AAV-C65S + siCtrl group showed the same pattern (Figure 17 D).

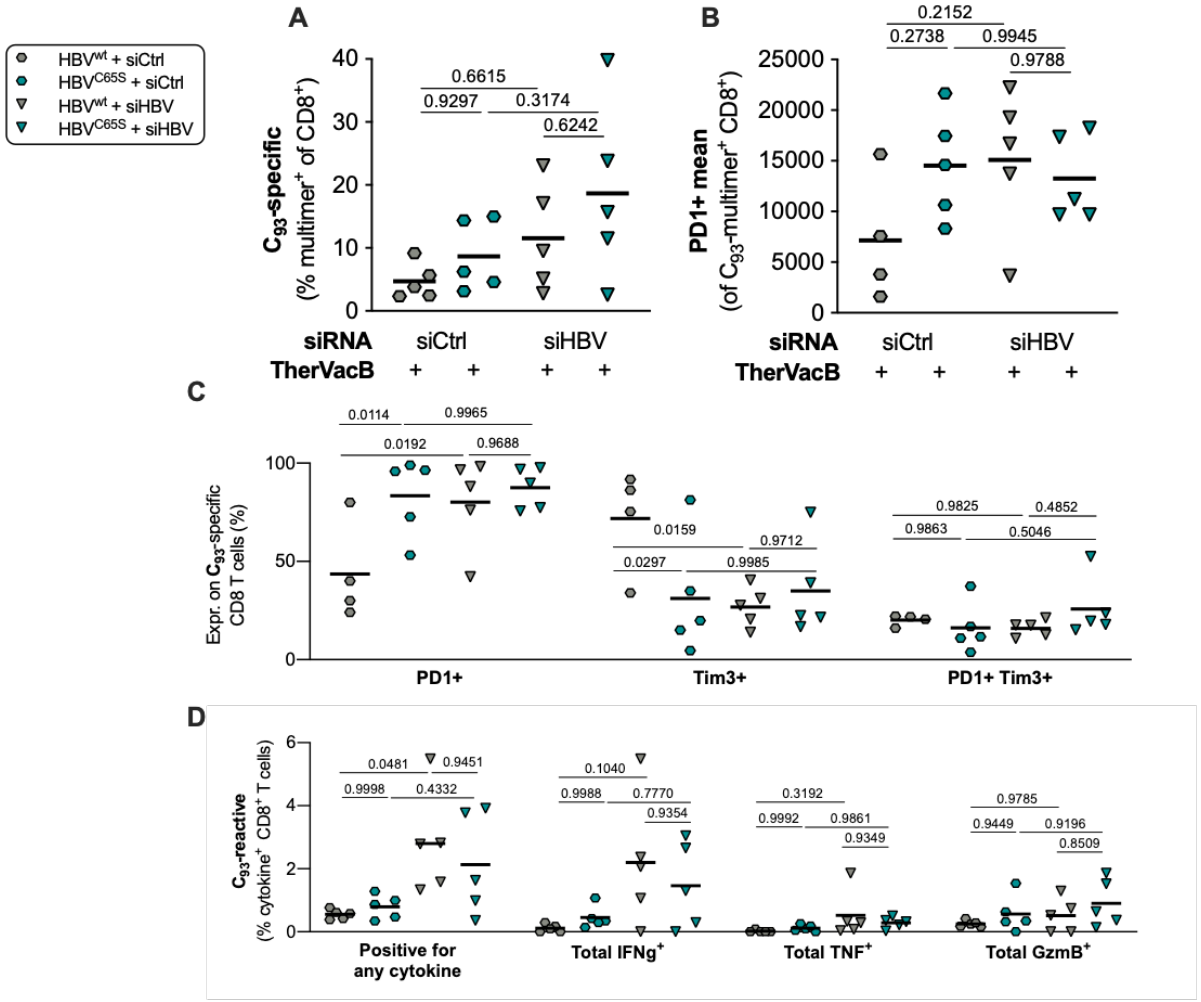

Figure 17 Effect of C65S mutation and siRNA treatment on vaccine-induced C_{93} specific CD8+ T cell response in liver
After sacrificing the mice and isolating liver associated lymphocytes, T cells were stained using (A) C_{93} -specific multimers and antibodies specific for co-inhibitory receptors. (A) The proportion of C_{93}-specific CD8 T cells (B) Mean fluorescence intensity of PD1. (C) Fraction of cells staining positive for PD1, Tim3 and both. After peptide restimulation, (D) TNFα, IFNγ and Granzyme B secretion and their combination was measured. For statistical analysis, the median is displayed, and significance is measured by multiple t-test.

The same staining strategy was performed for S190 specific CD8+ T cells and showed sufficient amounts of S_{190} specific cells for further analysis (Figure 18 A). The S_{190} specific CD8+ T cells performed quite similar to the C_{93} specific T cells in certain aspects. The PD1 expression showed similar distribution patterns. The percentage of PD1 expression of the siHBV groups and the AAV-C65S + siCtrl group was significantly higher than the AAV-WT + siCtrl group (Figure 18 C). The same was seen for the C_{93} specific CD8+ T cells. However, the S_{190} specific T cells showed no significant difference in the mean of PD1 expression (Figure

18 B). Moreover, S_{190} specific CD8+ T cells showed a high percentage of Tim3 expression (Figure 18 C). All groups except the AAV-WT + siCtrl showed a cytokine secretion for any cytokine (Figure 18 D).

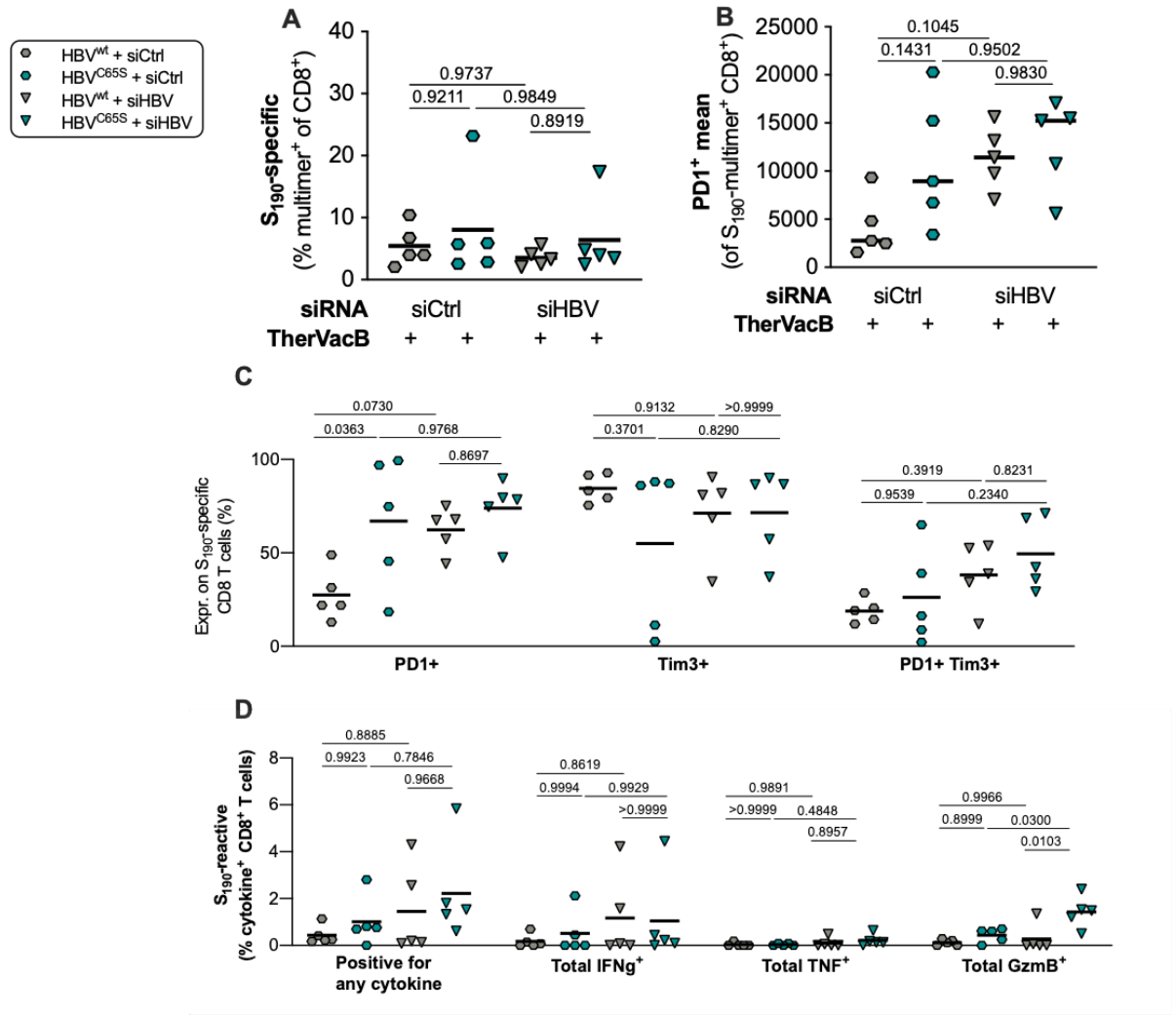

Figure 18 S_{190} specific CD8+ T cell response in liver
After sacrificing the mice and isolating liver associated lymphocytes, the CD8+ T cells were stained for (A) S_{190} specificity and afterwards for co-inhibitory receptor expression like (B) PD1 mean (C) PD1 as well as their combinations. After peptide restimulation, (D) TNFα, IFNγ and Granzyme B secretion and their combination was measured. For statistical analysis, the median is displayed, and significance is measured by multiple t-test.

As a control, T cells were also stained for MVA_{B8R}. There was no significant difference in the number of B8R-specific CD8+ T cells between the different treatments (Figure 19 A), and they were further analysed for co-inhibitory receptor expression. There was an expression of the

receptors seen on the surface of the CD8+ T cells. The percentage of PD1+ T cells was significantly lower in the AAV-WT + siCtrl group compared with the other groups. This correlates with the PD1 positivity of the C_{93} and S_{190} specific CD8+ T cells and relativizes the equally high PD1+ expression of the AAV-C65S + siCtrl group with the siHBV groups (Figure 19 B, C). After peptide restimulation, all the mice showed a sufficient cytokine positivity of their MVA_{B8R} specific CD8+ T cells (Figure 19 D), indicating an efficient immunisation and an HBV specificity of the T cells. Moreover, the immune response measured before was apparently a specific HBV immune response.

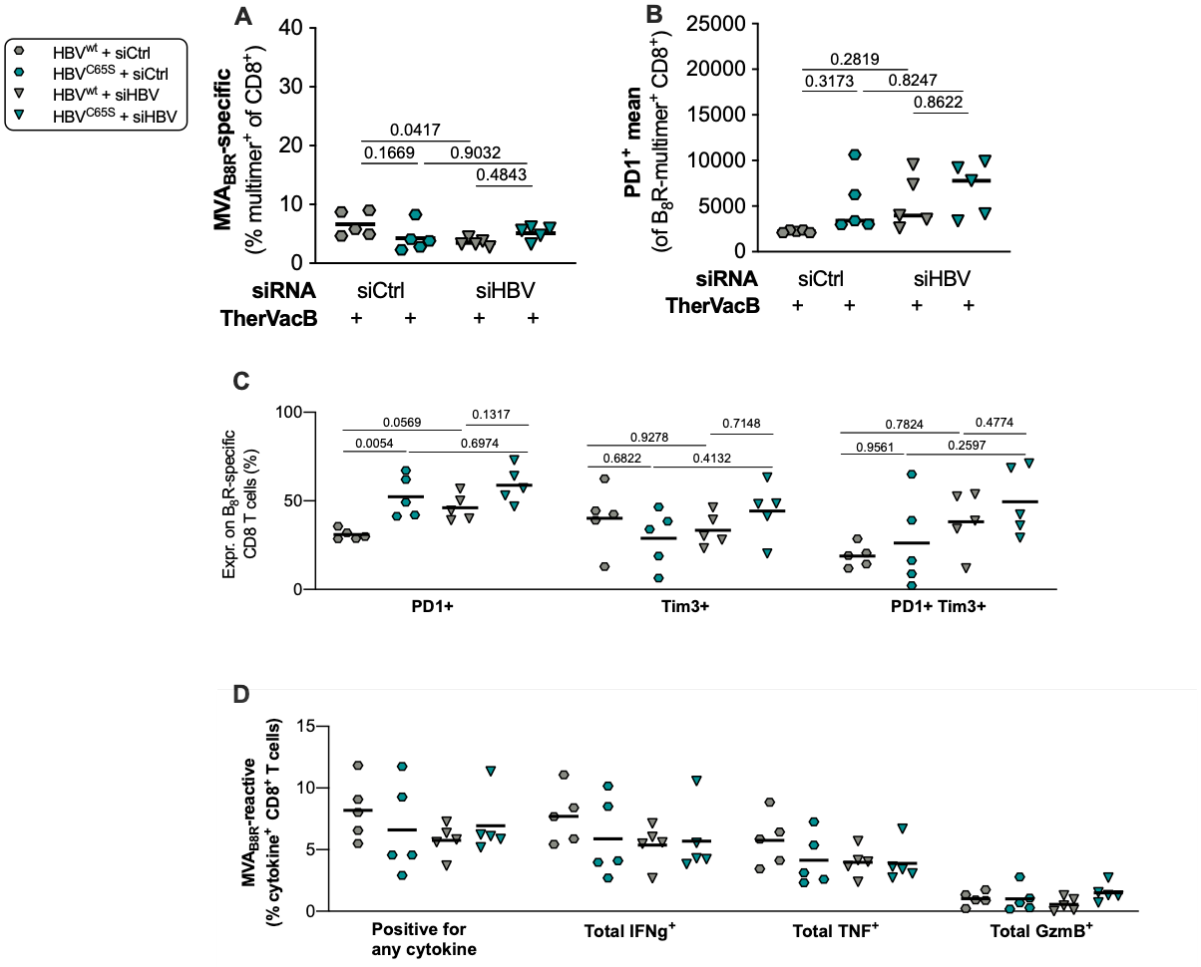

Figure 19 MVA_{B8R} specific CD8+ T cell response in liver
After sacrificing the mice and isolating liver associated lymphocytes, the CD8+ T cells were stained for (A) MVA_{B8R} specificity, and afterwards, for co-inhibitory receptor expression like (B) PD1 mean (C) PD1, Tim3 and CTLA4 as well as their combinations. After peptide restimulation, (D) TNFα IFNγ and Granzyme B secretion and their combinations were measured. For statistical analysis, the median is displayed, and significance is measured by multiple t-test.

In the immunohistochemical staining and PCR measurement of HBV pgRNA and HBV totalRNA, there was a big difference between the groups treated with siCtrl and siHBV siRNAs. The siHBV treated mice showed a significantly lower amount of core positive cells/mm^2 in the immunohistochemical staining of liver slices compared to the mice treated with siCtrl siRNAs (Figure 20 A, B). There was no core positive cell found in one individual of the AAV-WT + siHBV group at all (Figure 20 A). This correlates with the serum HBe antigen level found in this mouse, which dropped to zero before the termination of the experiment. There was also low amounts of HBV pgRNA (Figure 20 C) and total HBV RNA (Figure 20 D) found in this individual. The same was seen for one individual in the AAV-C65S + siHBV and the AAV-C65S + siCtrl group (Figure 20 A, C, D). Apart from these outliers, a clear pattern of antiviral activity was seen. There was no significant difference between the AAV-C65S and the AAV-WT group in the siCtrl treated mice regarding the core positive cells per mm^3, the HBV pgRNA and the HBV totalRNA relative to cyclophilin A expression (Figure 20 A, C, D). The same effect was seen comparing the AAV-C65S and the AAV-WT group of the siHBV group (Figure 20 A, C, D). However, there was a significant difference between the two AAV-WT groups treated with siHBV or siCtrl siRNA. The AAV-WT + siCtrl group showed a significantly higher amount of core positive cells per mm^2 and a significantly higher amount of HBV pgRNA and HBV totalRNA compared to the AAV-WT + siHBV group (Figure 20 A, C, D). The same was seen comparing the AAV-C65S + siCtrl and the AAV-C65S + siHBV groups (Figure 20 A, C, D). Summarised, the AAV-C65S infection had no impact on the antiviral efficacy of HBV specific T cells after TherVacB immunisation. In contrast, the treatment with siHBV siRNA before starting the TherVacB scheme led to a significant difference in antiviral efficacy of the HBV specific T cells.

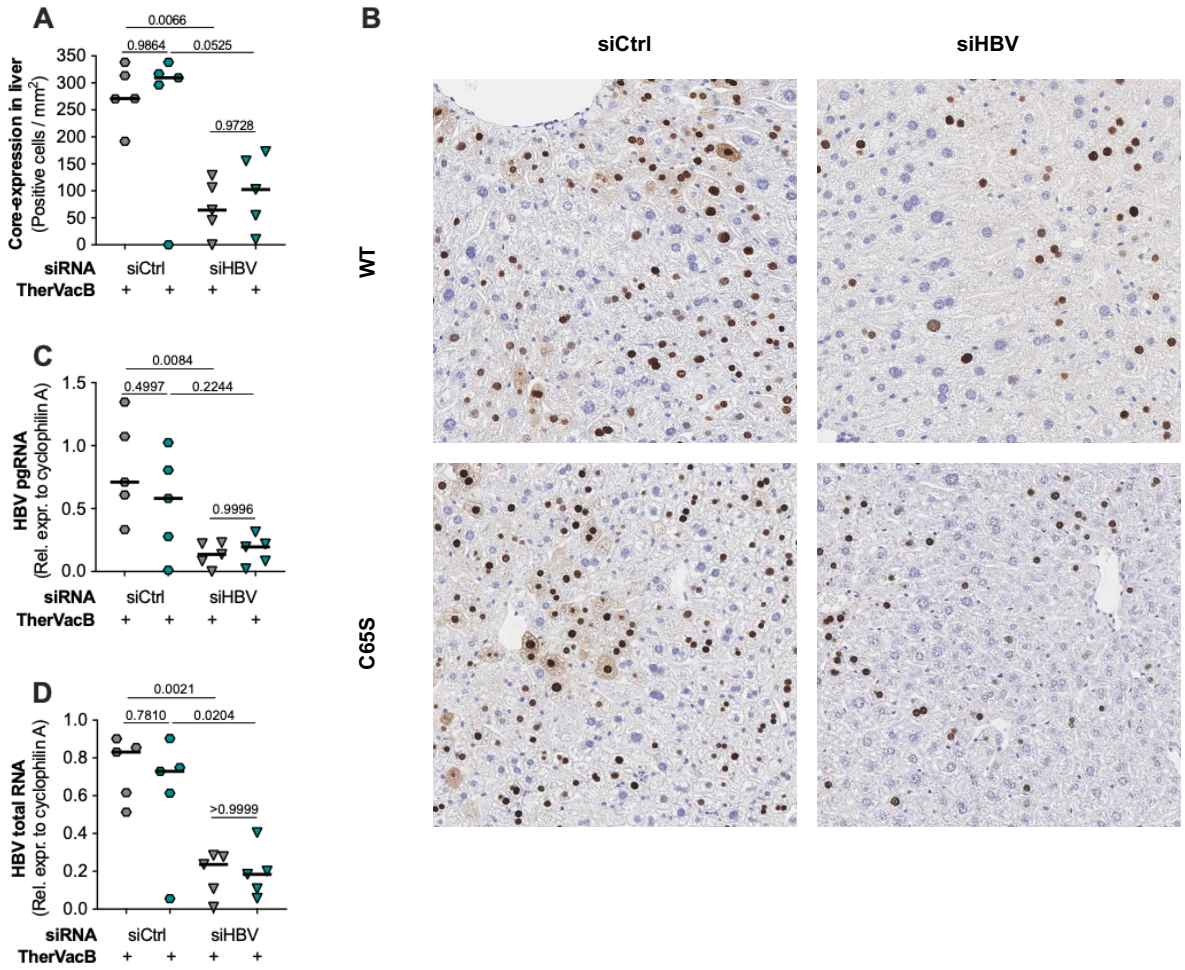

Figure 20 Effect of C65S mutation and siRNA treatment on the antiviral efficacy of therapeutic vaccination
Liver slices from the mice were made and (B) Immunohistochemically stained for core protein. (A) Core positive hepatocytes per mm^3 were counted and compared between the groups. Furthermore, liver slices were lysed, and PCRs were performed, measuring (C) HBV pgRNA and (D) HBV totalRNA relatively to cyclophilin A expression.

Apart from the immunohistochemical staining for HBc proteins, staining for HBs proteins was performed. It is clearly detectable that in the liver of the mice treated with HBV siRNA, the staining intensity of the HBs proteins was lower. Comparing the AAAV-WT + siCtrl and the AAV-C65S + siCtrl group, there is also a difference in the staining intensity. In the AAV-WT + siCtrl group, the intensity indicated the presence of more HBs proteins. Nevertheless, the

staining of the AAV-C65S group clearly shows that HBs proteins remaining intracellularly are detectable in vivo (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

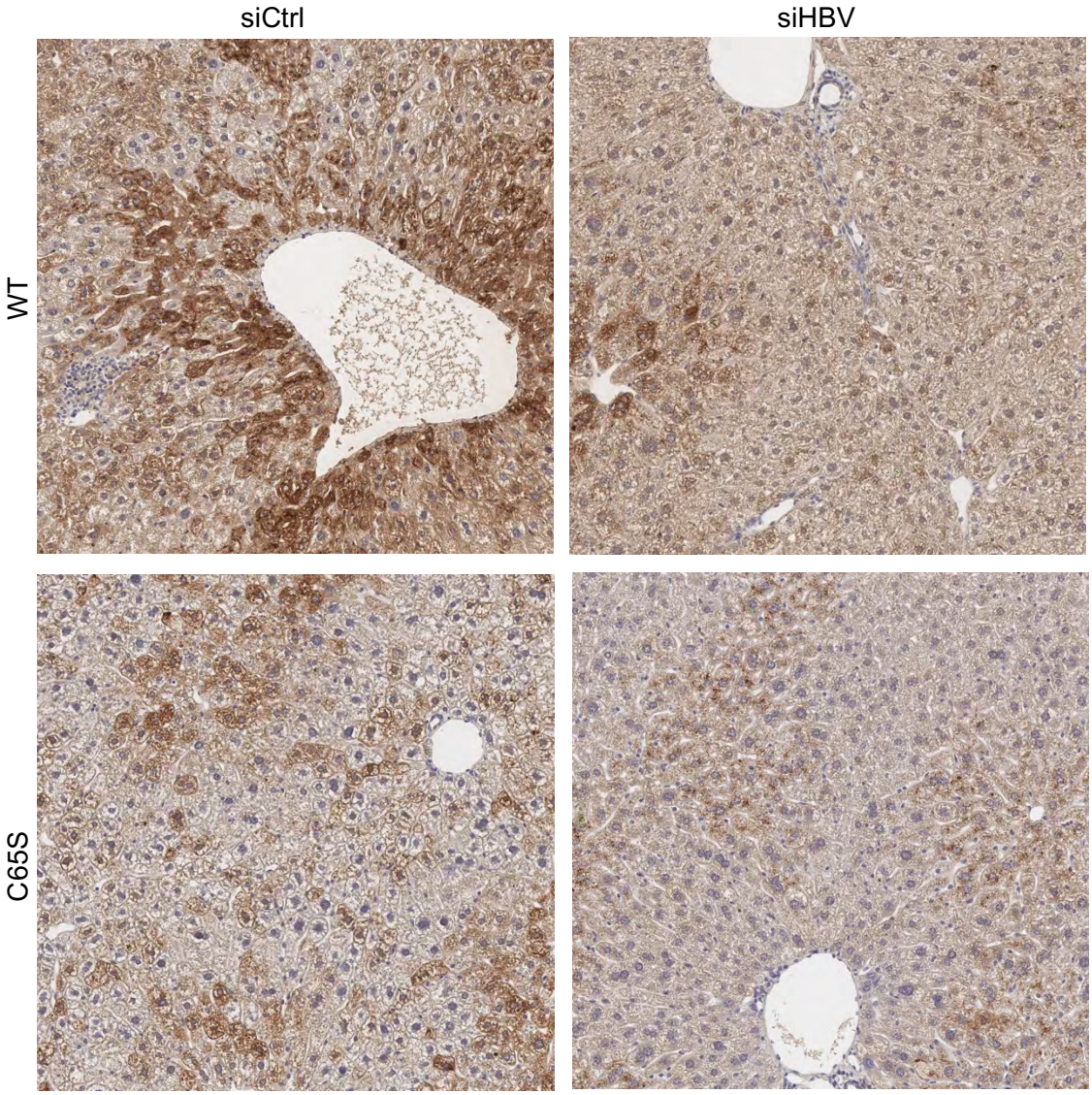

Figure 21 Immunohistochemical staining for HBs proteins in the liver of treated mice
After withdrawing the livers of the mice, slices were produced and immunohistochemically stained for HBs proteins. They are coloured brown. For each treatment group, one representative picture was chosen.

5 Discussion

Currently, a curative treatment for chronic HBV infection is not available, highlining the need for new therapeutic approaches. When developing therapy options, siRNAs are a promising technique to suppress the translation of viral proteins. Furthermore, it restores the responsiveness of mice to therapeutic vaccination (Michler, Kosinska et al. 2020). SiRNA

treatment followed by TherVacB is a concept for an HBV cure and is currently undergoing clinical trials.

It is essential to determine the role of secreted HBs antigens and locally expressed antigens in the liver in mediating HBV immune tolerance for the ongoing efforts regarding this therapeutic approach. HBs antigens are discussed to initiate and maintain HBV immune tolerance and suppress affective immune induction. Whether it is vital for effective immune induction to suppress the translation of locally expressed HBs antigens in the liver or whether it is sufficient to lower the HBsAg concentration in patients' blood was the subject of this work.

Immune tolerance was established by infecting mice and inducing high HBV antigen titers in the mice. Using AAV-WT vectors and AAV-C65S vectors for infection created the possibility to differentiate the immune response after treatment. SiRNA therapy was performed with HBV siRNA and ctrl siRNA. After therapy, immune induction was performed via TherVacB. By doing so, the therapy outcome between four groups was measured. Mice with AAV-C65S + ctrl siRNA translated the HBs antigen, but it was not secreted into the bloodstream, whereas AAV-WT + ctrl siRNA treated mice translated and secreted HBs antigen. In the HBV siRNA treated mice, locally expressed and secreted HBs antigens were suppressed before immune induction.

In summary, we found that not the secretion defective mutant led to a better therapy outcome, but the pretreatment with HBV siRNAs did. For clinical use of the therapy scheme, this work highlights the necessity of pretreatment with HBV siRNAs to suppress the locally expressed antigens in liver tissue. Our results support a recent report by Fumagalli, Di Lucia et al. that clearing extracellular HBsAg with antibodies is not suggested to equally support the induction of a strong T cell response against HBV (Fumagalli, Di Lucia et al. 2020).

To investigate the research question, several starting conditions of the experiment had to be achieved.

When investigating the functionality of the secretion defective mutant, a comparable pattern of antigen secretion was found in the cell culture and the mouse model. In both experimental setups, HBe antigen was secreted. After terminating the mouse experiment, the difference in HBe antigen secretion in the first mouse experiment correlated with a difference in core protein expression in unvaccinated animals. Therefore, it is attributable to a slightly varying number of functional AAV and not to an interference of the C65S mutation with the HBe antigen secretion. Equally high levels of HBe antigen support our assumption in the second mouse experiment.

In contrast, all experimental setups showed the expected difference in HBs antigen secretion. In the C65S mutation, no HBs antigen was detected in the supernatant of the cells and the

bloodstream of the mice. Interestingly, the western blot of lysed Huh7 cells showed intracellular HBs protein translation after HBV-WT transfection and HBV-C65S transfection. Moreover, immunohistochemical staining of HBs proteins was possible in the WT and the C65S mice treated with ctrl siRNA in our second mouse experiment. Since the locally expressed HBs proteins in the C65S mutant are critical to distinguish between the role of secreted HBs antigen and locally expressed antigen in mediating immune tolerance, these results demonstrated that our experimental setup is suitable for this research question.

Moreover, we investigated the effect of the vectors on the well-being of the mice to exclude a confounding effect on the results. During both mouse experiments, all of the individuals gained weight, which is a critical parameter for the condition of the mice. There was no significant difference seen between the WT and the C65S group, indicating an equal impact of the vectors on the organism of the mice. Furthermore, a hepatotoxic effect of the vectors was investigated. None of the mice developed high ALT levels before therapeutic vaccination. The rise in ALT levels after vaccination is most likely due to the priming of HBV specific CD8+ T cells and not due to a hepatotoxic effect of the vectors. The low ALT levels before treatment combined with high HBsAg titers and HBeAg titers define the chronic HBV infection of the mice. The establishment of chronic infection was indispensable for the experiment's performance and was achieved here.

In order to selectively investigate the effect of HBs antigen in the liver and the serum on immune tolerance, we tried to create mouse models which are as comparable as possible. Therefore, we defined the HBe antigen as particularly important. Since HBe antigen is an indirect marker for the viral load in the individuals, we tried to achieve equal levels of HBe antigen in all mice. The first mouse experiment evoked a difference in HBe serum antigen levels. Therefore, a different viral load was assumed, supported by the immunohistochemical staining of core proteins in the untreated group after terminating the experiment. In the Core staining of hepatic slices after terminating the experiment, the non-vaccinated C65S group showed an almost four times higher amount of core positive cells/mm^2. Additionally, the PCRs of HBV total RNA and HBV pgRNA detected a significantly higher viral load in the non-vaccinated C65S group. The results of the treated groups were not investigated here since these mice were under antiviral therapy, and the starting conditions of the experimental setup could not be evaluated.

To establish similar starting conditions in the next mouse experiment, the amount of injected vector was adjusted, and a comparably high titer of HBe antibodies was aimed. As we investigated in the supernatant of the experiment, HBe titers were comparable at the beginning of the experiment. In this experimental setup, though, the immunohistochemical staining of

liver slices after terminating the experiment was not suitable for interpreting the starting conditions since all mice were treated during the experiment. Therefore, we suggested the starting conditions suitable for the experiment and performed it as described.

5.1 HBV specific antibodies are secreted before therapeutic immunisation

The two mouse experiments showed a similar picture of anti-HBs antibody detection. After the first protein prime, respectively, after the first siRNA therapy, the HBsAg levels in the serum of the treated groups decreased until they were below the threshold at the end of the experiment. Simultaneously the anti-HBs antibodies rose after starting the therapy. There are probably two effects responsible for the detection of these antibodies. Firstly, TherVacB induces proliferation of B cells and massive induction of the secretion of anti-HBV antibodies. Secondly, once the HBs antigens are declining, the percentage of anti-HBs antibodies present in immunocomplexes with the corresponding antigen is lowering. The antibodies become detectable.

In both mouse experiments, in the AAV-C65S group, anti-HBs antibodies were detected even before treatment with TherVacB. These effects were seen when no anti-HBs antibodies could be detected in the equally treated wildtype mice. Therefore, these findings are not explainable with an HBV specific priming of B-cells and a massive production of anti-HBs antibodies. It is instead attributable to the missing immunocomplexes in the C65S group. HBs antigen is not secreted into the bloodstream in these mice, and the circulating anti-HBs antibodies become detectable. Therefore, it can be assumed that the HBs antibodies in the AAV-WT group are also secreted before immune stimulation by TherVacB but exist in immunocomplexes with HBsAg and are therefore not detectable. Since these antibodies are detectable before TherVacB therapy, priming of the B cells without external support is suggested. Apparently, the HBs specific B cells start to proliferate and develop further into plasma cells even before an external induction of the immune system. Still, this secretion of HBs antibodies itself appears not to be able to reduce the load of HBs antigens circulating in the bloodstream of chronically HBV infected individuals sufficiently for a restoration of the immune response. These results support the notion that CD8+ T cells are essential for HBV control and not the circulating antibodies, which is further described under 3.5.2 Adaptive immune response on HBV infection. The same effect of antibody secretion before immune induction could be assumed for anti-HBe antibodies and further investigated.

5.2 The difference in ex vivo cytokine secretion of HBs specific T-cells support the findings of Riedl, Reiser et al.

The staining of S_{190} specific CD8+ T cells and S_{209} specific CD8+ T cells support the findings of Riedl, Reiser et al. (Riedl, Reiser et al. 2014). They have already demonstrated the influence of the different presentations of endogenous vs exogenous HBs antigen on the priming of CD8+ T cells. Apparently, endogenous and exogenous HBs antigen is differently presented on MHC molecules and therefore, the priming of CD8+ T cells differs.

In our first mouse experiment, the staining of the CD8+ T cells of the immunised mice showed a contrary positivity of cytokines comparing the S_{190} and S_{208} specific CD8+ T cells after peptide restimulation. The S_{190} specific T cells of the mice replicating HBV-C65S secreted significantly lower amounts of Granzyme B, whereas S_{208} specific CD8+ T cells of the C65S group secreted significantly higher amounts of TNFα and IFNg. In the AAV-C65S group, the HBs proteins are not secreted. Therefore, the endogenous HBs protein is processed, leading to a presentation of mostly epitope S_{190}. In the AAV-WT mice, both epitopes are presented on MHC molecules. After immune induction, the primed T cells for the S_{190} and S_{208} epitope migrate to the liver and detect infected hepatocytes and APCs, where they get activated and start to secrete cytokines. During this process, they also start to express Co-inhibitory receptors like PD1 and Tim3 and are getting exhausted. This is not happening to the T cells specific to S_{208} epitope in mice infected with the AAV-C65S, where much less S_{208} is presented, and the T cells are getting less exhausted. Therefore, the S_{208} specific CD8+ T cells in the AAV-C65S mice appeared more functional in the analysis after terminating the experiment.

5.3 siRNA pretreatment significantly improves the therapy outcome of TherVacB, not the absence of HBs antigens in the bloodstream

To evaluate the influence of HBsAg in the serum on the therapy outcome after TherVacB immunisation, the pretreatment with AAV-C65S and AAV-WT vectors appeared suitable. Two different starting conditions were produced, with mice secreting the HBs antigen into the serum and mice only expressing it in the hepatocytes, as described before. Via the treatment with HBV siRNAs and ctrl siRNAs, three differently infected groups of mice were achieved and treated with TherVacB. All of the locally expressed HBV proteins in the liver were suppressed by using the HBV siRNA. By treating with ctrl siRNA, the mice infected with AAV-WT normally

translated and secreted their proteins and antigens, respectively. The AAV-C65S mice treated with ctrl siRNA translated all of their HBV proteins but only secreted the HBe antigen.

In the past, after HBV siRNA pretreatment, a robust immune response could be induced via TherVacB (Michler, Kosinska et al. 2020). In the state of chronic infection, TherVacB with ctrl siRNA pretreatment should not be able to break the immune tolerance and lead to a solid and polyclonal CD8+ T cell response. However, the pretreatment with AAV-C65S created a condition with no HBs antigen in the bloodstream. Thereby the role of secreted HBs antigen for HBV specific immune tolerance could be explored.

All mice developed a stable chronic HBV infection, which is indispensable for the experimental setup. As described before, it was indicated by high HBs and HBe antigen titers and low ALT levels. In the C65S group, no HBs antigen was detected in the bloodstream. Still, we supposed these mice to be chronically infected. The immune response results supported our assumption after TherVacB immunisation. In the C65S + ctrl siRNA group, the HBe antigen levels remained on a high level after immunisation, typical for a chronic infection. Furthermore, the immunohistochemical staining for HBc and HBs proteins in the liver stained the still present proteins. Combined with a weak CD8+ T cell response after the termination of the experiment, we assumed a stable chronic infection in all of the mice.

At first, we analysed the HBV siRNA pretreated mice. When starting the HBV siRNA treatment, the drop of HBe and HBs antigens in the serum indicated suppression of the HBV protein translation in these mice. Obviously, the suppression of HBs proteins could not be detected in the bloodstream of AAV-C65S pretreated mice. Still, we assumed the proteins were suppressed sufficiently. In the analysis of the immune response of HBV siRNA treated mice, a strong immune response was seen. The first change after immunisation was seen in the ALT concentration in the blood of immunised mice. The substantial rise of ALT in the immunised mice pretreated with HBV siRNAs indicated a cytolytic degradation of hepatocytes by HBV specific CD8+ T cells. It was not seen equally in the mice of the first experiment or the mice pretreated with ctrl siRNA. In conjunction with the drastically decreasing concentration of antigen levels after HBV siRNA treatment and TherVacB immunisation, a strong immune response before terminating the experiment is assumable. After termination, the examination of the CD8+ T cell response supported the assumption. In the mice treated with HBV siRNAs, the mean of PD1 positivity plus the positivity for any cytokine after ex vivo restimulation gave a hint towards activated, functioning HBV specific T cells. The described pattern was equally seen in C_{93} and S_{190} specific CD8+ T cells. The strong induction of the immune response apparently led to a sharp and significant reduction of HBc and HBs proteins in the immunohistochemical staining of liver slices. The same effect was seen in the analysis of HBV

pgRNA and total HBV RNA. Taken together, the immune induction of TherVacB after HBV siRNA treatment was solid and efficient. Therefore, a polyclonal immune response can be assumed, and an eventual cure of the mice might occur.

Still, at the point of the termination of the experiment, the HBV infection was present in the groups treated with HBV siRNA. HBe antigens were still secreted, and HBV proteins and HBV RNA were found in the liver. Interestingly, the infection with AAV-C65S or AAV-WT HBV led to no difference in these parameters. Apart from the HBs antigen concentration in the serum of the mice, no significant difference was found. The HBe antigen levels decreased equally, and HBc protein-positive cells were found equally in both groups. Also, the HBV RNA was detected on a similar level. We concluded that the absence of HBs antigen in the serum before starting the siRNA therapy had no impact on the therapy outcome and did not significantly support the breaking of the immune tolerance.

Secondly, we analysed the ctrl siRNA groups. Here we did not expect a strong and polyclonal immune response with a breaking of the immune tolerance only via TherVacB. Whether the absence of HBs antigen enables a breaking of the immune tolerance was analysed. After TherVacB immunisation, the HBe antigen titers showed a drop, compatible with an HBV specific immune response. The same was seen in the analysis of HBs antigen levels in AAV-HBV infected mice. Also, the ALT levels rose, indicating degradation of hepatocytes. However, when terminating the experiment, antigen levels remained high, not compatible with a breaking of the immune tolerance. Moreover, after the termination of the experiment, a high positivity for HBc proteins and HBs proteins was detected in the immunohistochemical staining. Coincided with a high positivity for HBV pgRNA and total HBV RNA, we assumed a still active infection. The immune tolerance apparently could not be broken only with TherVacB treatment. Interestingly, no significant difference was seen between the AAV-C65S and AAV-HBV treated mice in all of these parameters. Apart from the HBs antigen titers, the parameters developed comparably and kept high viral loads.

Surprisingly, these findings do not correspond with the CD8+ T cell response of these mice. In the FACS staining, the co-inhibitory receptor expression of the ctrl siRNA treated mice showed significant differences in PD1 and Tim3 expression on C_{93} and S_{190} specific CD8+ T cells. The co-inhibitory receptor expression patterns of the AAV-C65S infected mice looked similar to the co-inhibitory receptor expression of the HBV siRNA treated mice. In the functionality of the T cells, the mice treated with ctrl siRNA show similar results, though. A low positivity of cytokines was detected after ex vivo restimulation for all of them. Still, these findings raise the question of whether the AAV-C65S infection had a comparable effect on the CD8+ T cell response than the HBV siRNA therapy.

In summary of all results, especially the antigen titers and the analysis of liver tissue after termination, the conclusion was drawn that locally expressed HBs antigens in the liver tissue play a role in the immune tolerance of chronic HBV infection. The lack of circulating HBs antigen itself is insufficient to establish a strong and polyclonal immune response after TherVacB immunisation and control the infection. HBe antigen levels and the core expression in the liver remain at a high level. HBV pgRNA and HBV total RNA are still transcribed, indicating no sufficient cccDNA degradation after immune induction. In contrast, siRNA pretreatment is the key to a successful therapeutic approach. Preventing the translation of HBV proteins in the liver plays an important role for breaking the immune tolerance via TherVacB. The HBe antigen levels drop significantly, and the core expression in the liver remains at a minimum five weeks after the MVA boost. HBV pgRNA and HBV total RNA decreases, which might be attributed to cytolytic and non-cytolytic cccDNA degradation.

Our findings support the results of Fumagalli, Di Lucia et al., that HBs antigen in the serum has little impact on HBV specific CD8+ T cell response and is not the key to the establishment of HBV specific immune tolerance (Fumagalli, Di Lucia et al. 2020).

5.4 Suppression of serum HBs antigen levels is not sufficient to break HBV-immunotolerance

As already discussed in this thesis, the need for new therapies against chronic HBV infection is urgent. The different therapy approaches include promising strategies to reestablish a strong and polyclonal immune response leading to efficient infection control. As already proved in recent papers, the combination of HBV siRNAs and TherVacB as therapeutic immunisation is a promising approach leading to a strong and polyclonal immune response with viral control in vivo (Michler, Kosinska et al. 2020). We once again showed that the combination of these two therapies approaches efficiently diminishes the viral load in high titer mice and can break the immune tolerance. However, the absence of HBs antigen in the serum of AAV-C65S mice did not support TherVacB equally to a siRNA therapy and the suppression of viral protein translation. Our work shows that serum HBs antigen itself is not responsible for the immune tolerance of chronic HBV infection. The serum HBsAg does not turn out to be a dedicated target for HBV immune therapy. Therefore, therapy approaches via HBs specific antibodies or HBs secretion inhibitors might not allow to break HBV immune tolerance. Instead, the suppression of HBV proteins in the hepatocytes reverts their potential to establish HBV immune tolerance. On the path towards an HBV cure, this work highlights the need for specific

HBV siRNAs and the immense potential of combining them with therapeutic vaccination such as TherVacB.

6 Material and Methods

6.1 Materials

6.1.1 Devices

Product	Supplier
Architect™ platform	Abbott Laboratories
BEP III platform	Siemens Healthcare
Centrifuge 5920R	Eppendorf
CytoFLEX S	Beckman Coulter
Gel chambers (agarose gel electrophoresis)	Peqlab
High capacity centrifuge 4K15	Sigma
Incubator Heracell 150	Heraeus Holding GmbH
Leica Bond MAX system	Leica Biosystems
LightCycler® 480 II	Roche Diagnostics
Light Microscope Axiovert 25	Carl Zeiss
NanoDrop One	Thermo Fisher Scientific
Neubauer improved hemocytometer	Brand GmbH + CO KG
Optima L-90K Ultracentrifuge	Beckman Coulter
Pipette “Accu-jet pro”	BrandTech Scientific Inc.

Pipettes	Eppendorf
Reflotron® Revlovet Plus	Roche Diagnostics
SCN 400 slide scanner	Leica Biosystems
Shaker and incubator for bacteria	INFORS AG; Heraeus Holding GmbH
Sterile hood HERA safe	Thermo Fisher Scientific
T professional Trio Thermocycler	Analytik Jena
Table-top centrifuge 5417R	Eppendorf
Thermo Mixer F1.5	Eppendorf
Tissue Lyser LT	Qiagen
Ultracentrifuge SW Ti Rotor	Beckman Coulter
Ultracentrifuge SW 55 Ti Rotor	Beckman Coulter

6.1.2 Consumables

Product	**Supplier**
96 well white microtiter plates	Greiner Bio
96-well plates for qPCR, FrameStar 480/96	4titude
Cell culture flasks, dishes, plates	TPP
Cell strainer 100µm	Falcon
Centrifugation Tubes (13 x 51 mm)	Beckman Coulter
ELISA 96-well plates Nunc MaxiSorb	Thermo Fisher Scientific

E-Plate 96	ACEA Biosciences
FACS 96-well V-bottom plates	Roth
Falcon tubes 15ml/50ml	Greiner Bio One
Filter tips	Greiner Bio One
Microvette 1,1ml Z-Gel tube	Sarstedt
Needles	Braun
Non-tissue culture-treated plates (6-well, 24-well)	Falcon
PCR tubes	Thermo Fisher Scientific
Pipette tips 10µl – 1ml	Biozym/ Greiner Bio One/ Gilson
Pipettes (disposable) 2, 5, 10, 25, 50ml	Greiner Bio One
Reaction tubes 1.5ml, 2ml	Greiner Bio One, Eppendorf
Reagent reservoirs, sterile	Corning
Reflotron ALT (GPT) stripes	Roche Diagnostics
Surgical Disposable Scalpels	Braun
Syringes	Braun

6.1.3 Chemicals and reagents

Products	**Supplier**
Architect HBsAg Manual Diluent	Abbott

Bovine serum albumin (BSA)	Roth
Brefeldin A (BFA)	Sigma
Cyclic-di-AMP	Invivogen
CountBright Absolute Counting Beads	Thermo Fisher Scientific
Dimethyl sulfoxide (DMSO)	Sigma
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco
Ethanol	Roth
Fetal calf serum (FCS)	Gibco
Trypan blue	Gibco

6.1.4 Buffers and solutions

Buffer	**Ingredients**
40% Percoll buffer	36ml Percoll 4ml 10xPBS 60ml 1xPBS
60% Percoll buffer	54ml Percoll 6ml 10xPBS 40ml 1xPBS
ACK lysis buffer	50mM Tris 150mM NaCl

	5mM $MgCl_2$ x 6 x H2O in H2O
FACS buffer	BSA 0.1% in PBS

6.1.5 Kits

Product	Supplier
Architect anti-HBeAg Reagent Kit	Abbott
Architect anti-HBsAg Reagent Kit	Abbott
Architect HBeAg Reagent Kit	Abbott
Architect HBsAg Reagent Kit	Abbott
Cytofix/Cytoperm™ (+Perm/Wash™ Buffer)	Becton Dickinson
Enzygnost™ anti-HBe monoclonal test	Siemens Healthcare Diagnostics
LightCycler 480 SYBR green master mix	Roche Diagnostics
NucleoSpin Tissue DNA and RNA	Macherey-Nagel
Plasmid PlusMidi Kit	Qiagen
RNeasy® Mini Kit	Qiagen
SuperScript™ III First-Strand Synthesis	ThermoFisher Scientific

6.1.6 Enzymes

Product	Supplier

Collagenase IV	Sigma-Aldrich

6.1.7 Primer

Primer name	Sequence	Application
HBV 1745	GGAGGGATACATAGAGGTTCCTTGA	qPCR, HBV DNA
HBV 1844	GTTGCCCGTTTGTCCTCTAATTC	qPCR, HBV DNA
Cyclophilin A fw	GGCAAATGCTGGACCCAACACA	qPCR, reference
Cyclophilin A rev	TGCTGGTCTTGCCATTCCTGGA	qPCR, reference
HBV3.5kbRNA fw	GAGTGTGGATTCGCACTCC	qPCR, HBV RNA
HBV3.5kbRNA rev	GAGGCGAGGGAGTTCTTCT	qPCR, HBV RNA

6.1.8 Plasmids

Plasmid	Transgene product(s)	Source
345 pAAV-HBV-1.3WT	HBV 1.3 WT	Fuwang Chen
345 pAAV-HBV-C65S	HBV 1.3 C65S	Fuwang Chen

6.1.9 Peptides

Peptide	Specificity	Amino acid sequence	Presented on
B8R	MVA	TSYKFESV	H-2 k^b
C93	HBcAg	MGLKFRQL	H-2 k^b
OVA	Ovalbumin	SIINFEKL	H-2 k^b
S190	HBsAg (adw)	VWLSAIWM	H-2 k^b
S208	HBsAg (adw)	IVSPFIPL	H-2 k^b

Peptide pool	Specificity	
HBV core pool	HBcAg	Genotype D, covering the region between aa 70-157
HBV S pool	HBsAg	Genotype D, covering the region between aa 145-226

6.1.10 Cell lines and bacterial strains

Cell line	Description	Source
HepG2	Human hepatoblastoma derived cell line	AG Protzer
Huh7	Human hepatoma cell line	AG Protzer

6.1.11 Media

Medium	Ingredients	
Collagenase medium	Collagenase 250 IU/mg	0,1g
	RPMI 1640, Gibco	8ml
DMEM full medium	DMEM	500ml
	FCS	50ml
	Pen/Strep, 10 000 U/ml	5.5ml
	L-Glutamine, 200mM	5.5ml
	NEAA 100x	5.5ml
	Sodium pyruvate, 100mM	5.5ml
RPMI full medium	RPMI 1640-medium, Gibco	500ml
	FCS	50ml
	Penicillin/Streptomycin	5ml

6.1.12 Mouse strains

Mouse strain	Description	Source
C57Bl/6J	Wildtype C57Bl/6J	Charles River

6.1.13 Viral vectors

Vector Name	Source
AAV-HBV 1.3 WT	generated by J.Hasreiter

AAV-HBV 1.3 C65S	generated by J.Hasreiter
MVA-HBsAg	Provided by A. Kosinska, J. Su
MVA-HBcAg	Provided by A. Kosinska, J. Su

6.1.14 Antibodies

Antibody	Dilution	Article Number	Supplier
Fixable Viability Dye - eFluor780 (APC-Cy7)	1:2500	65-0865-14	Invitrogen
Granzyme B-PE	1:100	GRB04	Invitrogen
mCD4-APC	1:100	17-0041-83	eBioscience
mCD4-V500	1:200	560782	BD Bioscience
mCD8a-Pb	1:100	558106	BD Bioscience
mCTLA-4-PerCP	1:200	106316	Biolegend
mIFN-γ-FITC	1:300	554411	BD Bioscience
mTNF-α-PeCy7	1:200	557644	BD Bioscience
mPD-1-FITC	1:100	11-9981-85	eBioscience
mTim-3-PeCy7	1:100	ABE-10-4079-25	abeomics
Streptactin-PE	1:50	6-5000-001	IBA Lifesciences

6.1.15 Multimers

The multimers consisted of MHC I peptide complexes and were kindly provided by Prof. Dirk Busch (Technical University of Munich, Germany).

Multimer:

C_{93}: MGLFKFRQL

MVA_{B8R}: TSYKFESV

OVA_{S8L}: SIINFEKL

S_{190}: VWLSAIWM

6.1.16 Software

Software	**Supplier**
Aperio Image Scope	Leica Biosystems
CytExpert	Beckman Coulter
FACS Diva™	Beckton Dickinson
FlowJo 10.4	Tree Star
Graph Pad Prism 8.1	Graph Pad Software Inc
i.control™ software	Tecan
LightCycler 480 Software 1.5.1.62	Roche
macOS Mojave 10.14.5	Apple
Windows 7/8/10, MS Office	Windows

6.2 Methods

6.2.1 Molecular biological methods

6.2.1.1 Determination of DNA or RNA concentration

Plasmid DNA and RNA concentrations were measured on a NanoDrop One after calibration with a buffer solution in the appropriate concentration. A drop of one µl of the solution was placed on the nanodrop sensor for measuring DNA or RNA concentration.

6.2.1.2 quantitative polymerase chain reaction (qPCR)

Quantitative PCR was used to determine the total intracellular HBV RNA, the HBV pgRNA and the housekeeping gene Cyclophilin A. Therefore, RNA was extracted from liver tissue by using an RNeasy® Mini Kit (Qiagen) and reverse transcribed into cDNA using a SuperScript™ III First-Strand Synthesis kit (ThermoFisher Scientific, Darmstadt, Germany) following the manufacturer's instructions. Relative quantification was performed by normalisation with the housekeeping gene cyclophilin A.

Moreover, HBV genome copy numbers per cell were quantified. To calculate the exact HBV-genome copy numbers in the AAV mouse model, AAV-copies were subtracted as HBV-specific PCR also amplifies the HBV integrate.

PCR was performed on a LightCycler® 480 II (Roche Diagnostics, Mannheim, Germany), per reaction 6µl LightCycler 480 SYBR green master mix (Roche Diagnostics), consisting of 5µl SYBR green and 0.5µl of each primer, was added to 4µl sample.

For the measurement of HBV DNA and AAV DNA, the following qPCR program was performed:

	T [°C]	**T [sec]**	**Ramp [°C/sec]**	**cycles**
Denaturation	95	300	4.4	1
Amplification	95 60 72	15 10 25	4.4 2.2 4.4	45
Melting	95 65 95	10 60	4.4 2.2 0.11	1

Cooling	40	1	2.2	1

For the pgRNA and totalRNA, the template was the following:

	T [°C]	T [sec]	Ramp [°C/sec]	cycles
Denaturation	95	300	4.4	1
Amplification	95 60	3 30	4.4 2.2	45
Melting				
Cooling				

6.2.2 Cell culture

All cell culture work was performed under sterile conditions using a sterile hood HERA safe (ThermoFisher scientific) with laminar airflow. The cells were cultivated at 37°C, 5% CO2 and 95% humidity in an incubator Heracell 150 (Heraeus Holding GmbH).

6.2.2.1 Maintainance of cell lines

Cell lines were cultivated in DMEM full medium and split every three to four days in a ratio of 1:7 or 1:10, depending on the confluency. HepG2 and Huh7 cell lines were treated with 2ml trypsin (37°C, 4 min), and the dissolved cells were ingested in 8ml DMEM full medium. Culture flasks and plates for HepG2 cultivation were pretreated with collagen (collagen R, 1:10 in H_2O, 37°C, 30 min) and washed with phosphate-buffered saline (PBS) before seeding the cells. In cultivation flasks, the cells were kept in 15ml DMEM full medium.

6.2.2.2 Counting cells

After the cells were treated with 2ml trypsin (37°C, 4 min), they were extracted from the culture flasks and resuspended several times in DMEM full medium to obtain a single-cell suspension.

Counting was performed manually by using a Neubauer improved hemocytometer (Brand GmbH + CO KG, Wertheim, Germany) under a light microscope. Therefore, 10µl of the cell suspension was mixed with 10µl trypan blue to stain dead cells.

6.2.2.3 Transfection of cells

Before transfection, the cells were either seeded in 96 well plates or 6 well plates, depending on the further analysation procedure. For supernatant withdrawal, the cells were seeded in 96 well plates. For performing a western blot after transfection, the cells were seeded in 6 well plates. The transfection was performed when confluency of 60-80% was reached.

For the transfection of cells in six well plates, 4µg of plasmid DNA was diluted in OptiMEM medium up to a total volume of 250µl. Then 10µl of lipofectamine detergent was added to 240µl of OptiMEM medium to reach a total amount of 250µl. After incubating the mix for 5 minutes at room temperature, the DNA mix was added to the lipofectamine 2000 mix and incubated for another 20 minutes. Then the medium of the cells was removed, and 1ml of transfection medium was added. After incubation for one day, the transfection medium was replaced by 2ml fresh cell culture medium.

6.2.2.4 Cell culture supernatant extraction and analysis

At specific time points after transfection, the supernatant was taken from the cells, and antigen levels were measured. After the infection was performed using AAV-HBV 1.3 vectors, HBeAg was measured on a BEP III platform using Enzygnost™ anti-HBe monoclonal test, and HBsAg was measured on an Architect™ platform using the qualitative HBsAg test. Whereas the supernatant of cell lines infected with AAV-HBV 1.3 plasmids was analysed on an Architect™ platform using the qualitative HBsAg and HBeAg test.

6.2.3 Mouse experiments

Mouse experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). Experiments were approved by the local Animal Care and Use Committee of Upper Bavaria and controlled by veterinarians regularly. The mice were kept in a pathogen-free environment with appropriate biosafety levels

either in “Helmholz Zentrum Neuherberg” or “TU München – Institute of Virology/Microbiology”. The mice were controlled by a responsible experimenter every week and treated accordingly when a burden was seen.

6.2.3.1 Injections

The viral vectors for HBV infection were diluted in 100µl NaCl and injected intravenously in the tail vein of the mice. For protein vaccination, the diluted detergent was injected i.m. into the thigh, containing 10µg HBsAg and 10µg HBeAg using 10µg ci-di-AMP as an adjuvant. Diluted in 60µl NaCl, the injection volume for each leg was 30µl. The MVA boost was also injected i.m. in the thigh of the mice.

6.2.3.2 Bleeding

Mice were bled from the cheek vein (vena facialis), where at different timepoints approximately 100µl blood was taken and collected in a Microvette 1,1ml Z-Gel tube for analysation. Beginning in the fourth week after HBV infection, blood was taken and analysed every two weeks.

After termination, the remaining blood was withdrawn from the vena cava inferior and placed in a Microvette 1,1ml Z-Gel tube for analysis.

6.2.3.3 Serum analysis

The blood collected in a Microvette 1,1ml Z-Gel tube was centrifuged (10.000g, 5 min) and the serum, which was located above the gel matrix in the Microvette, was collected in another tube and used for further analyses. HBsAg, HBeAg and Anti HBsAg titers were quantified on an Architect™ platform using the quantitative HBsAg test (Ref.: 6C36-44; Cutoff: 0.25 IU/ml), the HBeAg Reagent Kit (Ref.: 6C32-27) with HBeAg Quantitative Calibrators (Ref.: 7P24-01; Cutoff: 0.20 PEI U/ml) and the anti-HBs antibody test (Ref.: 7C18-27; Cutoff: 12.5 mIU/ml) (Abbott Laboratories, Wiesbaden, Germany). Anti-HBe antibody titers were measured after 1:7 dilution with PBS on a BEPIII platform (Siemens Healthcare, Eschborn, Germany) using Enzygnost™ anti-HBe monoclonal test (data given in Relative Light Units [RLU]). Serum alanine aminotransferase (ALT) activity was measured in a 1:4 dilution with PBS using the Reflotron® Revlovet Plus GPT/ALT test (Roche Diagnostics, Mannheim, Germany).

6.2.3.4 Terminating experiment and processing of organs

On the day of the experiment's termination, the mice were sacrificed with CO^2 narcosis and opened to access the internal organs. First, the skin of the abdomen was opened, then the peritoneum was removed. The remaining blood was drawn from vena cava inferior with a 23G syringe and collected in a Microvette 1,1ml Z-Gel tube for further analysation. To remove non-associated lymphocytes from the liver, PBS was injected into the vena portae, and the liver was perfused until the lymphocytes were eroded. Once the liver became bright, the perfusion was successful. Before the liver was extracted, the gall bladder was removed. Moreover, the spleen was excised, and both organs were stored in RPMI full medium on ice until further processing.

Next, approximately 5mm cubic sections were removed from the liver with a scalpel and further processed to extract DNA, RNA and histological slices. The liver tissue was frozen at -20°C in T1 buffer (Nucleo Spin Tissue Kit) for DNA analysis. The slice was stored at -20°C in RNA later for RNA isolation. Another section was fixed in 4% paraformaldehyde (PFA) for 24 hours and then transferred to a PBS container, where it was stored until paraffin embedding. The livers were weighed before and after removing the cuts to calculate which portion of the organ was used for LAL isolation.

6.2.3.4.1 Analysis of HBV DNA in liver tissue

Frozen liver samples in T1 buffer were lysed using the Tissue Lyser LT (Qiagen, Hilden, Germany). After the tissue was sufficiently lysed, 20% SDS was added, and then the tissue was digested with Proteinase K and RNAse A. The remaining DNA was obtained with phenol-chloroform. DNA amplification and analysis was performed on a LightCycler® 480 II (Roche Diagnostics, Mannheim, Germany).

6.2.3.4.2 Analysis of HBV RNA

Fresh mouse liver tissue was stabilised in RNA later and stored at -20°C until further processing. According to the manufacturer's instructions, the tissue was thawed and

mechanically disrupted by a Tissue Lyser LT (Qiagen, Hilden, Germany) for RNA isolation. Afterwards, the RNA was obtained with an RNeasy® Mini Kit (Qiagen) following the manufacturer's protocol, and cDNA was reverse-transcribed using a SuperScript™ III First-Strand Synthesis kit (ThermoFisher Scientific, Darmstadt, Germany). By real-time RT-PCR on a LightCycler® 480 II (Roche Diagnostics, Mannheim, Germany), the transcripts were amplified with primers only detecting the HBV 3.5kb transcripts and normalised to cyclophilin A expression.

6.2.3.4.3 Histology and Immunohistochemistry

The liver tissue embedded in paraffin was cut into 2µm thin slices and stained with eosin hematoxylin following the standard protocol. For Immunohistochemistry, the slices were stained with rabbit anti-HBcAg (Diagnostic Biosystems, Pleasanton, CA, USA; #RP 017; 1:50 dilution; retrieval at 100°C for 30 min with EDTA) on a Leica Bond MAX system (Leica Biosystems). For analysation, the tissue slices were scanned with an SCN 400 slide scanner (Leica Biosystems), and core positive cells were quantified with Aperio Image Scope (Leica Biosystems) using five different tissue areas adding up to 2,6mm^2.

6.2.3.4.4 Isolation of murine liver associated lymphocytes

To obtain the LALs, the liver was crushed through a 100µm cell strainer, using the plunger of a 2ml syringe, and taken up in 40ml washing medium. After centrifuging the cells at 600g for five minutes, the sedimented cells were resuspended in 8ml collagenase medium and digested for 30 minutes at 37°C. By mixing the dilution every ten minutes, the tissue was in contact with the collagenase medium, and the intracellular matrix was digested. Next, 37ml of cold wash medium was added to the dilution to stop the reaction, and the cells were sedimented. Afterwards, the cell pellet was resuspended in 3ml 40% percoll buffer, carefully layered on top of 3ml 80% percoll buffer in a 15ml falcon tube and centrifuged with 1700g at room temperature and without breaks. Centrifuging concentrates the LALs between the 40% and 80% percoll phase, from where they are transferred into a new 50ml falcon tube. After another two washing steps with wash medium, the cells are diluted in 1800µl full medium and stored on ice for further processing.

6.2.3.4.5 Isolation of murine splenocytes

To gain the splenocytes, the spleen was crushed with the plunger of a 2ml syringe through a 100µm cell strainer into a falcon tube. The remaining cells on the cell strainer were removed by cold wash medium until all the cells were diluted in 40ml medium. Then the cells were centrifuged at 4 °C for 5 min at 600g, and the supernatant was discarded. To lyse the erythrocytes, the pellet was resuspended in 2 ml ACK buffer, and the suspension was incubated for 2 min at room temperature. Next, the cells are put on ice to stop the reaction and filled up to 45 ml with RPMI full medium. After the cells were pelleted (4 °C, 600g, 5 min), they were resuspended in 20 ml RPMI full medium and once more filtered through a cell strainer (100 µm). After one washing step with wash medium, the cells were taken up in 3ml RPMI full medium and placed on ice until further processing.

6.2.3.4.6 Multimer staining

The splenocytes and LAL were stained with MHC I multimers to detect HBV-specific T Cells. The multimers are conjugated with K^b-restricted HBV-derived peptides $S_{190\text{-}197}$ (S_{190}; VWLSAIWM), $core_{93\text{-}100}$ (C_{93}; MGLKFRQL), MVA-derived peptide $B8R_{20\text{-}27}$ (MVA_{B8R}; TSYKFESV) and as control ovalbumin-derived peptide $S8L_{257\text{-}264}$ (OVA_{S8L}; SIINFEKL). Per sample 0,4µg multimer was labelled with 0,4µg Streptactin-PE (IBA Lifesciences, Göttingen, Germany) and incubated in 30µl FACS buffer for 30 minutes on ice. Since the multimers are light-sensitive, the incubation took place in darkness and the light sensitivity was also taken seriously in all further steps. After incubation, 30µl of the labelled multimers were added to the cells, following another 30 minutes of incubation in darkness. The surface staining was performed using anti CD8 (clone 56.6-7; BD Biosciences, Heidelberg, Germany) and anti CD4 (clone L3T4; BD Biosciences) antibodies. Moreover, PD1 and TIM3 and CTLA4 expression was stained using the corresponding antibodies (anti-PD1: clone J43, Invitrogen, Karlsruhe, Germany; anti-TIM3: clone B8.2C12, BioLegend, San Diego, CA, USA; anti-CTLA4: clone UC10-4B9, BioLegend). Dead cells were detected and excluded from the analyses using fixable viability dye eFluor780 (Invitrogen, Karlsruhe, Germany). Once the antibodies were added, another 30-minute incubation followed, ending with two washing steps in FACS buffer. At last, the cells were resuspended in 200µl FACS buffer and analysed.

6.2.3.4.7 Intracellular cytokine staining

The LALs and splenocytes were first stimulated with the following peptides for intracellular cytokine staining. With a final concentration of 1µg/ml, the peptides MVA_{B8R}, OVA_{S8L}, HBV S_{190}, C_{93}, $S_{208\text{-}215}$ (S_{208}; IVSPFIPL), HBc peptide pools (genotype D, aa 70-157) and HBs peptide pools (genotype D, aa 145-226) containing both the dominant CD8 and the CD4 T cell epitopes were used. After one hour at 37°C, 20µl Brefeldin A (1mg/ml) was added to each sample. Stimulation continued for 14 hours at 37°C until intracellular cytokine staining was performed. Therefore, dead cells were first stained and later excluded from the analysation by using fixable viability dye eFluor780 (Invitrogen, Karlsruhe, Germany). Also, the regular surface staining was performed by using anti CD8 (clone 56.6-7; BD Biosciences, Heidelberg, Germany) and anti CD4 (clone L3T4; BD Biosciences) antibodies in a concentration of 1:100. Then, 20 minutes of incubation in darkness followed, ended by a washing step in FACS buffer. They were fixed and permeabilised by incubating the cells with 100µl Cytofix/Cytoperm™ (+Perm/WashTM Buffer) for 20 minutes. Subsequently, a washing step followed with 150µl Cytofix/Cytoperm™ (450g, 4 °C, 2,5 min) and intracellular staining. The staining was performed according to the surface staining, but antibodies were diluted in Cytofix/Cytoperm™. Anti-IFNγ antibody (clone XMG1.2; eBioscience), anti-TNF antibody (clone: MP6-XT22; BD Biosciences) and anti-Granzyme B antibodies were added to the cells and incubated for 25 minutes in darkness on ice, before washing two times and finally resuspending the cells in 200µl FACS buffer. The analysation on the CytoFLEX S cytometer followed.

6.2.3.4.8 Flow cytometry

The data from LALs and splenocytes was acquired on a CytoFLEX S (Beckman Coulter) flow cytometer. To calculate the absolute number of T Cells in the liver or spleen, 10µl CountBrightTM Absolute Counting Beads (Invitrogen, Karlsruhe, Germany) was added to the cell suspension directly before analysation. The data was analysed with FlowJo software (Tree Star, Ashland, OR, USA). Data from multimer staining and peptide stimulation was analysed after background subtraction determined by detecting OVA_{S8L} peptide in the flow cytometer.

6.2.4 Statistical analyses

The statistical evaluation was carried out with the program GraphPad Prism version 8.0, where different tests were run depending on the results. P-values <0,05 were considered statistically significant

7 Figures

8 References

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