Fakultät für Medizin

RNAi-mediated suppression of PD-L1 to restore Hepatitis B Virus-specific immunity and enhance the efficacy of therapeutic vaccination

Till Bunse

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität
München zur Erlangung eines

Doktors der Medizin (Dr. med. sci.)

genehmigten Dissertation.

Vorsitzender: Prof. Dr. Dirk Busch

Prüfende der Dissertation:
1. Prof. Dr. Ulrike Protzer
2. Prof. Dr. Dr. Stefan Engelhardt

Die Dissertation wurde am 06.10.2020 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 16.03.2022 angenommen.

Table of contents

Abstract

The World Health Organization estimates that approximately 257 million people are chronically infected with the hepatitis B virus (HBV) leading to 300,000 annually deaths due to HBV-related carcinoma and 450,000 deaths from liver cirrhosis or other HBV-related complications. HBV persistence has been shown to correlate with an inefficient virus-specific T cell response. Induction of HBV-specific immune responses by therapeutic vaccination is a promising strategy to cure HBV, but has shown limited efficacy so far. There is evidence that HBV establishes an immunosuppressive milieu in the liver and thus escapes an effective immune response. T cells isolated from the blood of chronic HBV infected patients show an increased expression of checkpoint molecules, such as PD-1, TIM-3 or LAG, preventing an effective T cell activation.

In the first part of this thesis the immunosuppression of HBV infection was investigated. It was shown that supernatant of HBV infected cells can inhibit a T cell response, most likely mediated by a soluble factor. Expression analyses of HBV infected mice showed a positive correlation of HBV antigens and the inhibitory molecule PD-L1. This data suggests a multifactorial etiology of the HBV mediated immunosuppression.

The preparation and validation of a siRNA against PD-L1 is the basis for the second part of the thesis. Different sequences and modifications were screened for effectiveness, stability and transfection efficiency. The most effective siRNA was further characterized and used for subsequent *in vivo* experiments.

The third part of this thesis focused on enhancing the effects of a therapeutic vaccine using the self-designed siRNA, compared to the conventional antibody-based approach of checkpoint inhibition. PD-L1 blockade showed a positive effect on the therapeutic vaccine: mice that received both treatments showed a stronger reduction of antigens in serum, higher antibody titers and had a lower viral load in liver and serum. This effect was more pronounced when siRNA was used for PD-1:PD-L1 inhibition compared to antibody-based therapy. An improved HBV-specific immune response was also shown in immune cells isolated from the liver. The number of HBV-specific CD8+ T cells and their

functionality, measured as intracellular cytokine expression, could be improved by the combinatory therapy.

In summary, the results of this work show siRNAs are a promising alternative to the current gold standard of blocking the PD-1:PD-L1 signaling pathway using antibodies. The successful development and optimization of a PD-L1 specific siRNA and its validation in vivo shows RNAi as a potential therapeutic adjunct to the therapeutic vaccine approach against chronic HBV infection

.

Zusammenfassung

Die Weltgesundheitsorganisation schätzt, dass etwa 257 Millionen Menschen chronisch mit dem Hepatitis-B-Virus (HBV) infiziert sind, was jährlich zu 300.000 Todesfällen durch HBV-assoziierte Karzinome und 450.000 Todesfälle durch Leberzirrhose oder andere HBV-assoziierte Komplikationen führt. Es hat sich gezeigt, dass die HBV-Persistenz mit einer ineffizienten virusspezifischen T-Zell-Reaktion korreliert. Die Induktion HBV-spezifischer Immunantworten durch eine therapeutische Vakzine ist eine vielversprechende Strategie zur Heilung von HBV, hat aber bisher nur eine begrenzte Wirksamkeit gezeigt. Es gibt Hinweise darauf, dass HBV ein immunsuppressives Milieu in der Leber generiert und damit einer effektiven Immunantwort entgeht. T-Zellen, die aus dem Blut von chronisch HBV-infizierten Patienten isoliert wurden, zeigen eine erhöhte Expression von Immuncheckpoints wie PD-1, TIM-3 oder LAG, die eine wirksame T-Zell-Aktivierung verhindern.

Im ersten Teil dieser Arbeit wurde die Immunsuppression, die im Rahmen einer HBV-Infektion auftreten kann, untersucht. Es konnte gezeigt werden, dass der Überstand von HBV-infizierten Zellen eine T-Zell Antwort hemmen kann, höchstwahrscheinlich durch einen löslichen Faktor vermittelt. Expressionsanalysen von HBV-infizierten Mäusen zeigten eine positive Korrelation von HBV-Antigenen und dem inhibitorischen Molekül PD-L1. Diese Daten deuten auf eine multifaktorielle Ätiologie der HBV-vermittelten Immunsuppression hin.

Die Herstellung und Validierung einer siRNA gegen PD-L1 ist die Grundlage für den zweiten Teil der Arbeit. Verschiedene Sequenzen und Modifikationen wurden auf Wirksamkeit, Stabilität und Transfektionseffizienz untersucht. Die effektivste siRNA wurde weiter charakterisiert und für nachfolgende *in vivo* Experimente verwendet.

Eine mögliche Effektsteigerung durch die Doppelbehandlung von therapeutischer Vakzine und der hier beschriebenen siRNA, im Vergleiche zu einer Antikörper

basierten Checkpoint Blockade, stand im Mittelpunkt des dritten Teils der Arbeit. Die PD-L1-Blockade zeigte eine positive Wirkung auf den therapeutischen Impfstoff: Mäuse, die eine Doppelbehandlung erhielten, zeigten eine stärkere Reduktion der Antigene im Serum, höhere Antikörpertiter und hatten eine geringere Viruslast in Leber und Serum. Dieser Effekt war ausgeprägter, wenn siRNA zur PD-1:PD-L1-Blockade eingesetzt wurde, im Vergleich zu einer antikörperbasierten Therapie. Eine verbesserte HBV-spezifische Immunantwort zeigte sich auch bei aus der Leber isolierten Immunzellen. Die Anzahl der HBV-spezifischen $CD8^+$ T-Zellen und ihre Funktionalität, gemessen als intrazelluläre Zytokinexpression, konnten durch die kombinatorische Therapie verbessert werden.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass siRNAs eine vielversprechende Alternative zum derzeitigen Goldstandard der Blockierung des PD-1:PD-L1-Signalweges mittels Antikörper darstellen. Die erfolgreiche Entwicklung und Optimierung einer PD-L1-spezifischen siRNA und ihre Validierung in vivo zeigt, dass RNAi eine potenzielle therapeutische Ergänzung zum therapeutischen Impfstoffansatz gegen chronische HBV-Infektionen darstellt.

Abbreviations

AAV	adeno-associated virus
ALT	alanine amino transferase
CHB	chronic hepatitis B
cccDNA	covalently closed circular DNA
DNA	deoxyribonucleic acid
E:T	effector to target ratio
eGFP	enhanced green fluorescent protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hours
HBc	HBV core protein
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBx	HBV X protein
HCC	hepatocellular carcinoma
IFN-α	interferon-α
IFN-γ	interferon-γ
LAL	liver-associated lymphocyte
mg	milligram
MHC	major histocompatibility complex
min	minutes
ml	milliliter
MOI	multiplicity of infection
NA	nucleoside analogue
ng	nanogram
nM	nanomolar
NTCP	sodium taurocholate cotransporting polypeptide
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PD-1	programmed cell death protein-1
PD-L1	programmed cell death ligand-1
PEG-IFN- α	pegylated IFN-α
Pen/Strep	penicillin/ streptomycin
pgRNA	pregenomic RNA
qPCR	quantitative PCR
rcDNA	relaxed circular DNA
RNA	ribonucleic acid
RNAi	RNA interference
Rpm	rounds per minute
RT	room temperature
sec	seconds
shRNA	short hairpin RNA
siRNA	short interfering RNA
TCR	T-cell receptor
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TNF-α	tumor necrosis factor-α
Treg	regulatory T cell
wt	wildtype
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar

1. Introduction

1.1 Hepatitis B virus (HBV)

The World Health Organization (WHO) estimated that approximately 257 million people live with a chronic Hepatitis B virus (HBV) infection. While the number of deaths caused by other infectious diseases like tuberculosis and human immunodeficiency virus infection are declining, those of viral hepatitis are rising. Chronic hepatitis B (CHB) can cause cirrhosis which is the leading cause of hepatocellular carcinoma (HCC). Despite having a safe and effective vaccine, many infections occur by vertical transmission from mother to child at birth or through contact with other infected young children. Furthermore, most of the people currently affected by CHB were born before a vaccine was widely available, underlining the importance of new therapeutic approaches (Indolfi et al. 2019; WHO 2017).

1.1.1 Classification

Human HBV, as part of a family of viruses known as hepadnaviridae, is a partially double stranded, enveloped DNA virus (Schaefer 2007). It is transmissible among humans and other hominids such as chimpanzees or macaques. For several animals, HBV-like viruses are known, e.g. woodchuck hepatitis virus (WHV) or duck hepatitis B virus (Hu 2016). The human HBV can be divided into nine genotypes, A to I, based on nucleotide divergence between viruses. Geographically, the composition of genotypes differs depending on the region. Overall genotype C is the most frequent genotype and mainly found in Eastern and Southeastern Asia, as well as Australasia and Oceania (Velkov et al. 2018).

1.1.2 Viral lifecycle

HBV is a non-cytolytic virus that exclusively replicates in hepatocytes. This tissue tropism is due to a liver specific receptor, sodium taurocholate cotransporting polypeptide (NTCP) which was identified as an important factor for virus entry (H. Yan et al. 2012). After initial interaction with heparan sulfate proteoglycans HBV subsequently binds to NTCP initiating viral entry. Upon viral internalisation the

nucleocapsid uncoates and is transported to the nucleus where the partially double stranded, relaxed circular DNA (rcDNA) is released. Here, the conversion to covalently closed circular DNA (cccDNA) occurs. As an episomal genome, it stays in the nuclei of infected cells and serves as a template for all viral transcripts (Lucifora and Protzer 2016). The HBV DNA has four open reading frames (ORF) encoding for seven proteins: the HBV envelope proteins, large (L), middle (M) and small (S), known as the HBV surface antigen (HBsAg), HBV core (HBc) the subunit of the nucleocapsid, HBV e antigen (HBeAg) is a proteolytically processed HBc protein with an additional N-Terminal peptide, a viral polymerase with a reverse transcriptase activity and a the HBV x antigen (HBx) (Seeger and Mason 2015). After transcription, newly synthesized RNA can, depending on the ORF that was used, either used for translation and production of viral proteins or in case of pregenomic RNA (pgRNA) it gets encapsidated and reverse transcribed into rcDNA. These infectious virions are secreted through multivesicular bodies from the cell together with subviral particles (SVPs), which mainly consist of HBsAg.

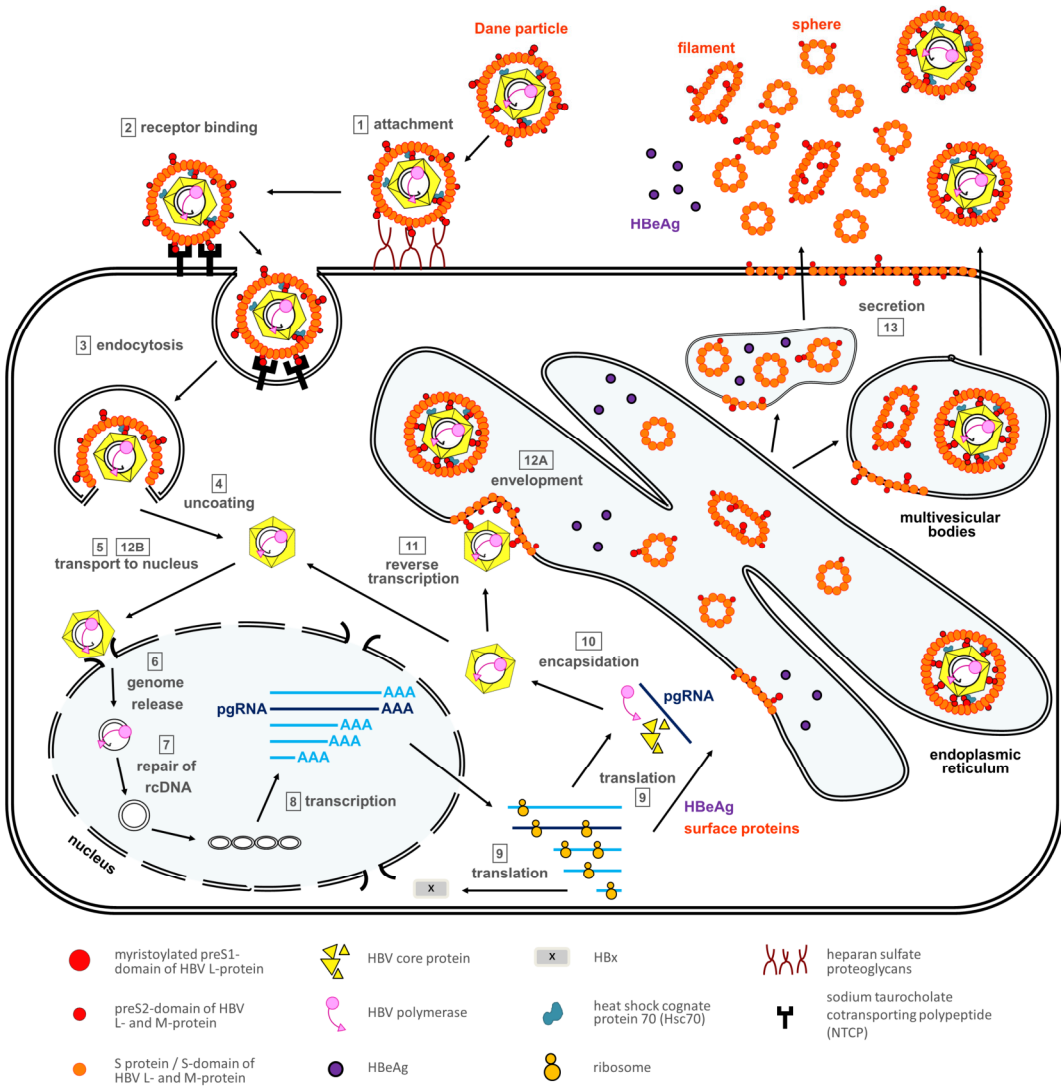


Figure 1: HBV life cycle: (1) the virion attaches via unspecific binding to heparin sulfate proteoglycan (HSPG), (2) NTCP serves as a specific receptor for the HBV PreS1 Domain, (3) clathrin mediated endocytosis, (4) uncoating and (5) transport of the viral capsid, (6) the viral genome is released into the nucleus, (7) conversion of rcDNA to cccDNA, followed by transcription (8), translation (9), encapsidation and reverse transcription of pregenomic RNA, then either envelopment (12A) or nuclear re-import of the mature capsid, (13) secretion of progeny virions. Adapted from Ko, Michler, and Protzer (2017).

1.1.3 Acute vs chronic infection

Once HBV infects the host it can cause an acute or chronic infection, mainly depending on the age of the patient when the infection occurs. Symptoms can range from mild symptoms and may be almost asymptomatic, especially in infants, to fulminant hepatitis that as a consequence lead to liver failure. Severe symptoms are common in acute courses of infection and are associated with viral

clearance. In 90% of the cases infection of neonates and infants result in persistent HBV infection and in less than 5% in adults (McMahon 2009). Persistent viral replication can lead in 8-20% after 5 years to progressive liver disease and cirrhosis, which is one of the main risk factors for HCC (Indolfi et al. 2019). Chronic infection with HBV is a dynamic interaction between the host´s immune system and the virus, reflected by the activity of the disease that can change during infection. Natural history of chronic HBV can be divided into five phases according to the European Association for the study of the Liver. Although these phases don't necessarily occur sequentially, they help in disease management and highlight the fact that persistent infection with HBV does not equal chronic hepatitis.

Phase 1: HBeAg-positive chronic HBV infection, formally known as "immune tolerant", characterized by high HBV DNA and HBeAg in the serum and almost no ALT elevation, indicating minimal or no necroinflammation or fibrosis. This phase is common in perinatal infected patients.

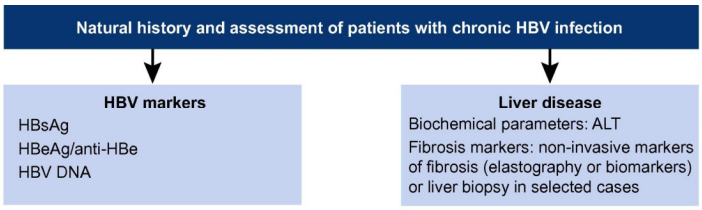
Phase 2: HBeAg-positive chronic hepatitis B, characterized by high HBeAg and viral DNA in the serum, together with elevated ALT. Necroinflammation and accelerated progression of fibrosis can be found in the liver. These patients can become HBeAg negative by seroconversion and eventually suppress HBV DNA and enter a HBeAg-negative phase.

Phase 3: HBeAg-negative chronic HBV infection, formally known as "Inactive carrier", characterized by low to undetectable HBV DNA levels and normal ALT in the serum. Antibodies against HBeAg (anti-HBe) and only minimal necroinflammation or fibrosis can be found. Usually HBsAg is present in the serum at low levels, but spontaneous loss can occur.

Phase 4: HBeAg-negative chronic hepatitis B, characterized by a lack of HBeAg and often detectable anti-HBe, as well as high HBV DNA levels in the serum. ALT is elevated as part of hepatic inflammation and fibrosis.

Phase 5: HBsAg-negative phase, also known as occult HBV infection, is characterized by negative HBsAg and antibodies against HBcAg (anti-HBc) and often also against HBsAg (anti-HBs). Serum HBV DNA levels are usually

undetectable, but cccDNA is frequently measured. HBV reactivation may occur in case of immunosuppression (European Association for the Study of the Liver. Electronic address and European Association for the Study of the 2017).



	HBeAg positive		HBeAg negative	
	Chronic infection	Chronic hepatitis	Chronic infection	Chronic hepatitis
HBsAg	High	High/intermediate	Low	Intermediate
HBeAg	Positive	Positive	Negative	Negative
HBV DNA	>10^7 IU/ml	10^4-10^7 IU/ml	<2,000 IU/ml°°	>2,000 IU/ml
ALT	Normal	Elevated	Normal	Elevated*
Liver disease	None/minimal	Moderate/severe	None	Moderate/severe
Old terminology	Immune tolerant	Immune reactive HBeAg positive	Inactive carrier	HBeAg negative chronic hepatitis

Figure 2: Natural history and assessment of patients with chronic HBV infection based on HBV and liver disease markers (European Association for the Study of the Liver. Electronic address and European Association for the Study of the 2017)

1.1.4 Current therapies

For acute hepatitis B the main goals of therapy are preventing liver failure, improving the quality of life and lowering the risk of chronicity. Due to the fact that in acute HBV infection 95% of adults recover and seroconvert to anti-HBs without antiviral therapy the majority of cases do not need specific treatment. Persistent symptoms, such as jaundice or coagulopathy, which can be signs of acute liver failure, should be treated with highly potent Nucleos(t)ide analogue (NA) (Terrault et al. 2016).

For CHB the main endpoint of treatment is the long-term suppression of HBV DNA levels and thereby disease progression. Additionally, the prevention of mother to child transmission, hepatitis B reactivation and treatment of HBV-associated extrahepatic manifestations are arguments for antiviral therapy. The two main options for treatment of CHB are NA or pegylated Interferonα (PegIFNα) (Terrault et al. 2016; Lok et al. 2016).

NA are inhibitors that interfere with the reverse transcriptase activity of HBV. Treatment leads to the reduction of HBV DNA levels in the blood, can prevent HBV reactivation in immunosuppressed patients and prevents HBV

transmissions of the virus. NAs can be divided into those with a high barrier to resistance, i.e. entecavir, tenofovir disoproxil fumarate and tenofovir alafenamide, and those with low barrier to resistance, lamivudine, adefovir dipivoxil and telbivudine (European Association for the Study of the Liver. Electronic address and European Association for the Study of the 2017). Since only reverse transcription is targeted by this therapy, already infected cells still continue to produce viral proteins. Thus, patients with persistent antigenemia, especially those with liver cirrhosis, remain at risk for HCC (Kumada et al. 2017).

PegIFNα is supposed to modulate the patient's immune response to clear the virus by IFN mediated pathways (Lucifora and Protzer 2016). A big disadvantage of this approach is the wide variability of response, together with an unfavorable safety profile, making many patients unqualified for this kind of treatment.

1.1.5 Therapeutic approaches for CHB

As HBV integrates into the hosts genome a "cure" is mostly functional. As an episomal structure, cccDNA is not replicated during cell mitosis. Even in Patients who recovered from acute HBV infection, cccDNA can still be detected in liver biopsies, which explains possible relapses during immunosuppression (Y. Chen, Sze, and He 2004).

To overcome this issue, several new therapeutic approaches have emerged and are currently in clinical and pre-clinical evaluation. They can be divided into direct antiviral agents, i.e. virus entry inhibitors, nucleocapsid inhibitors or drugs that target cccDNA, on the one hand and immunotherapeutic agents, i.e. Toll-like receptor 7 (TLR7) agonists, checkpoint inhibitors or therapeutic vaccination, on the other hand (Zhang et al. 2019; E.J. Gane 2017; Durantel and Zoulim 2016; Ishigami et al. 2015).

Direct antiviral agents aim to suppress disease progression by specifically targeting the virus. Either by interfering with steps in the viral life cycle or by targeting the viral genome. For instance, the entry step of HBV can be inhibited by Myrcludex-B, a synthetic lipopeptide derived from the PreS1 domain of the HBV envelope protein. Myrcludex-B binds to NTCP and blocks receptor mediated virus entry and therefor infection of new hepatocytes, as well as intrahepatic

spreading of HBV (Volz et al. 2013). Capsid assembly modulators (CAMs) interfere with nucleocapsid assembly, leading to exposed cccDNA which then can be degraded (Nijampatnam and Liotta 2019; Qazi et al. 2018). Another promising approach is the inhibition of viral transcripts using RNA interference (RNAi). Since all ORF of HBV share a common 3'-end, this region can be targeted to suppress all viral transcripts (Michler et al. 2020).

Immunotherapeutic agents stimulate innate pathways to deliver an antiviral effect. The goal is to eradicate the virus using the host's immune response, similar to the PegIFNα treatment. For instance, TLR7 agonists stimulate pathways usually induced by single stranded RNA, directly activating pro inflammatory systems. A different approach is to suppress inhibitory signals involved in the immune response. These inhibitory signals, designated immune checkpoints, regulate whether a T cell`s T cell receptor (TCR) binding leads to an activation. Therefore, drugs that interfere in this process are called checkpoint inhibitors.

Alternatively, to reconstitute the HBV specific immune response, the use of therapeutic vaccination has been studied. The goal is to stimulate T cell responses with HBV specific peptides or DNA. Different vaccination schemes have been published, currently in different stages of clinical development. The application of the prophylactic vaccine scheme in chronically infected patients failed to induce a sufficient immune response to HBV. Inducing preferentially antibodies, these vaccines did not induce a cytotoxic T cell response required for curing HBV. Current approaches aim at inducing antibodies and a T cell response in a first step and then boosting the T cell response using a viral vector. This heterologous prime/boost scheme has proven to be effective in different *in vivo* models for HBV infection (Michler et al. 2020; Backes et al. 2016).

1.1.6 Model systems to study HBV

An important prerequisite for the development of new therapeutic approaches is a model for HBV infection. Due to the discovery of NTCP as a key factor in HBV infection (H. Yan et al. 2012), it was possible to generate cell lines supporting the entire life cycle (Ko et al. 2018). However, when it comes to *in vivo* models, the

picture is different. The most common animals for *in vivo* studies are rodents, which are naturally not susceptible for HBV infection. Woodchucks and Peking ducks have been used as animal models due to their permissivity for woodchuck hepatitis virus (WHV) and duck hepatitis B (DHBV), respectively that resemble a HBV infection. But due to differences in the disease pathogenesis and progression, relating knowledge from these animal models to human HBV is not always possible (Mason 2015).

In 1995 the first HBV transgenic mice were introduced. It harbored a 1.3 overlength copy of the HBV genome leading to a high-level viral gene expression in the liver and kidney tissues of these mice (Guidotti et al. 1995). Although this model helped elucidating major characteristics of HBV infection, it comes with certain limitations: Viral entry and clearance cannot be studied, due to the integration of the viral genome into the host genome. Another constrain is the lack of cccDNA in transgenic mice, restricting the applicability as a model for the natural course of infection.

Different efforts have been made using several vectors to deliver the HBV genomes into mouse livers and induce HBV replication. The simplest method is the hydrodynamic injection of HBV plasmids. A plasmid that encodes for the viral genome is injected intravenously (i.v.) in a high volume. Eventually the DNA will be taken up by the liver, mimicking an acute infection. However, this process damages the hepatocytes and viral replication is only transient, due to the lack of cccDNA establishment (L.R. Huang et al. 2012). Recent cloning techniques allowed the construction of a minicircle, a circular DNA lacking almost all fragments unrelated to HBV. Hydrodynamic injection of these minicircles leads to a “cccDNA dependent” transcription (Protzer 2017), but injection derived liver damage still remains an issue (Z. Yan et al. 2017). As an alternative way, viral vectors have proven to be useful to transfer a HBV genome. Adenoviral or adeno-associated viral (AAV) vectors can, depending on the dose used, establish a chronic infection with HBV. Although, this model does not support cccDNA formation, stable antigenemia and a competent immune system allows studying new therapeutic approaches that aim for viral clearance (Dion et al. 2013).

1.2 Immune checkpoints

Activation of naïve T cells is accomplished by a variety of different stimuli, derived from either interaction with other cells or from soluble sources such as cytokines or chemokines. Taken together, one can differentiate between three kinds of signals. The first signal is generated by the interaction between a TCR and an antigen that is bound to a major histocompatibility complex (MHC). The distinct co receptor of the TCR determines whether MHC class I or class II is recognized. For complete activation and the start of proliferation, the T cell needs the secondary signal, otherwise the T cell will become anergic. The co-stimulatory signal occurs through the binding of CD28 to a B7 molecule, leading to clonal expansion of the T cell and the expression of cytokines. Eventually, cytokines drive the T cell into differentiation, which is known as the third signal. Depending on the molecule that CD28 binds to, the co-stimulatory signal can lead to an inactivation of the T cell. Under physiological conditions, these molecules help to maintain a balance between a necessary immune activation and minimizing collateral tissue damage by overactivation. Drugs that inhibit these inhibitory molecules are called checkpoint inhibitors and are currently used for various kinds of cancer therapies (Haanen and Robert 2015). In some cases, the tumor cells express high levels of these inhibitory molecules, evading an appropriate immune response. This feature is not exclusive to cancer but also known in the context of viral infection (E.J. Gane 2017; Schonrich and Raftery 2019).

1.2.1 CTLA4

The best described receptor involved in immune inhibition is cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a homologous protein to CD28. Upon T cell activation it is expressed on the surface and competes with CD28 on binding to costimulatory molecules such as B7.1 or B7.2. Due to a higher avidity of CTLA-4 to those ligands, CD28 ligation and therefore T cell activation is reduced. Knockout models show the importance of inhibitory signaling during immune responses. Mice lacking CTLA-4, die to uncontrollable T cell expansion emphasizing the vital role for lymphocyte homeostasis (Waterhouse et al. 1995).

1.2.2 PD-1

Another member of the CD 28 superfamily is the programmed cell death 1 (PD-1) receptor. Mainly expressed on T- and B-cells (He et al. 2015), it binds to two ligands, PD-L1 and PD-L2. Those ligands are expressed on all hematopoietic cells, endothelial cells and epithelial cells, and help maintaining peripheral tolerance and protecting the body from auto-reactivity (Okazaki et al. 2013; Bardhan, Anagnostou, and Boussiotis 2016; Sharpe et al. 2007). Ligation of the receptor leads to inhibitory signaling through an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) located in the cytoplasm. Several ways have been described how PD-1 can alter an immune response, from which the recruitment of phosphatases, inhibition of TCR signaling and induction of regulatory T cells seem to be the most important factors (Sheppard et al. 2004; Francisco et al. 2009). Upregulation of PD-1 in acute infection helps to balance strength and quality of the immune response, preventing excessive tissue damage during clearance of the pathogen. Studies suggest that the exploitation of these immune suppressive properties by viruses can favor the establishment of chronic infections (Schonrich and Raftery 2019; Maier et al. 2007; Sharpe et al. 2007). Virus-specific T cells isolated from CHB patients show upregulated PD-1 levels, associated with dysfunctional cytokine expression, indicating a dysfunctional phenotype (Dong et al. 2019; Peng et al. 2008). Experimental blockade of the PD-1:PD-L1 pathway, to overcome the T cell exhaustion, has shown some promising results (Liu et al. 2014; Jiang 2012).

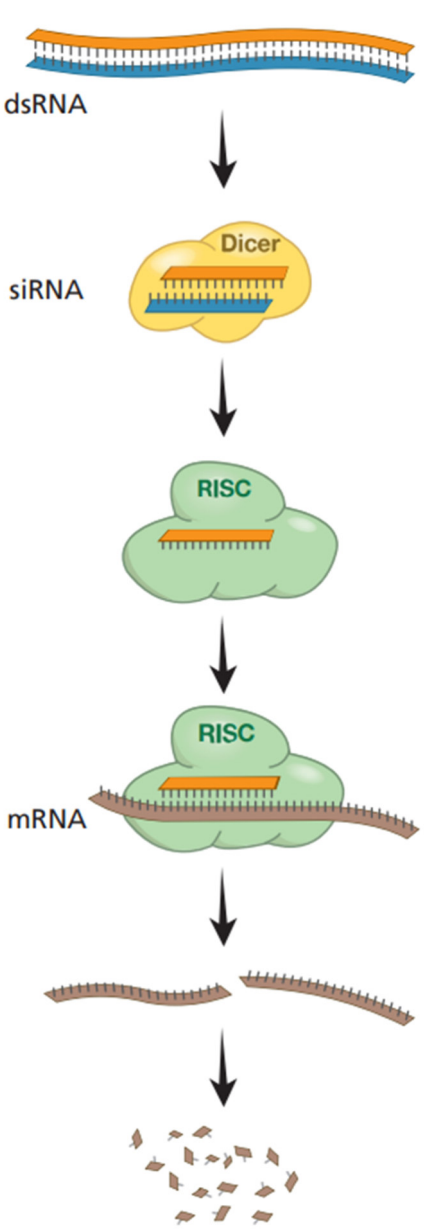
1.2.3 LAG3

Lymphocyte-activation gene 3 (LAG3) is another surface protein of activated T cells. It maintains T cell homeostasis in a similar fashion as PD-1 and CTLA-4. Through binding to MHC class II molecules, proliferation and activation of T cells, especially Tregs, is reduced (Huard et al. 1995; C.-T. Huang et al. 2004). Upregulated LAG3 levels are part of the exhausted phenotype in chronic virus infections, such as chronic HBV infection (Dong et al. 2019).

1.3 RNA interference

RNAi is a complex mechanism used by cells to regulate genes, fight viral infections and keep transposons integrated in the genome in check. First described in 1990 as a gene silencing phenomenon, it is triggered by either endogenous or exogenous molecules (Napoli, Lemieux, and Jorgensen 1990).

1.3.1 Molecular mechanism



The gene silencing by RNAi is mainly driven by the RNA-induced silencing complex (RISC). Double-stranded RNA is cleaved into smaller fragments, 21-23 nucleotides long, by the endonuclease Dicer (Fig. 3). This duplex, designated short interference RNA (siRNA), is formed by a guide strand and a passenger strand. Ago2, a complex associated with the RISC, then cleaves the passenger strand and the guide strand is loaded into the RISC providing a template for specific mRNA recognition (Matranga et al. 2005). After binding through complementary base pairing, the mRNA is cleaved and eventually degraded. RNAi plays a vital role in cell homeostasis, which is emphasized by the fact that the generation of siRNA is implemented in many pathways (Fig.4) (Daneholt 2006).

Figure 3: The basic principles of RNAi. The endonuclease Dicer cuts double-stranded RNA into short fragments of RNA (siRNA). One of the strands is loaded into the RISC complex and a complementary mRNA can bind and eventually gets degraded. (Daneholt 2006)

1.3.2 Small interference RNA

The generation of siRNA serves multiple purposes, as shown in Fig. 4.

First described in plants as an efficient defense against viral infection, siRNA derived from viral dsRNA can inhibit viral protein synthesis (Covey et al. 1997). Although this pathway was also validated in worms and flies (Swevers, Liu, and Smagghe 2018), the significance in humans is not fully understood yet.

Another important source of siRNA is the cleavage of transcribed transposons. While beneficial in general, transposons can also induce mutagenesis and therefore a regulating system is needed (Belancio, Hedges, and Deininger 2008).

Micro RNA (miRNA) are RNA molecules encoded in the genome regulating the posttranscriptional expression of a vast amount of mRNA. Since miRNA are involved in a lot of pathways, their expression profile have been linked to the development and progression of many diseases (Morishita et al. 2020; Vishnoi and Rani 2017).

Besides regulating posttranscriptional mRNA level, dsRNA can induce promoter methylation and condensation of heterochromatin, thus inhibiting mRNA on a transcriptional level (Volpe et al. 2002; Mette et al. 2000).

But not only endogenous generated siRNA can induce gene silencing. When treated with artificially generated siRNA, cells display specific gene silencing similar to dsRNA application. This led to the widespread use of RNAi as a powerful tool in experimental studies (Jiang 2012; Singh, Trivedi, and Jain 2018; Soutschek et al. 2004).

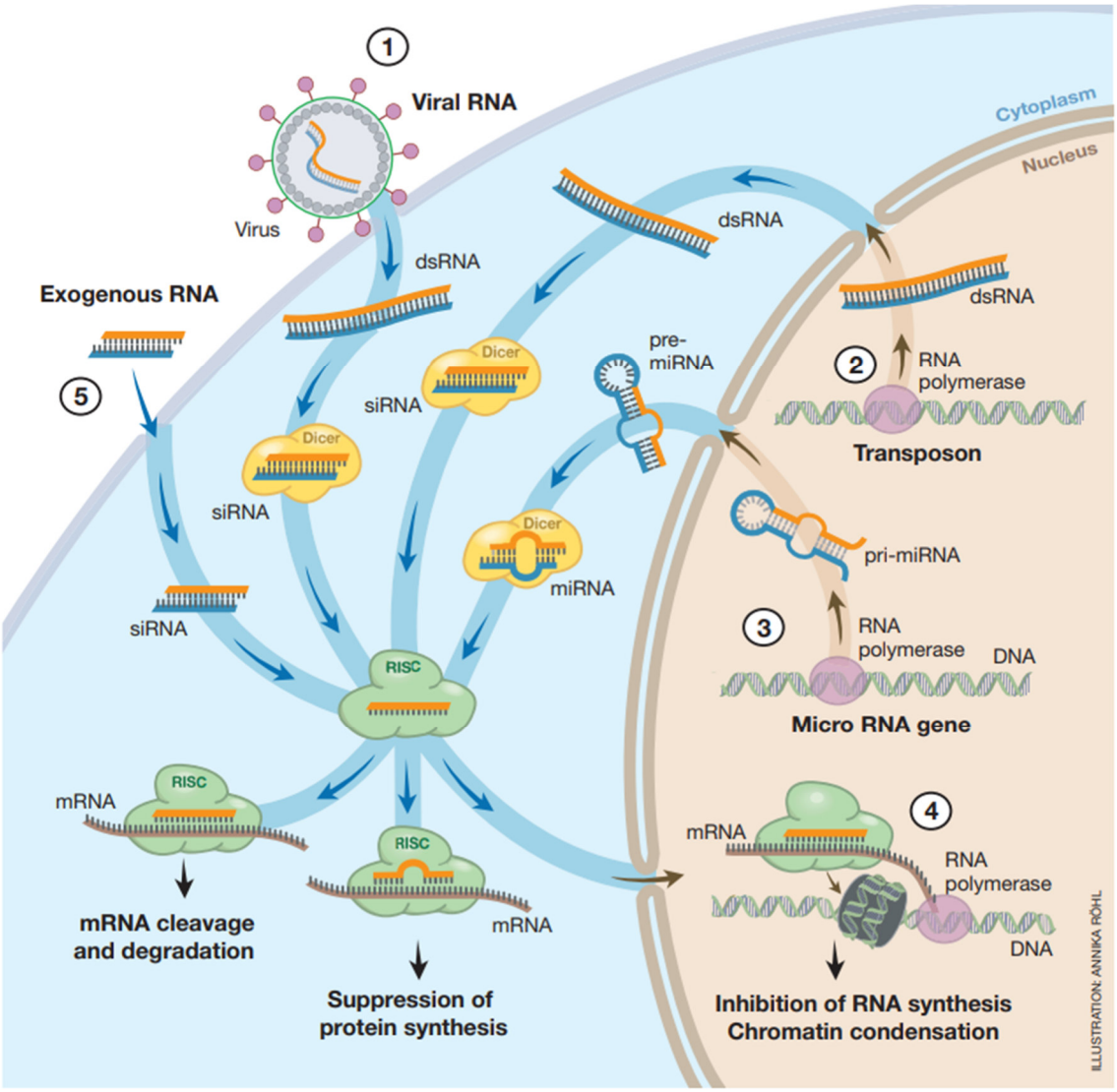


Figure 4: Pathways involved in RNAi. Virus derived double stranded RNA can trigger Dicer and RISC mediated degradation (1), transposons and repetitive DNA are degraded by RNAi (2), protein synthesis can be blocked by small RNA generated within the cell (3) and consequent RISC binding to the corresponding mRNA (4). This process can be utilized experimentally by providing exogenous RNA to induce specific gene silencing (5) (Daneholt 2006).

1.3.3 siRNA *in vivo*

With RNAi as an effective and easy way of knocking down genes, the therapeutic potential became soon obvious. A broad range of diseases can be targeted using RNAi, such as various types of cancer (Singh, Trivedi, and Jain 2018), liver cirrhosis (Schuppan et al. 2018) or infections with HBV (Konishi, Wu, and Wu 2003). But the efficacy and safety of siRNA as a potential drug for *in vivo* use depends on several factors.

Administered systemically the siRNA is transported via the circulatory system. Unmodified siRNA is prone to degradation by endogenous nucleases or the reticuloendothelial system, but siRNA modification can improve nuclease resistance (Chiu and Rana 2003).

Another concern when using siRNA are off-target effects. Sequence homologies to the gene that is targeted can lead to the undesired knock down of other genes. Thus, choosing a sequence that is specific for the gene of interest can reduce side effects. In addition, the delivery of the siRNA only to a specific cell type or organ is of preference. Organ specific delivery can be achieved by using different kinds of transfection reagents, formulation of siRNA or through linkage of the siRNA to a molecule that facilitates the uptake in a specific organ. Unformulated siRNA is hydrophilic and negatively charged, preventing it from crossing cell membranes. A lipid-based delivery system relies on the formation of lipoplexes from positively charged liposomes and the negatively siRNA (Santel et al. 2006). For *in vitro* studies commercially available Lipofectamine is often used, but the applicability is limited in *in vivo* studies, due to dose dependent toxicity and pulmonary inflammation (Dokka et al. 2000). Thus, instead of liposomes, cationic polymers; e.g. polysaccharide, can be used to form nanoparticles. Both methods protect the siRNA from degradation by endonucleases and mediate a rather undirected cellular uptake.

When linked to a molecule that binds to a specific receptor only expressed in a certain cell type, specific delivery can be ensured. For liver directed delivery N-Acetylgalactosamine (GalNAc) can be applied. GalNac is a derivate of galactose and binds specific to the asialoglycoprotein receptor on hepatocytes (Springer and Dowdy 2018). For uptake into non-parenchymal liver cells, the siRNA can be conjugated with derivatives of cholesterol to facilitate cell penetration by natural transport mechanisms (Lorenz et al. 2004).

1.4 Aim of the study

So far, the treatment options for CHB are limited. NAs are highly potent in inhibiting reverse transcriptase activity, have a good safety profile and are widely used in the clinics. They efficiently suppress HBV replication in the liver resulting

in diminished viral DNA in the sera. Nevertheless, the persistent form of the HBV genome, the cccDNA, stays in infected hepatocytes. Thus, these patients remain at risk of reactivation of CHB when therapy is discontinued, especially under immunosuppressive circumstances. To overcome this, efforts have been made to find new therapeutic approaches for CHB. The overall goal of this thesis is to evaluate the combination of these new immunotherapies and their impact on *in vitro* and *in vivo* infection models.

The first question we wanted to address in the thesis, is if we can recapitulate the HBV-induced immune suppression in an *in vitro* model. Patients suffering from CHB have been described to have an attenuated immune response, not only against HBV, but also against antigens not related to HBV. Characterizing the ways HBV impairs immune responses can help restore immune competence and improve curative therapies, such as therapeutic vaccination.

The second part of the thesis is designated to the design of siRNA against PD-L1. As a promising target for checkpoint inhibition, PD-L1 suppression holds the potential to improve HBV-specific immune responses.

The aim of the third part of the thesis is the evaluation of a combinatorial approach of therapeutic vaccination and checkpoint inhibition using PD-L1 siRNA. Although therapeutic vaccination showed promising results in low antigenemia infection models, it failed to induce robust immune responses in high antigenemia models. Thus, we tried combining vaccination and checkpoint inhibition to enhance the HBV specific immune response.

2. Results

2.1 Immune suppression in chronic HBV infection

Determining HBV-related/mediated immune evasion mechanisms which interfere with viral clearance is necessary for the development of new therapeutic approaches to cure chronic HBV infection.

It has been shown that circulating T cells in CHB B patients express significantly higher levels of exhaustion markers compared to healthy controls (Park et al. 2016). Furthermore, HBV-specific T cells showed an impaired effector function when re-stimulated with peptides *ex vivo* (Bertoletti and Ferrari 2016).

2.1.1 T cell inhibition by HBV infection

In order to screen for possible targets to suppress and subsequently enhance the HBV-specific immunity, the validation of an appropriate cell culture model resembling a natural infection *in vivo* was required. Therefore, a very well-established cell line from our lab, HepG2-NTCP K7 (Ko et al. 2018) was used for further experiments.

HBV infected or non-infected HepG2-NTCP cells were co-cultured with cytotoxic T cells. These T cells were transduced to express the TCR targeting Glypican 3, a protein ubiquitously expressed on HepG2 cells. Upon addition of the CD8+ T cells, in an effector to target ratio of 1 to 4, cell detachment was measured through real-time impedance measurement via the Xcelligence system. Non-transduced T cells were used as a control (Mock).

In both, infected and non-infected HepG2-NTCP cells, a cytotoxic effect was visible within the first 24 hours after addition of the T cells (figure 5). Although killing was observed in both groups, infected cells showed a delayed cell detachment compared to non-infected cells. No cytotoxic effect was measured in HepG2 co-cultered with non-transduced T cells.

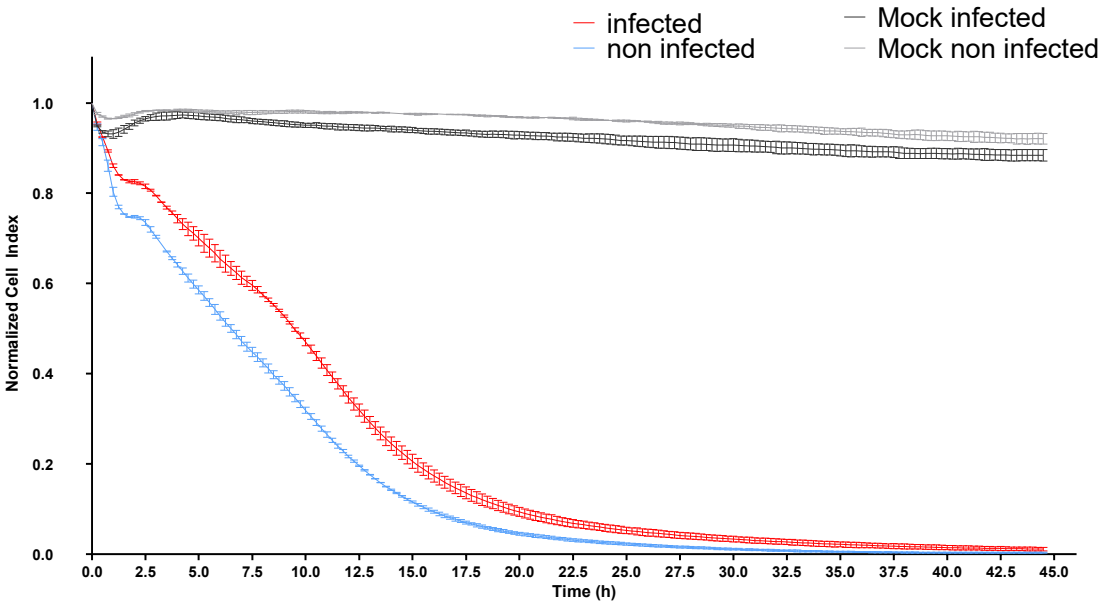


Figure 5: HBV-infection attenuates T cell cytotoxicity. HBV-infected or non-infected HepG2-NTCP cells were cocultured with CD8+ T cells expressing a TCR against Glypican-3. The viability of HepG2-NTCP cells was measured through the impedance differences on the bottom of the wells, which is caused by cell detachment, over a 45-h time frame.

The delayed killing of infected compared to non-infected HepG2-NTCP cells indicate that HBV infection renders the cell less susceptible to T cell mediated cytotoxicity.

2.1.2 Immune inhibition by a secreted factor

In order to differentiate whether this inhibition is mediated by a cell bound- or a soluble-factor, HepG2 cells were incubated with either HBs or HBc to promote MHC-I mediated presentation on the cell surface. These cells were then co-cultured with T cells either expressing a TCR specific for HBs or HBc antigen (Wisskirchen et al. 2017). As a negative control, HBc-loaded cells were co-cultured with T cells specific for HBs and vice versa. Supernatant derived from either HBV-infected or non-infected HepG2-NTCP was added to the co-culture and secreted IFNγ was measured from supernatant at 24h by ELISA.

T cells produced higher IFNγ levels when co-cultured with HepG2 cells loaded with a peptide matching their TCR specificity compared to control (figure 6).

Interestingly, when co-cultured in the presence of supernatant derived from HBV infected cells, IFNγ production was lower than in the presence of supernatant derived from non-infected cells.

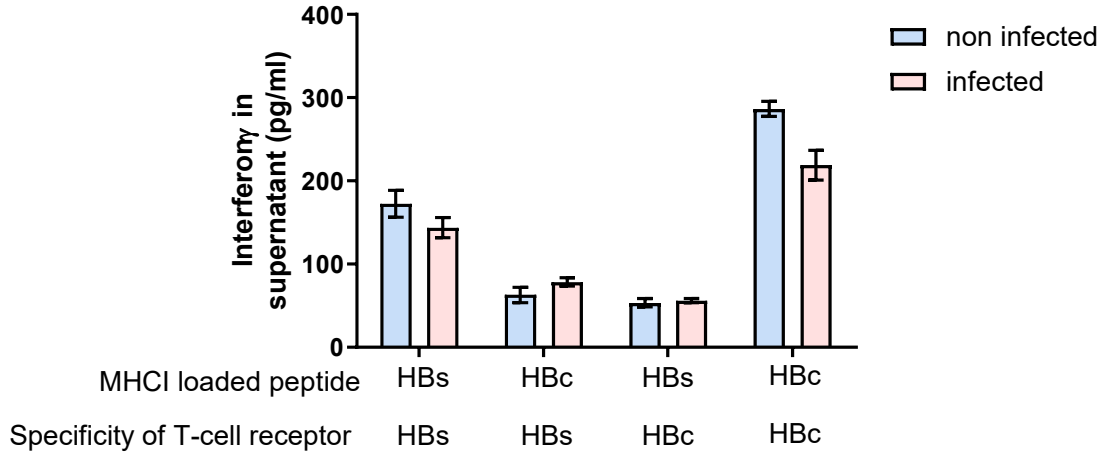


Figure 6: Reduced IFNγ expression in the presence of supernatant from HBV-infected cells. HepG2 cells were pre-loaded with *HBc and HBs derived peptides and kept in the supernatant of HBV-infected or non-infected HepG2-NTCP cells. After co-culture with T cells* either expressing a HBc- or HBs-specific TCR IFN-γ was quantified by ELISA.

In summary, T cells cultured in the presence of supernatant from HBV infected cells produce less INFγ indicating that infected cells produce a soluble factor that inhibits T cell responses.

2.1.3 PD-L1 in context of HBV infection

As one major factor in T cell suppression, PD-L1 is also known to play a crucial role in chronic HBV infection. It has been shown that the degree of inflammation in CHB correlates with the PD-L1 expression in the liver (J. Chen et al. 2011). To further investigate this, HepG2-NTCP were infected with HBV and the expression of PD-L1 mRNA was quantified via RT-qPCR. As a positive control, cells were cultured in the presence of IFNα or IFNβ. Cells were harvested 12h, 24h, 36h, 48h, 72h, 96h, 7 days post infection (d.p.i.), 10d.p.i., and IFN treated cells collectively after 96h.

An increasing PD-L1 expression was observed during the course of infection (figure 7). However, this upregulation was only minor compared to expression

levels in cells that were treated with either IFNα or IFNβ. In IFN treated cells HBV infection did not alter PD-L1 expression levels.

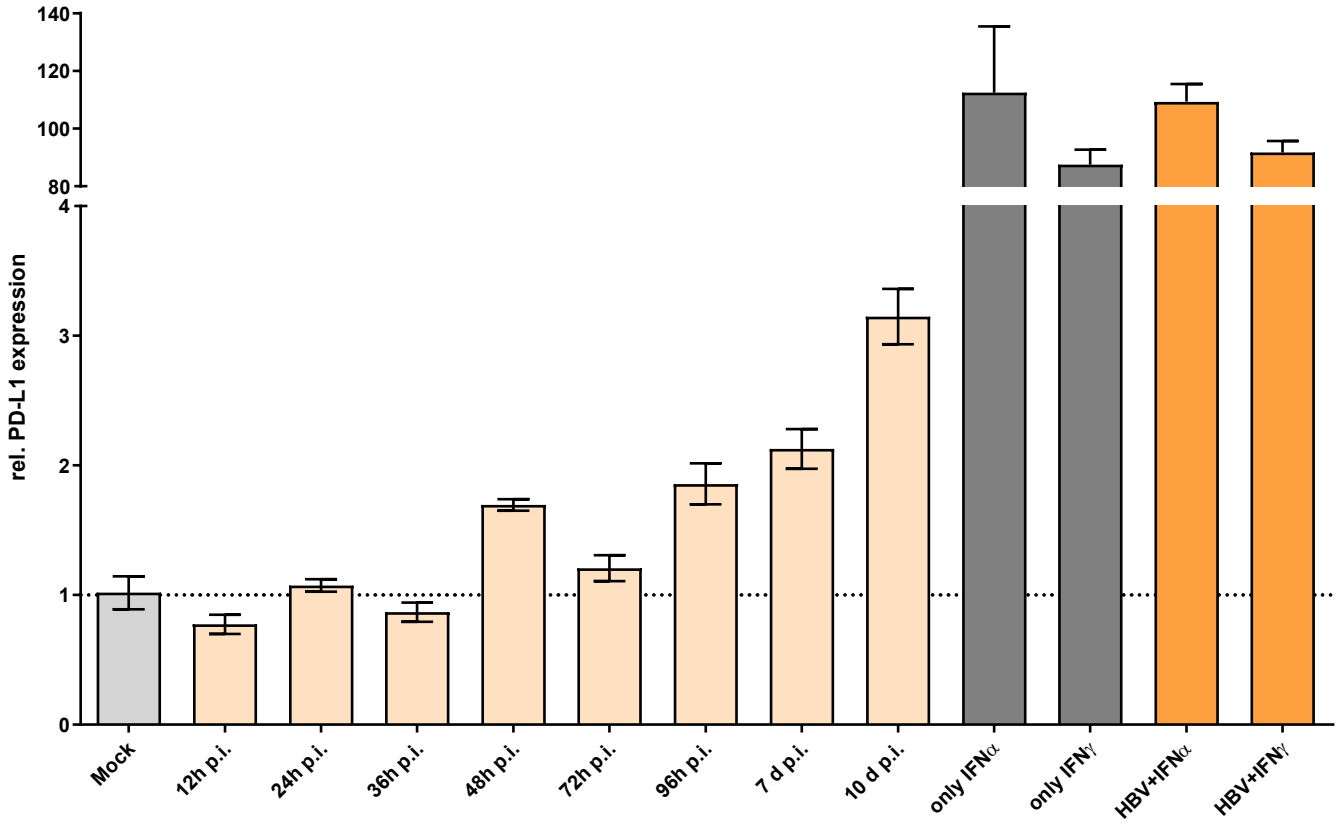


Figure 7: PD-L1 expression in HBV infection. HepG2-NTCP cells were seeded and pre-differentiated with 2.5% DMSO prior to infecting with HBV. 1000 U/ml IFNα or IFNβ was added 24h after infection. Cells were harvested 96 hours after seeding or at the indicated time points, e.g. 24 hours post infection (h.p.i.) or days post infection (d.p.i.). PD-L1 expression was quantified relative to TATA-box-binding protein mRNA by RT-qPCR.

Taken together, this data suggests that the in vitro infection model HepG2-NTCP is able to express PD-L1 and its expression is increased by inflammatory signals.

2.1.4 PD-L1 expression in HBV transgenic mice

To validate these results *in vivo*, we used the HBVxfs mouse line which is transgenic for an 1.3-overlength HBV genome containing a frame shift mutation in the X-protein (Dumortier et al. 2005). These mice were treated once with siRNA targeting the 3' end shared by all HBV transcripts (HBV-siRNA) or siRNA targeting transthyretin (TTR-siRNA) as a negative control. Levels of HBsAg and

HBeAg were measured in the serum and PD-L1 expression via RT-qPCR from whole liver lysate.

Mice treated with siRNA against HBV transcripts, showed a decrease in antigenemia up to three weeks after transfection (figure 8A+B). PD-L1 expression levels were lower in mice receiving HBV siRNA compared to TTR siRNA (figure 8C). Analysis of HBsAg levels in the serum and PD-L1 expression showed a significant linear correlation (R=0.9998; p-value: 0.0125) (figure 8D).

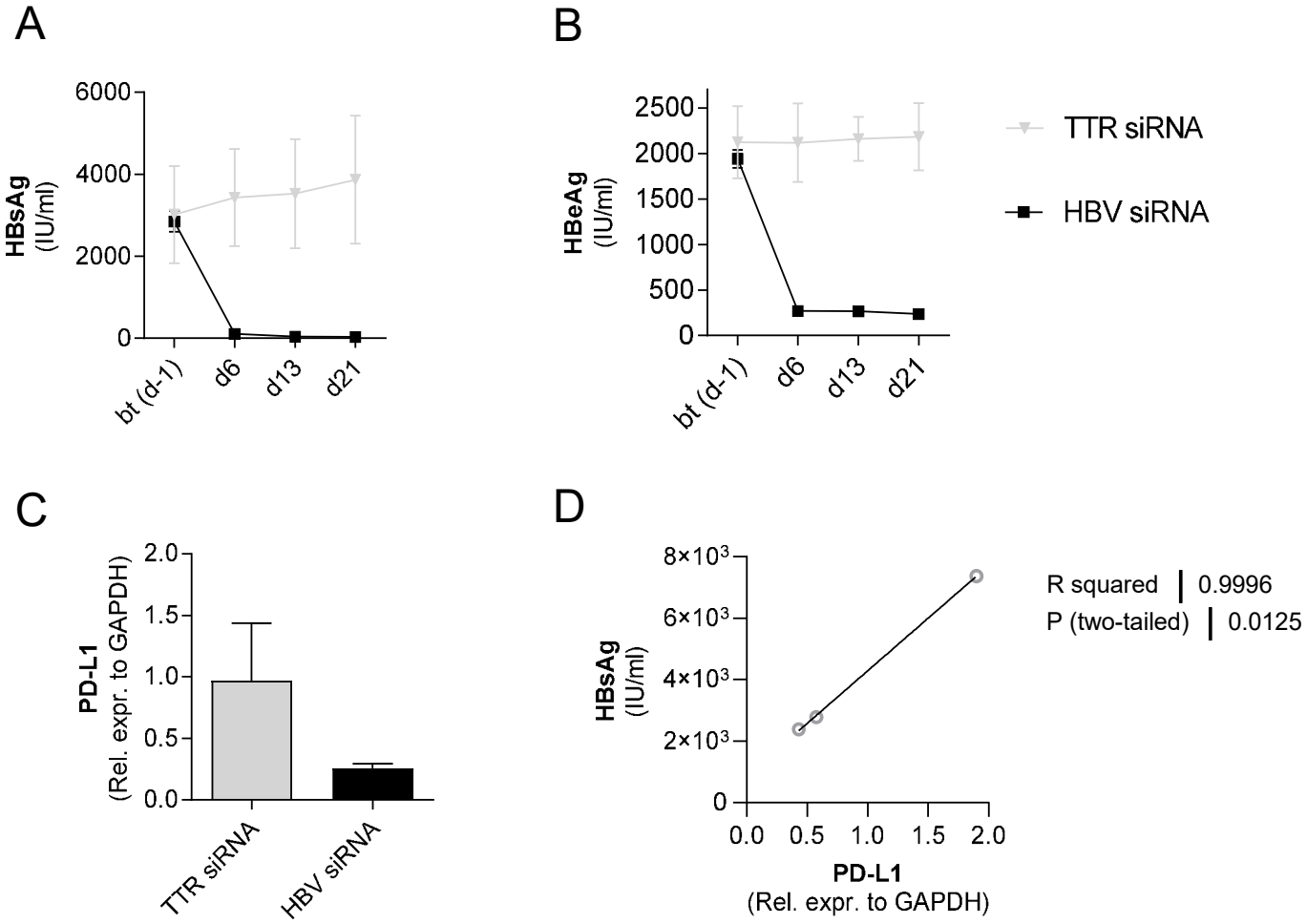


Figure 8: Correlation of HBV antigenemia and PD-L1 expression. HBVxfs transgenic mice treated with either HBV-specific siRNA or control siRNA. (A) HBsAg or (B) HBeAg was measured from serum at indicated time points. (C) qPCR from liver-lysate for PD-L1 expression relative to glyceraldehyde 3-phophate dehydrogenase (GAPDH). (D) Linear regression of HBsAg in the serum and PD-L1 expression in the liver.

Collectively, this data suggests that the high level of HBV antigen expression promotes a higher PD-L1 expression in the liver.

2.2 PD-L1 siRNA design

PD-1/PD-L1 interaction is a way of reducing T cell activation by healthy tissue to minimize cytotoxic damage. It is a delicate balance between clearing the cause of infection and preserve normal tissue (Meng and Lu 2017). Therefore, limiting the blockade of PD-L1 to the relevant tissue will be beneficial to reduce adverse events. Compared to antibody mediated checkpoint inhibition, which is the current gold standard of therapy, RNAi may enhance the efficacy of the approach by a more precise suppression of PD-L1:PD-1 interaction.

2.2.1 Proof of concept siRNA against PD-L1

As described in the introduction of this thesis, siRNA is known to have a short-term and transient effect on gene expression. In a proof of concept experiment, self-designed siRNA (Birmingham et al. 2007) against two different regions of PD-L1 mRNA, named siPD-L1_1 and siPD-L1_2, were used and their inhibitory effects against control siRNA and commercially available PD-L1 siRNA (ThermoFisher, Ambion stealth RNAi, named Ambion_1 and Ambion_2) were compared. HepG2 cell were transfected with different siRNAs and PD-L1 expression was stimulated by addition of INFγ. Subsequently, cells were lysed and a western blot for PD-L1 and β-actin was performed.

Analysis showed that one of the self-designed (siPD-L1_1) and both commercially available siRNAs suppressed PD-L1 expression (figure 9). Self-designed siPD_L1_2 and no siRNA resulted in similar expression levels, whereas, control siRNA led to a slightly less expression of PD-L1.

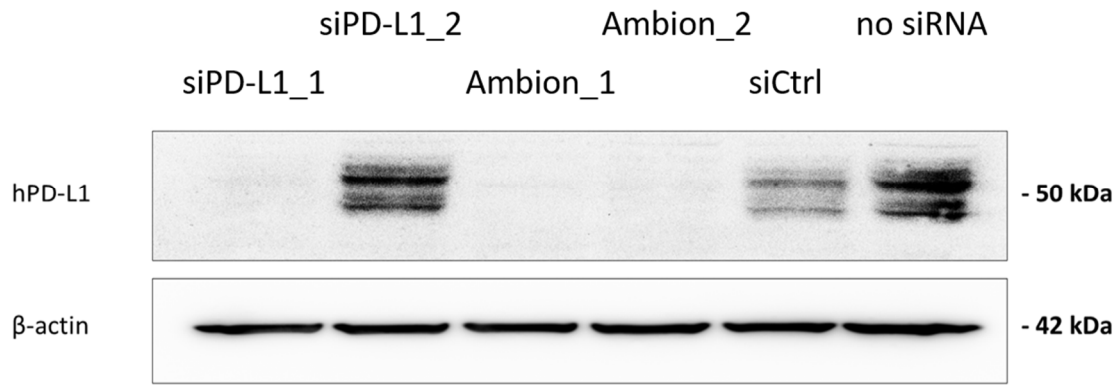


Figure 9: PD-L1 knockdown by siRNA in vitro. HepG2-NTPC cells were transfected with siRNA against PD-L1(siPD-L1_1+2, Ambion_1+2) or with siRNA targeting TTR (siCtrl) as a control. Subsequently, the cells were incubated with medium containing IFNγ to upregulate PD-L1.48 hours post transfection, protein expression was evaluated by western blot. β-actin was stained as loading control.

In summary, siRNA against PD-L1 suppresses PD-L1 protein expression in HepG2 cells. This suppression is sufficient to counteract the upregulation caused by IFNγ treatment.

2.2.2 Design and characterization of siPD-L1

To further enhance the effect of the self-designed siRNA, Shubhankar Ambike (PhD-candidate, Institute of Virology, TUM) designed siRNAs against PD-L1 targeting different sequences. To test the efficacy, the PD-L1 sequence was cloned into a plasmid reporter construct designated PsiCHECK2, using cDNA isolated from mice liver lysate. The plasmid contains two different luciferases, firefly luciferase and renilla luciferase, that each use different substrates. The gene of interest was cloned into a multiple cloning site downstream of the renilla luciferase stop codon leading to a fused mRNA during transcription. Targeting this construct using RNAi leads to degradation of both mRNA and therefore leads to a decreased renilla luciferase expression. To normalize for transfection efficiency, expression of the second firefly luciferase was measured.

Ten different siRNA were tested by co-transfecting with the PD-L1-PsiCHECK2 plasmid into HEK 293 cells. Luciferase activities were measured successively 48 hours after transfection.

The relative luciferase signal was normalized to values from siCtrl treated cells. A reduced signal was observed in almost all PD-L1 siRNAs but most prominently in siPD-L1_3, siPD-L1_4 and siPD-L1_7 (figure 10A). The signal was comparable to Ambion siRNA with almost 90% reduction. A subsequent cell viability assay performed with these siRNAs showed no cell toxicity (figure 10B). Since siPD-L1_4 showed the most promising effect on PD-L1 downregulation further improvements were made and used for subsequent experiments.

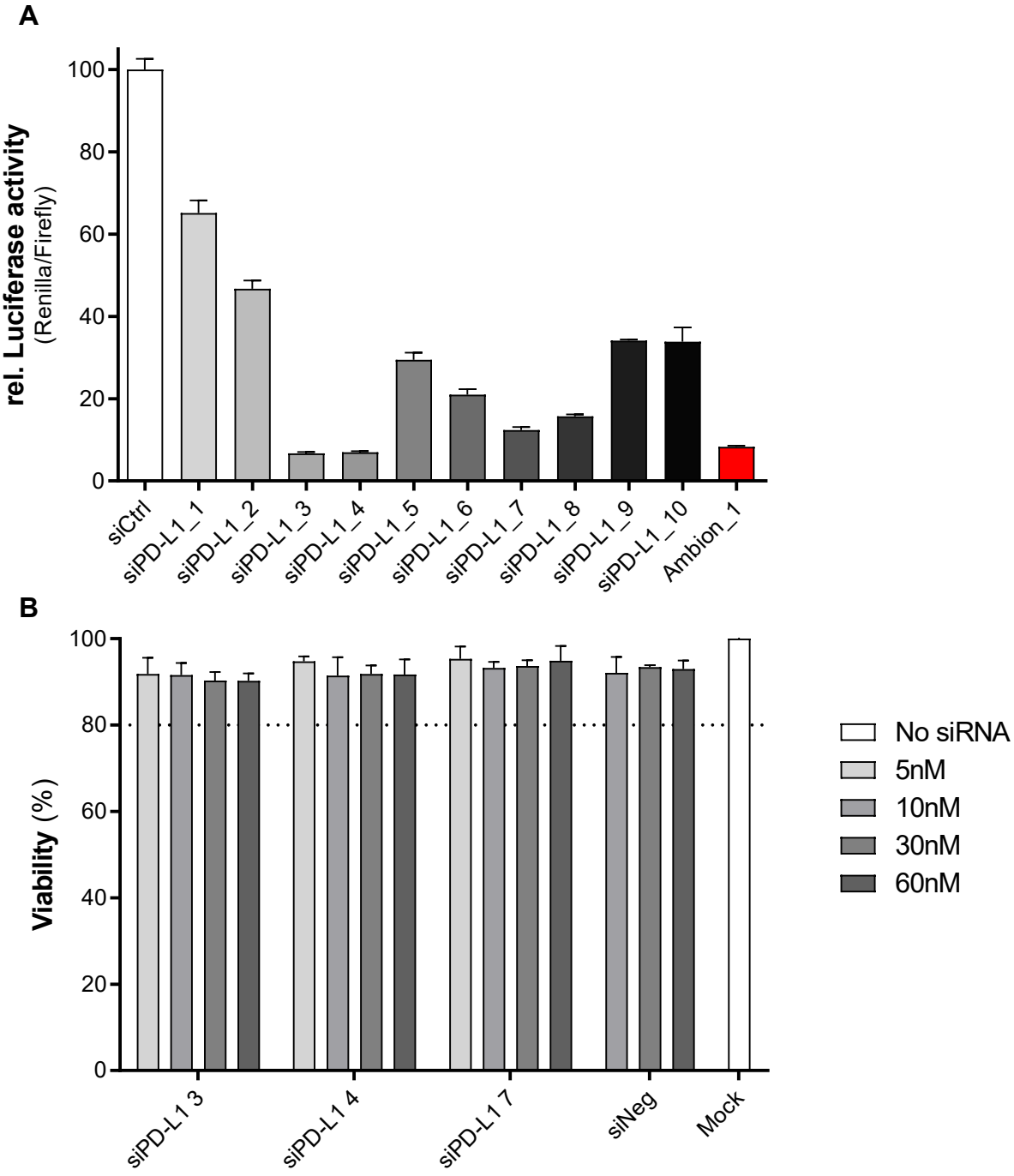


Figure 10: Screening of siRNA against PD-L1. (**A**) HEK293 cells were co-transfected with the reporter plasmid PsiCHECK2 and different siRNA against PD-L1 (siPD-L1_1-10) and 24 hours later renilla and firefly activity was measured sequentially. A commercially available siRNA was used as a positive control (Ambion_1) and siRNA not targeting PD-L1 as negative control (siCtrl). (**B**) For the most effective siRNA (siPD-L1_3,4 and 7) a cell titer blue viability assay was performed. Values are displayed relative to untreated mock which was set to 100%.

In prospect of using these siRNA for *in vivo* studies several modifications were introduced to enhance stability, bioavailability, and reduce off-target effects. There are many modifications described in literature e.g. 2' ribose modification,

substitution with fluor- or alkyl-groups, or using locked nucleic acids at the 5' end of the sense strand where the ribose has an extra bridge connecting the 2' oxygen and 4'carbon (Chiu and Rana 2003). To deliver siRNA to a specific organ or cell type it is beneficial to conjugate the duplex to a specific agent e.g. N-Acetylgalactosamine (GalNAc) which facilitates a cell type specific uptake into hepatocytes (Allerson et al. 2005; Chiu and Rana 2003; Dutkiewicz et al. 2008; Rothe et al. 2008; Soutschek et al. 2004).

We introduced 2'-OMe RNA bases, which enhances the binding affinity towards RNA, leading to higher nuclease resistance, reduced immunogenicity, and less off-target effects (Soutschek et al. 2004; Allerson et al. 2005). For an enhanced serum stability, we modified some nucleosides to 2'-Fluoro-nucleosides according to a pattern previously described to enhance effectivity *in vivo* (Foster et al. 2018). Furthermore, we replaced the non-bridging oxygen in some of the phosphate backbones with sulfur. These phosphorothioate linkages are used to increase nuclease resistance facilitate cellular uptake and bioavailability (Kurreck 2008).

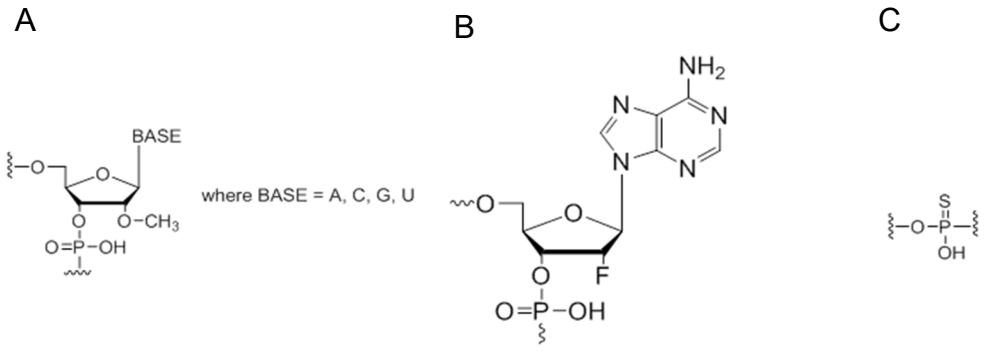


Figure 11*: siRNA modifications. (A) 2`-O-methyl modification, (B) 2`-Fluoro-adenosine, (C) Phosphorothioate backbone modification.*

To test whether the modification had any effect on the efficiency, the co-transfecting experiment with the PsiCHECK2-plasmid and the modified siRNA was repeated and both siRNAs were titrated.

Both versions of the siPD-L1 suppressed the luciferase signal in a dose dependent manner (figure 12A) with a maximum of 90% suppression. Titration

revealed an IC50 value of 218.3 pM for the unmodified (figure 12B) and 238,5 pM for the modified siRNA (figure 13C).

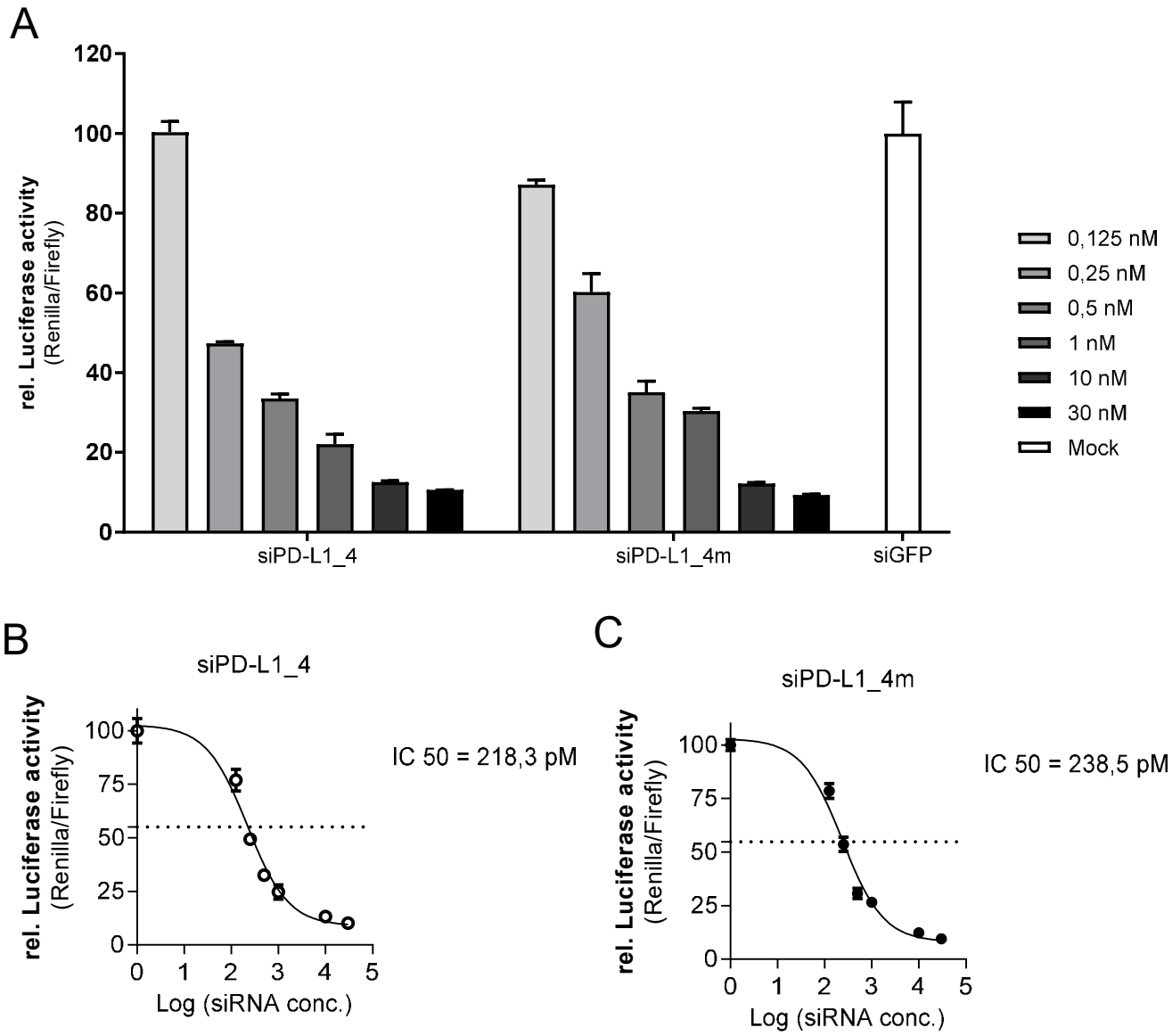


Figure 12: Titration of modified and unmodified siRNA. HEK293 cells were co-transfected with either unmodified (siPD-L1_4) or modified siRNA (siPD-L1_4m) against PD-L1, or siGFP as a negative control, together with the PsiCHECK2 plasmid. (A) Both siRNAs were titrated and rel. luciferase activity was measured 24 hours after transfection. IC50 values were calculated for the unmodified (B) and modified siRNA (C).

Modification of the siRNA should increase nuclease resistance and limit degradation. To test whether modification improved these properties, a comparable siRNA against enhanced green fluorescent protein (eGFP) was designed and co-transfected into cells with a plasmid encoding for eGFP. As a negative control siRNA targeting murine transforming growth factor beta 1 (TGF-β1), was included. GFP signal was monitored every day by fluorescence microscopy, up to a total of four days.

After one day no difference in fluorescence was observed (figure 13). Two days post transfection the functional siRNA treated cells displayed a lower signal compared to control. Three days post transfection this difference was still visible for the modified siRNA targeting GFP, however, cells treated with unmodified siGFP showed comparable fluorescence as control. This trend was even stronger after four days post transfection: the modified functional siRNA depleted the GFP signal, while all other siRNA failed to suppress GFP expression.

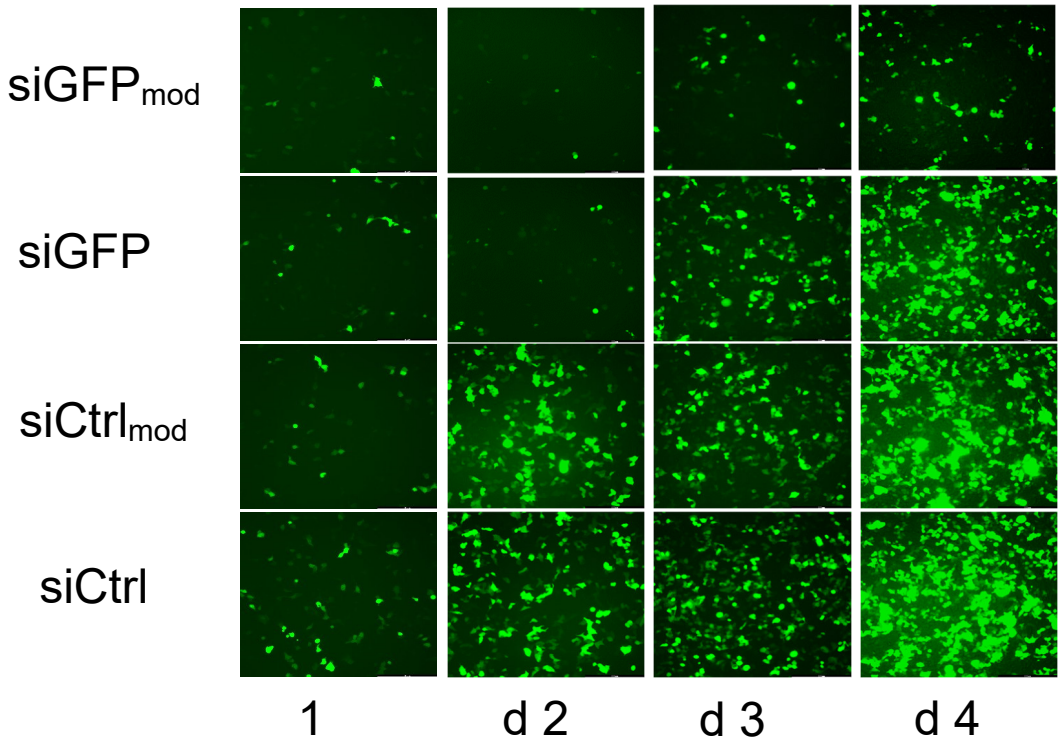


Figure 13: Prolonged protein knockdown by chemically modified siRNAs. HEK293 cells were co-transfected with a plasmid expressing eGFP and siRNA targeting GFP (siGFP) or TGF-β1 (siCtrl) as a control. Both siRNA were used either modified or unmodified respectively. Fluorescent signal was measured up to four days via microscopy.

Nucleic acids transfected into cells can act as potent activator of innate immune responses, e.g. binding to retinoic acid-inducible gene-I (RIG-I) or toll-like receptor (TLR) 3 (Meng and Lu 2017). To investigate possible immune stimulation, mRNA levels of different interferon stimulated genes (ISG) were analyzed by RT-qPCR, i.e. interferon beta (IFNβ), Interferon Induced proteins with Tetratricopeptide repeats (IFIT), 2'-5'-oligoadenylate synthetase (OAS). HEK 293 cells were transfected using RNAiMAX with either modified or unmodified siRNA, transfection reagent alone, and Polyinosinic:polycytidylic acid (Poly I:C) as positive control. Expression levels were normalized to untreated control.

For all siRNA, independent of specificity and modification, no upregulation in IFNβ, IFIT or OAS was observed (figure 14). Only Poly I:C treatment led to high induction of all analyzed ISG.

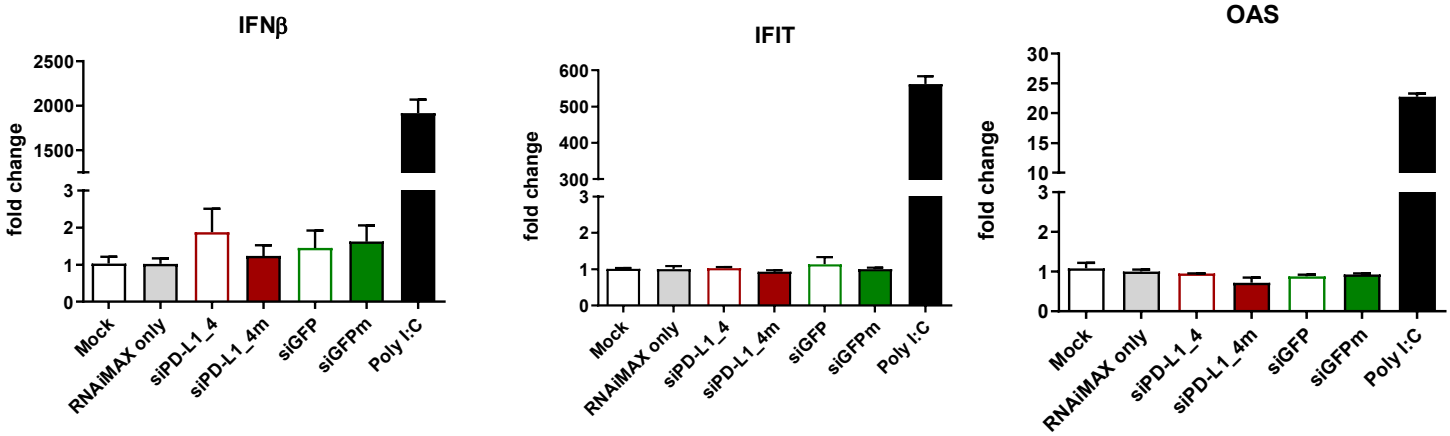


Figure 14: Innate immune response upon siRNA transfection. HEK 293 cells were transfected using unmodified (siPD-L1_4, siGFP) or modified (siPD-L1_4m, siGFPm) siRNA, Poly I:C was used as a positive control, and transfection reagent (RNAiMAX) only, as negative control. Interferon stimulated gene expression was measured by RT-qPCR.

Together, these results demonstrated a successful screening for an siRNA against PD-L1. The siRNA modifications were evaluated and led to prolonged suppression of the target protein while maintaining an acceptable IC50 value and without inducing an immune response.

2.3 Checkpoint inhibition *in vivo*

Previous work from our laboratory showed promising results for therapeutic vaccination (Michler et al. 2020). The approach was to induce HBV-specific T cell responses in mice replicating HBV using a heterologous prime/boost strategy termed TherVacB. While mice with low antigenemia usually cleared viral infection, mice with high antigen levels did not (Backes et al. 2016). With regards to enhanced immune responses following PD-1/PD-L1 blockade, we aimed for an enhanced efficacy of TherVacB by combining with siRNA against PD-L1.

2.3.1 PD-L1 suppression by siRNA *in vivo*

To test whether siRNA treatment can suppress PD-L1 expression *in vivo*, C57BL/6 mice were transfected with siRNA using a liposome-based transfection reagent either against PD-L1 or GFP as a control. 72h after transfection immunohistochemistry (IHC) staining for PD-L1 was performed by the department of Comparative Experimental Pathology (Klinikum rechts der Isar).

Although the PD-L1 expression in both groups was very low and mostly restricted to non-parenchymal cells, siPD-L1 treated mice showed even less PD-L1 signal compared to siCtrl (Figure 15A+B). RT-qPCR from whole liver lysate confirmed this finding displaying reduced PD-L1 mRNA levels in mice treated with siPD-L1 compared to siCtrl (figure 15C).

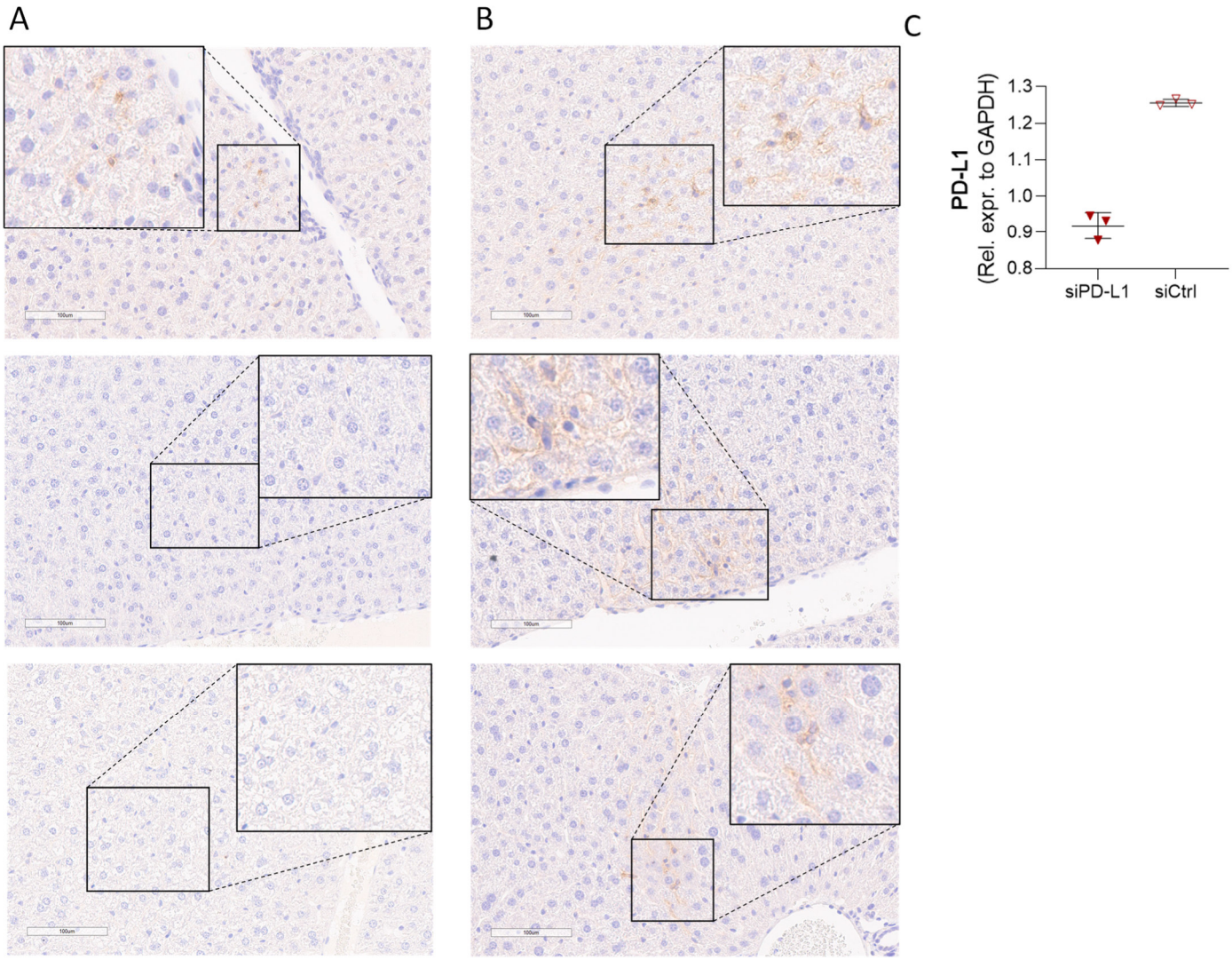


Figure 15: PD-L1 knockdown in vivo. Mice were transfected i.v. with siRNA against (**A**) PD-L1 or (**B**) GFP as a control complexed with a liposome-based transfection reagent. 72h post transfection IHC of the liver against PD-L1 was performed. (**C**) PD-L1 expression was measured by RT-qPCR from whole liver lysate and analyzed relative to GAPDH.

Collectively, this experiment demonstrates that siPD-L1_4 could lower PD-L1 expression in the liver of wildtype (wt) mice.

2.3.2 Combination of siRNA treatment and therapeutic vaccination

Next, the combination of siRNA treatment of PD-L1 and TherVacB was investigated. C57BL/6 wt mice were infected intravenously with an AAV-HBV. As an indicator for chronic infection, stable levels of HBsAg and HBeAg in the sera of these mice were observed and the TherVacB scheme was applied nine weeks

after transduction. Vaccination was performed by applying a two-time protein-prime with HBV-antigen (HBsAg, HBcAg) followed by a boost with the modified vaccinia virus Ankara (MVA) as displayed in the schematic representation in figure 16A. Two weeks after MVA boost these mice were sacrificed and the immune response was evaluated.

While untreated mice and mice receiving siRNA targeting PD-L1 only, showed stable antigen level, therapeutic vaccination led to a reduction of HBsAg and HBeAg (figure 16B). This effect was even more pronounced in mice that received the vaccination in combination with siRNA against PD-L1. In line with this observation, we saw that TherVacB was able to induce antibodies against HBsAg (anti-HBs) and HBeAg (anti-HBe) (figure 16C+D). An increase in serum ALT activity after MVA-boost was observed, most prominently in mice receiving both vaccination and siRNA (figure 16E+F).

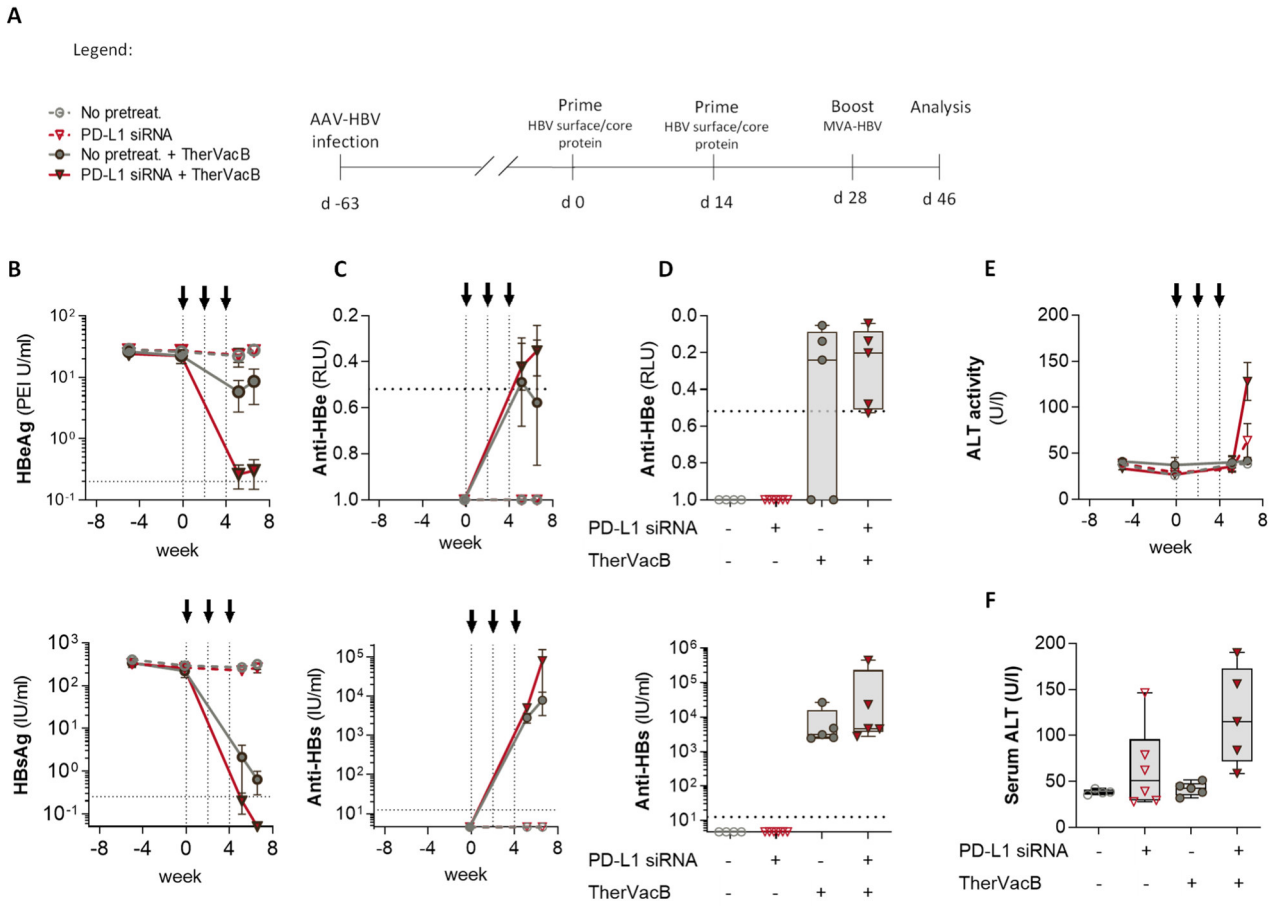


Figure 16*:* Experimental setup and serum parameters of siRNA in vivo **treatment.** (A) Experimental groups and setup: Mice were transduced with adeno-associated virus harboring a 1.2-overlength HBV genome. After 8 weeks these mice displayed stable HBV antigen levels indicating a chronic infection. We then treated these mice with a combination of siRNA against PD-L1 and TherVacB. The siRNA was administered with a dose of 100 µg one day prior to the protein prime and the MVA-boost. (B) Antigen levels were measured for 16 weeks, arrows in the graph indicate administration of protein priming and MVA boosting (C) Antibody levels were measured once prior to vaccination, at week -5 and week 0. (D) Antibody levels as measured on the day of analysis. (E) ALT activity was measured over the course of the experiment and (F) at the day of analysis. Box plots show median, interquartile ratio (IQR) (Box), and minimum to maximum (whiskers). Data shown are from five to six independent biological replicates per group.

Liver-associated lymphocytes (LAL) were isolated, re-stimulated with HBV specific peptides overnight and stained for intracellular cytokine expression (ICS). Overall, the percentage of T cells expressing INFγ and granzyme B (GzmB) was comparable in mice without treatment and mice only receiving PD-L1 siRNA (Figure 17A+B). Although TherVacB alone showed minimal influence on IFNγ expression, GzmB expression was higher when stimulated with the S or core peptide pool. When combined with siRNA against PD-L1, TherVacB led to higher numbers of IFNγ expressing T cells, especially when stimulated with the C93 peptide or the Core peptide pool.

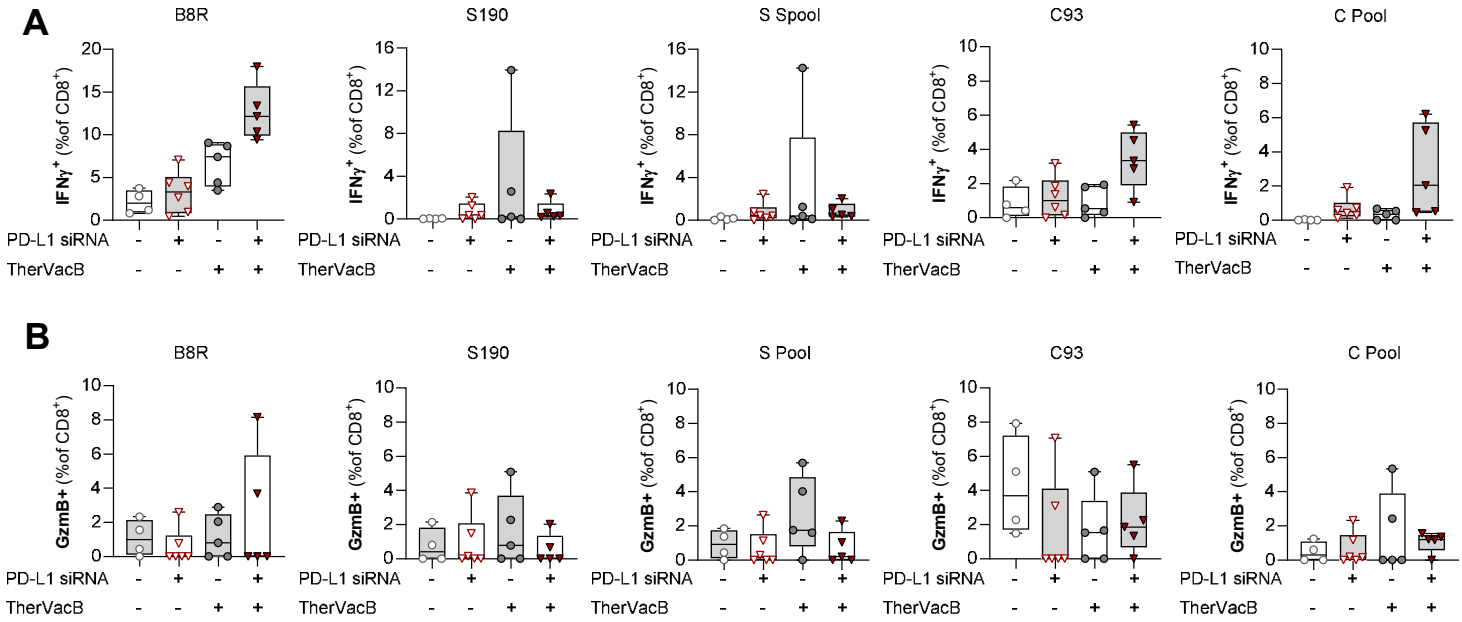


Figure 17: Intracellular cytokine expression upon HBV-specific re-stimulation. AAV-HBV infected mice were treated with siRNA against PD-L1 and TherVacB. Liver-associated lymphocytes were isolated and re-stimulated with the peptide depicted above the graph, i.e. B8R, S190, S pool, C93 and core pool. After stimulating for 12h, either (**A**) IFNγ or (**B**) GzmB expression of CD8+ T cells was measured. Box plots show median, interquartile ratio (IQR) (Box), and minimum to maximum (whiskers). Data shown are from four to six independent biological replicates per group.

Additionally, HBcAg was stained in the liver as an indicator for HBV infected hepatocytes. Higher frequencies of core-positive cells were observed in untreated and siRNA treated mice compared to vaccinated mice (figure 18A+B). When TherVacB was combined with PD-L1 blockade this effect was even stronger. As pgRNA measurements serves as an additional indicator for viral burden, mice treated with TherVacB in combination with siRNA showed ten-fold lower pgRNA levels compared to other treatment groups (figure 19C).

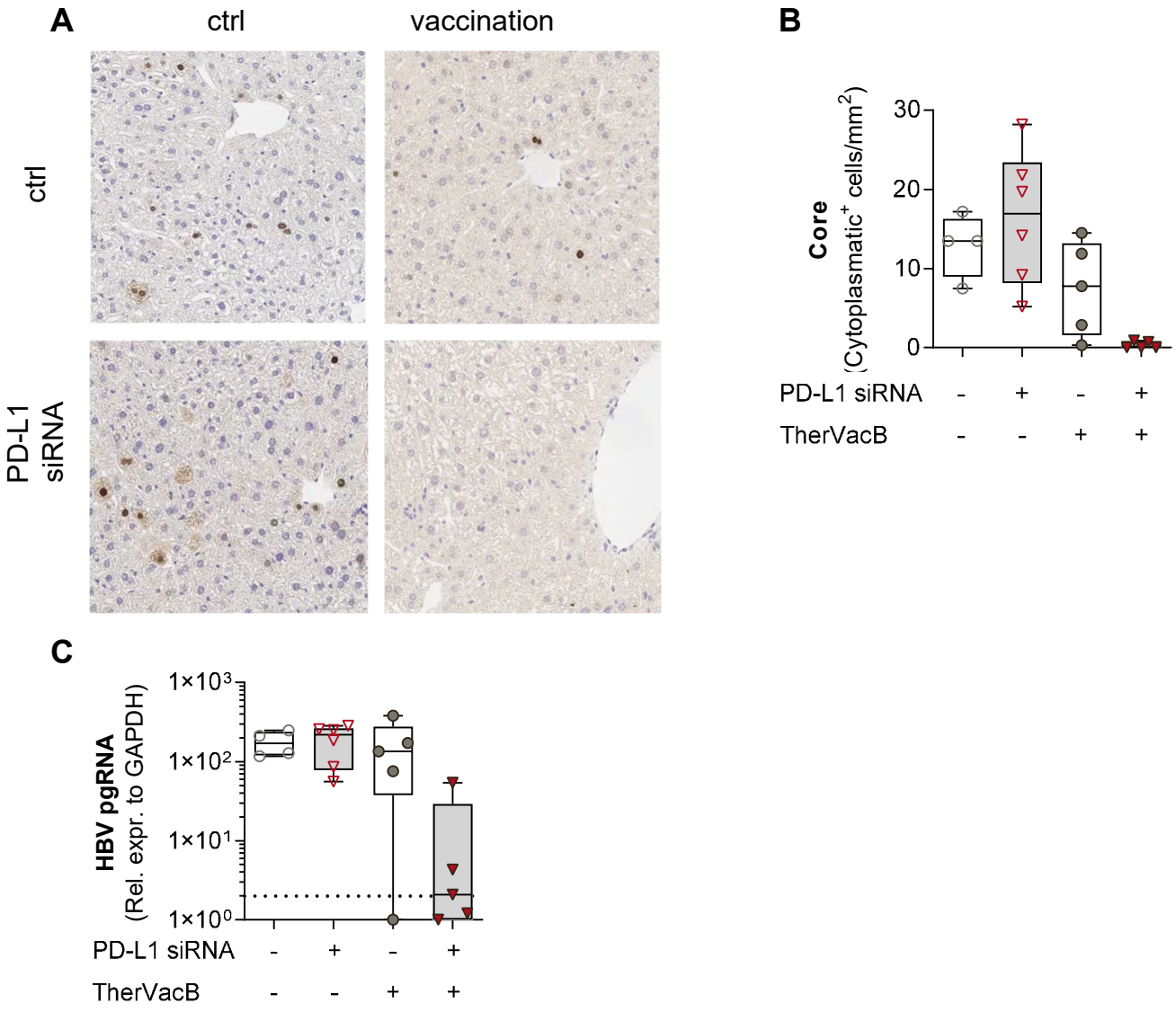


Figure 18: HBV parameters after combinatory treatment. (A) Immunohistochemistry staining was performed for HBc on liver samples, (B) HBc-positive hepatocytes were quantified in control (ctrl) and PD-L1 treated samples. (C) HBV pgRNA levels relative to GAPDH were measured by RT-qPCR from whole liver lysate. Box plots show median, interquartile ratio (IQR) (Box), and minimum to maximum (whiskers). Data shown are from four to six independent biological replicates per group.

In summary, the combination of PD-L1 silencing and TherVacB led to a higher reduction of HBV antigens and higher titers of HBV specific antibodies in AAV-HBV treated mice in comparison to TherVacB only. T cell responses displayed higher numbers of IFNγ producing $CD8^+$ T cells specific for C93. Elevation of ALT in the serum indicated hepatocyte directed T cell activity and a reduction in HBcAg-positive hepatocytes was observed in these mice. A decrease in pgRNA was detected in mice treated with siRNA and TherVacB compared to

vaccination alone confirming a depletion of AAV-HBV infected cells in the liver(figure 18C).

Collectively, this suggests that the application of siPD-L1 enhances the efficacy of TherVacB.

2.3.3 TherVacB combined with siRNA or antibody treatment

The current gold standard of PD-1/PD-L1 pathway blockade is the application of antibodies. While this approach has been proven to be effective in many refractory malignancies and in enhancing immune responses against several infections, adverse effects, such as autoimmunity, remains a possible risk due to the systemic effect of an antibody (Spain, Diem, and Larkin 2016). Thus, limiting the immune modulation to the site of infection is desirable. To test whether our siRNA treatment result in comparable effects to antibodies targeting PD-L1, we compared both methods in combination with TherVacB. Additionally, we compared self-designed siRNA to a commercially available siRNA.

AAV-HBV infected mice received TherVacB in combination with either control siRNA (siCTRL), our self-designed PD-L1 siRNA (siPD-L1), a commercially available siRNA (Ambion), a control antibody (AB Ctrl) or a PD-L1 antibody (AB PD-L1). One week after MVA-boost, the mice were sacrificed, and the immune response was analyzed.

A reduction in HBsAg level in all treatment groups after protein priming and MVA-boost was observed (figure 19A). In contrast to HBsAg, only few animals treated with the Ambion siRNA were able to suppress HBeAg levels at week 4 to the limit of detection. This effect was accompanied by an acute elevation of ALT activation in the serum in these mice (figure 19B), but diminished by week 5 (figure 19C). At week 5, most of the animals displayed a drop in HBsAg levels and few animals displayed a loss in HBeAg levels. These findings were in line with the detected antibody response in these mice. All mice had anti-HBs antibodies while only few developed anti-HBe antibodies. The AB PD-L1 group did not show (measurable) anti-HBe levels.

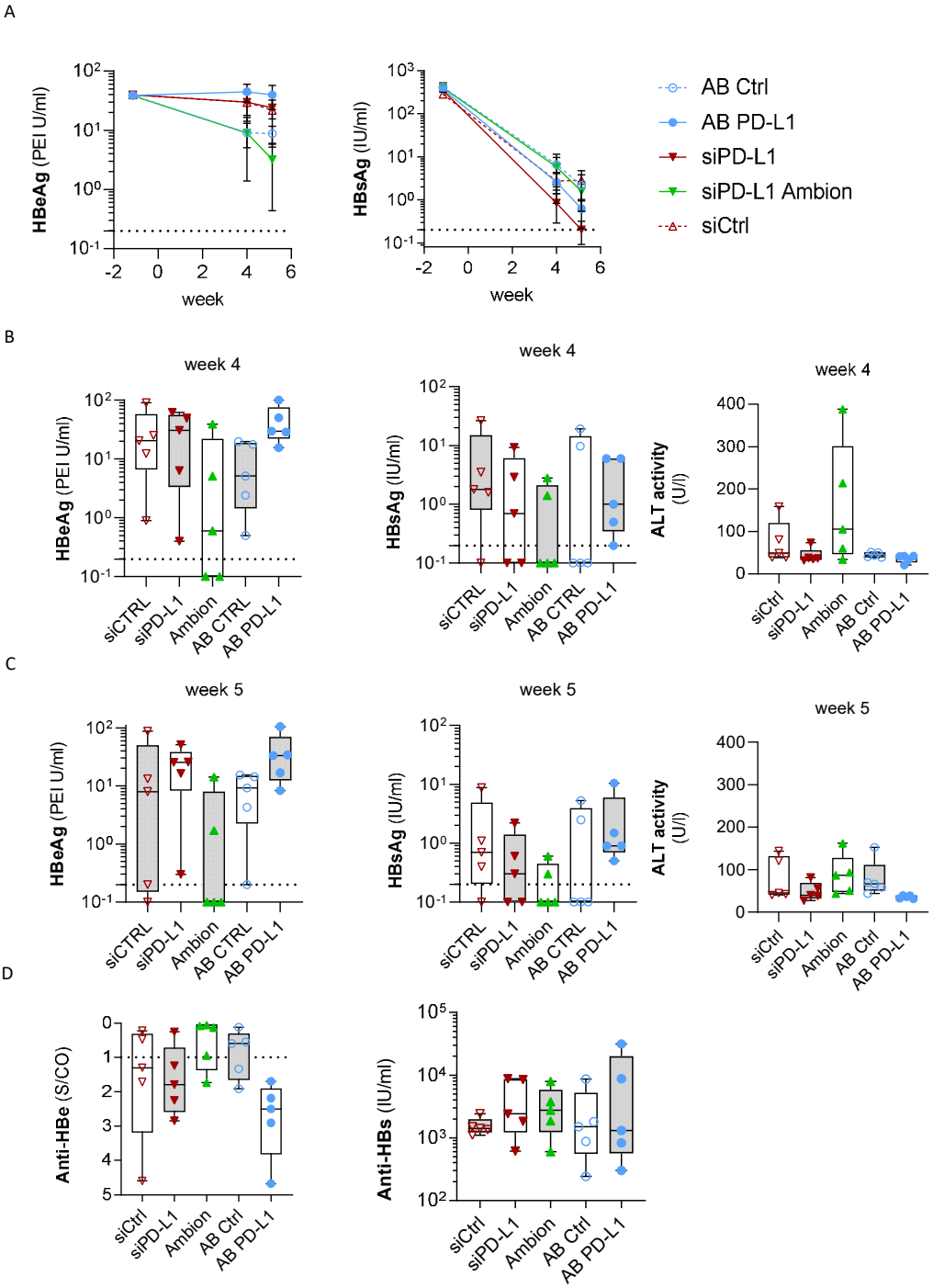


Figure 19: Serum parameters from mice treated with siRNA compared to antibody. AAV- HBV infected C57BL/6 mice were vaccinated according to the TherVacB scheme and received co-treated with a self-designed siRNA against PD-L1 (siPD-L1), a commercially available siRNA against PD-L1 (Ambion), control siRNA (siCtrl), an antibody against PD-L1 (AB PD-L1) or an isotype control antibody (AB Ctrl) when given the protein prime or the MVA-boost.(A) HBe and HBs antigens were measured from serum. (B) HBe and HBs antigen levels and ALT activity at the day of MVA boost were analyzed (week 4). (C) HBe and HBs antigens level and ALT activity at the day of analysis (week 5). (D) Antibody response measured at the last day of the experiment. Box plots show median, interquartile ratio (IQR) (Box), and minimum to maximum (whiskers). Data shown are five independent biological replicates per group.

For the functional analysis of LAL, an overnight re-stimulation of the cells with HBV-specific peptides was performed with a subsequent ICS. Almost all mice showed an IFNγ response when stimulated with the core peptide pool (figure 20A). Overall, the responses were diverse in mice receiving the siCtrl whereas siPD-L1 silencing led to a more robust response. The highest response was measured in mice receiving the Ambion siRNA. Although, the PD-L1 antibody enhanced the immune response when compared to the control antibody, the frequencies of IFNγ positive CD8+ T cells were still lower compared to siRNA treated groups. The same trend was observed in IFNγ expression when other peptides were used for stimulation. This higher cytokine expression through siRNA mediated PD-L1 blockade was even more pronounced when GzmB expression was analyzed (figure 20B). About twice the amount of $GzmB^+$ $CD8^+$ T cells in Ambion siRNA or siPD-L1 treated mice were measured compared to siCtrl, independent of the peptide used for stimulation. Mice treated with AB PD-L1 had higher frequencies of $GzmB^+$ $CD8^+$ T cells compared to AB Ctrl, but not as high as siRNA treated animals. As for IFNγ and GzmB, TNFα expression was higher in siPD-L1 and Ambion treated groups compared to siCtrl or antibody treated mice. The highest frequencies of $TNFα^+$ cells were in Ambion treated mice when stimulated with Core mix or S mix peptide pool.

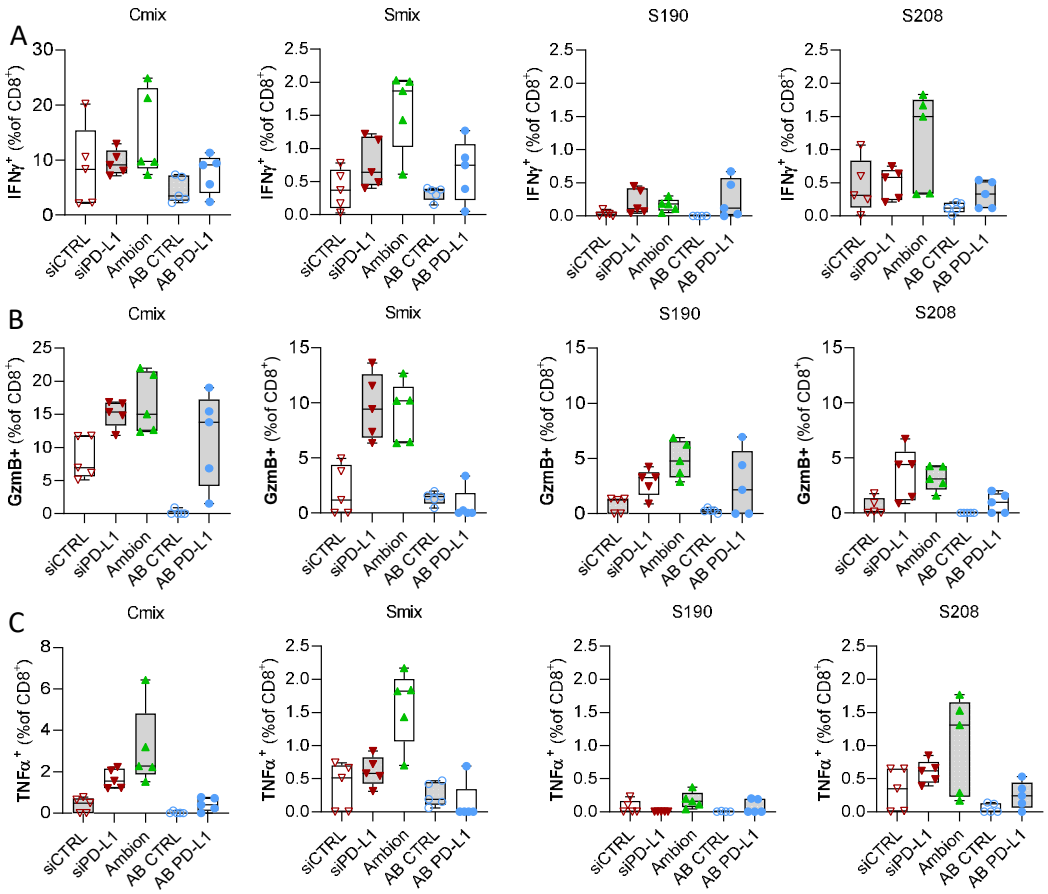


Figure 20: ICS from LAL in siRNA or antibody treated mice. LAL were isolated from the same experiment and stimulated overnight with HBV core peptide mix, S peptide mix, S190 and S208. ICS was performed after 16 hours measuring (**A**) INFγ, (**B**) GzmB and (**C**) TNFα. The peptide used for stimulation is depicted above the graph. Box plots show median, interquartile ratio (IQR) (Box), and minimum to maximum (whiskers). Data shown are five independent biological replicates per group.

To measure the frequencies of HBV-specific T cells, multimer staining was performed using tetramers specific for B8R, the HBV specific peptides C93 and S190 and as a negative control OVA. The frequencies for S190 and OVA were displayed low signal and thus could not be analyzed, however, the data for C93 and B8R is shown in figure 21. siCtrl and siPD-L1 treated mice showed similar numbers of C93-specific CD8+ T cells with the median at around 20% of $CD8^+$ T cells (figure 21A). Ambion treatment led to slightly higher numbers in $CD8^+$T cells compared to the other siRNAs. In contrast, numbers in mice treated with either one of both antibodies Ctrl and PD-L1 were lower compared to siRNA groups. We saw no difference in numbers of C93-specific $CD8^+$ T cells when comparing both antibody groups.

Next, the expression of different exhaustion marker on those tetramer+ cells was analyzed. Except of some variability in the Ambion group, almost all cells specific for C93 showed high levels of LAG3. This trend was even more striking when looking at PD-1 levels. Almost 100% of the C93-specific T cells showed expression of PD-1 on their surface. Only a minor difference was observed when analyzing cells that expressed high levels of PD-1, only two mice from the Ambion group and one from siPD-L1 had slightly lower expression levels. Some mice in the Ambion group and siPD-L1 treated mice showed lower frequencies of PD-1 high expressing cells compared to siCtrl and both antibody groups. TIM3 expression was at around 15% compared to siCtrl treated tetramer positive T cells and at almost 25% for siPD-L1. Mice from the Ambion group displayed similar levels of TIM3 compared to siCtrl, as did mice treated with the control antibody, whereas the functional antibody led to higher TIM3 expression compared to control.

A different trend was observed for B8R-specific cells. In all mice receiving a PD-L1/PD-1 blockade, slightly more cells were detected compared to their corresponding controls (figure 21B). LAG3 expression was comparable in all treatment groups with frequencies between 5% to 20%. As for C93-specific cells, most B8R-specific cells were positive for PD-1. Interestingly, when we checked whether these cells express intermediate or high levels of PD-1 we saw that the expression levels were similar to LAG3. Only 5%-20% of tetramer+ T cells expressed high levels of PD-1, with minor differences between the treatment groups. The same was true for TIM3: although siPD-L1 and AB PD-L1 treated mice had larger numbers of TIM3 positive T cells than their control, we generally saw low amounts of expression.

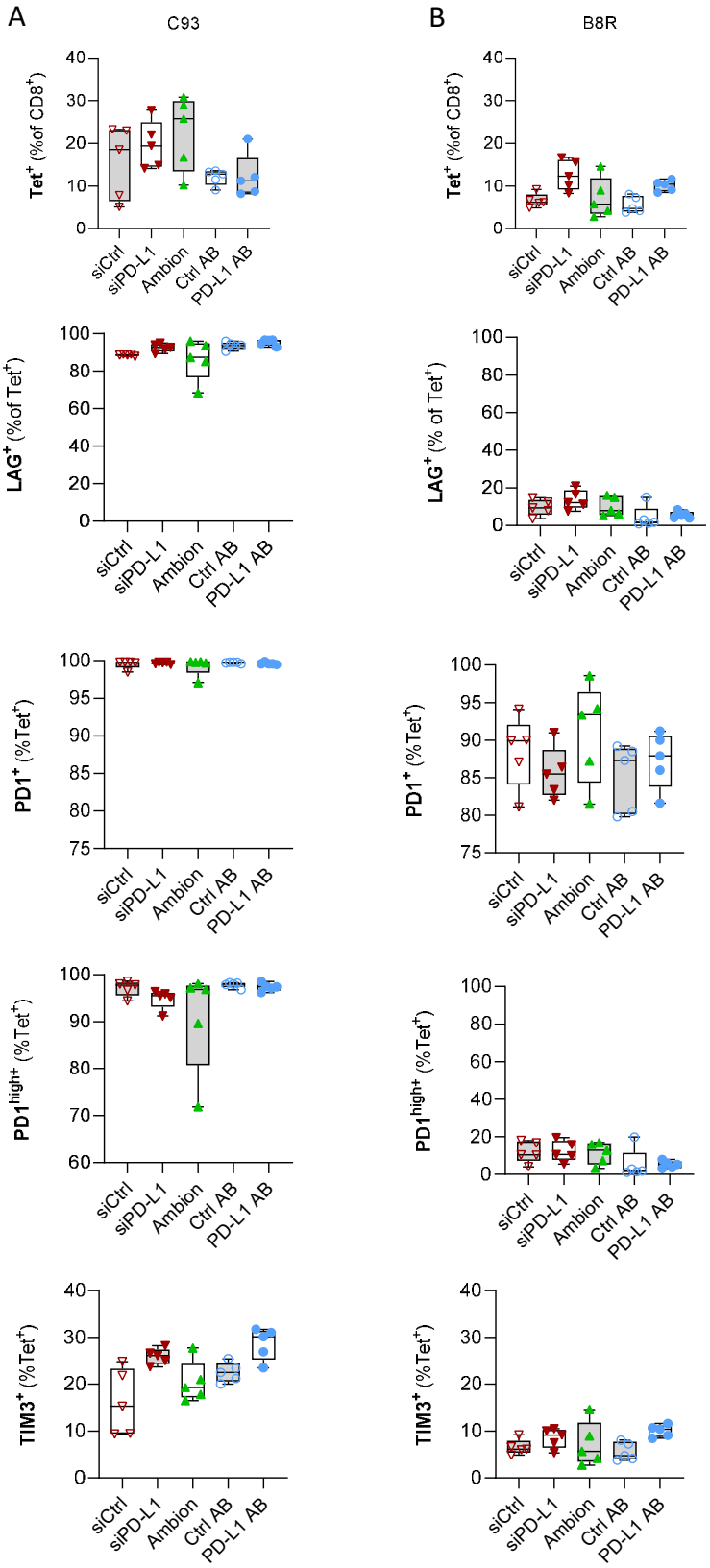


Figure 21: Phenotypical analysis of antigen specific CD8 T cells. LAL were isolated and stained with tetramers specific for (**A**) C93 and (**B**) B8R. Additionally, exhaustion markers, PD1, LAG3 and TIM3 were stained. Box plots show median, interquartile ratio (IQR) (Box), and minimum to maximum (whiskers). Data shown are five independent biological replicates per group.

In summary, PD-1:PD-L1 pathway blockade in combination with TherVacB led to a stronger reduction antigenemia and higher anti-HBs levels than vaccination alone. We detected more functionally active T cells in mice which received siRNA against PD-L1 when re-stimulated with HBV-specific peptides and analyzed for IFNγ, GzmB and TNFα expression. These T cells also varied in numbers confirmed by our multimer staining. We saw a slight change in the phenotype in anti PD-L1 treated cells compared to their respective controls.

Collectively, this data suggests that the application of siRNA against PD-L1 in combination with TherVacB is a promising approach for the treatment of chronic infection with HBV.

3. Discussion

Once a chronic infection with HBV in the liver is established, viral clearance is rarely achieved. Treatment involves the use of NA and PegIFNα, aiming at long-term suppression of HBV DNA levels (Terrault et al. 2016). Although the use of NA is usually well tolerated and more broadly applicable than PegIFNα, it fails to address the production of viral antigens by already infected cells, leaving them at risk for HCC (Kumada et al. 2017). Despite the fact that under certain circumstances drug discontinuation is possible, for most patients a lifelong therapy is mandatory (Lok et al. 2016). Although long-term treatment is proven to be safe (Darweesh et al. 2019), new approach aim at developing a finite treatment circumventing a lifelong administration of antiviral drugs. Immunotherapy aims to reconstitute the hosts immune system to achieve functional cure. One of those approaches is therapeutic vaccination. The aim of this thesis was to determine the relevant factors in viral clearance by therapeutic vaccination and translate this knowledge into a combinatory approach.

3.1 PD-L1 in the context of HBV infection

In order to escape viral clearance, HBV is able to render the innate immune response ineffective. T cell response in chronic HBV infection is characterized by depletion and impaired effector function, accompanied by an upregulation of inhibitory molecules on their surface (Dong et al. 2019).

We observed that HBV infection *in vitro* leads to resistance against T cell mediated cytotoxicity. Even though our data suggests that a factor excreted by infected cells is involved in inhibiting T cell function, PD-L1 expression also contributed to the immune evasion of HBV. Inflammatory conditions in the setting of CHB lead to high PD-L1 expression in the liver (J. Chen et al. 2011). This finding was confirmed in our infection model where a reduction of antigens correlated with decreased PD-L1 expression in the liver. This overexpression of inhibitory molecules establishes an immunosuppressive micromilieu.

The interaction of PD-L1 and PD-1 on an activated T cell can lead to an attenuated immune response. It was shown that antibody mediated blockade of either PD-1 or PD-L1 improved IFNγ upon *ex vivo* re-stimulation and increased the number of HBV specific cytotoxic T cells (Maier et al. 2007). Priming T cells by therapeutic vaccination and subsequentially enhance the immune response by PD-L1 blockade is a promising approach already successful in the setting of WHV (Liu et al. 2014).

3.2 Combination of siRNA treatment and TherVacB

The key for effective protein knockdown through siRNA application is high sequence specificity with minimal off-target effects. Sequence design, modifications, and delivery method among other things are crucial issues to address when using RNAi as a therapeutic approach. Optimized siRNAs against a variety of targets are available for purchase, so are siRNAs against PD-L1.

In this thesis the designs of siRNA against PD-L1 was evaluated, ranging from the sequence design, to the introduction of modifications, up to the evaluation in *in vivo* experiments. The Dual-Luciferase reporter assay experiments described in section 2.2.2. proofed the successful screening and optimization of siRNA targeting PD-L1 *in vitro*. Furthermore, application *in vivo* led to suppressed PD-L1 levels and enhanced efficacy of therapeutic vaccination.

It has been shown, that antibody mediated blockade of PD-1 or PD-L1 improved the outcome of therapeutic vaccination (E. Gane et al. 2019; Liu et al. 2014; Kosinska et al. 2015). Based on these findings, inhibition of PD-1:PD-L1 interaction with antibodies was compared to the use of siRNA. This comparison was investigated by applying the TherVacB scheme to the AAV-HBV infection model. Mice receiving PD-L1 blockade in combination with TherVacB showed stronger immune responses and lower viral parameters compared to controls. Upon peptide re-stimulation, siPD-L1 treatment led to higher numbers of $IFN\gamma^+$, $GzmB^+$ and $TNF\alpha^+$ T cells, higher antibody titers, and lower antigen levels compared to control siRNA. Although this effect was also observable for the antibody mediated PD-L1 blockade, its effect was inferior to siRNA treatment.

These results indicate an advantage of siRNA over antibody treatment in terms of an enhanced immune response following therapeutic vaccination.

3.3 Relevance for clinical application of checkpoint inhibitors and TherVacB

The use of immunotherapy in cancer is a success story. Refractory malignancies that used to be associated with a very low life expectancy, can now be treated with checkpoint inhibitors and improve the prognosis of the patient (Rodríguez-Cerdeira et al. 2017; Singh, Trivedi, and Jain 2018). Driven by these achievements, the interest in that field grew a lot and led to the development of several antibodies targeting a range of checkpoint inhibitors. Once approved for clinical use, researchers tried to broaden the range of application to other diseases such as viral infections (Barber et al. 2006).

Although promising results in the context of infectious diseases could be achieved, as discussed previously in this thesis, the treatment with checkpoint inhibitors comes with a price. Current gold standard of checkpoint inhibition is the administration of a specific antibody against the desired molecule. Due to the fact that immune checkpoints play a pivotal role in T cell inhibition, interference can lead to so called immune-related adverse events (irAEs) (Spain, Diem, and Larkin 2016). Most common irAEs are gastrointestinal complications, such as enterocolitis, diarrhea and hepatitis, skin toxicity, associated with rash, pruritus and vitiligo, and toxicity to endocrine organs, with thyroid dysfunctions as the most common disfunction (Spain, Diem, and Larkin 2016). Although these side effects are usually treatable and transient in nature, but cases of sever liver failure or lethal skin reactions have been described.

The idea of this thesis was to utilize the advantage of checkpoint inhibition without the drawback of systemic irAEs. The effect of siRNA transfected with an cell type specific transfection reagent is limited to the desired site (Altogen-Biosystems). siRNA mediated PD-L1 inhibition was compared antibody treatment as an alternative cotreatment to therapeutic vaccination. Data described in section 2.3.3 suggest that indeed siRNA is a viable option for substitution of an antibody. Although we saw an elevation in ALT levels in all groups receiving siPD-L1, this

effect was only mild and transient. We could not see any antiviral effect when administering the siPD-L1 alone, in some animals it even increased viral burden. The aggravation of a viral infection through PD-L1 suppression was already described for CHB patients and other chronic viral infections (Schonrich and Raftery 2019). Data published 2019 by Garcia-Bates et al. suggests that PD-1:PD-L1 pathway inhibition can inhibit the transition of naïve $CD4^+$ cells and $CD8^+$ cells to effecter memory phenotypes. When combined with a way to stimulate the immune system in the right direction, as in this case with therapeutic vaccination, an enhanced antiviral effect and a decrease in viral burden could be observed.

3.4 Design of siRNA for clinical application

Two main problems are currently limiting the use of siRNA for clinical application: the siRNA needs to be formulated to be taken up by the cell, preferable only into the desired cell type, and the so called off-target effects, caused by sequence homologies, leading to the undesired knock-down of other host proteins (Pecot et al. 2011).

Choosing a sequence which is specific for the desired target is crucial to reduce off-target effects. Due to differences in the sequence of corresponding proteins between species, designing siRNA which are cross-reactive against humans and the preclinical animal models is not always possible. Using different sequences in different phases of drug development can lead to unpredicted complications. Furthermore, sequence-specific immune activation has been described, emphasizing the importance of sequence design (Meng and Lu 2017).

During design of the siRNAs used in this thesis all these criteria have been addressed: siRNA Sequences were chosen using either a protocol published by (Birmingham et al. 2007) or according to (Boudreau and Davidson 2012) and sequences with homologies were excluded using the National Library of Medicine´s BLAST. Although murine and human sequence are 68,7% identical (Altschul 1993), no siRNA cross-reactive to both could be designed. Lastly elements known to be recognized by the innate immune system were excluded and modifications were introduced to enhance nuclease resistance and bioavailability.

In vitro data suggests that this workflow led to the production of a stable and efficient siRNA, which induces no significant immune response. The efficiency was compared to a commercially available siRNA in the AAV-HBV infection model and proved to achieve similar results.

When linked to high affinity antibodies or receptor ligands, siRNA can be delivered in a cell type specific manner (Wittrup and Lieberman 2015). Michler et al could show that GalNAc coupled siRNA, targeting all HBV transcripts, was efficiently transfected into hepatocytes when injected subcutaneously (s.c.).

This study showed that organ specific delivery is achieved by coupling the siRNA to an organ specific receptor and making the need for i.v. injections obsolete in the process. The route of administration is an important factor in drug development, highlighted by the fact that HBV prevalence and the spread of viral infections due to unsafe medical injection is co-localized (Pépin et al. 2014). When given injections, the amount of blood transferred from previous patients determines the risk of transfection. When performing i.v. injections the likelihood of remaining blood in the needle/syringe is higher compared to s.c. or i.m (Pépin et al. 2014).

PD-L1 expression evaluated by IHC, determined non parenchymal liver cells, i.e. liver sinusoidal endothelial cells, as the important target cells for suppression. Thus, a transfection reagent targeting a broader spectrum of cells in the liver had to be used. A liposome-base reagent was used to transfect the siRNA to the liver tissue, although it requires an i.v. injection. Future studies should address the drawback and compare alternative transfection methods.

3.5 Conclusion

Chronic HBV infection establishes an immunosuppressive milieu demonstrated by our *in vitro* and *in vivo* infection models. Although this immune suppression is not exclusively established by PD-L1 overexpression, the inhibition in the right context can alleviate viral burden.

Collectively, this thesis provides strong evidence that therapeutic vaccination in combination with PD-L1 checkpoint inhibition can reconstitute the HBV specific immunity. This data suggests that siRNA mediated checkpoint inhibition is even more potent than inhibition by antibodies. Further research is needed to address the specific side effects, most likely in the setting of clinical studies.

Materials and methods

3.6 Materials

3.6.1 Cell lines

Name	Description
HEK293	Human embryonic kidney cells, transformed with fragments of adenovirus type 5 DNA (Graham et al. 1977)
HepG2-NTCP	HepG2 cells with a stable expressing of sodium taurocholate co-transporting polypeptide (NTCP) (Ko et al. 2018)

3.6.2 Mouse strains

Mouse line	Description	Source
HBVxfs	C57BL/6, HBV-transgenic, 1.3 overlength genome, frame shift mutation in X protein	AG Protzer
Wildtype	C57BL/6J wildtype	Janvier

3.6.3 Cell culture media

Medium	Ingredients	Amount
DMEM full medium	DMEM	500 ml
	FCS	50 ml
	Pen/Strep, 10,000 U/ml	5.5 ml
	L-Glutamine, 200 mM	5.5 ml
	NEAA,100x	5.5 ml
	Sodium pyruvate, 100 mM	5.5 ml

Freezing medium	FCS DMSO	90 % 10 %
HepG2 Diff medium	DMEM FSC Pen/Strep, 10,000 U/ml L-Glutamine, 200 mM NEAA, 100x Sodium pyruvate, 100mM DMSO	500 ml 5 ml 5.5 ml 5.5 ml 5.5 ml 5.5 ml 10.5 ml
Human T cell medium	RPMI 1640 FSC Pen/Strep, 10,000 U/ml L-Glutamine, 200 mM NEAA, 100x Sodium pyruvate, 100 mM HEPES Gentamicin	500 ml 50 ml 5.5 ml 5.5 ml 5.5 ml 5.5 ml 5.5 ml 208 µl
LB medium pH 7.0	Tryptone Yeast extract NaCl in 1 liter H2O	10 g 5 g 10 g
RPMI full medium	RPMI 1640 FCS Pen/Strep, 10,000 U/ml L-Glutamine, 200 mM NEAA, 100x Sodium pyruvate, 100 mM	500 ml 50 ml 5.5 ml 5.5 ml 5.5 ml 5.5 ml
Transfection medium	DMEM FCS L-Glutamine, 200 mM NEAA, 100x Sodium pyruvate, 100 mM	500 ml 50 ml 5.5 ml 5.5 ml 5.5 ml

Wash medium	RPMI 1640 Pen/Strep, 10,000 U/ml	500 ml 5.5 ml

3.6.4 Buffers

Buffer	Ingredients
ACK lysis buffer	150 nM NH_4Cl 10mM $KHCO_3$ 0.1 mM Na_2EDTA pH 7.2 – 7.4 in H_2O
FACS buffer	0.1 % BSA in PBS
Lysis buffer	2.5ml 1M Tris 2.5ml 5M NaCl 0.3ml 0.5M EDTA 0.075g EGTA 0.5 Triton X100 250µl NP40 5ml Glycerol 0.558g $Na_4P_2O_7$
SDS-Loading dye	3.75 ml 1M Tris/Cl pH 6.8 6 ml glycerol 1.2g SDS 0.93g DTT 6 mg bromphenol blue in 10 ml H_2O
10x TBS	12.1g Tris 40g NaCl Adjust to pH 7.4 In 500 ml H_2O
TBS-T	100 ml 10x TBS 1ml Tween-20 In 1l H_2O
10x SDS Running buffer	30.3g Tris 145g Glycin 100 ml 10% SDS In 1l H_2O
10x Transfer buffer	30.3g Tris 144,1g Glycin In 1l H_2O
Transfer buffer	100ml 10x Transfer buffer 200ml Methanol in 1l H_2O

3.6.5 Kits

Product	Supplier
AllPrep DNA/RNA Kit	Qiagen, Valencia, CA, USA
CellTiter-Blue Cell Viability Assay	Promega BioSciences, CA, USA
Dual-Luciferase Reporter Assay	Promega BioSciences, CA, USA
GeneJet Gel Extraction Kit	Fermentas, St. Leon-Rot, Germany
GeneJet Plasmid Miniprep Kit	Thermo Scientific, Schwerte, Germany
Human IFN- γ ELISA MAX Standard Sets	Biolegend, San Diego, CA, USA
LightCycler 480 SYBR Green I Master mix	Roche, Mannheim, Germany
Liver In Vivo Transfection Kit	Altogen Biosystems, NV, USA
NucleoSpin RNA isolation Kit	Macherey-Nagel, Düren, Germany
NucleoSpin Tissue Kit	Macherey-Nagel, Düren, Germany
SuperScript III First-Strand Synthesis SuperMix for qRT-PCR	Invitrogen, Karlsruhe, Germany
TA Cloning Kit	Invitrogen, Karlsruhe, Germany

3.6.6 Laboratory equipment and consumables

Product	Supplier
BEP (HBeAg measurement)	GE Healthcare Life Sciences, Freiburg, Germany
Cell culture flasks and plates	TPP, Trasadingen, Switzerland
Cell culture incubator HERAcell 150i	Thermo Scientific, Rockford, USA
Centrifuge 5417C / 5417R	Eppendorf, Hamburg, Germany
Cryo vials	Geiner Bio One, Kremsmünster, Austria
ELISA 96well plates Nunc	Thermo Scientific, Rockford, USA
Falcon tubes 15ml, 50ml	Greiner Bio One, Kremsmünster, Austria

Fluorescence microscope CKX41	Olympus, Hamburg, Germany
Freezing container	Thermo Fisher Scientific, Waltham, USA
Fusion Fx7 (chemiluminescence detection; UV light system)	Peqlab, Erlangen, Germany
Gel chambers (agarose gel electrophoresis)	Peglab, Erlangen, Germany
Gel chambers (SDS-PAGE)	Bio-Rad, Hercules, USA
Heating block	Eppendorf, Hamburg, Germany
Hemocytometer	Brand, Wertheim, Germany
Hyperflask	Corning, Amsterdam, The Netherlands
Light Cycler 480 II	Roche, Mannheim, Germany
Light Cycler 96	Roche, Mannheim, Germany
Pipette “Accu-jet pro”	Brand, Wertheim, Germany
Pipette filter tips	Starlab, Ahrensburg, Germany
Pipette tips 2 – 50ml	Greiner Bio One, Kremsmünster, Austria
Pipettes	Eppendorf, Hamburg, Germany
PVDF membrane	Bio-Rad, Hercules, USA
qPCR 96-well plates	4titude, Berlin, Germany
Reaction tubes	Eppendorf, Hamburg, Germany
Reflotron ALT stripes	Roche, Mannheim, Germany
Reflotron Reflovet Plus (ALT reader)	Roche, Mannheim, Germany
Sterile hood	Heraeus, Hanau, Germany
Tecan plate reader Infinite F200	Tecan, Männerdorf, Switzerland
Ultracentrifuge Beckman SW40 rotor	Beckman Coulter, Brea, USA
Western Blotting Chamber	Bio-Rad, Hercules, USA
Whatman paper	Bio-Rad, Hercules, USA

3.6.7 Antibodies

Antibody	Supplier
Granzyme B-PE	Invitrogen
mCD3-PerCP-Cy5.5	eBiosciences
mCD4-APC	eBioscience
mCD4-PE-Cy7	eBioscience
mCD4-V500	BD Biosciences
mCD8-PE	BD Biosciences
mCD8-PE-Cy7	Biolegend
mCD8-PerCP-C7	eBioscience
mCD8-V500	BD Biosciences
mCTLA-4-PerCP-Cy5.5	eBioscience
mIFN-γ-APC	BD Biosciences
mIFN-γ-FITC	BD Biosciences
mPD-1-FITC	eBioscience
mPD-1-PacBlue	eBioscience
mTIM-3-APC	Biolegend
mTNF-α-PE-Cy7	BD Biosciences

3.6.8 Chemicals and reagents

Chemical or reagent	Supplier
Agar-agar	Roth, Karlsruhe, Germany
Agarose	Peqlab, Erlangen, Germany
Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare Life Sciences, Freiburg, Germany
Ampicillin	Roth, Karlsruhe, Germany
Collagen R	Serva Electrophoreses, Heidelberg, Germany
DMSO	Sigma-Aldrich, Steinheim, Germany
Dulbecco´s modified Eagle´s Medium	Gibco/Invitrogen, Carlsbad, USA
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Merck, Darmstadt, Germany

FCS (heat-inactivated)	Gibco/Invitrogen, Carlsbad, USA
Formaldehyde	Roth, Karlsruhe, Germany
Gentamicin	Ratiopharm, Ulm, Germany
Glutamine	Sigma-Adrich, Steinheim, Germany
Glycine	Roth, Karlsuhe, Germany
HBsAg, genotype A, recombinant	Dynavax
HBcAg, genotype A, recombinant	Dr. Dišlers,APP Latvijas Biomedicīnas (Riga, Latvia)
IFN-α	Roche, Vienna, Austria
IFN-γ	Boehringer Ingelheim, Vienna, Austria
Isopropanol	Roth, Karlsruhe, Germany
Lipofectamine 2000/3000	Life Technologies, Carlsbad, USA
Lipofectamine RNAiMAX	Life Technologies, Carlsbad, USA
Methanol	Roth, Karlsruhe, Germany
Milk powder	Roth, Karlsruhe, Germany
NaCl	Roth, Karlsruhe, Germany
NaOH	Roth, Karlsruhe, Germany
Non-essential amino acids 100x	Gibco/Invitrogen, Carlsbad, USA
OptiMEM	Gibco/Invitrogen, Carlsbad, USA
Page Ruler Plus Prestained protein ladder	Thermo Scientific, Waltham, USA
PBS	Gibco/Invitrogen, Carlsbad, USA
PEG6000	Merck, Hohenbrunn, Germany
Penicillin/streptomycin	Gibco/Invitrogen, Carlsbad, USA
Pierce RIPA buffer	Thermo Scientific, Rockford, USA
Polyacrylamide	Roth, Karlsruhe, Germany
Protease inhibitor (Comlpete)	Roche, Mannheim, Germany
RotiSafe	Roth, Karlsruhe, Germany
SDS	Roth, Karlsruhe, Germany
SmartLadder DNA (small fragment)	Eurogentec, Liege, Belgium
Sodium citrate	Roth, Karlsruhe, Germany
Sodium pyruvate	Gibco/Invitrogen, Carlsbad, USA

Sucrose	Roth, Karlsruhe, Germany
T5 exonuclease	New England Biolabs, Ipswich, USA
TEMED	Roth, Karlsruhe, Germany
Tris base	Roth, Karlsruhe, Germany
Tris HCL	Roth, Karlsruhe, Germany
Trypan blue	Gibco/Invitrogen, Carlsbad, USA
Trypsin	Gibco/Invitrogen, Carlsbad, USA
Tryptone	Roth, Karlsruhe, Germany
Tween 20	Roth, Karlsruhe, Germany

3.6.9 Software

Software name	Supplier
FlowJo, version 10.4	BD Biosciences
LightCycler 480 SW 1.5.1	Roche
Prism 8	GraphPad Software Inc.
ImageJ	NIH
RTCA Software 2.0	ACEA Biosciences
Serial Cloner	SerialBasics

3.6.10 siRNA

Name	Sequence sense (antisense)
siGFP	GCAGCACGACUUCUUCAAG
siPD-L1_1	CCUACUGGCAUUUGCUGAACGCAUU (AAUGCGUUCAGCAAAUGCCAGUAGG)
siPD-L1_2	AGACGUAAGCAGUGUUGAA (UUCAACACUGCUUACGUCU)
siPD-L1_3	GUGGAGAAAUGUGGCGUUG (uAACGCCACAUUUCUCCACAU)

siPD-L1_4	GGAGAAAUGUGGCGUUGAA (UUCAACGCCACAUUUCUCCAC)
siPD-L1_5	GCGGACUACAAGCGAAUuA (UGAUUCGCUUGUAGUCCGCAC)
siPD-L1_6	GGUGCGGACUACAAGCGAA (UUCGCUUGUAGUCCGCACCAC)
siPD-L1_7	GUGCGGACUACAAGCGAAU (AUUCGCUUGUAGUCCGCACCA)
siPD-L1_8	GAGUAUGGCAGCAACGUuA (UGACGUUGCUGCCAUACUCCA)
siPD-L1_9	GGUCAACGCCACAGCGAAU (AUUCGCUGUGGCGUUGACCCU)
siPDL1_10	GCUGGACCUGCUUGCGUUA (UAACGCAAGCAGGUCCAGCUC)
si403-425	AAUUCUUUGGUUGAUUUUGUU (CAAAAUCAACCAAAGAAUUUU)
si603-625	UAGAAAAUCUCAUUAGUUGUU (CAACUAAUGAGAUUUUCUACU)

3.7 Methods

3.7.1 Cell culture

All adherent cell lines were cultured under sterile conditions in DMEM full medium and incubated at 37°C, 5% CO_2 and 95% humidity. Cells were passaged every three to four days depended on their confluency. For HepG2 derived cells, a single cell suspension was obtained by treatment with trypsin (5-10 min, 37°C). Plates and culture flasks were collagenized prior to the seeding of the cells.

3.7.2 RNA isolation

Tissue was stabilized using RNA*later* and stored at -80°C. After thawing, samples were disrupted using a Tissue Lyser LT according to manufacturer´s instructions. For RNA isolation the Nucleo Spin RNA Kit was used according to manufacturer´s instructions.

3.7.3 DNA isolation

Cellular DNA was extracted using the NucleoSpin Tissue Kit, according to the manufacturer's protocol.

3.7.4 Determination of DNA or RNA concentration

DNA and RNA concentrations were determined using a NanoDrop One using the appropriate buffer solution as blank.

3.7.5 cDNA synthesis

For cDNA synthesis, RNA was reversely transcribed using the SuperScript III Kit according to manufacturer´s instructions. Obtained cDNA was diluted in H_2O.

3.7.6 quantitative PCR

Quantitative PCR was performed using the LightCycler 480 system and the SYBR green master mix according to manufacturer´s instructions.

3.7.7 Dual luciferase assay

All experiments containing the psiCHECK-2 plasmid from Promega, were analyzed by using the Dual-Luciferase Reporter Assay system from Promega HEK 293 cells were co-transfected with the psiCHECK-2 plasmid and siRNA using RNAiMAX according to the manufacturer's protocol.

3.7.8 Counting of cells

A single cell suspension was obtained and 10µl was diluted with trypan blue in a 1:1 ratio. Cells were counted on a Neubauer improved hemocytometer using a microscope.

3.7.9 Transfection of cells

Cells were seeded in 24-well plates according to 60-80% confluency at the day of transfection. Invitrogen Lipofectamin RNAiMAX transfection reagent was used according to the manufacturer's protocol.

3.7.10 Infection with HBV

HepG2-NTCP cells were differentiated using HepG2 Diff medium 48h prior to infection. The HBV stocks were kindly provided by Jochen Wettengel. Cells were infected with a MOI of 100 in the presence of 5% PEG in HepG2 Diff medium for 24h.

3.7.11 Isolation of primary immune cells

Splenocytes were collected by crushing through a 100µm cell strainer. Lysis of erythrocytes was performed using 2 ml of ACK lysis buffer for 2 min at RT. After

washing, the cells were resuspended in murine T cell medium and staining for flow cytometry was performed.

For collection of liver-associated lymphocytes (LALs), livers were perfused *in situ* using PBS and subsequently crushed through 100 µm cell strainer. After centrifugation, cell pellets were resuspended in 12 ml collagenase medium (wash medium + 10mg collagenase type IV) and digested for 20 minutes at 37°C. After washing of the cells, a Percoll gradient was performed using PBS-buffered 40% Percoll for resuspension layered onto 4 ml of 80% Percoll in a 15 ml falcon tube. After centrifugation at 1200g for 20 min, Lymphocytes located between Percoll layers were collected and cultivated in murine T cell medium.

3.7.12 xCELLigence experiments

The xCELLigence system is an impedance-based method to monitor cell viability in real-time. Microelectrodes on the bottom of a 96 cell culture plate detect changes in electrical impedance caused by cell growth or detachment. Cell viability is illustrated as a cell index normalized to 1 at the beginning of the co-culture experiment.

3.7.13 Mice experiments

All animal experiments were performed in accordance with the regulations of the Society for Laboratory Animal Science (GV-Solas). All mice were kept at the animal facilities of the Helmholtz Zentrum Munich in a pathogen-free (SPF) facility.

Protein vaccination was performed by intramuscular (i.m.) injection of 10µg HBsAg, 10µg HBcAg and 10µg ci-di-AMP diluted in an appropriate volume. For MVA-Boost, $1x10^7$ plaque-forming units (pfu) of MVA-HBsAg and $1x10^7$ pfu MVA-HBcAg were injected i.m.

3.7.14 Bleeding and serum analysis

Mice were bled from the cheek at the indicated time point. Blood was collected in Microvette 500 LH-Gel tubes and centrifuges at 10.000g for 5 min at RT.

Serum alanine aminotransferase (ALT) activity was measured using Reflotron Reflovet Plus according to the manufacturer's protocol.

HBsAg, HBeAg, anti-HBs and anti-Hbe levels were quantified using the ARCHITECT system according to the manufacturer's protocol.

3.7.15 *Ex vivo* peptide stimulation

LALs or Splenocytes were plated into 96-well plates and stimulated with indicated peptides in a concentration of 1µg/ml. After one hour, Brefeldin A was added and stimulation was continued for up to 16 hours. Subsequently an ICS was performed.

3.7.16 Flow cytometry

Surface staining was performed in 96- well plates using antibodies diluted in a final volume of 50 µl/well in FACS buffer. Cells were incubated with the antibodies for 30 min on ice and in the dark. After washing, cells were resuspended in 200µl FACS buffer and analyzed using a CytoFLEX S flow cytometer.

Dead cells were stained using fixable viability dye eF780 according to the manufacturer's protocol.

For multimer staining MHC I streptamers that bind to HBV-specific TCRs were used. The streptamers were labelled with Strep-Tactin according to the manufacturer's protocol. Labelled multimers were added to cell surface antibody mix, following the protocol for standard surface staining.

Intracellular cytokine staining (ICS) was performed after cell permeabilization using the Fixation/Permeabilization Solution Kit BD Biosciences according to the manufacturer's protocol. Antibodies for intracellular staining were diluted in Perm/Wash buffer and incubated the dark. After washing, cells were resuspended in 200µl Perm/Wash buffer and analyzed using a CytoFLEX s flow cytometer.

4. Figures

5. References

Allerson, C. R., N. Sioufi, R. Jarres, T. P. Prakash, N. Naik, A. Berdeja, L. Wanders, R. H. Griffey, E. E. Swayze, and B. Bhat. 2005. "Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA." *J Med Chem* 48 (4): 901-4. https://doi.org/10.1021/jm049167j. https://www.ncbi.nlm.nih.gov/pubmed/15715458.

Altogen-Biosystems. Accessed 23.07.2020. https://altogen.com/product/liver-in-vivo-transfection-kit/.

Altschul, S. F. 1993. "A protein alignment scoring system sensitive at all evolutionary distances." *J Mol Evol* 36 (3): 290-300. https://doi.org/10.1007/bf00160485.

Backes, S., C. Jager, C. J. Dembek, A. D. Kosinska, T. Bauer, A. S. Stephan, A. Dislers, G. Mutwiri, D. H. Busch, L. A. Babiuk, G. Gasteiger, and U. Protzer. 2016. "Protein-prime/modified vaccinia virus Ankara vector-boost vaccination overcomes tolerance in high-antigenemic HBV-transgenic mice." *Vaccine* 34 (7): 923-32. https://doi.org/10.1016/j.vaccine.2015.12.060. https://www.ncbi.nlm.nih.gov/pubmed/26776470.

Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. "Restoring function in exhausted CD8 T cells during chronic viral infection." *Nature* 439 (7077): 682-7. https://doi.org/10.1038/nature04444.

Bardhan, Kankana, Theodora Anagnostou, and Vassiliki A. Boussiotis. 2016. "The PD1:PD-L1/2 Pathway from Discovery to Clinical Implementation." *Frontiers in immunology* 7: 550-550. https://doi.org/10.3389/fimmu.2016.00550. https://pubmed.ncbi.nlm.nih.gov/28018338

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5149523/.

Belancio, Victoria P., Dale J. Hedges, and Prescott Deininger. 2008. "Mammalian non-LTR retrotransposons: For better or worse, in sickness and in health." *Genome Research* 18 (3): 343-358. https://doi.org/10.1101/gr.5558208. http://genome.cshlp.org/content/18/3/343.abstract.

Bertoletti, A., and C. Ferrari. 2016. "Adaptive immunity in HBV infection." *J Hepatol* 64 (1 Suppl): S71-S83. https://doi.org/10.1016/j.jhep.2016.01.026. https://www.ncbi.nlm.nih.gov/pubmed/27084039

https://www.journal-of-hepatology.eu/article/S0168-8278(16)00062-3/fulltext.

Birmingham, A., E. Anderson, K. Sullivan, A. Reynolds, Q. Boese, D. Leake, J. Karpilow, and A. Khvorova. 2007. "A protocol for designing siRNAs with high functionality and specificity." *Nat Protoc* 2 (9): 2068-78. https://doi.org/10.1038/nprot.2007.278. https://www.ncbi.nlm.nih.gov/pubmed/17853862

https://www.nature.com/articles/nprot.2007.278.

Boudreau, Ryan L., and Beverly L. Davidson. 2012. "Chapter fourteen - Generation of Hairpin-Based RNAi Vectors for Biological and Therapeutic Application." In *Methods in Enzymology*, edited by Theodore Friedmann, 275-296. Academic Press.

Chen, Ji, Xiao-Mei Wang, Xue-Jie Wu, Yan Wang, Hong Zhao, Bing Shen, and Gui-Qiang Wang. 2011. "Intrahepatic levels of PD-1/PD-L correlate with liver inflammation in chronic hepatitis B." *Inflammation Research* 60 (1): 47-53. https://doi.org/10.1007/s00011-010-0233-1. https://doi.org/10.1007/s00011-010-0233-1.

Chen, Y., J. Sze, and M. L. He. 2004. "HBV cccDNA in patients' sera as an indicator for HBV reactivation and an early signal of liver damage." *World J Gastroenterol* 10 (1): 82-5. https://doi.org/10.3748/wjg.v10.i1.82.

Chiu, Y. L., and T. M. Rana. 2003. "siRNA function in RNAi: a chemical modification analysis." *RNA* 9 (9): 1034-48. https://doi.org/10.1261/rna.5103703. https://www.ncbi.nlm.nih.gov/pubmed/12923253.

Covey, Simon N., Nadia S. Al-Kaff, Amagoia Lángara, and David S. Turner. 1997. "Plants combat infection by gene silencing." *Nature* 385 (6619): 781-782. https://doi.org/10.1038/385781a0. https://doi.org/10.1038/385781a0.

Daneholt, Bertil. 2006. "The Nobel Prize in Physiology or Medicine 2006." Nobel Media. Accessed 04.05.2020. https://www.nobelprize.org/prizes/medicine/2006/advanced-information/.

Darweesh, S. K., A. A. Gad, K. Akroof, and Z. A. ElLatif. 2019. "Entecavir and other nucleos(t)ide analogs prophylaxis in hepatitis B virus-related liver transplantation: long-term efficacy and safety." *Eur J Gastroenterol Hepatol* 31 (5): 607-612. https://doi.org/10.1097/meg.0000000000001377.

Dion, S., M. Bourgine, O. Godon, F. Levillayer, and M. L. Michel. 2013. "Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules." *J Virol* 87 (10): 5554-63. https://doi.org/10.1128/JVI.03134-12. https://www.ncbi.nlm.nih.gov/pubmed/23468504

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3648192/pdf/zjv5554.pdf.

Dokka, Sujatha, David Toledo, Xianglin Shi, Vincent Castranova, and Yon Rojanasakul. 2000. "Oxygen Radical-Mediated Pulmonary Toxicity Induced by Some Cationic Liposomes." *Pharmaceutical Research* 17 (5): 521-525. https://doi.org/10.1023/A:1007504613351. https://doi.org/10.1023/A:1007504613351.

Dong, Yuejiao, Xuefen Li, Lu Zhang, Qiaoyun Zhu, Chunlei Chen, Jiaqi Bao, and Yu Chen. 2019. "CD4+ T cell exhaustion revealed by high PD-1 and LAG-3 expression and the loss of helper T cell function in chronic hepatitis B." *BMC Immunology* 20 (1): 27. https://doi.org/10.1186/s12865-019-0309-9. https://doi.org/10.1186/s12865-019-0309-9.

Dumortier, J., K. Schönig, H. Oberwinkler, R. Löw, T. Giese, H. Bujard, P. Schirmacher, and U. Protzer. 2005. "Liver-specific expression of interferon gamma following adenoviral gene transfer controls hepatitis B virus replication in mice." *Gene Ther* 12 (8): 668-77. https://doi.org/10.1038/sj.gt.3302449.

Durantel, D., and F. Zoulim. 2016. "New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus." *J Hepatol* 64 (1 Suppl): S117-S131. https://doi.org/10.1016/j.jhep.2016.02.016. https://www.ncbi.nlm.nih.gov/pubmed/27084032.

Dutkiewicz, M., H. P. Grunert, H. Zeichhardt, S. W. Lena, J. Wengel, and J. Kurreck. 2008. "Design of LNA-modified siRNAs against the highly structured 5' UTR of coxsackievirus B3." *FEBS Lett* 582 (20): 3061-6. https://doi.org/10.1016/j.febslet.2008.07.051. https://www.ncbi.nlm.nih.gov/pubmed/18691577.

European Association for the Study of the Liver. Electronic address, easloffice easloffice eu, and Liver European Association for the Study of the. 2017. "EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection." *J Hepatol* 67 (2): 370-398. https://doi.org/10.1016/j.jhep.2017.03.021. https://www.ncbi.nlm.nih.gov/pubmed/28427875.

Foster, Donald J., Christopher R. Brown, Sarfraz Shaikh, Casey Trapp, Mark K. Schlegel, Kun Qian, Alfica Sehgal, Kallanthottathil G. Rajeev, Vasant Jadhav, Muthiah Manoharan,

Satya Kuchimanchi, Martin A. Maier, and Stuart Milstein. 2018. "Advanced siRNA Designs Further Improve *In Vivo* Performance of GalNAc-siRNA Conjugates." *Molecular Therapy* 26 (3): 708-717. https://doi.org/10.1016/j.ymthe.2017.12.021. https://doi.org/10.1016/j.ymthe.2017.12.021.

Francisco, Loise M., Victor H. Salinas, Keturah E. Brown, Vijay K. Vanguri, Gordon J. Freeman, Vijay K. Kuchroo, and Arlene H. Sharpe. 2009. "PD-L1 regulates the development, maintenance, and function of induced regulatory T cells." *The Journal of experimental medicine* 206 (13): 3015-3029. https://doi.org/10.1084/jem.20090847. https://pubmed.ncbi.nlm.nih.gov/20008522

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2806460/.

Gane, E. J. 2017. "Future anti-HBV strategies." *Liver Int* 37 Suppl 1: 40-44. https://doi.org/10.1111/liv.13304. https://www.ncbi.nlm.nih.gov/pubmed/28052637

https://onlinelibrary.wiley.com/doi/full/10.1111/liv.13304.

Gane, E., D. J. Verdon, A. E. Brooks, A. Gaggar, A. H. Nguyen, G. M. Subramanian, C. Schwabe, and P. R. Dunbar. 2019. "Anti-PD-1 blockade with nivolumab with and without therapeutic vaccination for virally suppressed chronic hepatitis B: A pilot study." *J Hepatol* 71 (5): 900-907. https://doi.org/10.1016/j.jhep.2019.06.028.

Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. "Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5." *Journal of General Virology* 36 (1): 59-72. https://doi.org/https://doi.org/10.1099/0022-1317-36-1-59. https://www.microbiologyresearch.org/content/journal/jgv/10.1099/0022-1317-36-1-59.

Guidotti, L. G., B. Matzke, H. Schaller, and F. V. Chisari. 1995. "High-level hepatitis B virus replication in transgenic mice." *J Virol* 69 (10): 6158-69.

Haanen, J. B., and C. Robert. 2015. "Immune Checkpoint Inhibitors." *Prog Tumor Res* 42: 55-66. https://doi.org/10.1159/000437178.

He, Jiabei, Ying Hu, Mingming Hu, and Baolan Li. 2015. "Development of PD-1/PD-L1 Pathway in Tumor Immune Microenvironment and Treatment for Non-Small Cell Lung Cancer." *Scientific Reports* 5 (1): 13110. https://doi.org/10.1038/srep13110. https://doi.org/10.1038/srep13110.

Hu, Jianming. 2016. "Hepatitis B Virus Virology and Replication." In *Hepatitis B Virus in Human Diseases*, edited by Yun-Fan Liaw and Fabien Zoulim, 1-34. Cham: Springer International Publishing.

Huang, Ching-Tai, Creg J. Workman, Dallas Flies, Xiaoyu Pan, Aimee L. Marson, Gang Zhou, Edward L. Hipkiss, Sowmya Ravi, Jeanne Kowalski, Hyam I. Levitsky, Jonathan D. Powell, Drew M. Pardoll, Charles G. Drake, and Dario A. A. Vignali. 2004. "Role of LAG-3 in Regulatory T Cells." *Immunity* 21 (4): 503-513. https://doi.org/10.1016/j.immuni.2004.08.010. https://doi.org/10.1016/j.immuni.2004.08.010.

Huang, L. R., Y. A. Gäbel, S. Graf, S. Arzberger, C. Kurts, M. Heikenwalder, P. A. Knolle, and U. Protzer. 2012. "Transfer of HBV genomes using low doses of adenovirus vectors leads to persistent infection in immune competent mice." *Gastroenterology* 142 (7): 1447-50.e3. https://doi.org/10.1053/j.gastro.2012.03.006.

Huard, Bertrand, Philippe Prigent, Muriel Tournier, Denis Bruniquel, and Frédéric Triebel. 1995. "CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins." *European Journal of Immunology* 25 (9): 2718-2721. https://doi.org/10.1002/eji.1830250949. https://onlinelibrary.wiley.com/doi/abs/10.1002/eji.1830250949.

Indolfi, Giuseppe, Philippa Easterbrook, Geoffrey Dusheiko, George Siberry, Mei-Hwei Chang, Claire Thorne, Marc Bulterys, Po-Lin Chan, Manal H. El-Sayed, Carlo Giaquinto, Maureen M. Jonas, Tammy Meyers, Nick Walsh, Stefan Wirth, and Martina Penazzato. 2019. "Hepatitis B virus infection in children and adolescents." *The Lancet Gastroenterology & Hepatology* 4 (6): 466-476. https://doi.org/10.1016/S2468-1253(19)30042-1. https://doi.org/10.1016/S2468-1253(19)30042-1

https://www.thelancet.com/journals/langas/article/PIIS2468-1253(19)30042-1/fulltext

https://www.thelancet.com/pdfs/journals/langas/PIIS2468-1253(19)30042-1.pdf.

Ishigami, M., Y. Ogura, Y. Hirooka, and H. Goto. 2015. "Change of strategies and future perspectives against hepatitis B virus recurrence after liver transplantation." *World J Gastroenterol* 21 (36): 10290-8. https://doi.org/10.3748/wjg.v21.i36.10290. https://www.ncbi.nlm.nih.gov/pubmed/26420956.

Jiang, W. 2012. "Blockade of B7-H1 enhances dendritic cell-mediated T cell response and antiviral immunity in HBV transgenic mice." *Vaccine* 30 (4): 758-66. https://doi.org/10.1016/j.vaccine.2011.11.076. https://www.ncbi.nlm.nih.gov/pubmed/22133510.

Ko, C., A. Chakraborty, W. M. Chou, J. Hasreiter, J. M. Wettengel, D. Stadler, R. Bester, T. Asen, K. Zhang, K. Wisskirchen, J. A. McKeating, W. S. Ryu, and U. Protzer. 2018. "Hepatitis B virus genome recycling and de novo secondary infection events maintain stable cccDNA levels." *J Hepatol* 69 (6): 1231-1241. https://doi.org/10.1016/j.jhep.2018.08.012. https://www.ncbi.nlm.nih.gov/pubmed/30142426.

Ko, C., T. Michler, and U. Protzer. 2017. "Novel viral and host targets to cure hepatitis B." *Curr Opin Virol* 24: 38-45. https://doi.org/10.1016/j.coviro.2017.03.019. https://www.ncbi.nlm.nih.gov/pubmed/28433762.

Konishi, M., C. H. Wu, and G. Y. Wu. 2003. "Inhibition of HBV replication by siRNA in a stable HBV-producing cell line." *Hepatology* 38 (4): 842-50. https://doi.org/10.1053/jhep.2003.50416.

Kosinska, A. D., J. Liu, M. Lu, and M. Roggendorf. 2015. "Therapeutic vaccination and immunomodulation in the treatment of chronic hepatitis B: preclinical studies in the woodchuck." *Med Microbiol Immunol* 204 (1): 103-14. https://doi.org/10.1007/s00430-014-0379-5.

Kumada, H., K. Koike, K. Suyama, H. Ito, H. Itoh, and W. Sugiura. 2017. "Efficacy and safety of tenofovir disoproxil fumarate rescue therapy for chronic hepatitis B patients who failed other nucleos(t)ide analogs." *Hepatol Res* 47 (10): 1032-1041. https://doi.org/10.1111/hepr.12842. https://www.ncbi.nlm.nih.gov/pubmed/27862721.

Kurreck, Jens. 2008. "Chapter 1 The Role of Backbone Modifications in Oligonucleotide-Based Strategies." In *Therapeutic Oligonucleotides*, 1-22. The Royal Society of Chemistry.

Liu, J., E. Zhang, Z. Ma, W. Wu, A. Kosinska, X. Zhang, I. Moller, P. Seiz, D. Glebe, B. Wang, D. Yang, M. Lu, and M. Roggendorf. 2014. "Enhancing virus-specific immunity in vivo by combining therapeutic vaccination and PD-L1 blockade in chronic hepadnaviral infection." *PLoS Pathog* 10 (1): e1003856. https://doi.org/10.1371/journal.ppat.1003856. https://www.ncbi.nlm.nih.gov/pubmed/24391505

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3879364/pdf/ppat.1003856.pdf.

Lok, A. S., B. J. McMahon, R. S. Brown, Jr., J. B. Wong, A. T. Ahmed, W. Farah, J. Almasri, F. Alahdab, K. Benkhadra, M. A. Mouchli, S. Singh, E. A. Mohamed, A. M. Abu Dabrh, L. J. Prokop, Z. Wang, M. H. Murad, and K. Mohammed. 2016. "Antiviral therapy for

chronic hepatitis B viral infection in adults: A systematic review and meta-analysis." *Hepatology* 63 (1): 284-306. https://doi.org/10.1002/hep.28280. https://www.ncbi.nlm.nih.gov/pubmed/26566246.

Lorenz, Christina, Philipp Hadwiger, Matthias John, Hans-Peter Vornlocher, and Carlo Unverzagt. 2004. "Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells." *Bioorganic & Medicinal Chemistry Letters* 14 (19): 4975-4977. https://doi.org/https://doi.org/10.1016/j.bmcl.2004.07.018. http://www.sciencedirect.com/science/article/pii/S0960894X04009084.

Lucifora, J., and U. Protzer. 2016. "Attacking hepatitis B virus cccDNA--The holy grail to hepatitis B cure." *J Hepatol* 64 (1 Suppl): S41-S48. https://doi.org/10.1016/j.jhep.2016.02.009. https://www.ncbi.nlm.nih.gov/pubmed/27084036.

Maier, H., M. Isogawa, G. J. Freeman, and F. V. Chisari. 2007. "PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific CD8+ T lymphocytes in the liver." *J Immunol* 178 (5): 2714-20. https://www.ncbi.nlm.nih.gov/pubmed/17312113

http://www.jimmunol.org/content/jimmunol/178/5/2714.full.pdf.

Mason, W. S. 2015. "Animal models and the molecular biology of hepadnavirus infection." *Cold Spring Harb Perspect Med* 5 (4). https://doi.org/10.1101/cshperspect.a021352.

Matranga, Christian, Yukihide Tomari, Chanseok Shin, David P. Bartel, and Phillip D. Zamore. 2005. "Passenger-Strand Cleavage Facilitates Assembly of siRNA into Ago2-Containing RNAi Enzyme Complexes." *Cell* 123 (4): 607-620. https://doi.org/10.1016/j.cell.2005.08.044. https://doi.org/10.1016/j.cell.2005.08.044.

McMahon, B. J. 2009. "The natural history of chronic hepatitis B virus infection." *Hepatology* 49 (5 Suppl): S45-55. https://doi.org/10.1002/hep.22898. https://www.ncbi.nlm.nih.gov/pubmed/19399792.

Meng, Z., and M. Lu. 2017. "RNA Interference-Induced Innate Immunity, Off-Target Effect, or Immune Adjuvant?" *Front Immunol* 8: 331. https://doi.org/10.3389/fimmu.2017.00331. https://www.ncbi.nlm.nih.gov/pubmed/28386261

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5362589/pdf/fimmu-08-00331.pdf.

Mette, M. F., W. Aufsatz, J. van der Winden, M. A. Matzke, and A. J. Matzke. 2000. "Transcriptional silencing and promoter methylation triggered by double-stranded RNA." *Embo j* 19 (19): 5194-201. https://doi.org/10.1093/emboj/19.19.5194.

Michler, T., A. D. Kosinska, J. Festag, T. Bunse, J. Su, M. Ringelhan, H. Imhof, D. Grimm, K. Steiger, C. Mogler, M. Heikenwalder, M. L. Michel, C. A. Guzman, S. Milstein, L. Sepp-Lorenzino, P. Knolle, and U. Protzer. 2020. "Knockdown of Virus Antigen Expression Increases Therapeutic Vaccine Efficacy in High-titer HBV Carrier Mice." *Gastroenterology*. https://doi.org/10.1053/j.gastro.2020.01.032. https://www.ncbi.nlm.nih.gov/pubmed/32001321.

Morishita, A., K. Fujita, H. Iwama, T. Chiyo, S. Fujihara, K. Oura, T. Tadokoro, S. Mimura, T. Nomura, J. Tani, H. Yoneyama, K. Kobayashi, H. Kamada, Y. Guan, A. Nishiyama, K. Okano, Y. Suzuki, T. Himoto, K. Shimotohno, and T. Masaki. 2020. "Role of microRNA-210-3p in hepatitis B virus-related hepatocellular carcinoma." *Am J Physiol Gastrointest Liver Physiol* 318 (3): G401-G409. https://doi.org/10.1152/ajpgi.00269.2019. https://www.ncbi.nlm.nih.gov/pubmed/31905024.

Napoli, C., C. Lemieux, and R. Jorgensen. 1990. "Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans." *Plant Cell* 2 (4): 279-289. https://doi.org/10.1105/tpc.2.4.279.

Nijampatnam, B., and D. C. Liotta. 2019. "Recent advances in the development of HBV capsid assembly modulators." *Curr Opin Chem Biol* 50: 73-79. https://doi.org/10.1016/j.cbpa.2019.02.009.

Okazaki, Taku, Shunsuke Chikuma, Yoshiko Iwai, Sidonia Fagarasan, and Tasuku Honjo. 2013. "A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application." *Nature Immunology* 14 (12): 1212-1218. https://doi.org/10.1038/ni.2762. https://doi.org/10.1038/ni.2762.

Park, J. J., D. K. Wong, A. S. Wahed, W. M. Lee, J. J. Feld, N. Terrault, M. Khalili, R. K. Sterling, K. V. Kowdley, N. Bzowej, D. T. Lau, W. R. Kim, C. Smith, R. L. Carithers, K. W. Torrey, J. W. Keith, D. L. Levine, D. Traum, S. Ho, M. E. Valiga, G. S. Johnson, E. Doo, A. S. Lok, K. M. Chang, and B. Research Network Hepatitis. 2016. "Hepatitis B Virus--Specific and Global T-Cell Dysfunction in Chronic Hepatitis B." *Gastroenterology* 150 (3): 684-695 e5. https://doi.org/10.1053/j.gastro.2015.11.050. https://www.ncbi.nlm.nih.gov/pubmed/26684441

https://www.gastrojournal.org/article/S0016-5085(15)01736-9/pdf.

Pecot, Chad V., George A. Calin, Robert L. Coleman, Gabriel Lopez-Berestein, and Anil K. Sood. 2011. "RNA interference in the clinic: challenges and future directions." *Nature reviews. Cancer* 11 (1): 59-67. https://doi.org/10.1038/nrc2966. https://pubmed.ncbi.nlm.nih.gov/21160526

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3199132/.

Peng, G., S. Li, W. Wu, X. Tan, Y. Chen, and Z. Chen. 2008. "PD-1 upregulation is associated with HBV-specific T cell dysfunction in chronic hepatitis B patients." *Mol Immunol* 45 (4): 963-70. https://doi.org/10.1016/j.molimm.2007.07.038.

Pépin, J., C. N. Abou Chakra, E. Pépin, V. Nault, and L. Valiquette. 2014. "Evolution of the global burden of viral infections from unsafe medical injections, 2000-2010." *PLoS One* 9 (6): e99677. https://doi.org/10.1371/journal.pone.0099677.

Protzer, U. 2017. "Viral hepatitis: The bumpy road to animal models for HBV infection." *Nat Rev Gastroenterol Hepatol* 14 (6): 327-328. https://doi.org/10.1038/nrgastro.2017.44. https://www.ncbi.nlm.nih.gov/pubmed/28400622

https://www.nature.com/articles/nrgastro.2017.44.

Qazi, Shefah, Christopher J. Schlicksup, Jonathan Rittichier, Michael S. VanNieuwenhze, and Adam Zlotnick. 2018. "An Assembly-Activating Site in the Hepatitis B Virus Capsid Protein Can Also Trigger Disassembly." *ACS Chemical Biology* 13 (8): 2114-2120. https://doi.org/10.1021/acschembio.8b00283. https://doi.org/10.1021/acschembio.8b00283.

Rodríguez-Cerdeira, Carmen, Miguel Carnero Gregorio, Adriana López-Barcenas, Elena Sánchez-Blanco, Beatriz Sánchez-Blanco, Gabriella Fabbrocini, Brunilda Bardhi, Ardiana Sinani, and Roberto Arenas Guzman. 2017. "Advances in Immunotherapy for Melanoma: A Comprehensive Review." *Mediators of inflammation* 2017: 3264217-3264217. https://doi.org/10.1155/2017/3264217. https://pubmed.ncbi.nlm.nih.gov/28848246

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5564072/.

Rothe, D., D. Werk, M. Dutkiewicz, S. Schubert, H. P. Grunert, H. Zeichhardt, V. A. Erdmann, H. Fechner, and J. Kurreck. 2008. "Inhibition of picornaviruses by means of RNA interference." *Nucleic Acids Symp Ser (Oxf)* (52): 63-4. https://doi.org/10.1093/nass/nrn032. https://www.ncbi.nlm.nih.gov/pubmed/18776254.

Santel, A., M. Aleku, O. Keil, J. Endruschat, V. Esche, G. Fisch, S. Dames, K. Löffler, M. Fechtner, W. Arnold, K. Giese, A. Klippel, and J. Kaufmann. 2006. "A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium." *Gene Therapy* 13 (16): 1222-1234. https://doi.org/10.1038/sj.gt.3302777. https://doi.org/10.1038/sj.gt.3302777.

Schaefer, S. 2007. "Hepatitis B virus taxonomy and hepatitis B virus genotypes." *World J Gastroenterol* 13 (1): 14-21. https://doi.org/10.3748/wjg.v13.i1.14.

Schonrich, G., and M. J. Raftery. 2019. "The PD-1/PD-L1 Axis and Virus Infections: A Delicate Balance." *Front Cell Infect Microbiol* 9: 207. https://doi.org/10.3389/fcimb.2019.00207. https://www.ncbi.nlm.nih.gov/pubmed/31263684

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6584848/pdf/fcimb-09-00207.pdf.

Schuppan, D., M. Ashfaq-Khan, A. T. Yang, and Y. O. Kim. 2018. "Liver fibrosis: Direct antifibrotic agents and targeted therapies." *Matrix Biol* 68-69: 435-451. https://doi.org/10.1016/j.matbio.2018.04.006.

Seeger, C., and W. S. Mason. 2015. "Molecular biology of hepatitis B virus infection." *Virology* 479-480: 672-86. https://doi.org/10.1016/j.virol.2015.02.031. https://www.ncbi.nlm.nih.gov/pubmed/25759099.

Sharpe, Arlene H., E. John Wherry, Rafi Ahmed, and Gordon J. Freeman. 2007. "The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection." *Nature Immunology* 8 (3): 239-245. https://doi.org/10.1038/ni1443. https://doi.org/10.1038/ni1443.

Sheppard, Kelly-Ann, Lori J. Fitz, Julie M. Lee, Christina Benander, Judith A. George, Joe Wooters, Yongchang Qiu, Jason M. Jussif, Laura L. Carter, Clive R. Wood, and Divya Chaudhary. 2004. "PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3ζ signalosome and downstream signaling to PKCθ." *FEBS Letters* 574 (1): 37-41. https://doi.org/https://doi.org/10.1016/j.febslet.2004.07.083. http://www.sciencedirect.com/science/article/pii/S0014579304009779.

Singh, Aishwarya, Piyush Trivedi, and Narendra Kumar Jain. 2018. "Advances in siRNA delivery in cancer therapy." *Artificial Cells, Nanomedicine, and Biotechnology* 46 (2): 274-283. https://doi.org/10.1080/21691401.2017.1307210. https://doi.org/10.1080/21691401.2017.1307210

https://www.tandfonline.com/doi/pdf/10.1080/21691401.2017.1307210?needAccess=true.

Soutschek, J., A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, and H. P. Vornlocher. 2004. "Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs." *Nature* 432 (7014): 173-8. https://doi.org/10.1038/nature03121. https://www.ncbi.nlm.nih.gov/pubmed/15538359.

Spain, L., S. Diem, and J. Larkin. 2016. "Management of toxicities of immune checkpoint inhibitors." *Cancer Treat Rev* 44: 51-60. https://doi.org/10.1016/j.ctrv.2016.02.001. https://www.ncbi.nlm.nih.gov/pubmed/26874776

https://www.cancertreatmentreviews.com/article/S0305-7372(16)00016-5/fulltext.

Springer, A. D., and S. F. Dowdy. 2018. "GalNAc-siRNA Conjugates: Leading the Way for Delivery of RNAi Therapeutics." *Nucleic Acid Ther* 28 (3): 109-118. https://doi.org/10.1089/nat.2018.0736.

Swevers, L., J. Liu, and G. Smagghe. 2018. "Defense Mechanisms against Viral Infection in Drosophila: RNAi and Non-RNAi." *Viruses* 10 (5). https://doi.org/10.3390/v10050230.

Terrault, N. A., N. H. Bzowej, K. M. Chang, J. P. Hwang, M. M. Jonas, M. H. Murad, and Diseases American Association for the Study of Liver. 2016. "AASLD guidelines for treatment of chronic hepatitis B." *Hepatology* 63 (1): 261-83. https://doi.org/10.1002/hep.28156. https://www.ncbi.nlm.nih.gov/pubmed/26566064.

Velkov, Stoyan, Jördis J. Ott, Ulrike Protzer, and Thomas Michler. 2018. "The Global Hepatitis B Virus Genotype Distribution Approximated from Available Genotyping Data." *Genes* 9 (10): 495. https://doi.org/10.3390/genes9100495. https://pubmed.ncbi.nlm.nih.gov/30326600

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6210291/.

Vishnoi, A., and S. Rani. 2017. "MiRNA Biogenesis and Regulation of Diseases: An Overview." *Methods Mol Biol* 1509: 1-10. https://doi.org/10.1007/978-1-4939-6524-3_1.

Volpe, T. A., C. Kidner, I. M. Hall, G. Teng, S. I. Grewal, and R. A. Martienssen. 2002. "Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi." *Science* 297 (5588): 1833-7. https://doi.org/10.1126/science.1074973.

Volz, T., L. Allweiss, MBarek M. Ben, M. Warlich, A. W. Lohse, J. M. Pollok, A. Alexandrov, S. Urban, J. Petersen, M. Lutgehetmann, and M. Dandri. 2013. "The entry inhibitor Myrcludex-B efficiently blocks intrahepatic virus spreading in humanized mice previously infected with hepatitis B virus." *J Hepatol* 58 (5): 861-7. https://doi.org/10.1016/j.jhep.2012.12.008. https://www.ncbi.nlm.nih.gov/pubmed/23246506.

Waterhouse, Paul, Josef M. Penninger, Emma Timms, Andrew Wakeham, Arda Shahinian, Kelvin P. Lee, Craig B. Thompson, Henrik Griesser, and Tak W. Mak. 1995. "Lymphoproliferative Disorders with Early Lethality in Mice Deficient in Ctla-4." *Science* 270 (5238): 985-988. https://doi.org/10.1126/science.270.5238.985. https://science.sciencemag.org/content/sci/270/5238/985.full.pdf.

WHO. 2017. "Global Hepatitis Report." World Health Organization. http://apps.who.int/iris/bitstream/10665/255016/1/9789241565455-eng.

Wisskirchen, Karin, Kai Metzger, Sophia Schreiber, Theresa Asen, Luise Weigand, Christina Dargel, Klaus Witter, Elisa Kieback, Martin F. Sprinzl, Wolfgang Uckert, Matthias Schiemann, Dirk H. Busch, Angela M. Krackhardt, and Ulrike Protzer. 2017. "Isolation and functional characterization of hepatitis B virus-specific T-cell receptors as new tools for experimental and clinical use." *PloS one* 12 (8): e0182936-e0182936. https://doi.org/10.1371/journal.pone.0182936. https://pubmed.ncbi.nlm.nih.gov/28792537

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5549754/.

Wittrup, Anders, and Judy Lieberman. 2015. "Knocking down disease: a progress report on siRNA therapeutics." *Nature reviews. Genetics* 16 (9): 543-552. https://doi.org/10.1038/nrg3978. https://pubmed.ncbi.nlm.nih.gov/26281785

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4756474/.

Yan, H., G. Zhong, G. Xu, W. He, Z. Jing, Z. Gao, Y. Huang, Y. Qi, B. Peng, H. Wang, L. Fu, M. Song, P. Chen, W. Gao, B. Ren, Y. Sun, T. Cai, X. Feng, J. Sui, and W. Li. 2012. "Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus." *Elife* 3. https://doi.org/10.7554/eLife.00049. https://www.ncbi.nlm.nih.gov/pubmed/25409679.

Yan, Zhipeng, Jing Zeng, Youjun Yu, Kunlun Xiang, Hui Hu, Xue Zhou, Lili Gu, Li Wang, Jie Zhao, John A. T. Young, and Lu Gao. 2017. "HBVcircle: A novel tool to investigate hepatitis B virus covalently closed circular DNA." *Journal of Hepatology* 66 (6): 1149-1157. https://doi.org/https://doi.org/10.1016/j.jhep.2017.02.004. http://www.sciencedirect.com/science/article/pii/S0168827817300727.

Zhang, B. Y., D. P. Chai, Y. H. Wu, L. P. Qiu, Y. Y. Zhang, Z. H. Ye, and X. P. Yu. 2019. "Potential Drug Targets Against Hepatitis B Virus Based on Both Virus and Host Factors." *Curr Drug Targets* 20 (16): 1636-1651. https://doi.org/10.2174/1389450120666190729115646. https://www.ncbi.nlm.nih.gov/pubmed/31362671.

Acknowledgement

I would like to sincerely thank my supervisor Prof. Dr. Ulrike Protzer for giving me the opportunity to do my thesis on this interesting topic. She supported me during the whole project and her scientific advice helped me become a better researcher.

My special thanks go to Prof. Dr. Stefan Engelhardt for being my second supervisor and his valuable discussions during the thesis committee meetings.

I am also grateful to Dr. Thomas Michler for being my mentor. I could always count on his scientific input and advice, without him I would have been lost most of the time.

A big thanks to all members of the Group who always helped me with my experiments, especially Dr. Anindita Chakraborty and Dr. Anna Kosinska.

I also want to thank all my friends for supporting me and being a big part of my life.

At last, I would like to thank my family for their continuous support along the way.