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HARNESSING T CELL IMMUNITY FOR THE PREVENTION AND TREATMENT OF LIVER CANCER

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Harnessing T cell Immunity for the Prevention and Treatment of Liver Cancer

Thesis for Doctoral Degree (Ph.D.)

By

Panagiota Maravelia

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Popular science summary of the thesis

Liver cancer ranks as the sixth most prevalent cancer on a global scale and the third leading cause of cancer-associated fatalities. With few improvements in the survival rates during the last decades, prevention is key in reducing its burden globally, together with development of novel therapies which are required for the advanced stages of the disease. Infection with hepatitis B virus (HBV) remains the main risk factor for developing liver cancer. Despite the existence of effective prophylactic vaccines against HBV, yet about 290 million people are chronic HBV carriers. Among those, up to 60 million carriers live with the risk of acquiring a hepatitis D virus (HDV) infection which if becomes chronic, triplicates the risk of developing liver cancer within 5-10 years after diagnosis.

During chronic infections and cancer, our immune system becomes compromised and is not capable of efficiently fighting chronic diseases. To address this problem, various therapies called immunotherapies, aiming at activating, boosting, or restoring the host's immune responses have been widely tested and employed in clinical practice. In this context, a type of white blood cells, called T cells play a major role as they are capable of killing infected and tumor cells. This requires that T cells recognize parts of a virus or tumor, that act as a unique flag of these infected/tumor cells and are called antigens. When the receptor of T cells (T cell receptor; TCR) recognizes and interacts with these antigens, then they become activated and capable of eliminating these threats.

In the current thesis, we have utilized two types of T cell-based immunotherapies for the prevention and treatment of liver cancer. The first type is based on a therapeutic vaccine for chronic HBV and HDV, as a strategy to prevent the onset of liver cancer. The second type is based on the identification of cancer-specific TCRs from patients with liver cancer. These TCRs can be used to genetically redirect a substantial amount of patients T cells to more efficiently recognize and kill tumor cells, hence offering a potential new treatment for advanced liver cancer.

The first two studies of the thesis focus on the development and optimization of a therapeutic vaccine targeting HBV and HDV infections. Following vaccinations in mice and rabbits, we show that our vaccine was safe and could efficiently activate T cells and antibody responses which are both required in order to eliminate infected cells and control the infection in the liver. Additionally, the vaccine was able to bypass the viral-induced T cell dysfunction and support antibody production in a setting of chronic infection. The antibodies induced by the vaccine could prevent HBV and HDV co-infection in liver-humanized mice (i.e. mice repopulated with human hepatocytes). Importantly, they could also protect HBV infected liver-humanized mice from superinfection with HDV, which is the most pathogenic hepatitis virus infection. Taken together, these findings suggest that the vaccine is safe and immunogenic and can efficiently complement current and future therapies stepping towards a functional cure for HBV and HDV infection. In the third study, we aimed to identify cancer-specific antigens from patients with liver cancer that can lead to activation of cancer-reactive T cells. We detected T cell responses against cancer-specific antigens in 4 out of 7 screened patients and isolated (putative) cancer-reactive TCRs for further evaluation of their expression and specificity. These cancer-specific TCRs could be utilized to genetically redirect a substantial quantity of patients T cells to more efficiently recognize and kill cancer cells when re-infused back to the patient.

In conclusion, this thesis illuminates two promising types of T cell-based immunotherapies and provides novel insights in the development of preventive and therapeutic tools aiming at conquering liver cancer.

Popular science summary of the thesis (in Greek)

Ο καρκίνος του ήπατος κατατάσσεται παγκοσμίως ως ο έκτος πιο συχνός καρκίνος και η τρίτη κύρια αιτία θανάτων από καρκίνο. Με λίγα επιτεύγματα στη βελτίωση των ποσοστών επιβίωσης τις τελευταίες δεκαετίες, η πρόληψη θεωρείται κλειδί για την μείωση των κρουσμάτων, καθώς και η ανάπτυξη νέων θεραπειών που απαιτούνται για την αντιμετώπιση των προχωρημένων σταδίων της νόσου. Η μόλυνση από τον ιό της ηπατίτιδας βήτα παραμένει ο κύριος κίνδυνος για την ανάπτυξη καρκίνου του ήπατος. Παρά την ύπαρξη ενός αποτελεσματικού προφυλακτικού εμβολίου κατά του ιού, περίπου 290 εκατομμύρια άνθρωποι είναι χρόνιοι φορείς ηπατίτιδας Β. Ανάμεσα σε αυτούς τους φορείς, έως 60 εκατομμύρια ζουν με τον κίνδυνο να συν-μολυνθούν από τον ιό της ηπατίτιδας δέλτα, η οποία μπορεί να τριπλασιάσει τον κίνδυνο ανάπτυξης καρκίνου του ήπατος εντός 5-10 ετών από τη διάγνωση.

Ασθένειες, όπως οι χρόνιες λοιμώξεις και ο καρκίνος, υποβαθμίζουν τη λειτουργία του ανοσοποιητικού μας συστήματος. Για την αντιμετώπιση αυτού του προβλήματος, διάφορες θεραπείες που ονομάζονται ανοσοθεραπείες έχουν δοκιμαστεί ευρέως σε κλινικές δοκιμές, και στοχεύουν στην ενεργοποίηση, ενίσχυση ή την αποκατάσταση των ανοσολογικών αποκρίσεων του πάσχοντα οργανισμού. Ένας τύπος λευκών αιμοσφαιρίων, που ονομάζονται Τ λεμφοκύτταρα, διαδραματίζουν καθοριστικό ρόλο στην καταπολέμηση ασθενειών, καθώς είναι ικανά να εξαλείφουν μολυσμένα και καρκινικά κύτταρα. Αυτό πραγματοποιείται όταν τα Τ κύτταρα διακρίνουν "μοτίβα" ενός ιού ή όγκου, τα οποία ονομάζονται αντιγόνα. Όταν λοιπόν ένας λειτουργικός υποδοχέας των Τ κυττάρων αναγνωρίσει αυτά τα αντιγόνα, τότε τα Τ κύτταρα ενεργοποιούνται για να εξαλείψουν παθογόνες και καρκινικές απειλές.

Οι δύο πρώτες μελέτες της παρούσας διατριβής επικεντρώνονται στην ανάπτυξη ενός νέου θεραπευτικού εμβολίου κατά των ιών της ηπατίτιδας Β και Δ, ως στρατηγική για την πρόληψη της εμφάνισης καρκίνου του ήπατος. Εμβολιασμοί σε ποντίκια και κουνέλια, έδειξαν ότι το εμβόλιο μας είναι ασφαλές και μπορεί να ενεργοποιήσει αποτελεσματικά τα Τ κύτταρα και αντισώματα που απαιτούνται για την εξάλειψη του ιού και για τον έλεγχο της μόλυνσης στο ήπαρ. Επιπλέον, το εμβόλιο μπόρεσε να

παρακάμψει τη δυσλειτουργία των Τ κυττάρων που προκαλείται από τον ιό και να υποστηρίξει την παραγωγή αντισωμάτων σε ένα περιβάλλον χρόνιας μόλυνσης. Τα αντισώματα που αναπτύχθηκαν από το εμβόλιο μπόρεσαν να αποτρέψουν τη συν–λοίμωξη από τους ιούς της ηπατίτιδας Β και Δ. Αξιοσημείωτο είναι το ότι τα αντισώματα αυτά μπόρεσαν επίσης να προστατεύσουν ποντίκια που έχουν ″ανθρωποποιημένο″ ήπαρ (ήπαρ με ανθρώπινα ηπατοκύτταρα) μολυσμένο με τον ιό της ηπατίτιδας Β, από το να συν–μολυνθούν με μια επιζήμια χρόνια λοίμωξη με ιό της ηπατίτιδας Δ. Συνολικά, αυτά τα ευρήματα αποδεικνύουν ότι το εμβόλιο μας είναι ασφαλές και ανοσογόνο και μπορεί να συμπληρώσει αποτελεσματικά τρέχουσες και μελλοντικές θεραπείες που βαδίζουν προς μια λειτουργική θεραπεία κατά του ιού της ηπατίτιδας Β και Δ.

Στην τρίτη μελέτη, στοχεύσαμε να ταυτοποιήσουμε καρκινικά αντιγόνα που μπορούν να αναγνωριστούν από τους υποδοχείς των Τ κυττάρων σε ασθενείς με καρκίνο του ήπατος. Ανιχνεύσαμε ανοσολογικές αποκρίσεις Τ λεμφοκυττάρων έναντι καρκινικών αντιγόνων σε 4 από τους 7 ασθενείς που υποβλήθηκαν σε διαλογή και απομονώσαμε τους υποδοχείς που αναγνωρίζουν καρκινικά αντιγόνα για περαιτέρω αξιολόγηση της έκφρασης και της ειδικότητας τους. Αυτοί οι υποδοχείς μπορούν να χρησιμοποιηθούν για να ανακατευθύνουν γενετικά ένα μεγάλο αριθμό κυτταροτοξικών Τ λεμφοκυττάρων των ασθενών, προκειμένου να αναγνωρίσουν και να εξαλείψουν καρκινικά κύτταρα, προσφέροντας επομένως μια πιθανή νέα θεραπεία για προχωρημένα στάδια καρκίνου του ήπατος.

Συμπερασματικά, αυτή η διατριβή φωτίζει δύο πολλά υποσχόμενους τύπους ανοσοθεραπειών με βάση τα Τ λεμφοκύτταρα και παρέχει καινοτόμες γνώσεις για την ανάπτυξη προληπτικών και θεραπευτικών εργαλείων με στόχο την καταπολέμηση του καρκίνου του ήπατος.

Abstract

Hepatocellular carcinoma (HCC) accounts for more than 80% of all diagnosed cases of liver cancer which is a major cause of cancer related fatalities worldwide. With few improvements in the survival rates during the last decades, prevention of HCC is key in reducing its burden globally. Infection with hepatitis B virus (HBV) remains the main etiological risk factor for developing HCC whilst in HBV patients co-infected with hepatitis D virus (HDV), the risk of developing HCC is triplicated due to the accelerated liver disease progression. In studies I and II, we aimed to develop a therapeutic vaccine for chronic HBV and HDV, as a preventive strategy for HCC. In study III, we sought to unlock novel T cell-based immunotherapies, as treatment for advanced HCC through isolation of neoantigen-driven T cell receptors (TCRs).

In study I, we show that a homologous preS1-HDAg DNA-based vaccine strategy was able to elicit robust T cell responses to HBV and HDV antigens and entry-inhibiting antibodies that could limit HBV monoinfection in liver-humanized mice. In study II, a heterologous DNA prime and protein boost preS1-HDAg vaccine strategy improved immunogenicity and could circumvent the HBV-induced tolerance present in the chronically infected host. Additionally, vaccine-induced antibodies protected liver-humanized mice against a chronic HBV/HDV co-infection and importantly they could protect HBV infected human-liver mice from HDV superinfection. In study III, we studied the cancer-specific T cell responses in patients with HCC, and we could detect T cell reactivity against mutated neoantigens in 4 out of 7 screened HCC patients. We isolated (putative) tumor-reactive TCRs for further evaluation of their expression and specificity. Neoantigen-specific TCRs could be utilized to genetically redirect a substantial quantity of T cells against tumor cells, thus offering a potential new treatment for advanced HCC.

Taken together, as we continue to unravel the dynamics of the immune system and refine therapies in the context of chronic diseases, this thesis illuminates two promising T cell avenues in the form of active and passive T cell immunotherapy

and provides novel insights in the development of preventive and therapeutic tools aiming at combatting liver cancer.

List of scientific papers

I. **Panagiota Maravelia**, Lars Frelin, Yi Ni, Noelia Caro Pérez, Gustaf Ahlén, Neetu Jagya, Georg Verch, Lieven Verhoye, Lena Pater, Magnus Johansson, Anna Pasetto, Philip Meuleman, Stephan Urban, and Matti Sällberg. *"Blocking Entry of Hepatitis B and D Viruses to Hepatocytes as a Novel Immunotherapy for Treating Chronic Infections"*. J Infect Dis. 2021 Jan 4;223(1):128-138

II. Rani Burm*, **Panagiota Maravelia***, Gustaf Ahlen, Sandra Ciesek, Noelia Caro Perez, Anna Pasetto, Stephan Urban, Freya Van Houtte, Lieven Verhoye, Heiner Wedemeyer, Magnus Johansson, Lars Frelin, Matti Sällberg, Philip Meuleman. *"Novel prime-boost immune-based therapy inhibiting both hepatitis B and D virus infections"*. Gut 2023 Jun;72(6):1186-1195

III. **Panagiota Maravelia**, Haidong Yao, Daniela Nascimento Silva, Curtis Cai, Yong-Chen Lu, Ola Nilsson, André Perez Potti, Francesca Gatto, Giulia Rovesti, Mattias Carlsten, Matti Sällberg, Per Stål, Carl Jorns, Marcus Buggert, Anna Pasetto. *"Unlocking Novel T Cell-Based Immunotherapy for Hepatocellular Carcinoma through Neoantigen-Driven T Cell Receptor Isolation"*. Manuscript.

*Shared contribution

Scientific papers not included in the thesis

I. Alejandro Fernandez, **Panagiota Maravelia**, Francesca Gatto, Giulia Rovesti, Chiara Chiavelli, Michael Chrobok, Daniela Nascimento Silva, Marcus Buggert, Per Stål, Carl Jorns, Ulrika Sandvik, Jonas Fuxe, Gustaf Ahlén, Matti Sällberg, Massimo Dominici, Guro Gafvelin, Hans Grönlund, Ola Nilsson and Anna Pasetto. *"In dept analysis of genetic alterations in solid cancers utilizing PIOR®: relevance for neoantigen discovery"*. Manuscript.

II. Daniela Nascimento Silva, Michael Chrobok, Giulia Rovesti, Katie Healy, Arnika Kathleen Wagner, **Panagiota Maravelia**, Francesca Gatto, Massimiliano Mazza, Lucia Mazzotti, Volker Lohmann, Margaret Sällberg Chen, Matti Sällberg, Marcus Buggert, Anna Pasetto. *"Process Development for Adoptive Cell Therapy in Academia: A Pipeline for Clinical-Scale Manufacturing of Multiple TCR-T Cell Products"*. Front Immunol 2022 Jun 16:13:896242

III. **Panagiota Maravelia**, Daniela Nascimento Silva, Giulia Rovesti, Michael Chrobok, Per Stål, Yong-Chen Lu, Anna Pasetto. *"Liquid Biopsy in Hepatocellular Carcinoma: Opportunities and Challenges for Immunotherapy"*. Cancers (Basel) 2021 Aug 27;13(17):4334

IV. Banu Batyrova, Fien Luwaert, **Panagiota Maravelia**, Yuria Miyabayashi, Neha Vashist, Julian M Stark, Sara Y Soori, Christopher A Tibbitt, Peggy Riese, Jonathan M Coquet, Benedict J Chambers. *"PD-1 expression affects cytokine production by ILC2 and is influenced by peroxisome proliferator-activated receptor-γ"*. Immun Inflamm Dis 2020 Mar;8(1):8-23

Contents

List of abbreviations

aa	Amino acid
ACT	Adoptive cell therapy
AFP	Alpha-fetoprotein
Bim	BCL2-interacting mediator
BTLA	B and T lymphocyte attenuator
CAR	Chimeric antigen receptor
cccDNA	Covalently closed circular DNA
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
COSMIC	Catalogue of Somatic Mutations in Cancer
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
ER	Endoplamic reticulum
FACS	Fluorence-activated cell sorting
GPC3	Glypican-3
gt	Genotype
HBsAg	HBV surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAg	Delta antigen
HDV	Hepatitis D virus
HLA	Human leukocyte antigen
ICI	Immune-checkpoint inhibitor
Ig	Immunoglobulin
IL	Interleukin
INDELs	Base insertions and deletions
IVS	In vitro stimulation

L-HBsAg	Large-HBsAg
L-HDAg	Large-HDAg
LAG-3	Lymphocyte-activation gene 3
M-HBsAg	Medium-HBsAg
MAGE	Melanoma-associated genes
MHC	Major histocompatibility complex
NAFLD	Non-alcoholic fatty liver disease
NAs	Nucleoside analogues
NASH	Non-alcoholic steatohepatitis
NTCP	Na^+-taurocholate co-transporting polypeptide
NY-ESO1	New York-esophageal squamous cell carcinoma-1
PD-1	Programmed cell death protein 1
pegIFN	Pegylated IFN
RNA sequencing	RNA-seq
ROS	Reactive oxygen species
S-HBsAg	Small-HBsAg
small HDAg	S-HDAG
SNVs	Single-nucleotide variants
SVPs	Subviral particles
TAAs	Tumor-associated antigens
TCR	T cell receptor
TERT	Telomerase reverse transcriptase
Tg	Transgenic
TGFβ	Transforming growth factor β
TILs	Tumor infiltrating lymphocytes
TIM3	T cell immunoglobulin and mucin domain-containing protein 3
TKIs	Tyrosine kinase inhibitors
TLR	Toll-like receptor

TMB	Tumor mutation burden
TNF	Tumor necrosis factor
TOX	Thymocyte selection-associated high-mobility group box protein
TRAIL	Tumor-necrosis factor related apoptosis-inducing ligand
TS	Targeted sequencing
VEGFR	Vascular endothelial growth factor receptor
WES	Whole-exome sequencing

1 INTRODUCTION

Our immune system offers defence mechanisms to protect us from pathogens such as viruses, parasites, fungi and bacteria. Additionally, it is capable of recognizing self- or mutated self-proteins (antigens) expressed by cancer cells. In this context, specific defence mechanisms of adaptive immunity are crucial. T cells are key players in this process, as they recognize foreign antigens through their T cell receptor (TCR), which interacts with major histocompatibility complex (MHC) molecules expressed on the surface of infected/cancer cells or antigen-presenting cells. Upon TCR recognition, T cells become activated and capable of killing infected and malignant cells or helping with orchestrating other immune cells and functions. Another important cellular component of adaptive immunity is B cells which secrete antibodies able to neutralize infected or malignant cells and generate immunological memory similarly to T cells.

Chronic diseases including viral infections and cancer can compromise the host's immune response leading to a T cell dysfunction. To address this problem, various immunotherapies aiming at activating, augmenting, or restoring the host's immune system have been widely tested and employed in clinical practice for the prevention and treatment of chronic diseases. Such immunotherapies include vaccines, adoptive cell therapies (ACT), immune checkpoint inhibitors (ICIs), cytokine-based therapies, or other immune modulators [1, 2]. Although, each of these immunotherapies have distinct mechanisms of action, they mainly affect immune responses in two ways; either by directly modifying them, as seen with ICIs targeting T cells, or by stimulating adaptive immune surveillance carried out by B and T cells, as exemplified by vaccines.

This thesis focuses on the prevention and treatment of hepatocellular carcinoma (HCC), the predominant form of liver cancer, by harnessing two types of T cell-based immunotherapies: vaccines and adoptive TCR-T cell therapy. The first strategy involves vaccination as a therapeutic tool for eradicating chronic viral hepatitis B and D, thereby preventing the onset of HCC. The second approach is based on the identification of cancer-specific TCRs from HCC patients, which can

be utilized to genetically redirect a substantial quantity of T cells, offering a potential new treatment.

1.1 Hepatocellular Carcinoma (HCC)

1.1.1 Incidence and mortality

HCC accounts for more than 80% of all diagnosed cases of liver cancer which stands as the sixth most prevalent cancer globally and the third leading contributor to cancer-associated fatalities [3, 4]. Its prevalence remains high in Eastern Asia, Northern Africa, and South-Eastern Asia with the highest mortality rates observed in countries of South-East Asia and sub-Saharan Africa [5]. Although much lower, the incidence of HCC in high income countries is expected to rise, as reflected by prevalence of obesity and metabolic complications together with demographic changes [6, 7] while the survival rates remain poor with no significant improvements over the 5-year survival in recent years [5, 8]. If current rates do not change, the incidence of liver cancer is predicted to increase by more than 50% in the next 20 years and 1.4 million new diagnoses forecast by 2040 [5]. With few improvements in the survival rates during the last decades, prevention of HCC is key in reducing its burden globally [5]. To achieve that, attempts are focusing to prevent its main etiological factors, as discussed in the following section.

1.1.2 Etiology: viral and non-viral

Chronic hepatitis B virus (HBV) infection is the primary causative factor behind over 50% of HCC cases while infection by hepatitis C virus (HCV) ranks as the second most significant contributor [9, 10]. Persistent liver inflammation resulting from chronic viral infections can lead to cirrhosis which progresses to HCC (Fig. 1). Notably, in HBV patients co-infected with the hepatitis D virus (HDV) the risk of developing HCC is triplicated due to the accelerated progression of liver disease [11, 12]. On the contrary, HCV-associated HCC has been significantly reduced due to the current treatment with anti-viral drugs that are very effective in achieving a sustained control and clearance of the infection [13]. Nevertheless, in the

presence of liver cirrhosis, the risk for developing HCC persists even in absence of HCV [14].

In addition to viral causes, various non-viral risk factors contribute to the development of HCC such as excessive alcohol consumption and exposure to aflatoxin B1, presence of metabolic disorders including the non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) or immune-related diseases such as autoimmune hepatitis and genetic conditions [15, 16]. Finally, demographic factors have also been associated with HCC incidence including advancing age and gender, with HCC incidence being more prevalent in males [17] (**Fig. 1**).

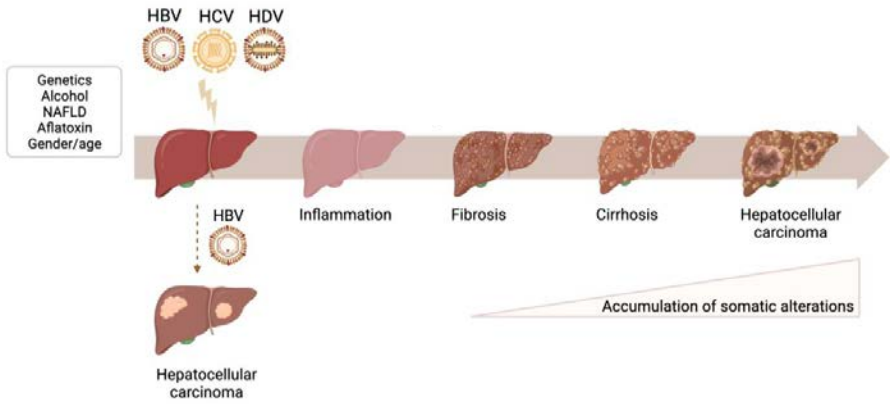

Figure 1. Development of hepatocellular carcinoma (HCC). Non-viral etiology (genetics, exposure to toxins, alcohol consumption, gender/age and metabolic disorders) as well as chronic infection with hepatitis B, C, and D viruses can influence liver disease progression. HCC arises from hepatocyte injury and the persistent, non-resolving chronic inflammation that advances through stages of fibrosis, cirrhosis and cell transformation. Independent of the etiology, cirrhosis remains the main risk factor for progression to HCC. In chronic HBV infection, HCC can also arise in the absence of cirrhosis due to direct oncogenic features of the virus. Chronic coinfection with hepatitis D virus in individuals who are chronic HBV carriers accelerates disease progression to liver failure and HCC. Many driver mutations (discussed in the following section) can occur and accumulate as liver disease progresses to HCC. HBV; hepatitis B virus, HCV; hepatitis C virus, HDV; hepatitis D virus, NAFLD; non-alcoholic fatty-liver disease. Inspired from [18]. Created with BioRender.com

1.1.3 Common somatic mutations

Although the events leading to HCC onset can be rather complex, an accumulation of somatic mutations in driver genes during progression to chronic liver disease has been reported [19, 20]. The most frequent mutations in driver genes identified in HCC involve alterations in several key genes including *TERT* promoter, *TP53*, *CTNNB1*, *ARID1A*, *ARID2*, *AXIN1*, *RPS6KA3*, *NFE2L2*, *KEAP1*, *RB1*, *VEGFA*, and *FGF19* [21, 22]. According to the COSMIC database, additional frequently mutated genes in HCC are *PTEN*, *KMT2C/D*, *SETD2*, *ATM*, *FAT4*, *PTPN13*, *ZNF521*, *CAMTA1*, *PTPRB*, *PREX2*, and *LRP1B* with mutation frequency ranging from 3% to 10%. Notably, *TP53* mutations are the most common occurring in 15%-40% of HCC cases, followed by *CTNNB1* at 10%-35%, *ARID2* at 3%-18%, *ARID1A* at 5%-17% and *AXIN1* at 5%-15%, among others [23]. *TERT* promoter mutations are also particularly important since they are present in approximately 20% of low and high-grade premalignant nodules and rising to as high as 60% in early-stage HCC [24]. These promoter mutations are considered key in HCC occurrence by promoting reactivation of telomerase in premalignant nodules in which there haven't been reported any additional recurrent genetic alterations in other key driver genes [23-25]. Also, no subclonal *TERT* promoter mutations in cirrhotic tissues have been reported so far, reinforcing the central role of *TERT* in initiating and driving HCC transformation in a cirrhotic background [23]. In contrast, in normal liver tissue, other driver genes, including *CTNNB1*, appear to be more influential in liver tumorigenesis [24, 26].

The genes mentioned above can play a major role in tumor progression by altering key signalling pathways and cellular functions including: cell cycle control (*TP53*, *CDKNA2*, *RB1*, *MDM2/MDM4*), the Wnt/β-catenin pathway (*CTNNB1*, *AXIN1*, *APC*, *FGF19*, *MYC*), oxidative stress (*NFE2L2*, *KEAP1*), chromatin modification (*ARID1A/B*, *ARID2*, *KMT2C/D*, *BAP1*, *CREBBP*, *IDH1/2*, *SMARCA4*), and the RTK/RAS/PI3K pathway (*PIK3CA*, *KRAS*, *PTEN*, *NF1*, *VEGFA*, *FGFR1*, *AKT1/2*, *TSC1/2*) [21, 22]. In addition, aberrant expression of genes (e.g. *SPTBN1*) involved in the transforming growth factor β (TGFβ) pathway contribute to HCC pathogenesis with one group of HCC patients showing up-regulation of genes contributing to inflammation and fibrosis,

and another HCC group showing down-regulation of genes with loss of TGFβ tumor suppressor activity [27]. Exome sequencing analysis of 243 liver tumors has revealed strong associations between mutational signatures and specific risk factors and environmental exposure [28]. Moreover, the landscape of driver genes and pathways can vary according to the stage of HCC [28]. Being able to identify genomic alterations in pathways that can be targeted by existing or novel drugs can significantly help to determine which HCC patient groups are the most likely to benefit from targeted treatments in future clinical trials [29, 30].

1.2 Viral-HCC

1.2.1 HBV and HDV infection

HBV is a non-cytopathic, hepatotropic DNA virus belonging to the Hepadnavirus family [31]. Despite the availability of highly effective preventive vaccines, infection with HBV still represents a major global health burden. It is estimated that more than 290 million people are currently HBV chronic carriers [32]. Apart from parenteral exposure, perinatal transmission during birth, early childhood exposure and immigration from highly endemic areas remain the main causes of HBV spread [33].

HDV is a circular, covalently closed single-stranded RNA which encodes for one protein, the delta antigen (HDAg) [34]. This is expressed in two isoforms, the small (S-) and the large (L-) HDAg both serving in different functions in virus replication and envelopment into the HBV surface proteins [35, 36]. HDV is a defective or as otherwise known a "satellite" virus because it requires HBV envelope proteins (**Fig. 2**) for its assembly, hepatocyte entry and subsequent propagation [37-39].

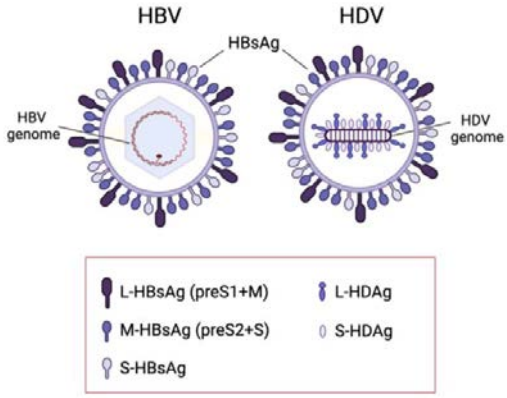

Figure 2. Hepatitis B and D viruses. HBV (DNA virus) and HDV (RNA virus) share the same envelope (HBsAg) protein. HBsAg is composed of three different size proteins, namely small; S-HBsAg protein that is comprised of only the S domain, middle; M-HBsAg that has the preS2 and S domains, and the large; L-HBsAg protein which has three domains in order, preS1, preS2 and S. PreS1 domain of the L-HBsAg is responsible for HBV and HDV hepatocyte entry and subsequent infection. HDAg is the only protein encoded by HDV and is expressed in two isoforms, the small (S-) and the large (L-) HDAg both serving in different functions in virus replication and envelopment into the HBV surface proteins. PreS1 and HDAg are the main components of the HBV/HDV therapeutic vaccine discussed in studies I and II. HBsAg; HBV surface antigen, HDAg; delta antigen. Image created with BioRender.com

Therefore, HDV infection can only exist in presence of HBV and can infect a healthy individual either simultaneously with HBV as coinfection, or after an already established HBV chronic infection as superinfection [40]. Major risk factors for HDV superinfection in chronic HBsAg carriers are intravenous drug use, and exposure to infected blood or blood products [41]. HBV and HDV coinfection is self-limited in more than 90% of immune competent individuals being infected as adults, similarly to an acute HBV monoinfection [35, 42]. In contrast, infections acquired in infancy or early childhood become chronic in the majority of cases [43]. Chronic HBV and HDV coinfection represents the most severe form of all viral hepatitis and significantly exacerbates the risk for developing cirrhosis and HCC [44-46].

1.2.2 Mechanisms of viral persistence: Impact on immune function

HBV enters the hepatocytes via the preS1 domain which is part of the large HBsAg (L-HBsAg) (**Fig. 2**) after binding to the sodium taurocholate co-transporting polypeptide Na^+-taurocholate co-transporting polypeptide (NTCP) receptor expressed by hepatocytes [47]. Following viral entry, the relaxed circular DNA (rcDNA) of HBV is transferred into the nucleus where it serves for the convertion to the covalently closed circular DNA (cccDNA) [48]. This cccDNA comprises the stable form of HBV DNA in infected hepatocytes that is used as template for translation of all viral RNAs and generation of new virions, therefore contributing to HBV persistence and transmission to descendant hepatocytes [49]. Integration of HBV sequences into the host's genome, although does not affect viral replication, supports the expression of viral antigens which can interfere with immune responses to promote persistence of infection [43]. Excessive production of soluble forms of HBsAg, HBeAg, and HBV virions has been shown to induce immune tolerance [50, 51]. In addition, HBV triggers only weak interferon responses, hence shielding recognition by the innate immune system [52], probably through sequestering cccDNA under the host cell machinery to avoid recognition by host-sensing receptors [53].

Adaptive immune responses in terms of both humoral [54] and cellular immunity [55] are crucial for clearance of HBV, as it is evident from resolution of acute infection [56]. However, in chronic infection efficient B cell neutralizing antibody responses are lacking, similarly to the virus-specific T cells which decline both quantitatively and qualitatively. HBsAg seems to play a key role in impaired B cell immunity through incorporation of non-infectious subviral particles (SVPs) that are released in great excess over infectious virions in order to inhibit recognition and neutralization by anti-HBs [57-59]. Another study suggests that in older chronic HBV patients, the progressive attrition of HBs-specific T cells in the blood may be more strongly associated with prolonged exposure to the virus rather than the levels of HBsAg [60]. This is likely due to the extended duration of infection in older patients compared to younger ones. These findings underscore the

importance of early treatment interventions for chronic HBV infection. Dysfunctional HBV-specific T cells have been also attributed to prolonged exposure to high loads of viral antigens [61], persistent viral antigen presentation in the infected liver [50], and the highly tolerogenic liver environment which tends to suppress the priming and function of T cells [62, 63].

Chronic activation of T cells leads to a state of exhaustion characterized by the expression of inhibitory receptors, impaired effector functions, reduced proliferation, and significant alterations in metabolic and transcriptomic profiles [64-69]. A key transcription factor in "programming" T cell exhaustion is TOX (thymocyte selection-associated high-mobility group box protein) which is induced upon high antigen TCR stimulation. TOX has been associated with reduced expression of cytokines and effector molecules and upregulation of inhibitory receptors such as programmed cell-death 1 (PD-1) [70]. In addition, exhausted HBV-specific T cells has been shown to be more prone to apoptosis characterized by upregulation of the death receptor; tumor-necrosis factor related apoptosis-inducing ligand (TRAIL-R2) and the pro-apoptotic BCL2-interacting mediator (Bim) [71-73]. Despite a characteristic phenotypic exhaustion profile based on (co-) expression of inhibitory receptors including PD-1, 2B4, lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and B and T lymphocyte attenuator (BTLA) [73], distinct T cell subsets with different functionality status have been identified [74] based on progression of chronic infection [75], antigen specificity [76], and tissue origin [77].

HDV is coated with the HBV envelope, therefore HDV hepatocyte entry is mediated by its preS1 domain using the NTCP entry receptor, similarly to HBV. Since HDV encodes only for the two isoforms of HDAg (S- and L-HDAg), finding targets that can be recognized by the immune system is challenging. HDV-specific CD4 and CD8 T cell responses have been observed, however towards limited number of epitopes within the L-HDAg and at low ex vivo frequencies for both MHC-class I and MHC-class II-restricted epitopes [78-80]. In addition, CD8+

HDV-specific T cells have been found in the blood of untreated chronic patients at low frequencies similarly to CD8+ HBV-specific T cells, however HDV-specific T cells do not appear to be terminally-differentiated and are less exhausted compared to other virus-specific T cells [80]. Instead, relative enrichment of a memory-like T cell subset in chronic HDV lacking activation capacity to its cognate antigen implies viral escape mutations to evade immune recognition based on reduced or lost Human-leukocyte antigen (HLA)-dependent antigen presentation [79-81]. Anti-HDV antibodies can be detected in acute-resolving HDV infection at rather low titers, but at higher levels during chronic infection [82]. However, no viral control or clearance has been achieved so far in in vivo studies, suggesting little contribution of HDAg-induced antibodies to confer protection against HDV [83, 84], probably due to lack of neutralizing activity.

1.2.3 HBV and HDV induced HCC

As mentioned previously, the majority of HCC cases arise from hepatocyte injury and the persistent, non-resolving chronic inflammation that advances through stages of fibrosis, cirrhosis and ultimately cell transformation [18]. HBV-related HCC can also occur without pre-existing cirrhosis, suggesting the presence of additional direct tumor-promoting mechanisms driven by the virus [10]. In addition, viral-driven HCC is considered the consequence of an interplay between direct oncogenic traits of the viruses, host's genetic and/or environmental factors and immune system-mediated mechanisms that occur progressively over time [18].

HBV genotypes (A-J) have been associated with different levels of likelihood of developing HCC [85]. HBV genotype C infections have been shown to predict risk for HCC development in 80% of cases [86, 87] followed by genotypes B, F, D, and A [88, 89]. Among the 8 HDV genotypes (1-8), genotype 1 has been associated with more severe clinical outcomes, including an elevated risk of HCC in comparison to genotype 2 [90], however the high genetic divergence among the different HDV genotypes and their dependency on HBV co-infection require further studies to elucidate more precisely their distinct clinical consequences. The levels of viral

replication and genomic mutations targeting specific regions, such as the basal core promoter/preCore region and the preS/S region of HBsAg also correlate with the risk of HCC development [91-94]. Accumulated mutations in envelope proteins due to for instance HBV DNA integration can lead to unbalanced production and retention of mutated HBV proteins in the endoplasmic reticulum (ER) of hepatocytes and activation of ER stress signaling pathway [18]. Activation of ER stress responses can in turn result to generation of reactive oxygen species (ROS), oxidative DNA damage and genomic instability which all favor tumor development [10, 93]. Additionally, HBV DNA integration into the host genome can induce chromosomal instability and insertional mutagenesis of HCC-related genes, therefore increasing the risk for developing HCC [95]. HBx protein is another important component supporting HBV viral replication and can contribute to hepatocyte transformation by dysregulating different pathways involved in cell proliferation, cell death and host DNA repair processes [96-98].

In the context of chronic HBV co-infection, HDV accelerates disease progression to cirrhosis partially due to the pre-existing HBV-associated liver damage, therefore increasing the risk of developing HCC. HDV can dysregulate immune-mediated pathways by inducing IFN-stimulated genes and cytokines such as TGFβ [99] and attract high numbers of cytotoxic T cells [100]. This suggests enhanced inflammation by HDV compared to HBV monoinfection which can subsequently accelerate liver damage. Moreover, activation of TGFβ signalling pathway by L-HDAg can promote fibrosis and hepatocarcinogenesis [101].

1.3 Current therapies for the prevention and treatment of HCC

Considering that chronic infection with HBV is the main risk factor for HCC and that co-infection with HDV can significantly exacerbate progression to HCC, therapeutic efforts aiming at controlling the spread of infection in the liver could help in preventing about half of all HCC cases [10]. However, yet there is not a cure for chronic HBV and HDV. Two formulations of IFN and nucleoside analogues (NAs) are used in the clinic for the treatment of HBV [102]. For chronic HDV, pegylated IFNα (pegIFNα) was until recently the only regimen recommended by international

guidelines [103]. Recently, a peptide-based entry-inhibitor named bulevirtide received conditional marketing approval by the European Union authorities for the treatment of chronic HBV and HDV patients [36]. Despite the encouraging clinical outcomes based on short-term studies, the possibility to lead to long-term off-therapy responses warrants further monitoring due to HBsAg persistence with or without risk of HDV RNA relapse in addition to long-term safety monitoring [36].

The goal in the clinical management of HBV is achieving a functional cure defined as sustained HBsAg loss in addition to undetectable HBV DNA and for HDV undetectable serum RNA six months after stopping treatment [104]. However, the currently used therapeutic regimens are not sufficient to achieve this. Although NAs are safe and can efficiently suppress HBV viral replication and slow disease progression during therapy, they are life-long and cannot always eliminate the risk of developing HCC [49, 105-107]. Since HDV replication and mRNA synthesis require host polymerases, usage of NAs as in the case of HBV is not feasible. Treatment with IFNα can suppress HDV replication, however, does not preclude the risk of RNA relapses off-therapy [108] and is contraindicated in patients with advanced or decompensated liver disease [36]. In addition, NAs combined with pegIFNα seems to offer limited improved clinical outcome for HBV [109] or HDV patients [110].

Ongoing efforts are focused on developing combination regimens for achieving a cure for chronic HBV and HDV infections. These regimens aim to target various stages in the virus life-cycle including inhibitors of HBV replication e.g. NAs and HBV core inhibitors [111], HBV/HDV entry inhibitors [112], translation inhibitors through RNA interference e.g. silencing RNA's [113] and (liver-directed) antisense oligonucleotides [114, 115], nucleic acid polymer inhibitors of HBsAg release [116, 117] and the currently under preclinical development cccDNA inhibitors [118]. Two additional to the entry-inhibitor bulevirtide novel anti-HDV regimens; lonafarnib (prenylation inhibitor) and REP2139Ca (nucleic acid polymer) have shown encouraging preliminary results in clinical trials [36, 117]. Finally, any of the novel aforementioned therapeutic approaches targeting HBV monoinfection could

ultimately be helpful in managing an HBV/HDV co-infection, such as RNA interference and antisense oligonucleotides which have shown substantial declines of HBsAg level in absence of pegIFNα [36].

The treatment options for HCC depend on the stage that is diagnosed. For early stages, surgical resection, liver transplantation and ablation are the standard-of-care options while for intermediate HCC stages, trans-arterial chemoembolization and radiotherapy are considered more effective treatments [119]. When diagnosed at an already advanced stage, the therapy options are limited to systemic treatment with tyrosine kinase inhibitors (TKIs) and palliative care [120, 121]. Additional to the first-line multi-kinase inhibitor sorafenib [122], new targeted therapies including other TKIs [123-126], vascular endothelial growth factor receptor (VEGFR)-directed therapies and ICIs [127, 128] have managed to significantly improve objective response rates and progression-free survival for advanced HCC patients, especially when used as combination treatments [129, 130].

However, HCC in its late disease stage remains hard to cure, probably owing to its high heterogeneity and clonal evolution leading to drug resistance [121, 131, 132]. This suggests that further therapeutic efforts are needed to ameliorate the currently insufficient clinical outcomes. Lymphocytic infiltration of tumor-specific effector T cells was associated with reduced risk of HCC recurrence following liver transplantation [133], highlighting the important role that T cells and T cell-based immunotherapy can have for the clinical management of HCC [134].

1.4 T cell-based immunotherapies for the prevention and treatment of HCC

In order to address one of the main therapeutic challenges in treating chronic infections and cancer, which is the T cell dysfunction, various immunotherapy strategies have been developed with the objective of either restoring or enhancing T cell function. One of the first proofs that harnessing the human immune system can result in long-lasting anti-cancer responses came with the introduction of interleukin 2 (IL-2) in clinical studies showing that its systemic

administration could treat patients with metastatic melanoma and renal carcinoma [135]. These findings set the stage for subsequent development of ACT of unmanipulated tumor-infiltrating lymphocytes or genetically-modified T cells following IL-2 expansion ex-vivo to improve the treatment effectiveness in other cancer types [136, 137]. Post-operative infusion with anti-CD3/IL-2 activated autologous T cells could significantly improve recurrence-free outcomes in patients that had undergone HCC resection compared to control/non-ACT treated group [138].

Blockade of T cell inhibitory signaling with ICIs aiming to block the interaction between checkpoint proteins and their ligands to prevent T cell inactivation [139] have shown remarkably efficacious clinical outcomes and nowadays they consist part of standard care for various cancer types including advanced HCC [139-141]. Nevertheless, there are still many cancer patients with metastatic epithelial tumors in whom ICI treatment does not lead to tumor regression [142]. In addition, only a small portion of HCC patients currently responds to ICI immunotherapy [127]. A correlation between the benefit from ICIs and the neoantigen load has been shown for various cancer types [143, 144] and suggests that tumors with a higher TMB are more likely to produce immunogenic neoantigens identified by T cells that are activated following treatment with ICIs [142]. However, further studies in HCC show no significant benefit in high TMB when compared to intermediate or low TMB HCC patients, overall indicating no apparent association between TMB/neoantigen load and ICI response rates or survival benefits [145, 146]. Instead, additional factors such as immune tumor infiltration [147-149], expression of PD-1/PD-L1 [150-152] and molecular traits [145, 153-156] have been considered as more valuable indicators for response to ICIs. Additionally, the etiology of HCC can influence the effectiveness of ICIs. According to a study by Pfister et al., in a preclinical model of NASH-associated HCC, treatment with PD-1 could expand intratumorally activated CD8+PD-1+ T cells without leading to tumor regression [157]. On the contrary accelerated disease progression was observed accompanied by increased tumor size and hepatic CD8+PD-1+CXCR6+, TOX+, and

TNF+ T cells [157]. According to the same study, patients with NASH-HCC who were treated with anti-PD-1/anti-PDL1 showed reduced overall survival compared to other HCC patients with different etiologies [157]. Similarly, another study interrogating the immune microenvironment between HBV-induced and non-viral-induced HCC revealed distinct immune signatures and supports the idea that PD-1 blockade is more likely to benefit HBV-induced HCC compared to non-viral HCC [158].

Altogether, given the fact that response rates to ICIs, the most clinically advanced T cell-based immunotherapy for advanced HCC so far, remain low and that multiple immunological and molecular factors can influence those responses, further exploration of more personalized treatment alternatives together with combinatorial approaches are warranted in order to benefit more patients and ameliorate the current clinical outcomes, especially for cold tumors which are characterized by low immune-infiltration.

In the following paragraphs, two types of immunotherapies that entail, respectively, active and passive antigen-specific T cell stimulation are discussed in more detail with regards to their role in chronic viral hepatitis and HCC.

1.4.1 Active T cell immunotherapy: Therapeutic vaccines

The aim of actively immunizing with a therapeutic vaccine in chronic HBV is to regain the magnitude and function of virus-specific T cells [63]. The administered vaccine regimen should be able to overcome virus-induced mechanisms of persistence to elicit antibody- and T cell-mediated immunity (**Fig. 3**), which are both crucial for virus control, as it is evident from self-resolution of HBV infection [50].

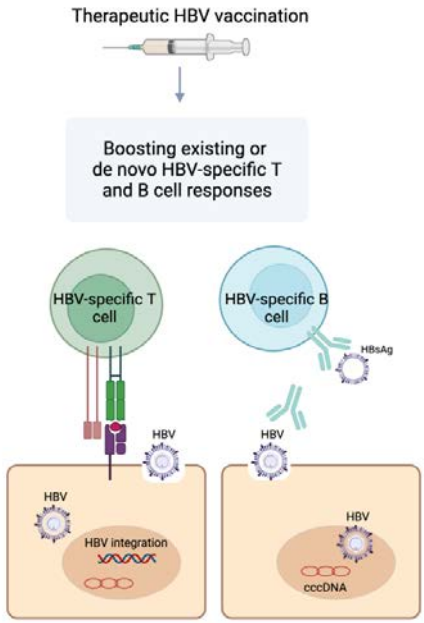

Figure 3. The concept of HBV therapeutic vaccination. Host adaptive immune responses (B and T cell immunity) can be harnessed using a therapeutic vaccine aiming to promote existing or prime de novo HBV-specific B and T cell responses that are protected from exhaustion. Both neutralizing antibodies and T cells are critical to control the infection based on cytotoxic dependent and independent mechanisms. HBV; hepatitis B virus, HBsAg; HBV surface antigen, cccDNA; covalently closed circular DNA. Inspired from [159]. Created with BioRender.com

Various vaccine platforms including peptide or protein vaccines, antigen-protein complexes, DNA, or adenoviral-based vectors have been developed targeting different HBV antigens, such as the S, preS2, C, P, X or combination of those [160-164] and some have shown promising results, mainly when combined with other antiviral or immunological regimens [165-171]. Despite encouraging preclinical data, the clinical efficacy observed so far remains poor and ongoing efforts are focusing on optimizing vaccine design in terms of choice and number of antigens, delivery platforms, choice of adjuvants, and route of administration [172, 173]. However, as long as the hurdle of T cell tolerance persists, additional combinatorial approaches are warranted to break or circumvent the HBV-specific impaired immune responses more efficiently [159], as supported by preliminary data from ongoing

clinical trials (reviewed in [102] and [172]). In chronic HBV patients treated with NAs, viral loads attributed to ongoing viral transcription from the cccDNA as well as the integration of HBV DNA into the host genome can remain elevated [95, 174]. Considering this, reduction of the viral loads based on pretreatment with small interference RNAs or nucleic acid polymers aiming to block secretion of HBsAg from infected hepatocytes can be beneficial prior to therapeutic vaccination for more efficacious anti-viral responses [113, 116, 175, 176]. Finally, concomitant use of ICIs with therapeutic vaccination could represent another promising strategy [159, 167, 177].

Similarly, the rationale for using anti-cancer vaccines is the generation of tumor-specific responses with enhanced potency [141]. This can be achieved by several means, including de novo priming of T cells against tumor antigens that would not naturally trigger an immune response, by enhancing an already existing response or by broadening the breadth and diversity of tumor-specific T cell responses [178]. For HCC, mainly peptide-based vaccines that target tumor-associated antigens (TAAs) have reached early clinical phase trials in humans [179-181]. TAAs are non-mutated self-derived proteins that can become immunogenic due to their aberrant expression or differentiation status within the tumor cells [182]. This class of antigens comprise a promising "off-the-shelf" therapeutic target since they are shared among cancer patients. Cellular responses against TAAs such as alpha-fetoprotein (*AFP*), glypican-3 (*GPC3*), *TERT*, melanoma-associated genes (*MAGE*)-1, 3 and 10, and New York-esophageal squamous cell carcinoma-1 (*NY-ESO1*) have been identified in blood and tumors of HCC patients and correlated with patient survival [183, 184]. Although early clinical and preclinical data based on TAA-vaccines showed that they can be well-tolerated, only sporadic T cell responses could be observed with limited clinical significance [141, 185]. The limited efficacy observed so far could be attributed to the fact that TAAs are not completely tumor specific, hence T cells targeted those can be subjected to central and peripheral tolerance mechanisms [142, 186].

An alternative approach comprises neoantigen-based vaccines [187, 188]. Neoantigens are mutated tumor-specific proteins that have gained increased interest in various types of cancer immunotherapies due to their putative low risk of toxicities on normal tissues and because they can elicit high avidity T cells [186, 189]. Neoantigens can arise in tumor cells through various mechanisms including genomic mutations (single-nucleotide variants; SNVs, base insertions and deletions; INDELs and gene fusions), aberrant transcriptomic variants, post-translational modifications, and virally-encoded open reading frames [186]. Neoantigen-based vaccines have shown promising clinical outcomes mainly in melanoma [190, 191] and glioblastoma [192]. In a small HCC cohort study including 14 patients, mutations in HCC tumors could generate immunogenic neoantigens and highlighted the potentials to be used in future combinatorial immunotherapy strategies [193]. Data on efficacy and safety from ongoing clinical trials in HCC based on different representative vaccine platforms including DNA (NCT04251117), mRNA (NCT05761717), dendritic cell (NCT04912765, NCT04147078), and peptides (NCT04248569, NCT05269381) are highly awaited to further assess their potentials.

1.4.2 Passive T cell Immunotherapy: ACT

ACT represents another promising type of immunotherapy in which autologous or allogeneic ex vivo expanded/sensitized cells are infused to the patient [194, 195]. One of the first successful ACT involved ex vivo expansion and re-infusion of tumor-infiltrating lymphocytes (TILs) in malignant melanoma patients [196-199]. TILs have also been tested in the context of HCC, however data from clinical studies are yet limited [200]. The extent to which TILs can be expanded to generate T cells specifically targeting tumor neoepitopes as well as the scalability of this expansion process remain major hurdles for clinical application [141]. Instead, increasing focus has been given on T cell engineering approaches based on chimeric antigen receptor (CAR)- and TCR-modified T cells designed to recognize a specific tumor or viral antigen.

An advantage of using CAR-T cell therapies is their ability to recognize surface antigens independently of HLA. This can be particularly beneficial for targeting tumors that employ HLA downregulation or loss as a mechanism to evade the immune system. CAR-T cells targeting GPC3 have shown efficacy in animal models with orthotopic xenografts and in patient-derived xenografts [201, 202] and are currently evaluated in clinical trials [203]. However a main concern with CAR-T cells is the on-target off-tumor toxicity that may arise following T cell infusion [204].

CAR-T cells recognizing HBV antigens have been tested as therapeutic option for HBV. According to a study by Bohne et al., CAR-T cells specific for HBsAg (S- and L-HBsAg) could selectively eliminate HBV cccDNA positive infected hepatocytes in vitro [205] while in another study performed in vivo S-HBsAg CAR-T cells could localize and function in the liver of mice and efficiently reduce HBV replication [206]. Despite the encouraging preclinical data, risks related to potential T cell mediated liver damage following infusion in a clinical setting should be carefully evaluated. The first evidence of clinical feasibility for targeting viral antigens in the context of HCC was based on a TCR-redirected T cell therapy against HBV [207]. According to this study, autologous T cells from an HBV-HCC patient were genetically engineered to express an HBsAg-specific TCR to treat metastatic extrahepatic lesions following liver transplantation after confirming that only the HCC metastases expressed HBsAg and not the donor's liver [207]. Although clinical efficacy could not be determined in this study due to that the patient was in terminal-stage disease, a robust reduction in HBsAg levels without exacerbation of liver inflammation or other on or off-target toxicities were observed [207]. A subsequent clinical trial in metastatic HCC patients following liver transplantation, identified HCC-derived cells expressing short HBV DNA fragments which encoded epitopes recognized by T cells [208]. The infusion of autologous T cells transiently expressing TCRs targeting these epitopes was shown to be safe following a dose escalation protocol, however among the two patients who underwent this treatment, one exhibited a clinical response, characterized by a reduction in the

size of the metastatic tumors [208]. In addition to HBV-induced HCC clinical cases, preclinical studies have used transiently expressed TCRs (targeting the HBV envelope and core) and could achieve viremia reduction without triggering subsequent liver inflammation in vivo [209]. Altogether, these data suggest that using viral-specific TCRs in the context of viral-induced HCC could be safe and beneficial to prevent HCC recurrence following liver transplantation, and that transiently expression of TCR genes can be a safer option to confer protection against viral infections or viral-induced HCC based on a dose escalation strategy for a better control and monitoring for potential side-effects.

TCR modified T cells have been also employed against TAA such as AFP, which is overexpressed in HCC [210] and have shown encouraging preclinical results [211]. Nevertheless, AFP-specific TCRs have shown low affinity and modest antitumoral responses due to central and peripheral tolerance mechanisms [141]. An alternative promising target for T cell-based ACT are neoantigens that are shared among multiple cancer patients. While neoantigen-based immunotherapies play a major role in the context of personalized treatments, targeting recurrent or otherwise called "hotspot" mutations in oncogenes like *TP53* is a promising strategy. Mutated versions of *TP53* are present in almost 50% of all cancers, including HCC, therefore targeting them can offer therapeutic benefits to multiple cancer patients who share the same mutations [212]. Studies have indeed shown that p53 harbors immunogenic epitopes derived from both unique and "hotspot" mutations across various cancer types [213-215]. Similarly, the identification of immunogenic hotspot mutations in *KRAS*, a commonly mutated oncogene in human cancers [216], represents another opportunity to create a library of TCRs. These TCRs, when matched with the appropriate HLA types, could be employed in ACT trials as an "off-the-shelf" therapy for multiple patients and/or tumor histologies. This approach of creating TCR libraries can be promising also for HCC [217] and warrants further investigation for applicability against HCC-driver mutations such as *PIK3CA* and *CTNNB1*, among others.

2 RESEARCH AIMS

The aim of this thesis was to investigate two immunotherapy approaches for the prevention and treatment of HCC. Particularly, studies I and II focus on the evaluation of a therapeutic vaccine for chronic HBV and HDV infection which comprises the main risk factor for developing HCC. Study III aims to identify immunogenic neoantigens that can be targeted with specific T cell receptor and therapeutic vaccination. The specific aims for each study are summarized below.

Study I: A main obstacle towards achieving a functional cure for chronic HBV and HDV infection is the dysfunctional immune response present in chronically infected patients. Combinational therapies able to both target different steps in the virus life-cycle and to restore the host's immune system are required in order to achieve sustained off-therapy responses. In this study, we evaluated a therapeutic DNA vaccine designed to block hepatocyte entry of HBV and HDV and to bypass the T cell tolerance during chronic infection. The objective was to determine its capacity to induce adaptive immune responses against HBV and HDV both in vitro and in vivo.

Study II: In this study we expanded our findings from study I by developing a heterologous DNA prime-protein boost vaccine strategy to additionally test the feasibility of i) circumventing the HBV-induced T cell tolerance present in the chronically infected host and ii) precluding HDV superinfection in human-liver chimeric mice infected with HBV and HDV.

Study III: T cells and their receptor repertoire play a crucial role in orchestrating anti-tumoral responses in several cancers. Nevertheless, the precise role of neoantigen-reactive T cells in HCC remains largely undefined. In the present study, we aimed to identify neoantigens that can lead to activation of reactive T cells from intra- and extra-tumoral origins in HCC patients. In addition, we aimed to characterize the clonality and transcriptomic landscape of infiltrating T cells in order to identify signature markers that can facilitate the identification and

isolation of neoantigen reactive T cells and/or their TCRs as potential immunotherapy for HCC.

3 MATERIALS AND METHODS

This section provides an overview of the main methods utilized in studies I , II and III with particular focus on the rationale behind selecting each methodology. More detailed description is provided in the methods section of each study.

3.1 Ethical considerations (studies I, II and III)

The studies presented in the current thesis were conducted considering ethical aspects for animal experimentation (studies I and II) and handling of clinical samples from patients (study III).

In studies I and II we evaluated different vaccine regimens in various animal models with regards to efficacy and safety, parameters that need to be assessed in vivo prior to reaching a clinical trial. For all animal experiments, we ensured that the principles of 3 Rs (Replacement, Reduction, and Refinement) were followed. Accordingly, we first performed in vitro experiments that enabled us to assess aspects of vaccine protein expression, potential toxicities, and virus neutralization in cell culture models, prior to designing animal experiments. In addition, before progressing to larger animal experiments, we initially conducted small-scale evaluations, such as dosing assessments. This approach aimed to minimize both the number of animals needed and the potential risk of animal suffering. We also ensured the welfare of animals during maintenance and all experimental procedures. Humane endpoints were established to euthanize mice before end of experiments in case of experiencing severe pain or distress. Complete documentation of experimental procedures in animal facility journals and data records in institutional electronic notebook were followed to ensure transparency, reduce need for duplicate studies and to help refining research methods. All animal procedures were reviewed and approved by local ethical committees for animal research to ensure they meet ethical standards and legal requirements.

In study III, we used clinical samples from patients with liver cancer undergoing liver transplantation at the Karolinska University Hospital. Ethical permits were acquired prior to initiating the study and the Declaration of Helsinki guidelines

were followed. Written informed consent was provided by all study participants and samples were anonymized. Data related to sensitive information such as genetic sequencing results were handled following General Data Protection Regulation (GDPR) and stored using encryption for authorized access to ensure protection and confidentiality. An ethical question that could be raised for this study, is how we should handle genetic findings that may reveal health-related complications for the participants and their relatives. We intended to use the findings for research purposes, solely with the aim to identify mutations that could be used as antigenic targets for T cell-based immunotherapy in the future. Any lateral genetic findings indicating direct clinical complication for the participants should be closely discussed with the clinicians who have the experience to evaluate the clinical relevance of the findings and decide whether additional counseling would be beneficial for the participants.

Finally, it is important to consider that the type of immunotherapy that we are aiming to develop in this study, could not be equally accessible for all communities. This is because despite the significant clinical benefit, personalized-adoptive cell therapies require well-resourced medical centers with high-standard infrastructure requirements and substantial specialized labor to tailor each manufacturing batch to a single patient. Therefore, the high financial cost associated with manufacturing and scalability, limits access to such therapies to only a minority of patients or communities who have adequate financial resources. Even in high-income countries, well-resourced medical centers are usually easier accessible to bigger cities. Therefore, multifaceted efforts should focus on reducing the costs of cell therapy production to make it affordable for more patients. This could be achieved for instance based on efforts for streamlined manufacturing processes and automation process optimization, point-of-care manufacturing and gene engineering innovations (e.g novel non-viral strategies over viral vectors) [218, 219]. Beyond manufacturing hurdles, a better mapping of patient populations demographics based on current clinical footprints could further help to improve clinical trial equity. Finally, careful patient stratification and

selection based on reliable biomarkers could help to mitigate certain financial hurdles in low or middle income countries [220].

3.2 HBV-HDV vaccine design (studies I and II)

The herein described HBV-HDV vaccine contains antigens targeting the consensus preS1 domain (aa 2-48) of the large HBsAg envelope protein of HBV and HDV fused with antigens encoding for the L-HDAg of HDV genotypes 1 and 2 (**Fig. 4**). PreS1 is the domain utilized by HBV and HDV to enter hepatocytes upon binding to the NTCP receptor [47]. Therefore, by including preS1 in the vaccine construct, we aimed at raising endogenous preS1 antibodies in order to block entry of HBV and HDV and thus prevent infection of new hepatocytes. Another benefit of including preS1 is that preS1-induced antibodies can more efficiently target and neutralize infectious virions whose surface is denser in the L-HBsAg compared to non-infectious SVPs which are mainly enriched for S-HBsAg and sequester S-induced antibodies [59, 221-223].

In chronic HBV infection the HBV-specific T cell responses are impaired, therefore we included HDAg as a heterologous T cell epitope carrier able to bypass the HBV-induced T cell tolerance to induce healthy naive HDAg-specific T cells. In the setting of HBV monoinfection, these HDAg-specific T cells can support priming of HBV-specific responses and sustained endogenous production of preS1 antibodies. Moreover, HDV-specific T cells and preS1 antibodies can protect HBV monoinfected patients from acquiring an HDV superinfection. In order to induce broad T cell responses, we included two strains of HDAg corresponding to the major genotypes of HDV (genotypes 1 and 2) [224]. The vaccine design concept is illustrated in **Fig. 4**.

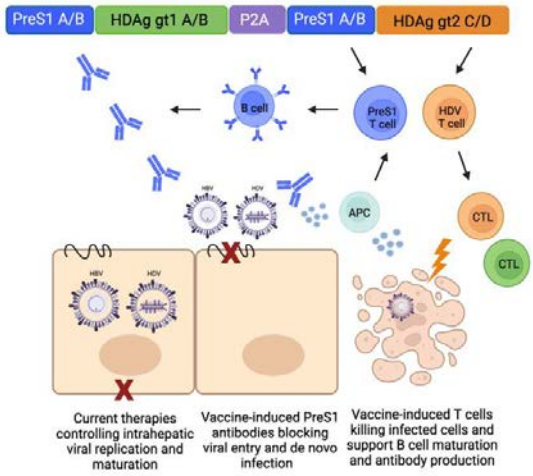

Figure 4. HBV/HDV vaccine concept. PreS1 antibodies induced by the vaccine inhibit entry of HBV and HDV, therefore preventing de novo infection of hepatocytes. HDAg sequences linked to preS1 act as a heterologous healthy B- and T-cell epitope supporting generation of naïve T cells and maturation of B cells. This supports endogenous production of preS1 antibodies that neutralize HBV and HDV and preS1/HDAg T cells able to kill infected cells. Shuttling of viral proteins to antigen-presenting cells further boosts priming of host's T cell machinery. HDAg; delta antigen, gt; genotype, APC; antigen-presenting cell, CTL; cytotoxic T cell, HBV; hepatitis B virus, HDV; hepatitis D virus. Modified from **paper I**. Created with BioRender.com

3.3 Immunizations (studies I and II)

Mice and rabbits were immunized with HBV and HDV encoding antigens as plasmid DNA (study I) and/or recombinant proteins (study II) with the purpose of generating antibodies and T cells responses to the vaccine antigens. Particularly, in study I we evaluated a homologous vaccine approach based on a DNA prime-boost strategy. We used DNA as vaccine platform because it has previously been shown to be safe and immunologically effective against HBV [165, 170]. All DNA vaccines were delivered intramuscularly in the tibialis anterior muscle by needle injection followed by in vivo electroporation. Application of electrical pulses in the muscle area following DNA injections is known to increase endogenous expression of the encoded genes and to enhance immunogenicity as a consequence of local

tissue injury and inflammation [225-227] which is why we used in vivo electroporation as DNA adjuvant.

In study II, we assessed whether a heterologous vaccine strategy based on DNA prime and protein boost could improve immunogenicity of the homologous DNA vaccine approach following one or two booster doses. For protein immunizations, the vaccine regimens were delivered subcutaneously in the mouse tail base and various protein adjuvants (incomplete Freund, alum, MF59, and QS21) were evaluated in order to identify the most immunogenic and at the same time clinically applicable protein-adjuvant combination [228].

3.4 Animal models (studies I and II)

Wild-type C57BL/6 (H-2^b), transgenic HHD-HLA-A2 and HBsAg mice (B6;SJL-Tg(Mt1-HBV)28Bri/ChiJ) [229] were used to evaluate the ability of the vaccine regimens to induce antibodies and T cell responses. New Zealand white rabbits were used in order to evaluate vaccine-induced antibody titers in a larger animal species and in order to generate high-yield serum preS1 antibodies to use in HBV and HDV in vitro and in vivo neutralization assays. Human-liver chimeric uPA+/+-SCID mice [230, 231] infected with HBV and HDV were used to assess the neutralization ability of preS1 antibodies following passive immunizations.

3.5 Evaluation of vaccine efficacy (studies I and II)

3.5.1 Detection of vaccine-induced antibodies and T cell responses

Two weeks after last vaccination boost, blood and splenocytes were collected to determine the intrinsic immunogenicity of the various prime-boost strategies based on enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunosorbent spot (ELISpot), respectively. ELISA was performed to assess the ability of the vaccine candidates to induce preS1-IgG antibodies from sera of vaccinated mice and rabbits. Anti-sera were evaluated for reactivity against preS1A and preS1B consensus peptides (aa 2-48) as well as for cross-reactivity to HBV (sub-) types A1, A2, B, B2, C, D1, E1, and F using pools of 20mer preS1-peptides. We conducted ELISpot assays using splenocytes from vaccinated mice to identify

T cells specific to HBV and HDV antigens. This was accomplished by measuring IFN-γ secretion after 48 hours of in vitro recall-antigen stimulation with HBV and HDV peptides.

3.5.2 In vitro and in vivo neutralization assays

In order to study if the vaccine-induced preS1 antibodies could neutralize HBV and HDV in vitro, the HepG2-hNTCP expressing cell line was used [47]. The in vitro neutralization was assessed for HBV monoinfection (study I) as well as for HBV and HDV co-infection (study II) prior to performing in vivo neutralization assays. For in vivo neutralization, the human-liver chimeric uPA+/+-SCID mouse model was generated as described previously [230] and human albumin quantification was performed to assess the level of liver humanization. Protection against HBV monoinfection (study I), HBV/HDV co-infection and HDV superinfection (study II) was evaluated upon adoptive-transfer of the vaccine-induced preS1 antibodies in the liver-humanized mouse model.

3.6 HCC patient cohort (study III)

In study III, our objective was to isolate neoantigen-specific T cell receptors, with the aim to later develop a T cell-based immunotherapy for HCC (**Fig. 5**). For this purpose, we collected clinical samples including HCC tumors, draining lymph nodes, liver flush, and peripheral blood lymphocytes from 16 HCC patients undergoing liver transplantation at Karolinska University Hospital.

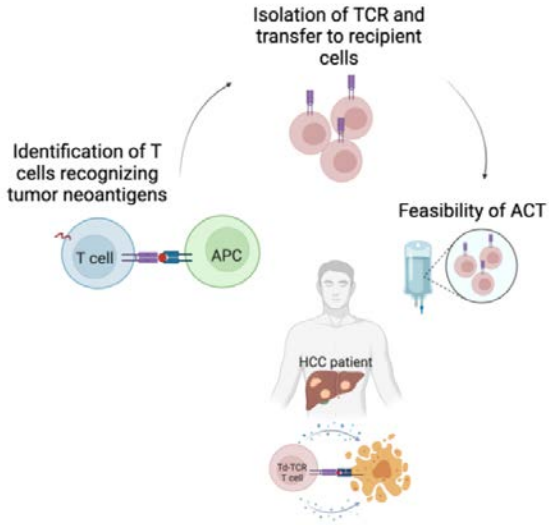

Figure 5. Illustration of objective for study III. The first step is to identify T cells that recognize neoantigens derived from HCC patients tumors. Reactive T cell receptors are then isolated and reconstructed for subsequent transfer to recipient cells. The ultimate goal is to create a library of tumor-reactive T cell receptors which in the context of matched HLA could be further assessed for adoptive cell therapy in patients with HCC. TCR; T cell receptor, ACT; adoptive cell therapy, APC; antigen-presenting cell, Td-TCR T cell; transduced-T cell receptor T cell. Created with BioRender.com

3.7 Neoantigen selection (study III)

In order to identify somatic mutations that give rise to tumor-specific neoantigens, we performed whole-exome sequencing (WES) and RNA sequencing (RNA-seq) on extracted nucleic acid from tumor and matched peripheral blood biopsies. Targeted sequencing (TS) was performed on tumor samples for the first HCC01, HCC02, and HCC04 patients based on a panel of 523-cancer related genes [232]. To evaluate and approve variants derived from WES/RNA-seq data, we fine-tuned the criteria using an internally developed personalized immuno-oncology ranking (PIOR ©) analysis tool. This tool assesses variants by considering their copy number status and the confidence level of the variant caller, as well as by integrating data from public repository datasets (unpublished data from submitted manuscript). All identified variants were additionally manually curated

and inspected after checking the BAM coverage from an integrated Jbrowser function. To determine which variants from both TS and WES to prioritize for immunological screenings, we considered the following parameters: i) the biological relevance of the variants i.e. if mutations occur in driver genes or frequently HCC mutated genes, ii) if the mutations are re-occurring at the same protein position among multiple patients ("hotspot" mutations), iii) the frequency of allelic fraction as indication of clonality, and iv) the quality of the tumor samples (i.e. if the tumors were viable, necrotic due to previous treatment, or with presence of viable HCC tumor cells despite treatment).

3.8 Identification of neoantigen-reactive TCRs (study III)

Our approach for selecting neoantigen-reactive TCRs was based on i) immunological screenings in which we tested T cell reactivity against different mutated proteins, ii) comparison of neoantigen-reactive versus non-reactive T cells based on single-cell RNA-seq, and iii) identification of expanded (putative-reactive) clones from memory/antigen-experienced T cells derived from tumors, draining lymph nodes and liver flush from HCC patients.

3.8.1 Immunological screenings

To investigate the presence of T cells reactive to the identified neoantigens we performed in vitro stimulation screenings (IVS) using autologous B cells as target cells [233, 234], which were isolated and expanded from liver flush. These B cells were loaded with neoantigen-encoding peptides. As source of effector cells, we utilized T cells isolated from liver flush, tumor, and/or draining lymph nodes of patients. Following in vitro stimulation of co-cultured T cells with B cells, flow cytometry was performed to assess the T cell reactivity based on expression of CD137 (4-1BB) which is a T cell activation marker [235, 236]. We proceeded with sorting of the CD137+ T cells in order to perform bulk RNA-seq of the variable V-J or V-D-J regions of the TRA and TRB genes.

In a second approach, to enhance enrichment of the neoantigen-specific T cells during IVS, we proceeded with an additional rapid expansion round following

sorting of the CD137+ T cells. After about three weeks in culture, the IVS-enriched T cells expanded during this time were screened again for reactivity to their cognate antigens. Upon confirmation, cells were sorted based on CD3+CD137+ (reactive) and CD3+CD137- (non-reactive) marker and proceeded with single-cell RNA-seq of the two T cell compartments to compare their TCR repertoire and facilitate isolation of the expanded TCR clonotypes in the reactive T cell population.

3.8.2 Single-cell RNA-sequencing on enriched antigen-experienced (memory) T cells

In order to extend the possibility of identifying neoantigen-driven TCRs beyond IVS screenings, we sorted antigen-experienced (memory) T cells directly from the patients' tumors based on CD3, CD45RA and CCR7 expression and performed single-cell RNA-seq. To compare the clonality and transcriptomic signatures of these T cells we also included liver flush and tumor-draining lymph nodes under the same sorting pipeline for subsequent immune-profiling based on 10X Genomics. Sequencing data were first processed according to Cell Ranger pipeline (v7.1; 10X Genomics). Sequencing data were mapped to human genome reference (GRCh38). TCR (CDR3) clonotype analysis was performed using Loupe VDJ Browser (v5.1; 10X Genomics).

3.9 TCR reconstruction and assessment (study III)

For reconstructing full length TCRs from RNA bulk-sequencing data, we paired the most dominant alpha and beta chains based on available full length CDR3 clonotypes for TRAV and TRBV genes using the international ImMunoGeneTics (IMGT)/V-Quest and IMGT/Junction Analysis tool. Only productive TRA and TRB rearranged sequences (no stop codons or out of frame shifts) were considered for calculations. We used modified murine TRAC and TRBC sequences for the constant regions to enhance stability of the generated TCRs and to avoid mismatches with the endogenous human TCR after genetic transfer to human T cells [237, 238]. The murinized TCRα and TCRβ chains were linked with a RAKR-SGSG and a P2A sequence to ensure equivalent efficiency in expressing both

chains [213, 233, 238]. The resulting TCRB-TCRA gene blocks were synthesized as RNA (GenScript) for subsequent genetic modification of patients T cells using mRNA electroporation. Following TCR-encoding mRNA electroporation, the TCR-engineered T cells were assessed for TCR expression and ability to recognize their cognate antigen based on flow cytometry analysis for murine TCRβ chain and CD137 antibody markers.

4 RESULTS AND DISCUSSION

4.1 Study I

Till now there is no cure for chronic HBV and HDV infections. Current HBV therapeutic regimens can efficiently suppress viral replication during treatment but they cannot eliminate the risk of developing HCC or acquiring an HDV co-infection [36, 105]. The main hurdle in achieving effective anti-viral responses remains the impaired host immune response due to chronic exposure to viral antigens and mechanisms induced by the viruses in order to escape immune surveillance [49, 59, 73]. To complement current and under development therapies that target different steps in the virus life-cycle, we aimed to develop an immunotherapy targeting both HBV and HDV infections. This immunotherapy was based on a homologous prime-boost DNA vaccine strategy. Ten different HBV-HDV combinations (**paper I, Fig. 1A; Fig. 6**) were evaluated for their ability to induce antibodies and HBV-HDV specific T cells. In addition, the induced preS1 antibodies were evaluated for their potential to inhibit HBV monoinfection both in vitro and in vivo. The main findings of this study are discussed below.

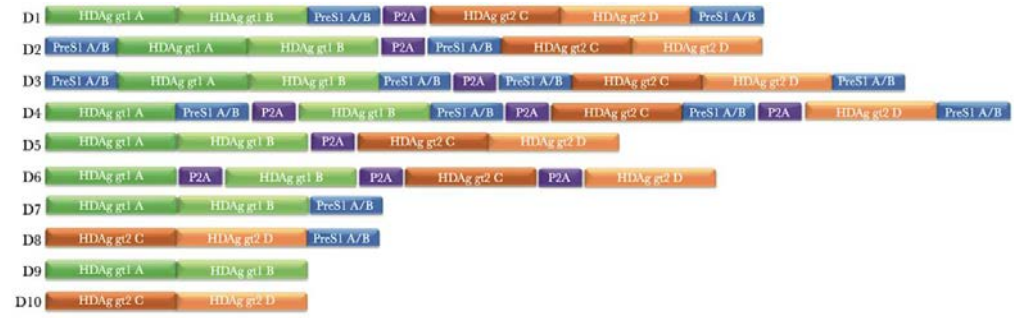

Figure 6. Ten different combinations of preS1-HDAg constructs (D1-D10) were evaluated for their immunogenicity based on a homologous DNA prime-boost strategy. Adapted from **paper I**.

4.1.1 Induction of HBV and HDV specific T cells following preS1-HDAg immunizations

Ten DNA-based vaccine regimens named D1-D10, as shown in (**Fig. 6**) containing different combinations of preS1 and HDAg sequences were assessed for their

ability to induce T cells to preS1 and HDAg antigens (**paper I, Fig. 2**). We could show that following two preS1-HDAg DNA-based vaccinations in mice with different genetic backgrounds, functional T cells specific for preS1 and/or HDAg genotypes 1 and 2 could be induced based on IFN-γ secretion (**paper I, Fig. 2, Fig. 4C** and **Suppl. Fig. 1**). These results confirm that active preS1-HDAg immunization is able to induce strong T cell responses to both preS1 and HDAg of two major HDV genotypes. They also suggest that a broad and functional T cell-based immunotherapy should contain antigenically distinct epitopes based on both HBV and HDV antigens in order to avoid a potential non-responding T cell status in humans.

Previous studies have characterized the B cell and T cell epitopes of preS1 as well as its potential role in overcoming the immunotolerance status mainly based on anti-preS1 mediated responses [239-241]. However, it still remains uncertain if therapeutic vaccines targeting only HBV antigens can bypass or restore the impaired HBV-specific T cell immunity during chronic infection based only on occasionally elicited T cell responses [170, 242, 243]. In our approach, we included HDAg linked to preS1, as a heterologous healthy T cell epitope carrier able to recruit naïve T cells that support endogenous production of preS1 antibodies and can efficiently activate host's T cell machinery. An additional benefit about inclusion of HDAg is that chronic HBV monoinfected patients immunized with preS1-HDAg vaccine could become immune against a subsequent HDV superinfection based on induction of HDV-specific T cell immunity.

4.1.2 Broadly cross-reactive preS1 antibodies can inhibit HBV monoinfection in vitro

Next, we show that the preS1-HDAg vaccine regimens were able to induce high anti-preS1 titers in mice (**paper I, Fig. 3A-B**) and rabbits (**paper I, Fig. 3C-D**). Sera from mice and rabbits vaccinated with the D4 construct had higher anti-preS1 titers, compared to the other tested vaccine regimens, which were cross-reactive against the tested HBV sub-types; A1, A2, B, B2, C, D1, E1, and F (**paper I, Fig. 4A** and **Suppl. Fig. 2**). Importantly, we could show that these vaccine-induced preS1

antibodies neutralize HBV in vitro (**paper I, Fig. 5**) in a well-established assay that supports full viral life-cycle [244]. Sera obtained from mice following two vaccinations (6 weeks) showed superior neutralization effect on HBV compared to sera obtained after one immunization (2 weeks) (**paper I, Fig. 5B**). Here D4-induced antibodies had the strongest neutralization effect, followed by construct D3 (**paper I, Fig. 5B**). However, in sera obtained from vaccinated rabbits, titers >1:1000 required to neutralize HBV in vitro which was achieved with D4 vaccination (**paper I, Fig. 6B**). Table 1 summarizes the ability of each vaccine construct D1-D10 to induce HDAg-specific T cells and cross-reactive preS1 antibodies. In vitro neutralization of HBV was also assessed for all vaccine candidates.

Table 1. Summary evaluation of D1-D10 vaccine candidates used in **paper I**. D4 construct is superior in terms of eliciting T cell responses to both HDV genotypes 1 and 2 and highly cross-reactive preS1 antibodies which can neutralize HBV in vitro.

Vaccine candidate	**HDV genotypes**	**Anti-PreS1 titers** (mouse)	**Cross-reactive antibodies** (HBV genotypes)	**In vitro HBV neutralization** (IC50)	**Anti-PreS1 titers** (rabbit)
D1	1 and 2	<1:10000	C, D, E	1:500	-
D2	1 and 2	<1:10000	A1, A2, B2, C, D, E	1:500	1:60
D3	1 and 2	1:2160-1:12960	D, E	1:500	<1:1000
D4	**1 and 2**	**1:12960**	**A-F**	**1:500**	**>1:1000**
D5	1 and 2	neg	neg	neg	-
D6	1 and 2	neg	neg	neg	-
D7	1	1:2160	A-E	1:500	-
D8	2	1:2160	C, D, E	1:500	-
D9	1	neg	neg	neg	-
D10	2	neg	neg	neg	-

4.1.3 D4-induced preS1 antibodies (partially) inhibit HBV monoinfection in vivo

Following our previous observations that titers >1:1000 were required to neutralize HBV in vitro (**paper I, Fig. 6B**), we used these anti-preS1 rabbit sera to further determine if they could inhibit HBV infection in a human-liver chimeric mouse model [231]. Three human-liver uPA-SCID mice were injected with preS1-IgG

antibodies derived from the D4-vaccinated rabbit and 3 days later they were challenged with HBV. We could show that a single injection of these antibodies could protect or significantly delay peak viremia in all challenged mice compared to the control group over a period of 8 weeks (**paper I, Fig. 6C; Fig. 7**).

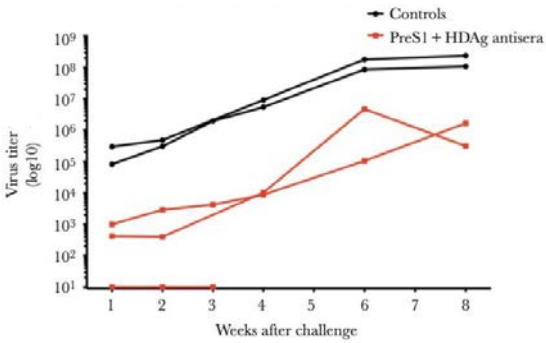

Figure 7. Protective effect of preS1-HDAg antisera against HBV infection, as determined by HBV titers at each time point following first inoculation. Each line represents 1 mouse. Black lines indicate control mice receiving naive IgG and red lines indicate mice immunized with D4 preS1 IgG. Adapted from **paper I**.

PreS1 antibodies can neutralize HBV [245]. Importantly, the most recent clinical advancement for the treatment of chronic HBV and HDV patients comprises a preS1 peptide-based entry inhibitor which when accompanied with pegIFNα has shown improved anti-viral efficacy for both HBV and HDV [246]. Despite the encouraging clinical outcomes based on short-term studies, further long-term monitoring is required to evaluate the possibility to achieve durable off-therapy responses with regards to both HBsAg persistence and risk of HDV RNA relapse in addition to dose-escalation safety monitoring [36].

4.2 Study II

In this study, we aimed to further investigate whether a heterologous DNA-prime protein-boost strategy involving preS1-HDAg could improve immunogenicity of the previous described preS1-HDAg vaccine with the goal of overcoming T cell tolerance present in the chronically infected host. Moreover, we sought to determine whether the antibodies induced by this approach could prevent HDV superinfection in vivo.

4.2.1 *Improved immunogenicity of heterologous prime-boost preS1-HDAg vaccine strategy*

The vaccine regimens evaluated for adaptive immunity were based on homologous prime-boost with D4 DNA construct (described in study I) or prime-boost with preS1-HDAg protein regimens, or heterologous DNA-prime protein-boost with preS1-HDAg protein regimens (**paper II, Fig. 1; Fig. 8**).

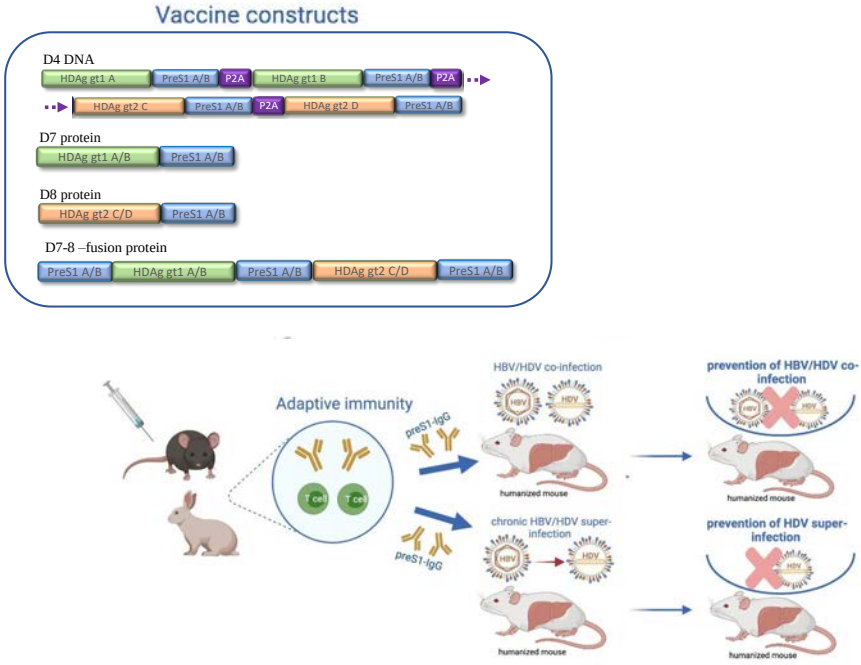

Figure 8. Graphical summary of study II. Four different preS1-HDAg fusion vaccine regimens were assessed for immunogenicity in mice and rabbits using a heterologous (DNA prime-protein boost) or homologous (DNA prime-boost and protein prime-boost) vaccine scheme. Neutralization effect of preS1 antibodies on HBV and HDV was assessed in vitro and in liver-humanized mice. Adapted from **paper II**.

We could demonstrate that mice primed with D4 DNA and boosted twice with the protein constructs elicited higher T cell responses compared to the homologous vaccine schemes, as it is evident from the higher number of IFN-γ secreting cells to both preS1 and HDV antigens following a genotype-specific manner (**paper II, Fig. 2B** and **Suppl. Fig. 3**). With regards to humoral responses, we showed that either the homologous prime-boost vaccination with preS1-HDAg proteins in

adjuvant, or the heterologous D4 DNA prime and preS1-HDAg protein boost strategy consistently induced 10^4–10^5 preS1 titers following two boosts in both mice and rabbits (**paper II, Fig. 2A, Fig. 3A, Fig. 5A** and **Fig. 6A**). Altogether, these results show that the homologous preS1-HDAg protein-based vaccine scheme could induce equally or comparably high anti-preS1 titers (10^4-10^5 sera end point dilution) as the heterologous DNA-prime protein-boost approach, however T cell responses were enhanced following the heterologous DNA-prime protein-boost vaccination strategy confirming its intrinsic immunogenicity.

4.2.2 PreS1-HDAg induced antibodies prevent HBV/HDV co-infection in vivo

Considering that HBV and HDV share the same envelope, induction of preS1 antibodies could in theory inhibit de novo infection of hepatocytes by both HBV and HDV. In study I, we had seen that a single injection of D4-induced anti-preS1 (end titers 10^3 at log scale) could limit HBV monoinfection in vivo, however we did not assess the impact on both HBV and HDV in a co-infection setting. Here, we used anti-preS1 end titers 10^4–10^5 (log scale) to evaluate if they can neutralize both HBV and HDV, first in vitro (**paper II, Fig. 3B-C**), and then in human-liver chimeric uPA-SCID mice challenged with HBV/HDV. One day after being passively immunized with the preS1-HDAg anti-sera, mice were challenged with HBV and HDV and the viremia levels were assessed over a period of twenty weeks post-infection. We could demonstrate that all mice receiving the preS1-HDAg anti-sera remained protected against HBV and HDV at all assessed time points, while control mice injected with naïve anti-sera developed high levels of viremia (**paper II, Fig. 4B-C)**.

4.2.3 PreS1-HDAg vaccination promotes pres1 antibody production and HDV-specific T cells in a model of chronic HBV infection

Over-secretion of HBsAg is a hallmark of HBV/HDV chronic infection and a main hurdle towards mounting sufficient anti-viral immune responses. Therefore, we aimed to explore if active immunizations with preS1-HDAg in a mouse model that resembles tolerance in the HBsAg-chronic carrier (HBsAg-Tg) would be able to elicit antibody and T cell responses [229]. Following one vaccination boost, we

could show that HBsAg-Tg mice developed preS1 antibodies, although much lower compared to the vaccinated wild-type C57BL/6J mice (**paper II, Fig. 5A**). This was reversed following two vaccine boosts, then equally high anti-preS1 levels could be achieved in the two groups (**paper II, Fig. 5A**). Interestingly, we also saw that active immunization with preS1-HDAg elicited HDV-specific T cells to both genotypes, but no preS1-directed T cells in HBsAg-Tg mice following one vaccine boost, compared to the responses in C57BL/6 vaccinated mice (**paper II, Fig. 5Bi-ii**). These findings suggest that our vaccine strategy can indeed circumvent the HBV T cell dysfunction through induction of preS1 antibodies and HDV-specific T cells. They also underscore the importance of HDAg inclusion as a naïve B- and T-cell epitope carrier able to support endogenous production of preS1 antibodies in the setting of chronic infection.

4.2.4 Passive immunizations with preS1-HDAg antisera protect HBV infected mice from acquiring HDV superinfection

Chronic HBV infected patients are at high risk of acquiring a detrimental HDV superinfection which accelerates disease progression to liver cirrhosis [36]. Till now, there is no prophylactic treatment available for these patients and current therapies are not efficient to protect them from developing a chronic HBV/HDV co-infection [35]. Therefore, we aimed to determine whether our vaccine-induced preS1 antibodies could prevent human-liver mice infected with HBV from acquiring an HDV superinfection. After establishing HBV infection and one day prior to challenging with HDV, mice were passively immunized with anti-preS1 (end titers 10^4, log scale). We could show that mice receiving preS1 IgG antibodies were protected from developing HDV superinfection while the control group that received naïve anti-sera developed high levels of viremia (**paper II, Fig. 6C; Fig. 9**).

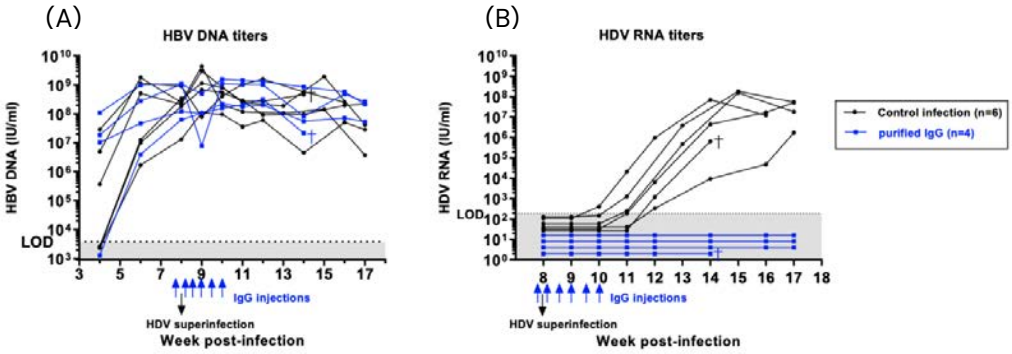

Figure 9. Prevention of HDV superinfection in HBV infected liver-humanized mice. (A) Although no effect was observed on HBV most likely due to the already established infection, (B) all mice passively immunized with preS1-HDAg anti-sera (blue lines) remained protected against HDV superinfection. Adapted from **paper II**.

Similar result was observed in a second independent experiment, in which mice repopulated with human hepatocytes from another donor could be at least partially protected (either complete protection or delayed kinetics) from developing HDV chronic infection (**paper II, Suppl. Fig. 4**). Therefore, these findings highlight the benefit of our vaccine which is to protect chronic HBV patients from getting a detrimental HDV superinfection.

4.3 Study III

Currently there is no cure for advanced HCC. T cell-based immunotherapies, mainly in the form of ICIs have managed to significantly improve overall survival rates, however still many patients do not respond to these treatments [127]. In this study, we aimed to study the presence of T cell responses to neoantigens in order to identify tumor mutation-driven T cell receptors that could be used as potential future immunotherapy for patients with HCC.

4.3.1 Mutational analysis and (putative) T cell reactivity

We manually curated and approved more than 650 coding variants arising from missense mutations in 16 out of 33 total sequenced tumors, as determined by the quality of the tumors i.e if they were viable or necrotic due to treatment and the

type of sequencing (targeted or WES) (**paper III, table S2**). Of those, we selected and prioritized SNV-derived neoantigens to test in T cell immunological assays considering i) the biological relevance of the variants i.e. if mutations occur in driver genes or frequently HCC mutated genes, ii) if the mutations are re-occurring at the same protein position among multiple patients ("hotspot" mutations) and iii) the frequency of allelic fraction as indication of clonality.

Out of the total 7 screened HCC patients with available good quality tumor samples and sufficient cells to perform functional screenings, putative T cell reactivity was detected in 4 patients (**paper III, table 1, table S1** and **Fig. 1C**). The T cell reactivity was identified based on expression of the T cell activation marker 4-1BB. Reactivity detected mainly against patient-specific mutations, in addition to one shared (hotspot) mutation in *PIK3CA* gene (**paper III, table 1** and **Fig. 1C**). We also included in the screenings peptide pools from viral hepatitis epitopes for the patients with previous history of infection, but we did not detect any reactivity (data not shown). Table 2 below summarizes clinical information for HCC samples with putative T cell reactivity to mutated proteins. Only reactivity to the mutated and not wild-type peptide sequences was assessed at this point.

Table 2. Summary of clinical information for HCC patients who were screened for reactivity in functional assays and showed putative T cell reactivity to mutated proteins. Adapted from **paper III**.

Patient ID	Age/Sex	Underlying disease	Fibrosis/cirrhosis	Pathology report	Immunogenic mutated protein
HCC02	59/M	HCV treated, HBV recovered, HAV antibodies	Cirrhosis	Moderately differentiated HCC	BCORL1^{S1530I}
HCC04	52/F	HCV treated, HBV recovered	Cirrhosis	Necrotic area after ablation, no remaining HCC cells	BRCA2^{E1593D} ERCC5^{A1119V} RANBP2^{N2068S}

HCC05	71/M	NASH	Cirrhosis stage 3-4	T1: moderately to well differentiated HCC T3: moderately to well differentiated HCC	YTHDF3^{K285E} CERS2^{Y296C} SBNO2^{L916R} CTNNB1^{H475Y} FANCA^{R825S} SNTG2^{T315N}
HCC14	73/M	alcoholic cirrhosis, portal thrombosis	Cirrhosis	T2: moderately differentiated HCC with viable cells	PIK3CA^{H1047R} HK3^{A845V} ADAT2^{A14V}

Following FACS sorting of reactive T cells based on 4-1BB expression, we performed bulk RNA-seq of the variable V-J or V-D-J regions of the TRA and TRB genes in order to pair the most dominant alpha and beta chains (**paper III, Fig. 2**). We reconstructed 21 (putative) neoantigen-reactive TCR pairs (**paper III, Fig. 2C**) and their evaluation regarding expression in new recipient cells and reactivity to their cognate antigen is currently ongoing.

4.3.2 Implementation of single-cell RNA-seq potentiates isolation of previously unidentified neoantigen-reactive TCRs

In a parallel approach, we performed an additional round of rapid expansion of the 4-1BB+ FACS sorted samples and proceeded with single-cell RNA-seq of the reactive (4-1BB positive) population while as control we used the non-reactive (4-1BB negative) T cell population (**paper III, Fig. 3**). Although, we observed that most of the samples lost their reactivity during the post-FACS sorting second expansion (**paper III, Fig. 3A**), likely due to stochastic outgrowth of non-reactive T cell clones, one sample remained positive and was enriched for its antigen recognition as is indicated by the increased expression of 4-1BB (**paper III, Fig. 2A** and **Fig. 3A**). Reactivity was specific to the mutant SBNO2 peptide while 4-1BB

expression to wild-type SBNO2 peptide remained at comparable levels as the no peptide (unstimulated) sample (**paper III, Fig. 3B**). Importantly, single-cell RNA-seq revealed three TCR clones that were solely expanded in the SBNO2-reactive sample, suggesting their antigen-specific reactivity (**paper III, Fig. 3C** and **table S4**) which was confirmed following in vitro immunological screenings for two out of three reconstructed TCRs (**paper III, Fig. 4** and **Fig. S2**).

Other studies have shown the limitation of ex vivo expanded T cells in detecting neoantigen reactivities and recognizing the potential of single-cell TCR sequencing in facilitating the detection of neoantigen-reactive T cells [247]. Accordingly, we conducted single-cell RNA-seq on memory-enriched (CD45RA/CCR7) T cells obtained from tumor, lymph nodes, and liver flush from two HCC patients (HCC01, HCC16) for whom we could not detect in vitro reactivity and from two additional patients (HCC05, HCC14) with good quality tumors and availability of cell sources in whom neoantigen-reactivity could be detected (**paper III, table S1**). The purpose was to increase the likelihood of identifying neoantigen-reactive T cells and/or their TCRs by prioritizing the characterization of most expanded T cell clones in the tumor compared to the clonotypes identified in draining lymph nodes and liver flush.

5 CONCLUSIONS & POINTS OF PERSPECTIVE

In this thesis, we aimed to investigate two immune-based approaches in the form of active (therapeutic vaccine) and passive (ACT) T cell-based immunotherapies for the prevention and treatment of HCC. Particularly, in studies I and II the goal was to evaluate a therapeutic vaccine for chronic HBV and HDV infections which comprise the main risk factor for developing HCC. In study III, we aimed to identify immunogenic neoantigens that can be targeted with specific T cell receptors as well as therapeutic vaccination for the treatment of HCC.

In **study I**, we could show that a homologous DNA-based vaccine strategy was able to elicit robust T cell responses to HBV and HDV antigens and preS1 entry-inhibiting antibodies that could limit HBV monoinfection in liver-humanized mice infected with HBV. Considering that current therapeutic regimens are not able to lead to a functional cure for chronic HBV and subsequently HDV infection, new therapeutic interventions should focus on overcoming, or circumventing, immune evasion mechanisms elicited by the virus in order to efficiently prompt the host to control the infection. The herein described approach shows the potential of bypassing two major hurdles during chronic infection; overexpression of viral antigens that block neutralizing antibodies and the induced T cell impairment.

In the clinical setting, a growing consensus implies that combinatorial approaches based on both viral-targeting and immune-directed therapies are utterly required in order to achieve a functional cure. Such combinatorial therapies should be able to i) completely suppress intrahepatic virus production and HBsAg secretion (from both cccDNA and integrated viral sequences), ii) inhibit de novo hepatocyte infection, and iii) enhance host's immune responses to promote virus-specific adaptive responses while ensuring a safe immune preservation in the liver [248, 249]. In line with this, co-treatment with NAs should be considered as part of the combinatorial strategy not only because they can reduce intrahepatic production of new virions but also importantly since they can reduce liver inflammation [249]. Preservation of liver function can in turn increase HBV-immune cells targetability and functionality, hence is important for successful immune-based therapies.

Additional anti-viral compounds such as capsid-assembly inhibitors [249], or RNA-directed therapies that are currently under clinical investigation and are aiming to silence the activity of cccDNA [250] will be most likely required in a co-treatment setting with immune-therapies in order to leap towards virus eradication.

In **study II,** we extended our previous findings by exploring different homologous and heterologous preS1-HDAg vaccine strategies to identify the most immunogenic approach capable of i) circumventing the HBsAg-induced tolerance present in the chronically infected host and ii) protecting against a chronic HBV/HDV co-infection. We could show that a heterologous preS1-HDAg vaccine scheme was able to bypass the HBsAg-specific T cell tolerance and support preS1 antibody production in a chronically infected host setting. In addition, the vaccine-induced preS1 antibodies were highly effective at preventing HBV/HDV co-infection in vivo and importantly they could protect HBV infected human-liver mice from acquiring an HDV superinfection. These findings highlight the benefit of our therapy as prophylactic strategy against HDV which is currently lacking. In addition, it may efficiently complement existing or emerging therapies targeting viral maturation, as described earlier, in order to bolster host's immune responses; a needed step towards achieving a functional cure.

In order to bring this vaccine therapy closer to the clinical setting, efforts in our group are currently focusing on optimizing the delivery method of the vaccine, comparing heterologous versus homologous strategies, optimizing protein manufacturing and scalability processes, as well as investigating mRNA delivery platforms. The goal is to have a highly immunogenic, safe and cost-effective therapeutic vaccine for chronic HBV and HDV ready to enter a phase I clinical trial in the near future.

In **study III**, we aimed to unlock novel T cell-based immunotherapies for the treatment of advanced HCC through isolation of neoantigen-driven T cell receptors. We were able to detect T cell responses against mutated neoantigens in 4 out of 7 screened HCC patients who had good quality tumor samples and

sufficient cells to perform functional screenings. The majority of the observed T cell reactivities were directed towards patient-specific mutations. Following FACS sort and bulk RNA-seq of the (putative) reactive T cell populations, we assembled TCR pairs corresponding to T cells recognizing different antigens. A major challenge here is the technical difficulty in pairing each high frequent TCRB chain with the correct TCRA [238]. Accounting for that, we additionally implemented single-cell RNA-seq for samples with confirmed reactivity to characterize their genetic profile and facilitate isolation of neoantigen-reactive TCRs. Ongoing screening of the selected TCRs confirmed reactivity for two neoantigen-specific TCRs (**paper III**, **Fig. 4**). In addition, through single-cell analysis, we are currently evaluating the immune-profile of memory-enriched T cells directly isolated from the tumor, liver flush, and draining lymph nodes. This approach can increase the likelihood of identifying T cell clones reactive to tumor antigens, as such reactive T cell populations may become skewed during ex vivo expansions and, consequently, their reactivity not being detected in vitro. It is plausible to hypothesize that most expanded T cell clonotypes within the tumor microenvironment represent "truly" tumor reactive T cell clones [238], however this remains to be confirmed through TCR screenings and identification of immune-gene signatures that can guide distinguishing bystander from mutation-specific T cells, as it has been shown in other solid cancers [247, 251].

Identifying neoantigens that could be used either in cancer vaccines or/and ACT mandate highly personalized therapies which can be very promising but also imply high manufacturing costs and scalability hurdles. Hence, concomitant efforts in our group focus on identifying T cell reactivities to public (shared) neoantigens from healthy donor samples. This can help creating a library of neoantigen-reactive TCRs which when HLA-matched could be used "off-the-shelf" in adoptive cell therapies for cancer patients.

In conclusion, this thesis has explored two promising T cell avenues in the fight against liver cancer: i) the development of a therapeutic vaccine for chronic viral hepatitis as a preventive strategy, and ii) the exploitation of neoantigen-driven

TCR-based immunotherapies as treatment for HCC. As we continue to unravel the benefits and complexities of the immune system and refine therapies, the convergence of these strategies not only underscores the potentials of T cell immunotherapy but may in future also contribute to the growing arsenal of tools aiming at conquering liver cancer and improving the quality of life for the many of patients in need.

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