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Adoptive immunotherapy for treatment of chronic hepatitis B

Nina Böttinger

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Vorsitzender: Univ.-Prof. Dr. Roland M. Schmid

Betreuerin: Univ.-Prof. Dr. Ulrike Protzer

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1. Univ.-Prof. Dr. Markus Gerhard

2. apl.Prof. Dr. Bettina Kempkes

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I Abstract

The human hepatitis B virus (HBV) is a small, enveloped, non-cytopathic DNA virus with a strong liver tropism. Infection with HBV can cause acute and chronic necroinflammatory disease, which in turn can lead to liver cirrhosis and hepatocellular carcinoma. Approximately 2 billion people have been infected worldwide, and about 240 million are currently chronically infected, representing a major public health problem. The available antiviral therapies can control HBV replication, but do not lead to an elimination of the viral transcription template (covalently closed circular DNA; cccDNA) from infected hepatocytes, which is required for virus clearance. Thus, there is an urgent need for new approaches towards an effective therapy. Since a chronic course of infection is accompanied by a weak and oligoclonal T cell response, a possible immunotherapeutic approach could be the induction of an artificial T cell response by adoptively transferred receptor-modified T cells. The present study therefore dealt with the evaluation of different strategies for treatment of chronic HBV infection using adoptive cell transfer.

In the first part, chimeric antigen receptors derived from T cell receptor-like antibodies were constructed to combine the antigen-binding properties of a natural T cell receptor with the features of a chimeric antigen receptor (CAR). CARs mediate antigen binding via an antibody-derived domain, and can induce activation of the grafted T cell in an MHC- and coreceptor-independent way. However, the newly cloned CAR constructs were found to have lost their antigen specificity, which led to the exclusion of TCR-like CARs from further evaluations.

Our group has already constructed and evaluated a chimeric antigen receptor directed against the HBV surface proteins, named S-CAR, which enables T cells to kill HBV-infected cells. In the second part of this study, natural killer cells were tested for their ability to specifically eliminate HBV-replicating hepatocytes when equipped with the S-CAR. However, S-CAR grafted NK cells exhibited a strong unspecific killing and S-CAR expression did not lead to redirected lysis of HBV+ target cells, rendering NK cells inapplicable for this treatment approach. T cells, on the other hand, even if derived from HBV-transgenic mice worked very efficiently. The latter did not show compromised effector functions when grafted with the S-CAR and compared to HBV-naïve S-CAR T cells upon adoptive transfer, representing an important finding with regard to a future clinical application.

The ability of S-CAR grafted human and murine T cells to exert specific antiviral effector functions provided the rationale for the major part of this study, representing a thorough analysis of S-CAR T cells and their properties upon transfer into HBV-transgenic mice. It could be shown that S-CAR T cells can survive over long term *in vivo*, without the acquisition of a generally exhausted phenotype despite the tolerogenic influence of the hepatic

environment. Reduction of viral load, though, was found to be limited to the first one to two weeks post transfer, and contraction of the S-CAR T cell population due to induction of apoptosis was identified as a major reason. In addition, remaining T cells down-regulated surface-located S-CAR in a partially antigen-dependent way, which further explained the *in vivo* silencing of S-CAR T cells. Importantly, S-CAR T cells could be efficiently re-activated *ex vivo* even at late time points post transfer, demonstrating a possible rescue of effector functions. This and the fact that S-CAR T cells were found to change to a memory phenotype *in vivo* indicates the establishment of a long-lived T cell population that would be able to counteract recurrent viral replication in the setting of chronic infection. In the clinical setting, the dampening of S-CAR T cell responses could even be beneficial for the patient because it limits potential pathogenesis and ensures maintenance of liver integrity.

The direct comparison of S-CAR T cell effector functions with those of vaccine-induced HBV-specific T cells revealed that S-CAR T cells were superior in our model, since they induced hepatocyte killing in addition to the antiviral effects elicited by non-cytopathic mechanisms. Importantly, liver damage in recipient mice was only transient, and treated mice did not exhibit severe side effects upon S-CAR T cell transfer.

In summary, adoptive T cell therapy of chronic hepatitis B using a chimeric antigen receptor was analyzed in detail and important insights into the fate and phenotypic changes of S-CAR T cells *in vivo* were obtained, which represents a crucial step towards the successful clinical application of this approach in the future.

II Abbreviations

µm	Micrometer
µM	Micromolar
AA	Amino acid
Ab	Antibody
ALT	Alanine Aminotransferase
APC	Antigen presenting cell
BFA	Brefeldin A
bp	Base pair
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
cccDNA	Covalently closed circular DNA
CD	Cluster of differentiation
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CMV	Cytomegalovirus
ctrl	Control
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTPs	Desoxyribonucleosidtriphosphates
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbant assay
EMA	Ethidium monoazide
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
GMP	Good manufacturing practice
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus

HBVtg	Hepatitis B virus transgenic
HCC	Hepatocellular carcinoma
HCMV	Human cytomegalovirus
HSCT	Hematopoietic stem cell transplant
ICS	Intracellular cytokine staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-β-D-thiogalactopyranosid
kb	Kilo base
kD	Kilo Dalton
LAL	Liver associated lymphocytes
LAMP-1	Lysosomal-associated membrane protein 1
LCMV	Lymphocytic choriomeningitis virus
LTR	Long terminal repeat
MCP	Monocyte chemotactic protein-1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MVA	Modified vaccinia virus Ankara
NaPyr	Sodium pyruvate
NEAA	Non-essential amino acids
NK cell	Natural killer cell
nm	Nanometer
nM	Nanomolar
NTCP	Na+-taurocholate cotransporting polypeptide
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-1	Programmed Death-1
Pen/Strep	Penicillin / Streptamycin
pgRNA	pregenomic RNA

polyA	Polyadenylation
PRR	Pattern recognition receptor
qPCR	Quantitative PCR
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
scFv	single chain variable fragment
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAA	Tumor associated antigen
TCR	T cell receptor
tEGFR	Truncated epidermal growth factor receptor
Tim-3	T cell immunoglobulin- and mucin-domain-containing molecule-3
TNF	Tumor necrosis factor
w/o	Without
WHV	Woodchuck hepatitis virus
WMHBV	Woolly monkey hepatitis B virus

1 Introduction

1.1 The Hepatitis B virus

1.1.1 Classification

The hepatitis B virus (HBV) was first described in 1970 (Dane et al., 1970) and is the prototype member of the hepadnavirus family. *Hepadnaviridae* are enveloped, double-stranded DNA viruses that can cause liver infections in humans and animals. They can be divided into the genera *orthohepadnaviridae* and *avihepadnaviridae*, depending on their ability to infect mammals or birds. Important characteristics of hepadnaviruses are their narrow host range, their high tropism for hepatocytes and the replication through an RNA intermediate involving a reverse transcription step. Hepatitis B virus is classified into eight genotypes A-H that differ in their sequence by 8% or more. Furthermore numerous subgenotypes have been described that differ by at least 4% (Schaefer et al., 2007).

1.1.2 Particle structure

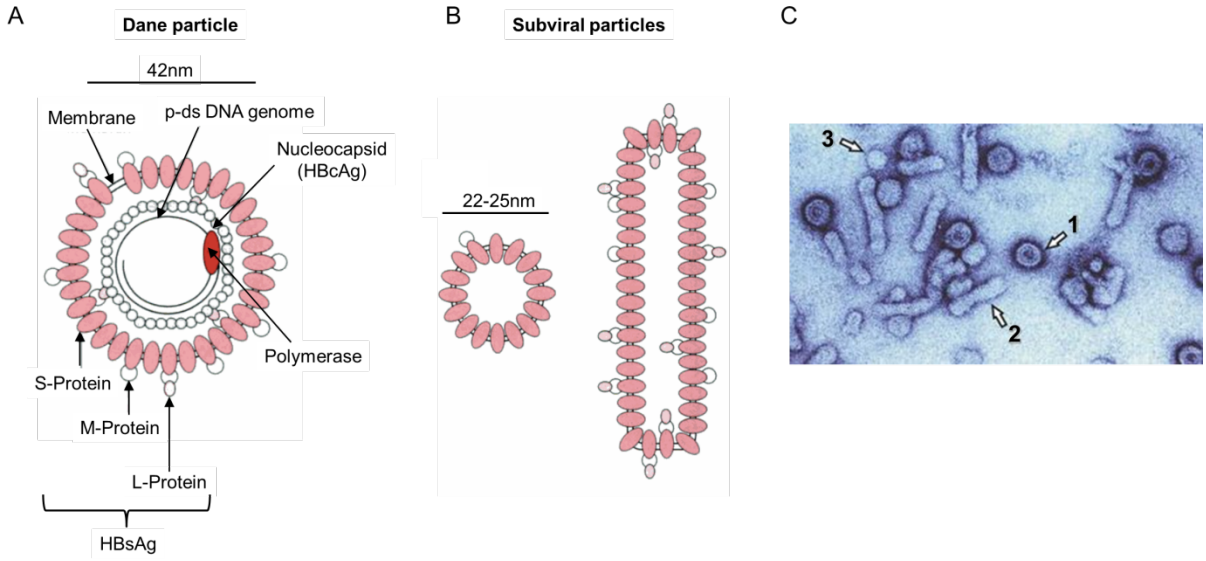


Figure 1.1 Structure of HBV particles. A) Structure of a Dane particle. The lipid envelope contains the small, middle and large (S-, M-, L-) surface proteins. The nucleocapsid is formed by the core antigen (HBcAg) and contains the partially double-stranded (p-ds) relaxed circular genome of about 3.2kb. Associated with the genome is the viral polymerase at the 5' end of the minus strand, as well as a primer domain at the 5' end of the plus strand. **B)** Structure of subviral particles. SVPs can be spherical (left) or filamentous (right), with the spheres mostly consisting of the small surface proteins and host-derived lipids. *Modified from: Hepatitis B – Infektion, Therapie, Prophylaxe; Georg Thieme Verlag 2006.* **C)** Electron microscope presentation of HBV particles. The three different particle types are marked with arrows (1: Dane particle; 2: filament; 3: sphere). *Modified from: H.-W. Zentgraf, Universitätsklinikum Heidelberg.*

Figure 1.1 A depicts a complete, infectious HBV particle. The virions, also called Dane particles, have a diameter of around 42nm and can be found in the sera of HBV infected individuals (Gerlich and Glebe 2010; Figure 1.1 C). The icosahedral nucleocapsid formed by the viral core protein (HBcAg), which harbours the viral genome in complex with the virus polymerase, is protected by a lipid envelope containing the HBV surface proteins (HBsAg). The surface proteins exist as small, middle and large surface proteins (S, M, L) that share the same carboxyterminus, but differ in the size of the N-terminal domain. They contain four helical transmembrane domains; and TM1 of the L-protein has been shown to exist in two different conformations (Bruss et al., 1994) (Figure 1.2 A+B). The a-determinant located in the extracellular loop is targeted by neutralizing antibodies and determines the serotype of the respective genotype (Figure 1.2 C; Bowden and Locarnini 2006).

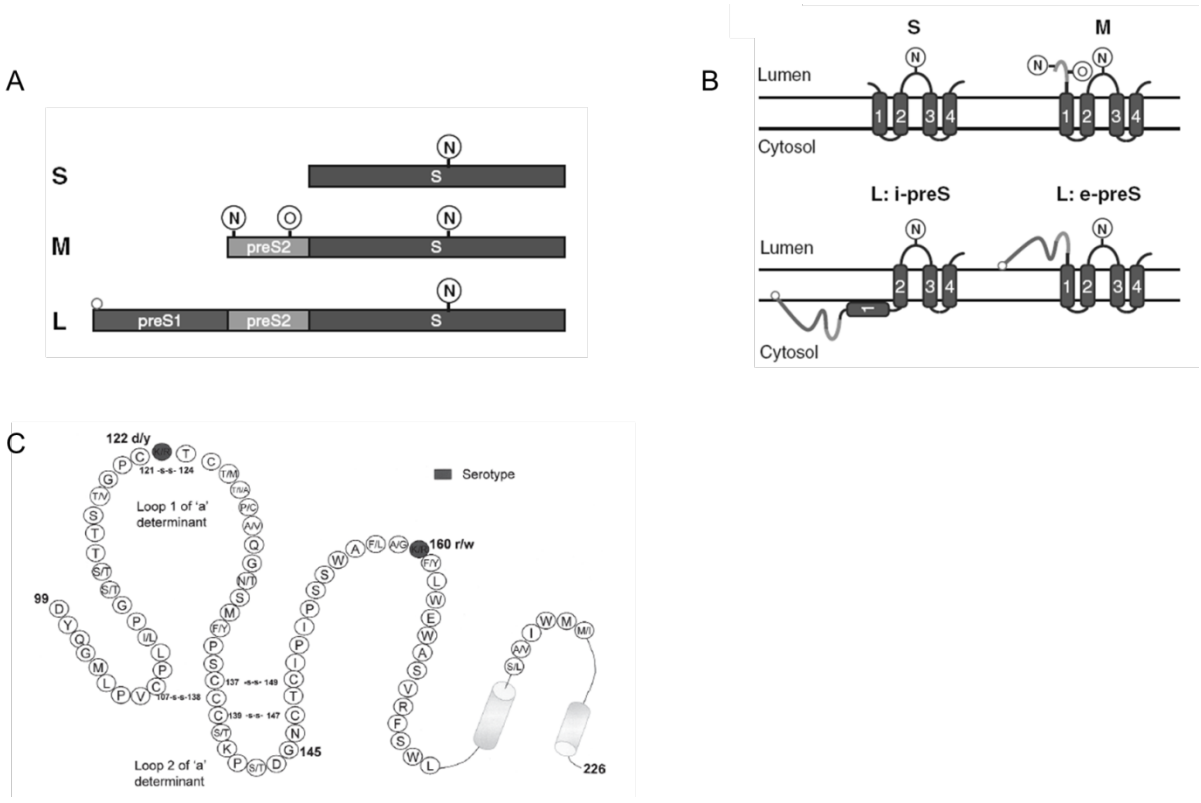


Figure 1.2 HBV surface proteins. A) Domain structures of the S, M and L envelope proteins. All three proteins contain the S-domain (S) consisting of 226 aa. The M and L proteins are extended to the 55aa preS2 domain (M) and the N-terminal preS1 domain (L) of 108 or 119aa. N: N-glycans; O: O-Glycans. A myristic acid is attached to the N-terminus of L (small circle). **B)** Predicted topology of the surface proteins. 4 transmembrane (TM) domains generate two cytosolic and one luminal loop. The L-Protein can be present in different topologies: TM1 is located in the cytosol upon co-translational membrane insertion (i-PreS) and partly translocates to the luminal space during maturation (e-PreS). *Modified from: Prange 2012*. **C)** Schematic model of the HBV S-protein. The immunogenic a-determinant located in the extracellular loop is comprised of two loops that are stabilized by cystein bonds (-s-s-). Mutations in the amino acids marked in dark grey determine the respective HBV serotype (d/y and r/w →adw, ayw, adr, ayr). *From: Bowden and Locarnini 2006.*

Besides infectious virions, excessive amounts of subviral particles (SVP) can be detected in the serum during an HBV infection (Figure 1.1 B+C). SVPs can be spherical, with a diameter of around 22nm, or filamentous with variable length, and only consist of host-derived lipids and HBV surface proteins with mainly S-proteins present (Ganem and Schneider 2001). The spheres outnumber the Dane particles by a factor of 10^4-10^6 (Rehermann and Nascimbeni, 2005). There are indications that soluble HBsAg impacts the host immune response by sequestering neutralizing antibodies and by direct impairment of cellular responses (Maini et al., 2010; Kondo et al., 2013).

1.1.3 Genomic organization and structural proteins

The genomic organization of HBV is schematically depicted in Figure 1.3. In the infectious viral particle, the ~3200bp DNA genome is present in a partially double-stranded form, the relaxed circular DNA (rcDNA). The viral polymerase (P) is covalently linked to the 5´ end of the complete minus-strand, and a short hairpin RNA serving as a primer for transcription initiation is bound to the 5´ end of the incomplete plus-strand. Upon infection of the cell and entry into the nucleus, the plus-strand is repaired, leading to the formation of the supercoiled covalently closed circular DNA (cccDNA), which serves as transcription template for viral RNAs of different lengths. Viral proteins are generated from the 3.5kb pre-genomic/pre-core RNA or subgenomic RNAs (2.4, 2.1, 0.7kb) using four overlapping open reading frames (ORF; preS/S, preC/C, P, X).

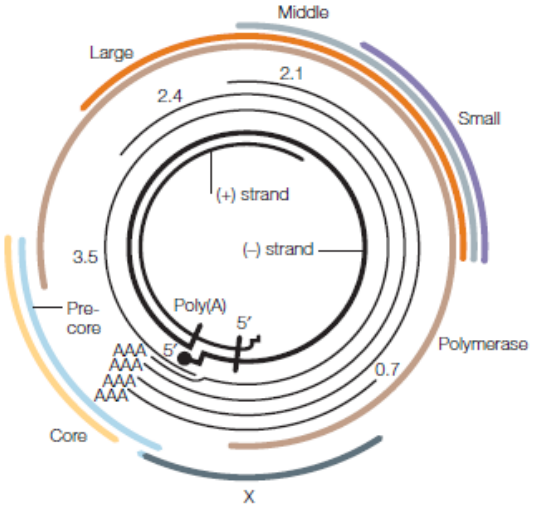


Figure 1.3 Genomic organization of HBV. The full-length minus (-) strand with the terminal protein (TP) of the polymerase attached and the incomplete plus (+) strand are represented by the inner circles. The thin black lines represent the mRNA transcripts of 3.5, 2.4, 2.1 and 0.7 kb length, terminating near the polyadenylation (poly(A)) signal. The translated HBV proteins, namely the large, middle and small surface proteins, the polymerase, the X protein and the core and pre-core proteins are represented by the outermost colored lines. *From: Rehermann and Nascimbeni, 2005.*

The S and C genes contain multiple in-frame translation initiation codons that give rise to related but functionally distinct proteins. The ORF S/preS can be structurally and functionally divided into the pre-S1, pre-S2, and S regions leading to the translation of the different envelope proteins (described in 1.1.2). The preC/C ORF encodes for the viral capsid protein HBc and the secreted hepatitis B e antigen (HBeAg). HBeAg as a surrogate marker for viral replication (Liaw et al., 2009) is dispensable for the viral life cycle but is thought to mediate immunosuppressive functions during infection (Guidotti and Chisari, 2006). Its described capacity to cross the placenta might be involved in induction of neonatal tolerance as shown in studies in HBV-transgenic (HBVtg) mice (Milich et al., 1990). HBeAg also has been shown to suppress antibody and core-specific T cell response to HBcAg in adult TCR-transgenic mice (Chen et al., 2004). The viral X protein encoded by the X ORF is required for initiation and maintenance of virus replication (Zoulim et al., 1994; Lucifora, Arzberger et al., 2011) and has been described as a strong transcriptional activator of several cellular genes associated with growth control (Chisari et al., 2010).

1.1.4 Replication cycle

An overview of the replication cycle of HBV is given in Figure 1.4. In the initial phase of HBV infection, virions bind to the cell surface and are subsequently internalized, probably via endocytosis (Kann and Gerlich, 2005). The receptors and factors involved in the entry process have been extensively studied, revealing the pre-S1 domain of the L-Protein as a key determinant for entry of HBV by interaction with cellular receptors on hepatocytes (Gripon et al., 1995). Binding to heparansulfate proteoglycans as low-affinity receptors has been shown to be important for the initiation of virus entry (Schulze et al., 2007), and addition of heparins can block HBV infection *in vitro* (Leistner et al., 2008). The main molecule supporting viral infection remained elusive until 2012, where Yan and colleagues described a functional receptor for HBV. A sodium taurocholate cotransporting polypeptide (NTCP), which is predominantly expressed in the liver, was found to specifically interact with the preS1-region of the L-Protein, thereby mediating HBV infection of hepatocytes (Yan et al., 2012).

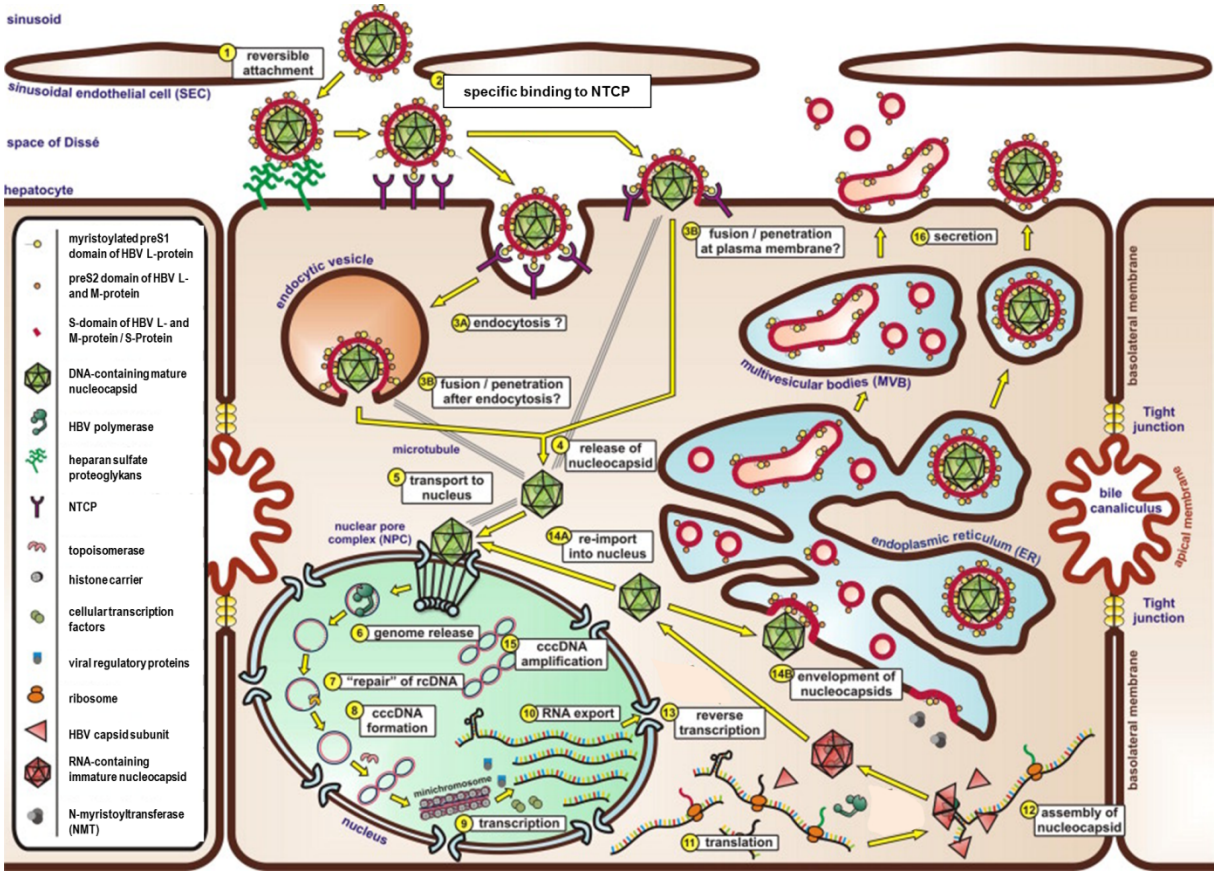


Figure 1.4 HBV life cycle. After entry mediated by sodium taurocholate cotransporting polypeptide (NTCP) and so far unknown cellular factors, the virus particle gets dissembled and the capsid is transported to the nucleus. Nuclear entry of viral DNA is followed by complementation of the plus strand, leading to cccDNA formation. Polyadenylated RNA-transcripts of different sizes are generated, with the pre-genomic 3.5kb RNA serving as template for replication. Upon encapsidation with the viral polymerase, reverse transcription takes place, forming the minus strand. After plus-strand synthesis and rcDNA formation, capsids are either recycled to the nucleus, or enveloped with HBV surface proteins, thereby forming mature capsids that are released through multivesicular bodies (MVBs). *Modified from: Urban 2010.*

Following host cell entry, the virus is disassembled and the viral genome is transported to the nucleus. The underlying mechanisms of the intracellular release, uncoating, decapsidation and nuclear transport are still poorly understood. After entry into the nucleus, cccDNA is formed by removal of the viral polymerase from the minus strand, removal of the RNA primer bound from the plus strand, and ligation of the DNA extremities for both strands after rcDNA completion. Both cellular factors as well as the viral polymerase are thought to be involved in this process (Beck and Nassal, 2007; Urban 2010). Transcription of HBV cccDNA by the cellular RNA polymerase II generates unspliced, polyadenylated RNAs with a 5´cap structure. As already mentioned, the viral proteins are translated from RNAs of different lengths using the respective ORF and translation initiation codons (see Figure 1.3). Replication starts with the encapsidation of the pre-genomic RNA via a cis-acting element on the RNA (epsilon) containing a stem-loop structure. The terminal protein of the polymerase

(TP) interacts with epsilon as well as newly formed core proteins, thereby forming the nucleocapsid. After encapsidation, reverse transcription of the pgRNA and subsequent positive-strand synthesis is mediated by the polymerase. Several steps of strand transfer lead to the formation of rcDNA, the DNA form present in infectious virions.

The assembly of mature virions with HBV envelope proteins produced in the endoplasmic reticulum (ER) has been shown to be dependent on the L-Protein (Bruss et al., 1995) and its interaction with the viral capsid (Böttcher et al., 1998). Recent studies proposed that HBV virions bud into so-called multivesicular bodies (MVBs) and exit the cell via the exosomal pathway (Watanabe et al., 2007). The release of subviral particles (SVPs) is thought to be mediated by budding via the ER and Golgi compartment (Prange 2012). Since there is a continuous membrane exchange between the ER and the plasma membrane, viral surface proteins that were not incorporated in viral or subviral particles are likely to reach the plasma membrane during this process. Nucleocapsids containing rcDNA can undergo nuclear re-import and cccDNA conversion, leading to an intracellular amplification of cccDNA. In naturally infected hepatocytes, 50 or more copies of cccDNA can be found due to this process, which, together with the expected long half-life (35-57d for DHBV, Addison et al., 2002; 33 to 50d for WHV, Zhu et al., 2001) ensures persistence of the viral genome (Beck and Nassal, 2007).

1.1.5 Epidemiology of HBV infection

Hepatitis B virus infection is a major public health problem, with approximately 2 billion people worldwide having been infected. Around 240 million people are currently chronically infected (*source: World Health Organization, WHO*) and therefore at high risk to develop hepatic decompensation, cirrhosis or hepatocellular carcinoma (HCC), with an estimated mortality of 0.5 to 1 million deaths per year (Lai et al., 2003; Liaw et al., 2009). The prevalence of chronic HBV infection is around 4% worldwide, but differs markedly between regions (Figure 1.5; Lavanchy et al., 2004; Ott et al., 2012). Infection rates are low in Western Europe and USA, intermediate in Mediterranean countries and Japan, and high in Southeast Asia and the sub-Saharan area.

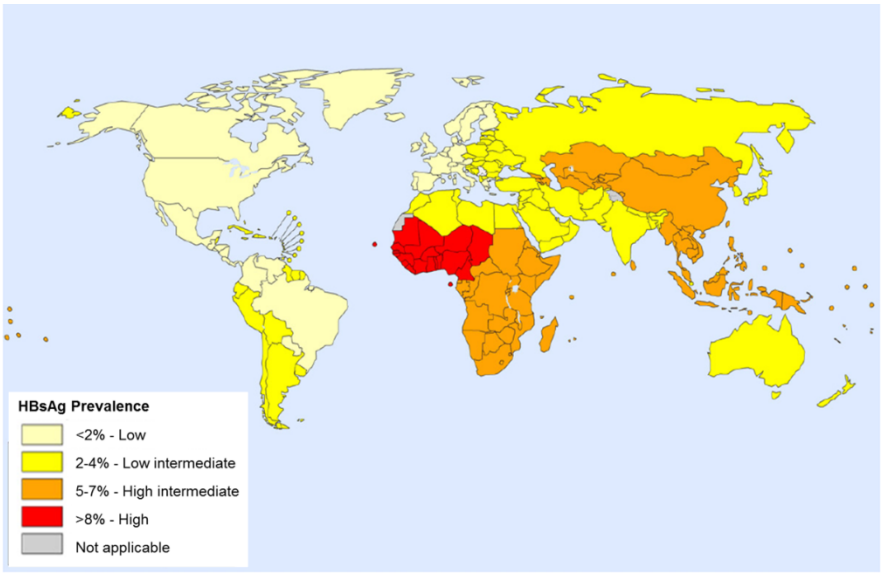


Figure 1.5 Epidemiology of HBV infection. Worldwide prevalence of HBV presented as HBsAg seroprevalence. Shown is the prevalence for adults (age 19-49) in 2005. Of note, the prevalence among children is slightly different (Ott et al., 2012). Regions colored in dark red show the highest prevalence with more than 8% of the population infected, followed by high intermediate prevalence (5-7%) and low/low intermediate endemic areas presented in lighter colors. *Modified from: Ott et al., 2012.*

The eight different genotypes A-H are geographically distributed as well, with genotype A prevalent in northwestern Europe and the USA, genotype B and C in Asia, and genotype D as the most spread in Southern Europe and the Mediterranean basin (Kao et al., 2007). Transmission of HBV occurs through blood and infected bodily fluids. In high-endemic areas, most infections occur by vertical transmission from mother to child or during early childhood. Around 90% of the HBeAg seropositive mothers transmit the virus to their offspring, leading to a high probability of perinatal infection. In areas of low prevalence, HBV infection occurs mostly in young adults, with risk factors including transfusions of unscreened blood, sexual promiscuity, sharing of syringes between drug users, working in a health care setting and renal dialysis (Liaw et al., 2009).

1.1.6 Pathogenesis of HBV infection

HBV is considered to be a non-cytopathic virus (Shimizu et al., 2012; Chisari et al., 2010). HBV infection can lead to a wide spectrum of liver disease including acute hepatitis, chronic hepatitis, cirrhosis and hepatocellular carcinoma. The disease outcome is strongly influenced by the host immune response with the virus-specific T cell response as one of the key determinants (Guidotti and Chisari, 2006).

1.1.6.1 Acute HBV infection

Acute HBV infection can range from asymptomatic infection to self-limited hepatitis and fulminant hepatitis. In adults, one third of acute infections are symptomatic. In less than 1% of cases a fulminant hepatitis develops, with a mortality rate of 70% (McMahon et al., 1985; Liaw et al., 2009). The serological and clinical changes after acute hepatitis B virus infection are shown in Figure 1.6 A. During a typical acute infection, HBV DNA remains at relatively low levels (10^2 – 10^4 genome equivalents per ml) for up to 4-7 weeks (Rehermann and Nascimbeni, 2005; Bertoletti and Ferrari, 2012). After this so-called incubation phase, HBV DNA as well as the secreted virus antigens HBeAg and HBsAg increase to peak titers. This is accompanied by the appearance of virus specific antibodies. HBcAg-specific IgM can be found early in infection and is considered to be a seromarker for ongoing acute disease (Liaw et al., 2009). Around 10 to 15 weeks post infection, serum alanine aminotransferase (ALT) levels begin to rise; indicating T cell mediated killing of HBV infected hepatocytes (Guidotti et al., 1999). There are indications that also non-cytopathic mechanisms elicited by the host immune system play an important role for clearance of viral DNA in serum and liver before the onset of clinical hepatitis (Chisari et al., 2010). Seroclearance of HBsAg occurs later and is followed by the appearance of anti-HBs antibodies, which have a virus-neutralizing character (Liaw et al., 2009). Apparent clearance of the virus can still be accompanied by persistence of cccDNA in a small fraction of hepatocytes, bearing the risk of re-activation even after a long time (Rehermann and Nascimbeni, 2005).

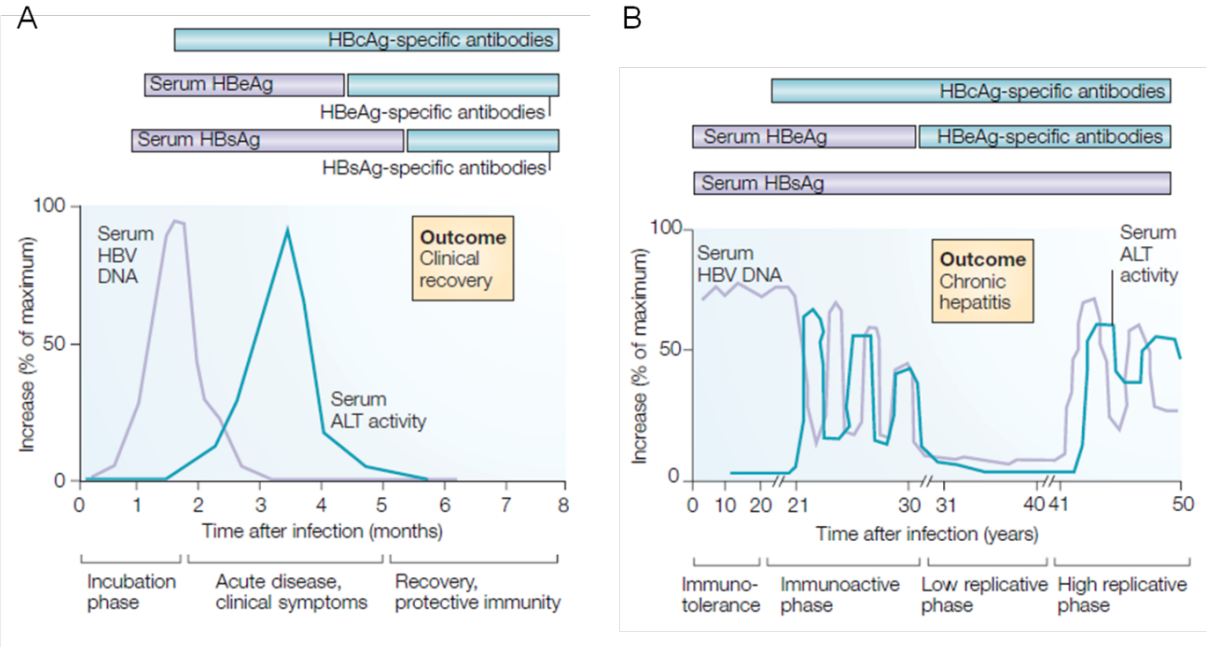


Figure 1.6 Clinical and virological course of acute and chronic HBV infection. A) Acute hepatitis B after horizontal transmission. A peak of virus replication is followed by immune-mediated clearance of the virus. HBV-specific T cells and HBsAg-specific antibodies confer lifelong protection from re-infection. **B)** Chronically evolving hepatitis B resulting from vertical transmission. Characteristic are several phases of variable length with fluctuating levels of virus replication and immune responses. See text for details. *From: Rehermann and Nascimbeni, 2005.*

1.1.6.2 Chronic HBV infection

The risk of developing chronic infection is closely correlated with the patient´s age at the time of infection: whereas the risk of chronicity for patients infected as adults is only around 1 to 5%, persistence of HBV is detected in 90% of infections after vertical transmission (Liaw et al., 2009) from mother to child. Chronic infection can be divided into several stages of variable length, starting with a clinically unapparent phase that is characterized by high levels of circulating HBV DNA and HBeAg (Figure 1.6 B). ALT levels are often normal during this phase, which can last for several years, until ALT levels rise, inflammatory liver disease becomes obvious and HBV DNA titers decline (Rehermann and Nascimbeni, 2005). Alternatively, there is a switch to a low replicative phase, during which HBeAg is cleared from the serum and HBeAg specific antibodies occur. HBV DNA is typically at low levels and ALT values are normalized. This stage of infection might last for a lifetime, but in some patients, recurrent high level HBV replication can occur, accompanied by necroinflammatory liver disease. Especially patients under immunosuppressive treatment or patients harboring certain HBV variants (core/pre-core mutations leading to increased replication / HBeAg negativity) are at risk to convert to a high replication hepatitis (Rehermann and Nascimbeni, 2005). Eventually, continuous virus replication leads to liver cirrhosis and hepatocellular carcinoma (HCC), with high viremia as important risk factor for HCC development (Chen et

al., 2006). Worldwide, around 50% of hepatocellular carcinomas are related to HBV (Lai et al., 2003; Liang et al., 2009).

1.2 The impact of the immune response on the course of HBV infection

1.2.1 Innate immune response

The role of the innate immune system during HBV infection is still controversially discussed (Bertoletti and Ferrari, 2012). Studies in HBV-infected chimpanzees (Guidotti et al., 1999; Wieland et al., 2004) and humans (Dunn et al., 2009) have shown that HBV infection does not lead to detectable changes in the expression pattern of intrahepatic genes in the first weeks of infection. In contrast, other viruses like HIV-1 induce a classical type I Interferon (IFN)/pro-inflammatory response in the infected livers (Stacey et al., 2009). This apparent lack of induction of IFN responses was interpreted as an ability of HBV to escape innate recognition, thereby acting as a “stealth virus” (Wieland et al., 2005; Chisari et al., 2010). The escape was explained by the replication strategy of HBV, with the cccDNA sequestered within the nucleus, the production of polyadenylated mRNA and the protection of newly transcribed genomes within viral capsids (Wieland et al., 2005). However, innate responses might be below the detection limit, and there is growing evidence that HBV can be sensed by the innate immune system via pattern recognition receptors (PRRs; Hösel et al., 2009; Lucifora et al., 2010; Protzer et al., 2012). HBV might also directly suppress innate immune responses via the viral proteins polymerase and X protein and by secreting large amounts of HBsAg and HBeAg into the circulation (Ait-Goughoulte et al., 2010; Bertoletti and Ferrari, 2012).

The contribution of natural killer cells as cells of the innate immune system is controversially discussed as well (Mondelli et al., 2010). NK cells are present in large amounts (40-60% of intrahepatic lymphocytes) in the normal liver (Doherty et al., 2000) and have been shown to increase within the circulation during early infection, but the effector functions did not appear concomitantly with viral clearance and were suppressed as viral load increased (Webster et al., 2000; Dunn et al., 2009; Fisicaro et al., 2009). NK cells probably play a more pathogenic than protective role in the course of infection (Dunn et al., 2007).

1.2.2 Adaptive immune response

While the contribution of the innate immune system to the development of early infection is still debated, the adaptive immunity is considered to be crucial for the course of infection, with virus specific T cell responses as a key player (Bertoletti and Ferrari, 2012). HBV-

specific CD4 and CD8 T cell responses can usually be detected after the exponential increase of HBV replication (Webster et al., 2000; Fisicaro et al., 2009) that follows the incubation phase with low replication levels, which probably prevent T cell priming. Although $CD4^+$ HBV specific T cells can be detected during the course of infection, it has been shown that they are indispensable for viral clearance during early infection, but depletion at the peak of liver disease has no impact on viral clearance in HBV infected chimpanzees (Baumert et al., 2007; Guidotti and Chisari, 2006). This indicates an important role of $CD4^+$ T cells as helper cells for $CD8^+$ T cell priming, with the $CD8^+$ T cells as main effector cells during infection (Thimme et al., 2003; Baumert et al., 2007; Yang et al., 2010; Chisari et al., 2010).

Importantly, $CD8^+$ T cells exert two different and non-related functions that contribute to viral clearance (Bertoletti et al., 2003). The decrease of viremia before the onset of liver disease is largely mediated by cytokines, most importantly IFNγ and TNFα, which have been shown to be secreted by $CD8^+$ T cells (Guidotti et al., 1996; Bertoletti and Ferrari, 2012) and lead to elimination of nucleocapsid particles as well as destabilization of viral RNA (Guidotti et al., 1996). After this initial decline of viral load, a cytolytic immune response ensues, causing both clinical hepatitis as well as final virus control (Guidotti et al., 1996; Yang et al., 2010). By secretion of cytokines and production of IFNγ-inducible chemokines CXCL9 and CXCL10, cytotoxic T cells further recruit antigen-non-specific inflammatory cells into the liver, thereby initiating a cascade of immunological events (Guidotti and Chisari, 2006). Viral clearance is associated with a vigorous, polyclonal and multispecific CD4 and CD8 T cell response (Maini et al., 2000; Chisari et al., 2010), which can be detected in the peripheral blood of patients with acute self-limited hepatitis B (Michel et al., 2002).

In chronic infection, this T cell response is weak, antigenically restricted or undetectable (Michel et al., 2002; Maini et al., 2000) and can lead to a persistent inflammation by low-level cytokine secretion without elimination of the virus (Bertoletti et al., 2003). Even if HBV-specific CD8 T cells can be detected in chronically infected patients, they are apparently not able to mount an adequate response to eliminate the virus (Maini et al., 2000, Bertoletti et al., 2003). Reasons for that could be a functional exhaustion of HBV specific T cells, rendering them dysfunctional with regard to exerting their effector functions. Indeed, intrahepatic HBV specific T cells during chronic infection have been shown to express higher levels of the exhaustion marker Programmed Death-1 (PD-1), and upregulation of PD-1 is associated with liver inflammation and ALT elevation (Fisicaro et al., 2010). In line with that, also the ligand for PD-1 (PD-L1) is upregulated on hepatic cells in patients with chronic viral hepatitis (Kassel et al., 2009). Recent work suggests a contribution of multiple inhibitory receptors to CD8 T cell exhaustion (Blackburn et al., 2009). Apart from PD-1, Tim-3 (T cell

immunoglobulin- and mucin-domain-containing molecule-3; Nebbia et al., 2012) as well as cytotoxic T lymphocyte antigen 4 (CTLA4, Schurich et al., 2011) as other markers of exhaustion have been described as being upregulated on CD4 and CD8 T cells in chronic HBV infection. Furthermore, CD8 T cells of chronically infected individuals show a pro-apoptotic phenotype (Lopes et al., 2008) that is thought to be mediated by insufficient activation by liver-located antigen-presenting cells (Bowen et al., 2004). Apoptosis may also be promoted by co-inhibitory signals delivered through CTLA4 (Schurich et al., 2011) or by T cell-intrinsic transforming growth factor-β (TGFβ; Tinoco et al., 2009).

Besides T cell intrinsic factors, the influence of extrinsic factors on HBV specific T cells is also likely to mediate disease outcome. HBV specific T cells could be suppressed by other immune cells such as regulatory T cells that have been found to be present in a higher proportion in livers of chronically infected patients with high viral load (Stoop et al., 2008) and suppress HBV specific T cells (Stross et al., 2012). Similarly, secretion of the immunosuppressive cytokine IL-10, that can be induced by HBcAg is also thought to negatively regulate T cell effector functions during chronic infection (Li et al., 2010). Moreover, an impaired function of dendritic cells (DCs) as specialized antigen-presenting cells could be involved in insufficient T cell priming (Wang et al., 2001). The mechanism causing DC dysfunctionality is unknown, but might involve HBV particles as well as soluble HBsAg (Op den Brouw et al., 2009).

1.3 Model systems for HBV infection

Since *in vitro* models are fast and offer well-controlled experimental settings, much work has been done to develop highly sophisticated systems based on cell lines or primary hepatocytes. For example, transfection of linear full-length HBV genomes leads to intracellular formation of cccDNA in hepatoma cell lines and allows for the investigation of virus and host factors on the transcriptional activity of cccDNA (Günther et al., 1995). The isolation of a cell line susceptible for HBV infection, the HepaRG cells (Gripon et al., 2002), represented an experimental breakthrough by allowing for the study of early infection steps and treatment approaches (Lucifora et al., 2011). However, its infection efficiency is limited. Although the discovery of NTCP as receptor for HBV (Yan et al., 2012) enabled the generation of additional cell culture models, they cannot completely replace *in vivo* model systems. Only appropriate animal models allow for studying of the virus within the complex liver environment and its interactions with the immune system, thereby making them indispensable for the confirmation and extension of results generated in cell culture experiments.

Yet, the strict species specificity of HBV, with humans as only natural hosts, greatly impairs *in vivo* investigations. The fact that chimpanzees can be experimentally infected with HBV has been used to gain important insights into the pathogenesis and immune responses during HBV infection. The chimpanzee model, however, is limited by high costs, low availability and strong ethical considerations (Rehermann and Nascimbeni, 2005; Dandri et al., 2013). Therefore, the investigation of HBV-related viruses in their natural hosts or in cell culture is the basis of many important advances in HBV research. Studies involving the duck hepatitis virus (DHBV) with the Pekin duck as the natural host generated substantial information about the hepadnaviral replication mechanism (Beck and Nassal, 2007), but the low sequence homology of HBV and DHBV as well as differences in the course of the infection limit further use of this model. The Woodchuck hepatitis virus (WHV) is more similar to HBV with regard to the genomic organization and the establishment of a chronic infection, and studies with experimentally infected woodchucks were fundamental for the preclinical development of antiviral drugs. Disadvantages of the woodchuck model are the limited availability of reagents, the difficult handling and the fact that woodchucks cannot be bred and have to be captured. Primary hepatocytes isolated from the tree shrew *Tupaia belangeri* represent a valuable source for HBV-permissive cells, and are even more susceptible to infection with the woolly monkey hepatitis B virus (WMHBV) that has been isolated from a new world primate. Tree shrews can also be experimentally infected with HBV, however at a low efficiency, which further compromises *in vivo* experiments in addition to the difficulties concerning the handling of the animals (Dandri et al., 2013).

The fact that all of the mentioned animal models suffer from different constraints has led to the development of several mouse models for HBV infection. Hydrodynamic injection of naked HBV DNA into murine livers can induce fast, high-level viral replication followed by rapid clearance of the virus by the murine immune system (Yang et al., 2002). This allows for the investigation of certain antiviral approaches as well as immune responses during acute infection. Injection of a modified plasmid DNA could delay the rapid immune clearance of the virus (Huang et al., 2006), thereby developing a model for HBV persistence. However, an important disadvantage of hydrodynamic injection is the strong discomfort caused in the animals as well as injection-induced liver damage that might already influence immune responses in a virus-independent way (Dandri et al., 2013). The intravenous injection of adenoviral vectors containing 1.3-fold HBV genomes leads to HBV replication in the liver of the treated mice followed by an acute, self-limited infection allowing for the investigation of immune-mediated viral clearance *in vivo* (Sprinzl et al., 2001; von Freyend et al., 2011). By administration of low doses of adenoviral vectors, it is possible to generate persistent infection similar to a chronic hepatitis B (Huang et al., 2012).

An approach to investigate effects on infected human hepatocytes *in vivo* is the generation of chimeric mouse models, with the urokinase-type plasminogen activator (uPA) transgenic mouse as the most widely used one. The transgene induces hepatocyte damage which creates the space for transplanted human hepatocytes to reconstitute the liver, and crossing with an immunodeficient mouse strain like the SCID (Severe Combined Immune Deficient) mouse model circumvents rejection of human cells. The generated chimeric liver tissue is susceptible for HBV infection and thereby offers potential for the investigation of viral entry and spreading and preclinical drug testing (Dandri et al., 2013), but lacks a functional immune system. In addition, the liver microenvironment constituted by murine chemokines and cytokines cannot crosstalk to the human hepatocytes, representing an important disadvantage of this model.

By integration of a terminally redundant, over-length 1.3 HBV-DNA construct into the murine genome, a transgenic mouse model (HBV1.3tg) with high titer virus expression was generated (Guidotti et al., 1995) and used for studying mechanisms of HBV pathogenesis and drug-mediated antiviral suppression (Dandri et al., 2013). In the work presented here, an HBV1.3tg-derived mouse strain was used that lacks functional HBx expression (HBVxfs; Dumortier et al., 2005). A clear disadvantage of this model is the fact that virus replication is stably driven by the transgene, which precludes the complete eradication of the virus by any therapeutic approach. However, this model was chosen because it allows for the investigation of antiviral effects in the presence of a functional immune system and liver microenvironment.

1.4 Prophylaxis and therapy of HBV infection

The infection rate with HBV can largely be diminished by avoiding or interrupting transmission. For example, routinely done serological screening of donor blood has led to a substantial reduction of transmission via blood transfusions. The fact that HBsAg-specific antibodies can neutralize the virus has led to the development of a vaccination with recombinantly produced HBsAg in the early 1980s (Liaw et al., 2009). Since the vaccination was recommended to be added into all national vaccination programmes by the WHO in the early 1990s, worldwide rates of infection have fallen. The administration of HBV-specific immunoglobulins is effectively used for newborn infants of HBV-infected mothers, as post-exposure prophylaxis in the health care setting or for the prevention of reactivation after liver transplantation (Kann and Gerlich, 2005; Liaw et al., 2009). However, HBV remains a major healthcare problem worldwide. It is still highly endemic in low-income countries where the vaccination is given less commonly or transmission occurs during early childhood (Liaw et al., 2009). In Germany, around 0.6% of the population (representing around 500.000

persons) are HBsAg positive (*source: Robert-Koch-Institut*); with the major part belonging to one of the risk groups discussed in 1.1.5. Effective treatment approaches are therefore indispensable for the control of HBV infection.

The first line treatment of chronic HBV infection consists of the administration of antiviral drugs that target the reverse transcriptase activity of the viral polymerase, comparable to antivirals used for HIV therapy. Entecavir and Tenofovir are nucleos(t)ide analogues that interact with viral replication and thereby effectively and safely lead to suppression of viral DNA and normalization of ALT levels (Liaw et al., 2009). Telbivudine, Lamivudine and Adefovir also belong to the same group of therapeutics, with Lamivudine, initially used for HIV treatment, being the first antiviral that was administered during chronic HBV infection in the mid-1990s (Lai et al., 2003, Hadziyannis et al., 2011). Entecavir is considered to be the most potent of those drugs, followed by Tenofovir and Telbivudine (Liaw et al., 2009). Responses to those antivirals can be strong and efficient, with a drop of HBV markers, amelioration of liver pathology and reported HBeAg seroconversion in around 20% of patients treated with Lamivudine for 1 year. Since such antivirals are usually well tolerated, a lifelong administration is generally possible (Liaw et al., 2009). However, administration frequently leads to the emergence of resistant virus strains that can rapidly outnumber the susceptible strains, leading to possible exacerbation of the disease. The newer antivirals Entecavir and Tenofovir have been designed in a way that avoids viral escape; thereby mutation rates during treatment are very low in those cases (Petersen et al., 2012). Lamivudine monotherapy, however, causes high drug resistance (Kann and Gerlich, 2005) and is still frequently used especially in developing countries due to lower costs. Apart from the fact that resistant virus strains emerge during treatment, treatment with nucleoside analogues does not achieve a final clearance of the virus. Although virus specific T cell responses can partly be enhanced under treatment (Boni et al., 2003; Kondo et al., 2013), this effect is only transient and complete elimination of infected cells is not achieved, bearing the risk of reactivation (Kann and Gerlich, 2005; Liaw et al., 2009).

A second clinically accepted treatment approach is the administration of Interferon-alpha (IFNα), which is available for treatment since the 1980s. In 2005, pegylated IFNα was introduced, which is more effective and can be administered once a week subcutaneously (Hadziyannis et al., 2011). IFNα as a type I IFN has immune-modulating capacities as well as direct antiviral activity. Engagement of the IFNα/β receptor complex upon binding activates the intracellular Jak/Stat signaling pathway and thereby leads to transcription of Interferon-stimulated genes (ISGs) that stimulate the host immune response (Sadler et al., 2008). An advantage of IFNα therapy is the absence of drug-resistant mutants. However, the response rate to IFNα is rather low (30-40% in HBeAg-positive patients) and differs

between the genotypes, with genotype A being for example more susceptible than other genotypes. Another drawback of IFNα therapy is the association with many side effects including influenza-like symptoms, anorexia, weight loss, fatigue and bone marrow suppression (Liaw et al., 2009).

1.5 New therapeutic approaches

Since currently available treatment options are not satisfying with regard to virus elimination, there is a strong need of better therapy options for chronic HBV infection. One focus of new therapeutic approaches is the targeting of different steps of the viral life cycle. For example, substances blocking virus entry and thereby re-infection are under current investigation. A synthetic lipopeptide derived from the pre-S1 domain of the L-Protein, named Myrcludex B, has been described to block HBV infection *in vitro* (Gripon et al., 2005) and *in vivo* in the uPA SCID model (Petersen et al., 2008) and is currently investigated in clinical trials. The discovery of NTCP as HBV receptor (Yan et al., 2012) has also revealed the target molecule of Myrcludex B.

Activation of the innate immune response using TLR agonists can lead to the induction of pro-inflammatory cytokines/type I interferons that have been shown to counteract HBV replication (Isogawa et al., 2005; Guo et al., 2009). The host adaptive immune response is, however, a key factor for the outcome of the disease as already described. Therefore, a variety of new therapeutic approaches aims at the activation and restoration of the patient´s dysfunctional immune response via various routes. One way is the application of a therapeutic vaccination strategy, which could be vaccination with peptides, proteins or DNA. Several studies have already employed those approaches, with partly encouraging results (Shimizu et al., 2012). Besides, different strategies aim for the activation of dendritic cells for induction of HBV specific immune responses, for example by antibody-induced *in vivo* activation or injection of DCs loaded with HBV-specific peptides/proteins (Shimizu et al., 2012). As already mentioned, one factor of the dysfunctional T cell response during chronic hepatitis B is thought to involve T cell intrinsic inhibitory signals via receptors like PD-1, CD244 (Raziorrouh et al., 2010) or Tim-3 (Nebbia et al., 2012). Therefore, new therapeutic approaches aim at the blockade of those signals. Indeed, antibodies against PD-L1 have been shown to partially restore T cell functions (Fisicaro et al., 2010). Along the same line, anti-apoptotic drugs could protect HBV-specific T cells from programmed cell death (Lopes et al., 2008). However, none of these approaches has been used in the clinical setting yet to prove safety. With regard to the PD-1 pathway, studies in animal models have demonstrated that the absence of PD-11/PD-L1 causes severe autoimmune liver damage (Iwai et al.,

2003; Barber et al., 2006), which could further enhance liver pathology. A precise evaluation of beneficial versus adverse effects is therefore indispensable when such pathways are blocked therapeutically.

The targeted delivery of cytokines to the liver would be another possibility to induce an immune response. An elegant way to specifically target HBV-infected hepatocytes represents the coupling of the respective cytokine to an antibody recognizing liver cells that express HBV proteins. Sastry et al. have described the production of a so-called T cell receptor-like (TCR-like) antibody directed against the HBV Env183-91 peptide presented on HLA-A2 by immunizing mice with soluble, peptide-loaded MHC molecules. The generated antibody was shown to specifically recognize HBV peptide/MHC complex on hepatocytes naturally infected with HBV (Sastry et al., 2011), thereby providing a tool for visualization of HBV infection as well as for targeted delivery of cytokines and antiviral drugs. In addition, an antibody directed against the HBV core18-27 peptide in complex with HLA-A2 was developed using the same strategy (Ji et al., 2012). Further studies have to address the question whether such antibodies are applicable in the clinical setting.

1.6 Redirection of cytotoxic T cells

Besides the mentioned approaches to re-activate the intrinsic T cell response, other strategies aim at artificially directing the otherwise dysfunctional T cells of a chronically infected individual to HBV-infected hepatocytes.

1.6.1 Cytotoxic T cells

With the $CD8^+$ T cells being the most important effector cells during HBV infection, redirection of cytotoxic T cells is the focus of many therapy approaches. The characteristics of $CD8^+$ T cells and their mode of antigen recognition are depicted in Figure 1.7. As an important part of the adaptive immune system, cytotoxic T cells are responsible for the recognition and elimination of foreign intracellular antigens like viruses. They circulate between the blood and the peripheral lymphoid organs where they get in contact with their cognate antigen via the T cell receptor (TCR). The TCR is composed of two chains (95% α and β chain; 5% γ and δ chain) forming a heterodimer, which is stabilized by cysteine bonds (Figure 1.7 A). The extracellular variable domains mediate specific antigen recognition. In contrast to antibodies that recognize native antigen, TCR recognition is directed against a peptide in complex with an MHC (Major Histocompatibility Complex) molecule on an antigen-presenting cell (APC). In the case of $CD8^+$ T cells, the TCR targets processed intracellular antigens presented on MHC I molecules that can be found on every nucleated cell of the body.

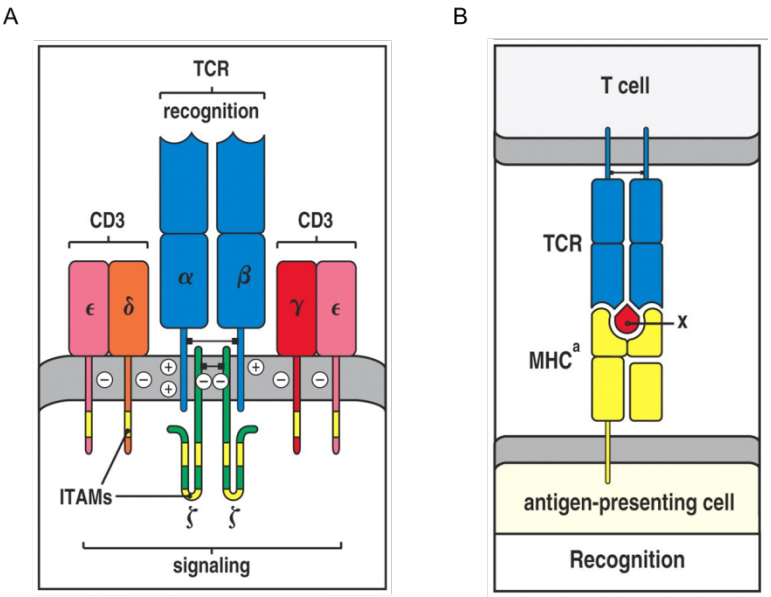


Figure 1.7 Cytotoxic T cells. A) Structure of the TCR/CD3 complex. Antigen recognition is mediated by α and β chain, whereas the CD3 complex mediates T cell activation upon antigen binding. The signal cascade is initiated by phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the intracellular CD3 chains. **B)** TCR/APC interaction. CD8+ T cells recognize endogenously processed peptides in a MHC-restricted fashion. Not shown: Interaction of co-receptors (see text for details). *Modified from: Janeway et al., 2005.*

The signal transduction after specific antigen binding is conducted by the CD3 complex associated with the TCR. It consists of two extracellular heterodimers composed of ε and δ or γ chain and an intracellular homodimer composed of two ζ chains. Importantly, cognate recognition of peptide and MHC leads to activation of the T cell only if a second co-stimulatory signal is present that can be generated by the interaction of CD28 on the T cell with its ligand B7 on the APC. The presence of both signals will lead to phosphorylation of the intracellular CD3 heterodimers and the CD3ζ chain and subsequent activation of intracellular signaling pathways in the T cell. Binding of the co-receptor CD8 further enhances signal strength (Janeway et al., 2005). Finally, this activation leads to cytokine secretion and target cell lysis, which can take place by two mechanistically distinct mechanisms: the receptor-mediated pathway via CD95 (Fas) and the secretory membranolytic pathway involving perforin and granzyme (Lowin et al., 1994).

1.6.2 T cell redirection with bispecific antibodies

A strategy to recruit the endogenous T cells to HBV infected cells could be the administration of bispecific antibodies. These are recombinant protein constructs that link the specificities of two antibodies in one molecule, as depicted in Figure 1.8. Technically, the variable domains of heavy and light chain of an antibody are cloned and covalently linked, thereby generating a so-called single-chain variable fragment (scFv) that retains the original antibody specificity.

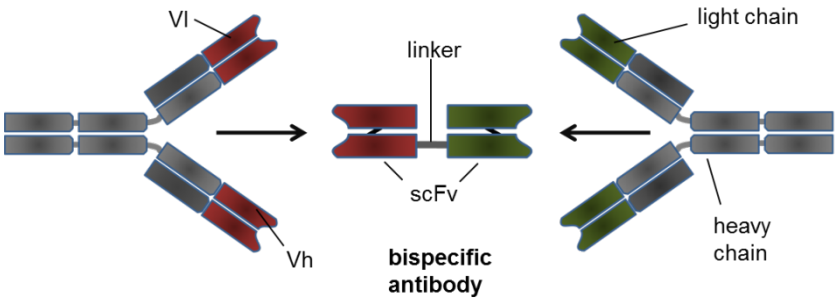


Figure 1.8 Structure of bispecific antibodies. The variable domains of an antibody derived from light and heavy chain (Vl and Vh) are covalently fused, forming a scFv (**s**ingle **c**hain **v**ariable **f**ragment). The connection of two scFvs with different specificities with a linker generates a bispecific antibody.

A huge variety of scFvs recognizing proteins, but also carbohydrates and glycolipids, have been developed (Sadelain et al., 2009). The fusion of two scFvs of different specificities with a linker creates a bispecific molecule (Baeuerle et al., 2009). T cell redirection can be achieved by fusing a CD3-specific scFv with a second scFv recognizing the desired antigen; a strategy that has been successfully employed for the targeting of several tumor antigens. Infusion of bispecific antibodies has been shown to mediate redirected tumor cell lysis *in vivo* for lung, gastric and colorectal cancer (Brischwein et al., 2006) and very efficiently for non-Hodgkin´s lymphoma in clinical trials (Bargou et al., 2008). An important advantage of bispecific antibodies concerning clinical use is the fact that they can be easily produced under GMP conditions and well controlled after administration, with a serum half-life of only several hours (Baeuerle et al., 2009). Regarding HBV, HBsAg represents a potent antigen for targeting with bispecific antibodies due to its supposed presence on the surface of an infected cell. The scFv C8 recognizing HBsAg was generated in a phage display, a selection process using human B cell-derived cDNA and recombinantly produced HBsAg. It is currently tested in combination with T cell- as well as NK cell-specific scFvs (Bohne et al., unpublished).

1.6.3 T cell redirection by adoptive transfer of antigen-specific T cells

The adoptive transfer of specific immune cells is already well established for treatment or prevention of opportunistic virus infections in allogeneic hematopoietic stem cell transplant (HSCT) recipients. Acute infection with or reactivation of Human Cytomegalovirus (HCMV) in those individuals has a high morbidity and mortality despite available antiviral treatment

(Boeckh et al., 2003). Similarly, Epstein Barr virus (EBV) infection or reactivation can lead to the development of a lymphoproliferative disease (EBV-LPD) in severe immune-compromised solid organ or HCT recipients (Berger et al., 2009). Isolation of HCMV-specific or EBV-specific T cells from the allogeneic donor, and subsequent adoptive transfer following *ex vivo* expansion can restore the patient´s immune response (Rooney 1998, Einsele et al., 2002; Peggs et al., 2003; Haque et al., 2007). The same strategy has been employed also in the setting of tumor therapy: isolation, *ex vivo* expansion and re-infusion of tumor-infiltrating lymphocytes (TIL) following depletion of endogenous lymphocytes has shown remarkable results for the treatment of metastatic melanoma (Dudley et al., 2002; Dudley et al., 2008). Yet, the selective isolation of appropriate amounts of tumor-specific T cells, the side-effect prone recognition of self-antigens as well as a low persistence after adoptive transfer complicates the development therapies for a bigger spectrum of malignancies based on the described approach (Berger et al., 2009).

Similarly, the transfer of endogenous HBV-specific T cells in the setting of chronic HBV infection is hardly feasible. As already described, HBV-specific T cells in chronic infection are only detectable in low frequencies, are antigenically restricted and exhibit an exhausted phenotype (Maini et al., 2000), which complicates direct use for therapy. However, the fact that around two thirds of HBV-infected patients safely clear the infection after having received allogeneic stem cell transplants from individuals with immunity to HBV (Ilan et al., 1993; Hui et al., 2005) supports the development of adoptive transfer strategies. To circumvent the above-mentioned obstacles involving endogenous HBV-specific T cells, virus-specific T cells with potent effector functions could be generated *ex vivo* and subsequently be transferred into the patient. To achieve this, gene-transfer approaches can be used, that graft T cells with receptors of desired specificity. Figure 1.9 depicts this treatment approach; the different engraftment strategies are presented in the next part.

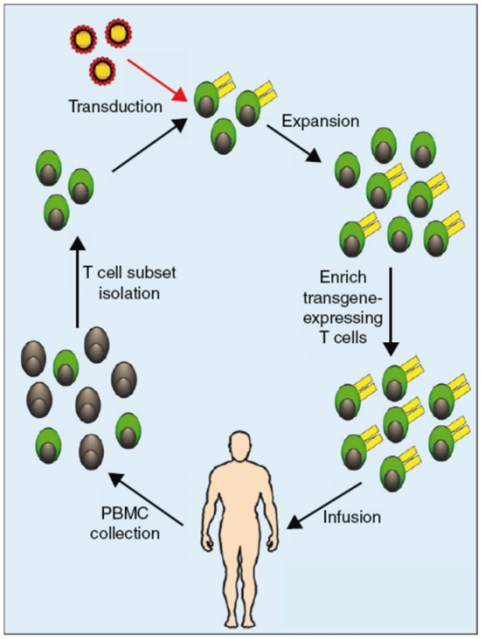


Figure 1.9 Principle of adoptive immunotherapy with genetically modified T cells. Autologous PBMC from the patient are collected and enriched for the desired cell type, i.e. CD8+ cells and genetically modified to express the transgene (TCR/CAR). Further steps could consist of an expansion and enrichment of receptor-positive cells, followed by adoptive transfer back into the patient. *Modified from: Turtle et al., 2012.*

1.7 Strategies for T cell engraftment

1.7.1 Expression of natural T cell receptors

A helpful tool for the direct isolation of T cells with certain specificity is the employment of soluble MHC-molecules loaded with the respective peptide, so called MHC multimers (Knabel 2002). T cells with the desired specificity and high avidity can be specifically stained with those molecules and isolated in a sorting process. To generate artificially redirected T cells, the respective T cell receptor alpha and beta chains can be amplified from cDNA of the T cell of interest and cloned into a vector for expression in patients T cells (Clay et al., 2002). This method has been already applied in the tumor therapy setting, with the cloning of high-avidity T cell clones targeting immunogenic peptides of tumor-associated antigens (TAA) present in melanoma (MART-1; Johnson et al., 2009), colorectal cancer (CEA; Parkhurst et al., 2011) and synovial sarcoma (NY-ESO-1; Robbins et al., 2011) with partially promising results (Morgan et al., 2006; Turtle et al., 2012).

In chronic HBV infection, the patient's T cell response is too weak to mediate appropriate recognition of infected cells as it has been described for clearance of the virus in a self-limited infection (Chisari et al., 2010). Since the T cells in the latter case are apparently very efficient, T cells from patients able to clear infection could be isolated, followed by isolation and cloning of the sequences encoding for the HBV-specific receptors. In 2011, Gehring and colleagues have described the isolation of T cell receptors from T cell lines directed against HBV peptides HBc18-27, HBs183-91 and HBs370-79 in complex with HLA-A2 and their

subsequent expression on human T cells which led to specific redirection to HBV-infected cells *in vitro* (Gehring et al., 2011). Krebs and colleagues in our laboratory have isolated a set of T cell clones from HBV convalescents against the same peptides that are very potent *in vitro* and soon to be tested in an HBVtg mouse model (Krebs et al., in preparation). A clear disadvantage of employing natural / physiological TCRs for adoptive therapy is the MHC-restricted antigen recognition. Although selection of the most abundant MHC subtype (HLA-A2 present in 50% of the population in Germany and the US; *source: www.allelefrequencies.net*) ensures the possibility of treatment for the majority of patients, individuals with different MHCI subtypes will not profit from this therapy. Furthermore, a down-regulation of MHCI on the target cell might affect the therapy success, as already shown in tumor settings where loss of MHC molecules happens upon transformation of the cells (Garrido et al., 1997). Unfortunately, hepatocytes in general have low MHC expression levels, which are also detected during chronic viral infection (Gehring et al., 2007).

1.7.2 Expression of chimeric antigen receptors (CARs)

As a possibility to circumvent the restrictions of physiological TCRs, T cells can be equipped with a chimeric antigen receptor. Such a molecule, as depicted in Figure 1.10, consists of one single polyprotein with an extracellular antigen-binding domain, a spacer domain, a membrane-spanning part and an intracellular signaling domain; a principal that was first described in 1989 (Gross et al., 1989) and subsequently termed “T-body” (Eshhar et al., 1993). The specialty of the antigen-binding domain is that it, in contrast to a natural T cell receptor, consists of a single-chain antibody which recognizes native, unprocessed antigen. This scFv is connected via an IgG-Fc-derived spacer determining the distance of effector and target cell with a signaling domain that can have different characteristics. The first generation of chimeric antigen receptors possessed an intracellular signaling domain derived from the CD3ζ chain or the Fc-receptor γ part (Sadelain et al., 2009). Such molecules conferred upon the engineered T cells the ability to secrete cytokines and to lyse target cells expressing the respective antigen (Park 2011). Clinical trials with first generation CARs directed against several tumor antigens have been conducted, i.e. for treatment of B cell lymphomas (Jensen et al., 2007) and neuroblastoma (Park et al., 2007; Pule et al., 2008). The therapeutic effects, however, were rather modest, due to limited survival and lacking proliferation of grafted T cells *in vivo* (Sadelain et al., 2009, Park et al., 2011). To improve strength and quality of antigen-induced signaling, so-called second generation CARs were engineered to display a combination of two signaling domains, with the prototype consisting of the signaling domains derived from the CD3ζ chain and the CD28 co-receptor (Hombach

et al., 2001). In third-generation CARs, the intracellular domain is completed with a third signaling part, i.e. 41BB or OX40 (Park et al., 2011). The insertion of multiple signaling domains led to increased cytokine secretion and permitted expansion in response to antigen in the absence of co-stimulation (Maher 2002). Improved survival allowed for sequential target cell killing that is mainly mediated via perforin/granzyme (Protzer and Abken, 2010; Chmielewski and Abken, 2012). Accumulating numbers of clinical trials for tumor malignancies are performed using newer generation CARs, with especially good results in trials employing CD19-CARs (Kalos et al., 2011; Porter et al., 2011) for treatment of B cell malignancies. However, frequent side effects due to the on-target/off-tumor toxicity cannot be ignored (Park et al., 2011). The administration of a third-generation CAR recognizing ERBB2 even lead to a lethal adverse event after a single administration in one trial, presumably due to targeting of healthy tissue by CAR T cells (Morgan et al., 2010).

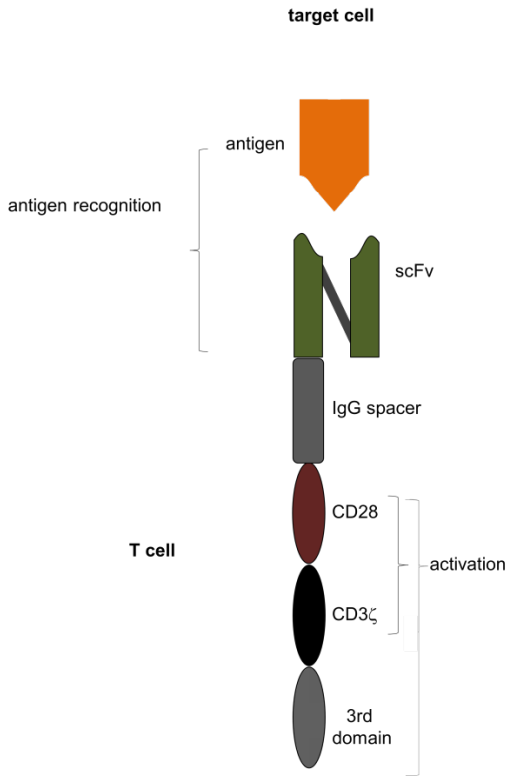


Figure 1.10 Structure of a chimeric antigen receptor. The chimeric antigen receptor (CAR) is composed of the extracellular antibody-like antigen binding domain (scFv), the spacer domain Fc and the intracellular signaling part. The signaling domain is constructed of the CD3ζ chain and frequently of the CD28 co-receptor signaling domain. The addition of a third signaling domain is optional (see text for details). By binding to the cognate antigen present on the target cell surface, an immunological synapse is formed, leading to T cell activation without the need of further co-stimulation and subsequent target cell killing.

With regard to those problems, targeting of virus-derived antigens seems more feasible, since cross-reactivity to self-antigens should be low, thereby minimizing the risk to induce autoimmunity. However, viral antigens have to be present on the infected cell in an appropriate amount to allow for clustering of CARs, which is probably needed for T cell activation. The HBV surface proteins seem to fulfill this criterium (Protzer and Abken, 2010;

Bohne, unpublished), which led to the development of a chimeric antigen receptor targeting HBsAg.

1.8 Development of a chimeric antigen receptor targeting HBsAg

By introducing the C8 scFv targeting presumably a conformational epitope in the a-determinant of the HBsAg, a second-generation chimeric antigen receptor specific for the HBV surface proteins (S-CAR) has been developed (see Figure 1.11). Engraftment of human T cells with the S-CAR using a retroviral vector system lead to specific recognition and lysis of HBV-replicating hepatoma cell lines as well as HBV-infected primary hepatocytes *in vitro* (Bohne et al., 2008).

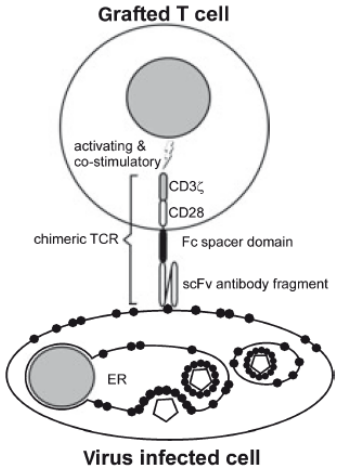


Figure 1.11 Engraftment of T cells with a chimeric T cell receptor directed against HBsAg. The S-CAR mediates binding to native HBsAg on the surface of HBV-infected hepatocytes via the C8-scFv. *From: Bohne and Protzer, 2007.*

To test whether S-CAR grafted T cells are able to reach the liver tissue and perform effector functions *in vivo*, murine T cells expressing the S-CAR were adoptively transferred into HBVtg mice. Indeed, S-CAR T cells were found to effectively home to the liver and exert profound antiviral effects, as detected by decrease of viremia as well as reduction of HBV DNA and cytoplasmic HBcAg in the liver (Krebs, Böttinger et al., 2013). Those *in vivo* data further suggested adoptive therapy using chimeric antigen receptors as a promising strategy to treat chronic Hepatitis B or HBV-related HCC.

1.9 Aim of this study

So far, there is no therapeutic option available that reliably leads to HBV eradication from chronically infected individuals. Therefore, there is a strong need for new treatment strategies. This study dealt with the overall aim to establish a safe and efficient adoptive cell therapy that allows virus elimination in chronic HBV infection and prevents the development of liver disease and HCC.

In the first part of this study, T cell receptor-like antibodies should be cloned as single chain antibodies (scFvs) and evaluated in the context of a chimeric antigen receptor with regard to their ability of redirecting T cells to HBV-replicating hepatocytes. Construction of a CAR would therefore combine coreceptor-independent signaling and prevention of TCR mispairing with the ability of a T cell receptor to recognize endogenously processed and presented antigen in context with MHC I, and thereby provide a new, additional tool for adoptive T cell therapy.

As an alternative to T cells, the study aimed at grafting natural killer cells (NK cells) with the chimeric antigen receptor targeting HBsAg (S-CAR) and subsequently testing those cells for their ability to recognize and specifically eliminate HBV^+ target cells. Background of this approach was the finding that NK cells modified to express a CAR recognizing the tumor antigen HER-2 were able to specifically eliminate HER-2-expressing target cells (Kruschinski et al., 2008). Ideally, NK cells grafted with the S-CAR could therefore complement S-CAR T cell effector functions in an adoptive cell therapy approach.

Studies in HBVtg mice had demonstrated that murine S-CAR T cells efficiently home to the liver and induce profound reduction of viral load *in vivo*. However, information about fate as well as phenotypic changes of S-CAR T cells *in vivo* was lacking. To obtain a complete picture, the third and major part of this thesis focused on a detailed phenotyping and long-term monitoring of S-CAR T cells *in vivo*. In parallel, possible side effects induced by S-CAR T cells in HBVtg mice were addressed. Moreover, murine $CD8^+$ T cells derived from HBVtg mice should be analyzed with regard to their effector potentials when grafted with the S-CAR. This represents an important aspect concerning a clinical use of S-CAR T cells in the future, since a clinical setting of adoptive transfer would imply the isolation and transduction of the patients' T cells that could possibly have been tolerized by HBV. In addition, the exerted antiviral effects of S-CAR T cells with those of naturally developed HBV-specific T cells should be compared side by side to investigate possible differences that could be important with regard to future clinical applications.

2 Results

2.1 Cloning and evaluation of chimeric antigen receptors with TCR-like specificity

2.1.1 Confirmation of specificity of TCR-like antibodies

TCR-like antibodies represent a combination of a classical T cell receptor and an antibody. The murine TCR-like antibodies generated by the Bertoletti group (Sastry et al., 2011; Ji et al., 2012) recognize either a peptide derived from the HBsAg (Env183A2) or the HBcAg (C18A2) in complex with the MHCI molecule HLA-A*0201. The aim of the first part of this study was the generation of chimeric antigen receptors with single chain antibodies derived from both the Env183A2 as well as the C18A2 TCR-like antibody (Figure 2.1.1).

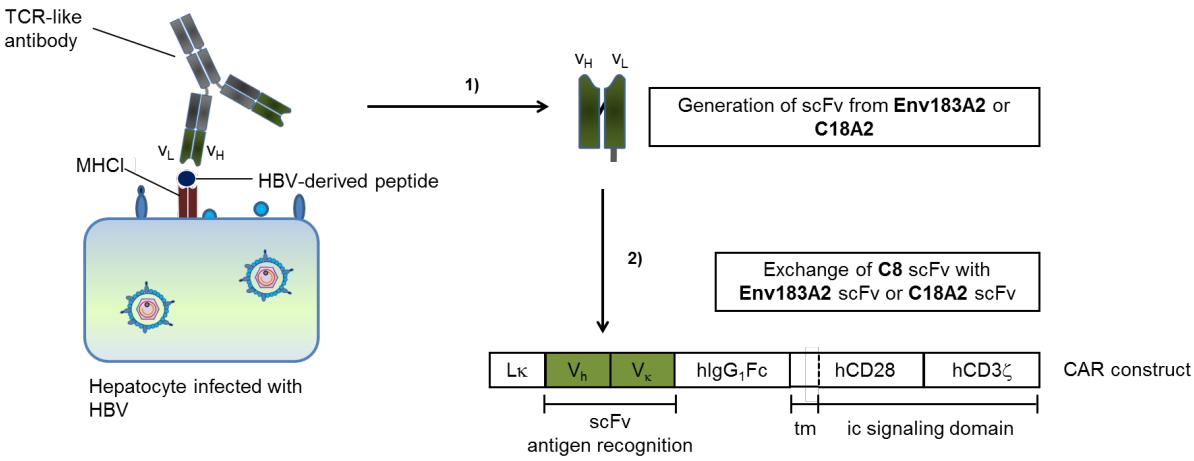


Figure 2.1.1 Outline for the cloning of TCR-like antibodies into the context of chimeric antigen receptors. Left: The antigen-binding domain of TCR-like antibodies is comprised of the variable domains of heavy (vH) and light chain (vL) and recognizes the complex of an HBV-derived peptide presented on MHCI (HLA-A2). **1)** Single chain antibodies were generated by cloning of vH and vL that are subsequently fused with a glycine-serine-linker. **2)** TCR-like scFvs were cloned into the CAR expression construct by replacing the C8 scFv which determines S-CAR specificity. LK: kappa chain leader sequence; hIgG$_1$Fc: Spacer derived from human IgG$_1$ fragment crystallizable region; hCD28: signaling domain of human CD28; hCD3ζ: signaling domain of hCD3ζ; tm: transmembrane; ic: intracellular.

To confirm TCR-like antibody specificity, supernatants of both hybridoma cell lines producing the antibodies were used for staining of HepG2-H1.3 cells. This cell line has been generated by stable transfection of the HLA-A2$^+$ hepatoma cell line HepG2 with an HBV 1.3-fold overlength genome (Jost et al., 2007), so HBV proteins should be endogenously processed and presented on the cell surface. However, when HepG2-H1.3 cells were stained with

hybridoma supernatants and a labelled secondary antibody against murine IgG, no specific signal compared to cells only stained with the secondary antibody or to HepG2 cells could be detected (Figure 2.1.2 A).

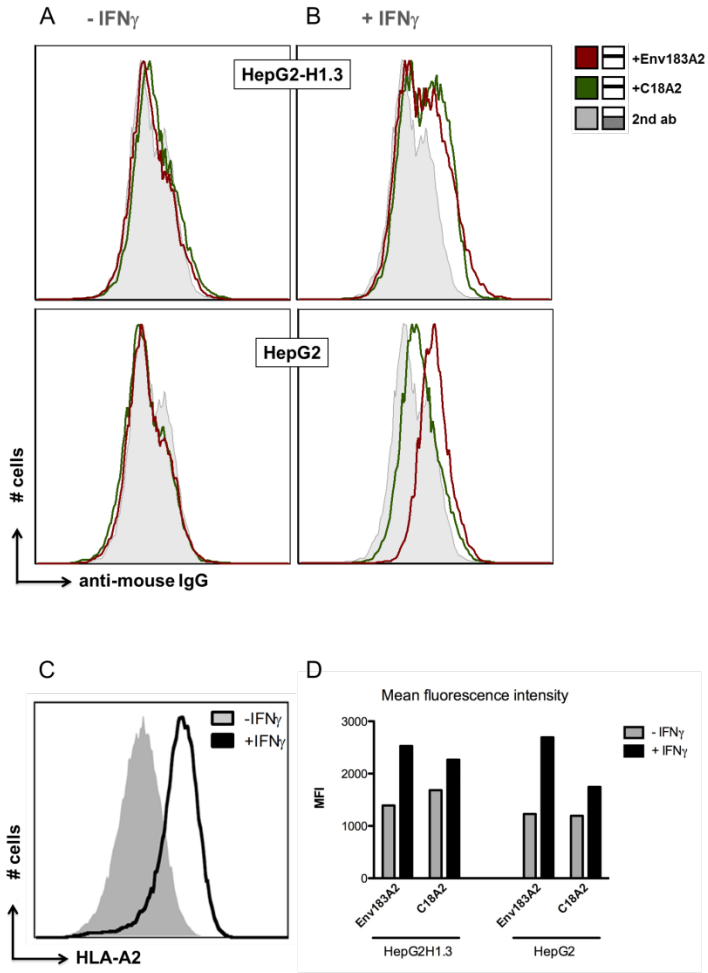


Figure 2.1.2 Staining with TCR-like antibodies. A) & **B)** Upper row: HBV-replicating HepG2-H1.3 cells (differentiated). Lower row: HBV-negative parental cell line HepG2. To visualize TCR-like antibody binding, a secondary labeled antibody targeting mIgG1 was added and samples were analyzed via flow cytometry. **A)** Cells were not treated before staining. **B)** Cells were treated with IFNγ for 48h before staining. **C)** HLA-A2 staining on HepG2-H1.3 cells without or after IFNγ treatment. **D)** Mean fluorescence intensity of labelled anti-mouse IgG (without or after IFNγ treatment).

Since hepatoma cell lines are known to show low MHC-I surface expression, cells were treated with IFNγ to up-regulate MHC-I expression (Figure 2.1.2 C). As shown in Figure 2.1.2 B and D, staining with the TCR-like antibodies following IFNγ treatment of HepG2-H1.3 cells resulted in a detectable shift of the mean fluorescence intensity (MFI) when Env183A2 or C18A2 were added. However, also IFNγ-treated HepG2 cells added as HBV negative control cell line stained positive especially with the Env183A2 antibody and led to increased mean fluorescence intensities (Figure 2.1.2 B and D); which implemented that IFNγ treatment enhanced also unspecific antibody binding. Therefore, the binding of the

antibodies to endogenously loaded MHC:peptide complexes could not be shown conclusively.

The second approach was to exogenously load HLA-A2^{+} cells with the respective peptides (Env183A2: S-peptide 183-91 or **FLL**TRILTI; C18A2: Core-peptide 18-27 or **FLP**SDFFPSV) to achieve a higher density of surface-located MHC:peptide complexes. When either T2 cells or HepG2 cells, both cell lines expressing HLA-A2, were loaded with the respective peptides, they stained specifically with the hybridoma supernatants (Figure 2.1.3 A). Furthermore, a cross-reactivity against other MHC:peptide complexes could be excluded (Figure 2.1.3 B), thereby confirming the respective antibody specificity.

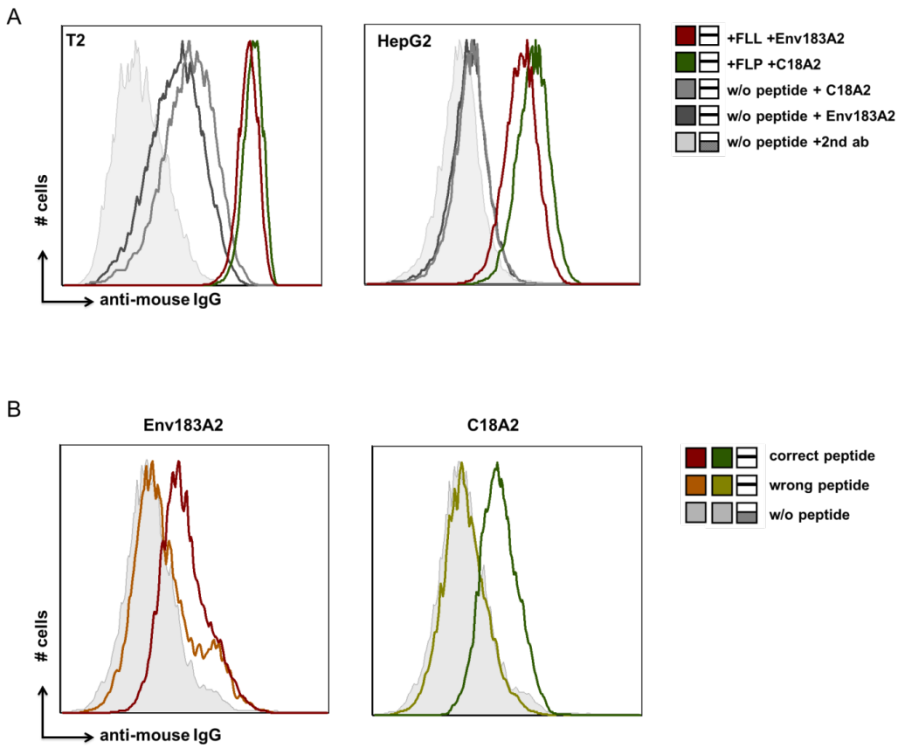


Figure 2.1.3 Staining of exogenously loaded peptide-MHC complexes with TCR-like antibodies. A) T2 (TAP-deficient human lymphoblast cell line) and HepG2 cells were loaded with respective peptides (FLP = HBcAg-peptide / FLL= HBsAg-peptide). Stainings were done sequentially, with the incubation with hybridoma supernatant (C18A2 / Env183A2) followed by staining with the labeled secondary antibody. Non-loaded cells (w/o peptide) served as control. **B)** Exclusion of cross-reactivity of TCR-like antibodies. Env183A2 / C18A2 hybridoma supernatant was incubated with HepG2 cells loaded with FLL or FLP peptide. Only the right combinations led to a positive signal.

2.1.2 Cloning of single-chain variable fragments and chimeric antigen receptors

Having confirmed the specificities of the TCR-like antibodies, the respective variable domains of heavy and kappa light chain (V_h and V_κ) had to be amplified and cloned in order to construct single chain antibody fragments (scFv) (Figure 2.1.4 A). Since the sequences were not known at this time point, variable domains were amplified using a set of degenerated primers (Dübel et al., 1994), kindly provided by Amgene. Sequencing of amplification products revealed consistent sequences that could also retrospectively be confirmed by the Bertoletti group (Figure 2.1.4 B).

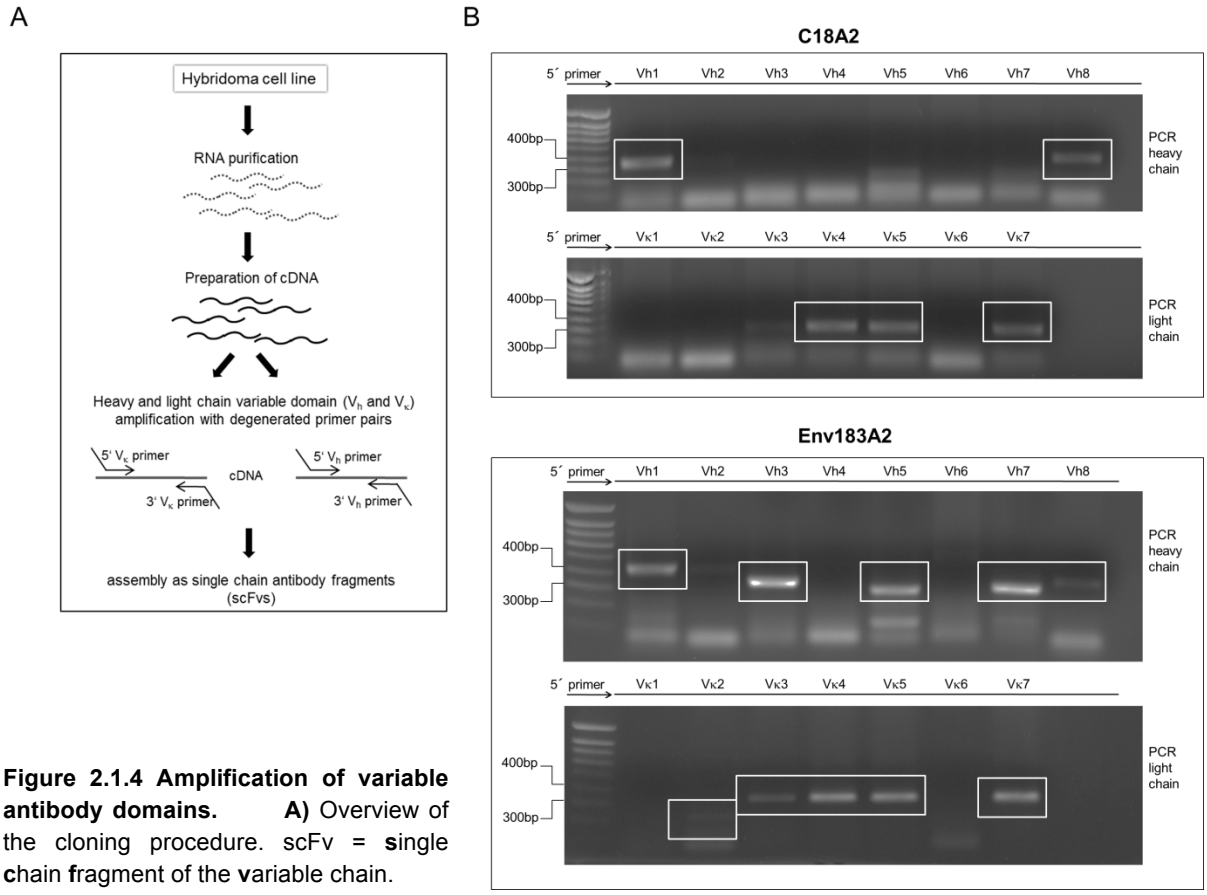


Figure 2.1.4 Amplification of variable antibody domains. **A)** Overview of the cloning procedure. scFv = **s**ingle **c**hain **f**ragment of the **v**ariable chain.

B) Detection of PCR products via gel electrophoresis. Variable chains should have a size around 350bp as verified by the marker. The labeling above the lanes describes the respective 5' degenerated primer used. In total, 8 different 5' primers were used for heavy chain amplification; 7 for the light chain. The 3' primers for heavy or light chain, respectively, were the same in each reaction.

Subsequently, each pair of variable chains was fused with a glycine-serine-linker to obtain complete single chain antibody constructs, and scFvs were cloned into the CAR expression construct by replacing the C8 scFv (Figure 2.1.1). Staining of 293T cells transfected with the new CAR constructs revealed expression of both newly constructed CARs, however less efficient than with the well-established S-CAR that harbors the C8 scFv (Figure 2.1.5 A).

To answer the question whether the generated CARs might still be able to recognize the original antigen, namely HLA-A2 in complex with the respective HBV peptide, transfected 293T cells were directly stained with specific streptamers. Such molecules are soluble MHC molecules that can be peptide-loaded and detected via Streptavidin-PE. If the Env183A2-CAR and the C18A2-CAR would have kept the specificity of the TCR-like antibodies, they should have bound the tetramers loaded with the Env183-91 or the C18-27 peptide, respectively. However, whereas staining for the CAR-backbone was positive (Figure 2.1.5 A), no streptamer binding could be observed for both of the receptors (Figure 2.1.5 B). Taken together, those results indicated that the newly generated constructs might have been inefficiently expressed and furthermore did not exhibit the same antigen specificities as the original antibodies.

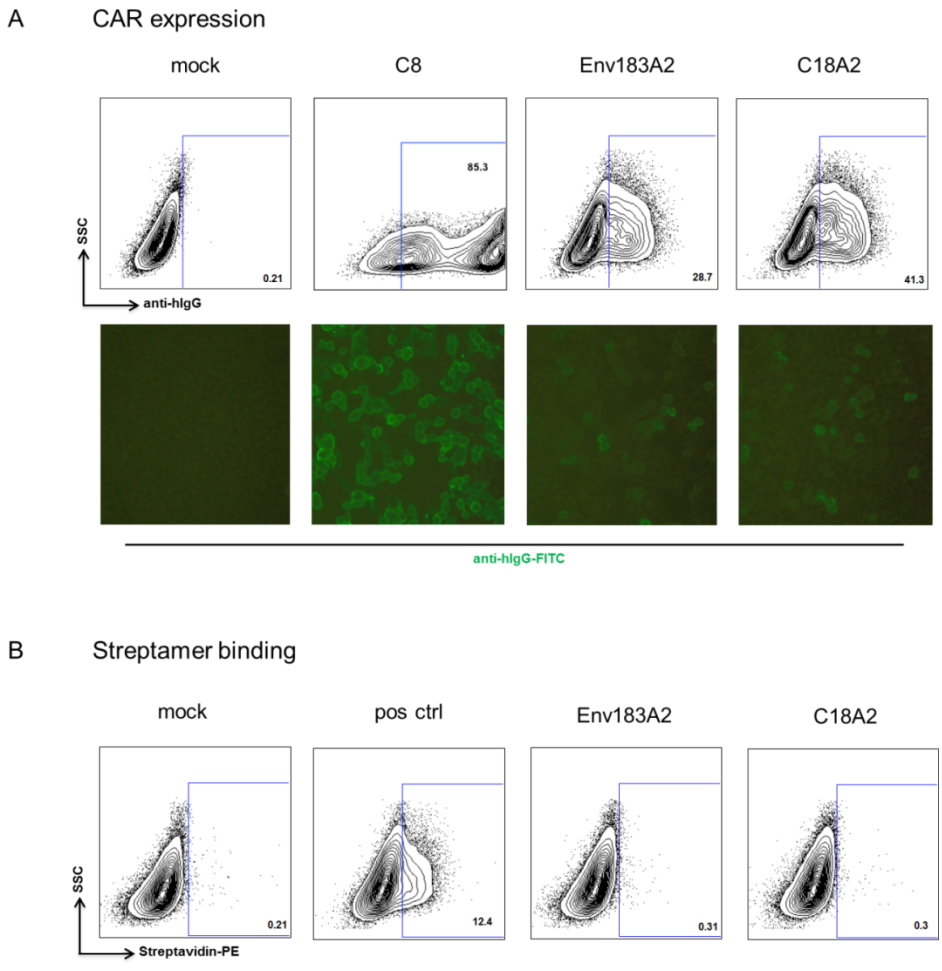


Figure 2.1.5 Expression of chimeric antigen receptors with scFvs C18A2 and Env183A2 and specificity testing. A) Staining reveals expression of CARs. Shown are 293T cells transfected with the C18A2-CAR/Env183A2 CAR and the C8-CAR (S-CAR) as control. Upper lane: flow cytometry; lower lane: immunofluorescence. **B)** Staining of CAR-expressing cells with specific streptamers. Pos ctrl: 293T cells transfected with a T cell receptor recognizing the FLP peptide and stained with the respective streptamer. SSC: sideward scatter. Streptavidin-PE: labeling of streptamers.

In parallel, retroviral supernatants were produced by co-transfection of retroviral packaging plasmids into 293T cells. When those supernatants where used for transduction of primary human T cells, receptor expression was undetectable for the newly generated CAR constructs, as detected by staining with an anti-IgG-antibody (Figure 2.1.6). Likewise, transduction of the human T cell line Jurkat as well as of murine T cells failed (data not shown), indicating that the generated receptors cannot be expressed in a stable way to be detectable upon transduction. Therefore, the newly cloned chimeric antigen receptors were excluded from further experiments.

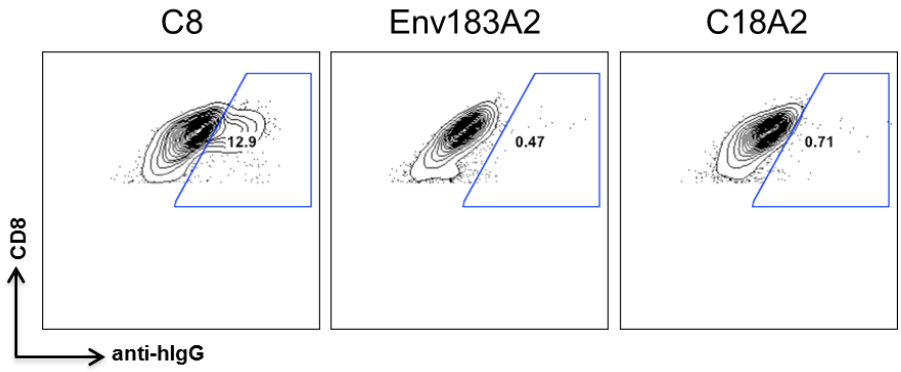


Figure 2.1.6 Transduction of primary human T cells. Human PBMC were transduced with supernatants derived from 293T cells transiently transfected to produce the respective retrovirus. Transduction with C8 (retrovirus encoding for S-CAR) served as control. Cells were gated on CD8 first.

2.2 Engraftment of primary human NK cells with the S-CAR and subsequent testing

2.2.1 S-CAR expression in primary human NK cells

NK cells were expanded from human PBMC using an optimized protocol. After 6 days of co-culture with the feeder cell line T2 and IL-2 stimulation, the NK cell population expanded from 5-10% to up to 70% of total living cells (Figure 2.2.1 A). Two rounds of retroviral transduction led to expression of the S-CAR with an efficiency around 15% (Figure 2.2.1 B). To avoid unwanted effects by T and NK T cells present in the population, CD3 depletion was performed prior to functional assays, leading to a highly pure NK cell population (Figure 2.2.1 C).

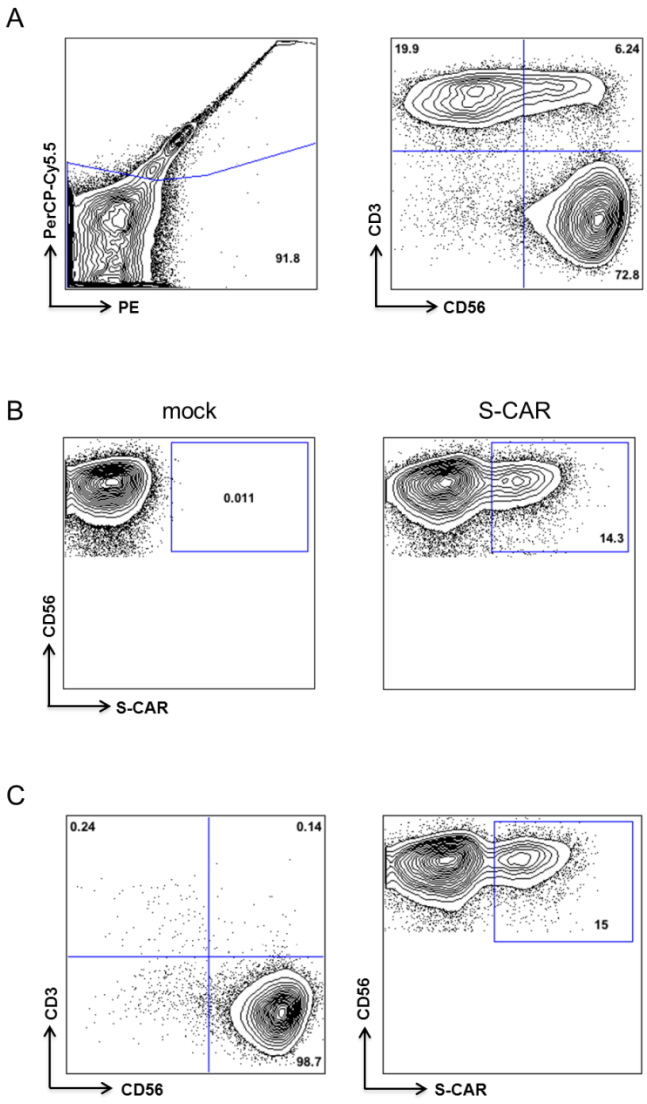


Figure 2.2.1 S-CAR expression in primary human NK cells. A) Expansion of NK cells from complete PBMC after 6 days of culture in the presence of feeder cells and IL-2. Gating was done on living lymphocytes by exclusion of dead cells (PE/PerCP-Cy5.5 double positive). **B)** Transduction rates of primary human NK cells. Shown is the frequency of S-CAR$^+$ cells among the CD3$^-$CD56$^+$ population. **C)** NK cell purity after depletion of CD3$^+$ cells.

2.2.2 NK cells show strong unspecific killing activity on hepatoma cells

To test the potential of S-CAR grafted NK cells to specifically kill HBsAg$^+$ hepatocytes, the transduced cells were co-cultivated with either HBV-negative hepatoma cells (HepG2) or HBV replicating HepG2.2.15 cells. Subsequently, an XTT-Assay was performed to check for cell lysis by measuring target cell viability. After 24h of co-culture, a large fraction of the target cells was lysed (Figure 2.2.2 A). However, killing did not seem to be specific, since it was also detectable when S-CAR grafted cells were cultivated with HBV-negative HepG2 cells (Figure 2.2.2 A and 2.2.2 B).

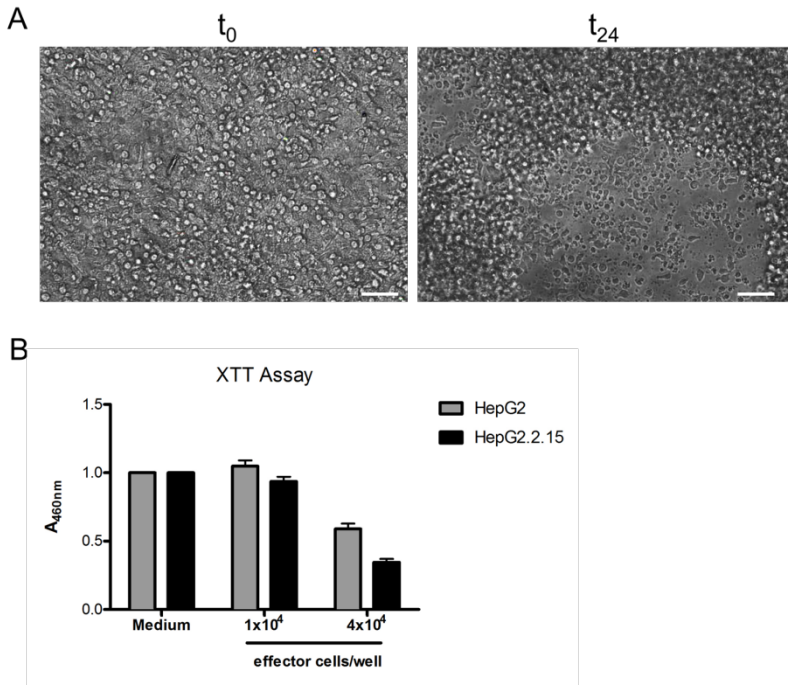


Figure 2.2.2 Hepatocyte killing by S-CAR NK cells. A) Light microscopy of S-CAR NK cells co-cultivated with HepG2 cells. t0 (left) represents the time point at the start of the co-culture, showing a confluent hepatocyte cell layer with round-shaped NK cells on top. After 24h of co-culture (right), the former confluent cell layer is largely disrupted. Scale bars: 20µm. **B)** XTT assay after co-culture of $1x10^4$ or $4x10^4$ S-CAR$^+$ NK cells with HepG2 or HepG2.2.15 cells. A_{460nm} represents the viability of the target cells. The addition of medium alone served as control.

2.2.3 S-CAR NK cells do not specifically lyse HBsAg expressing hepatoma cells upon rescue of MHC-I expression

Since NK cells kill MHC-I negative cells, and liver cells and hepatoma cells in particular are known to have low MHC-I surface expression, target cells were stimulated with IFNγ to up-regulate MHC expression (Figure 2.2.3 A). As shown in Figure 2.2.3 B, enhanced HLA-A2 expression on hepatoma cells could rescue unspecific killing by NK cells. However, S-CAR grafted NK cells did not have clear additional, specific effects on HBV$^+$ cells, indicating that S-CAR signaling might not be functional in this setting. This led to the decision to focus on T cell therapy in further experiments.

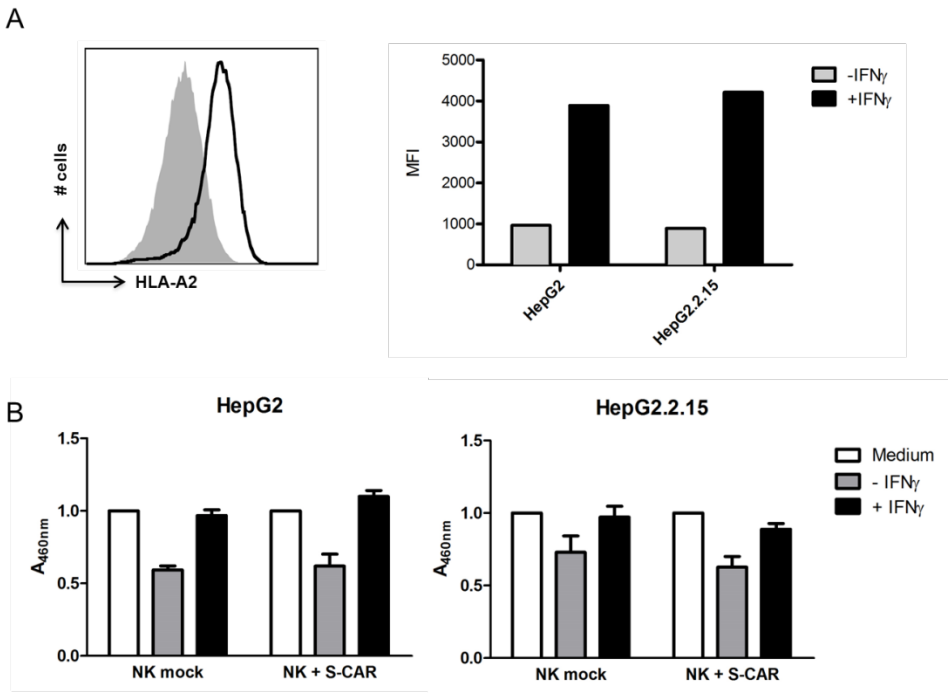


Figure 2.2.3 Hepatoma cell killing upon HLA-A2 rescue. A) HLA-A2 rescue on hepatoma cells. HepG2 and HepG2.2.15 cells were treated with IFNγ or left untreated. After 48h, HLA-A2 surface staining was performed. Left: histogram of stained HepG2 cells as example. Right: Mean fluorescence intensity for HLA-A2 of HepG2/HepG2.2.15 cells with or without IFNγ treatment. **B)** XTT assay after 24h co-culture of mock-transduced or $4x10^4$ S-CAR$^+$ NK cells with HepG2 or HepG2.2.15 cells. Target cells were either left untreated (-IFNγ) or pre-treated with IFNγ before co-culture.

2.3 Construction and evaluation of a stable retrovirus-producing cell line

The preparation of S-CAR$^+$ T cells for adoptive transfer depended on the production of fresh retrovirus that is capable of achieving good transduction rates. However, the efficiency of the transient transfection of Plat-E cells for vector production varied with every experiment, causing a critical point in the transduction protocol. To circumvent this, a cell line that is continuously secreting retroviral particles was established.

Platinum-E (Plat-E) cells stably express the retroviral-derived viral structural genes gag-pol and env, which are required for packaging, processing, reverse transcription, and integration of recombinant genomes (Morita et al., 2000). For stable production of retroviral vectors, the Plat-E cell line itself was transduced with amphotropic retroviral vectors produced in 293T cells and delivering the gene of interest (S-CAR) in combination with a packaging signal (Figure 2.3.1).

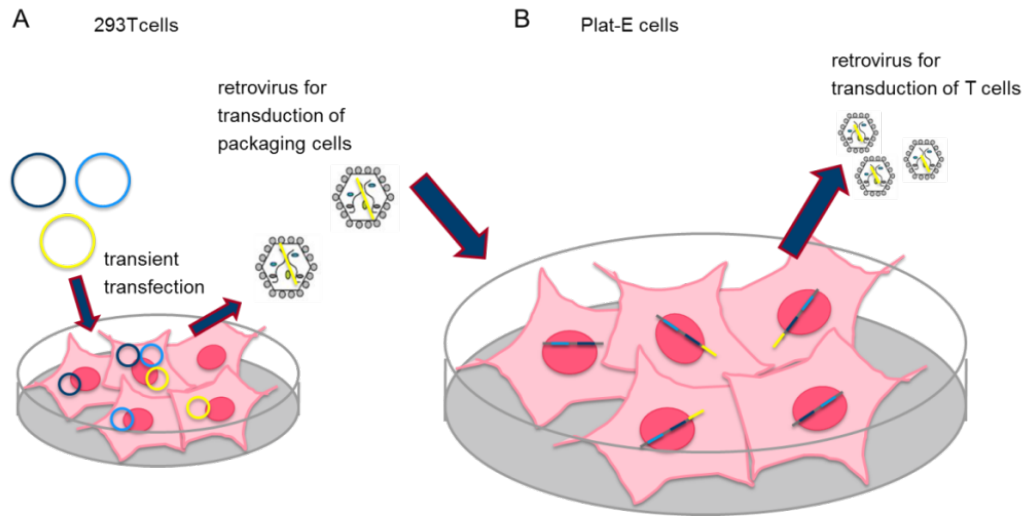


Figure 2.3.1 Schematic procedure for construction of a stable retrovirus-producer cell line. A) Transient transfection of HEK 293T cells (Human Embryonic Kidney cell line). The 3 colored circles represent the different plasmids encoding for the retroviral gag and pol proteins, the amphotropic surface proteins (env) and the retroviral genome encoing for the gene of interest to be included in the retroviral particles. **B)** Transduction of the packaging cell line Platinum-E. Since the gag, pol and env-genes are already integrated in the genome, stable integration of the transgene with a packaging signal leads to continuous virus production.

Figure 2.3.2 shows Plat-E cells transduced with 293T supernatant, which expressed the S-CAR to a high extent. Among the S-CAR$^+$ cells, two populations with either a rather low, or a high amount of surface S-CAR expression could be distinguished, probably indicating different amounts of transgene copies in those cells. In the next step, both S-CAR$^+$ populations were sorted via flow cytometry and further kept in culture as Plat-E_S-CAR$_{low}$ or _S-CAR$_{high}$, respectively. One week after the sort, both cell lines as well as non-transduced Plat-E cells were again stained for S-CAR expression. Interestingly, only S-CAR$_{high}$ derived cells showed stable expression of the S-CAR (Figure 2.3.3 A).

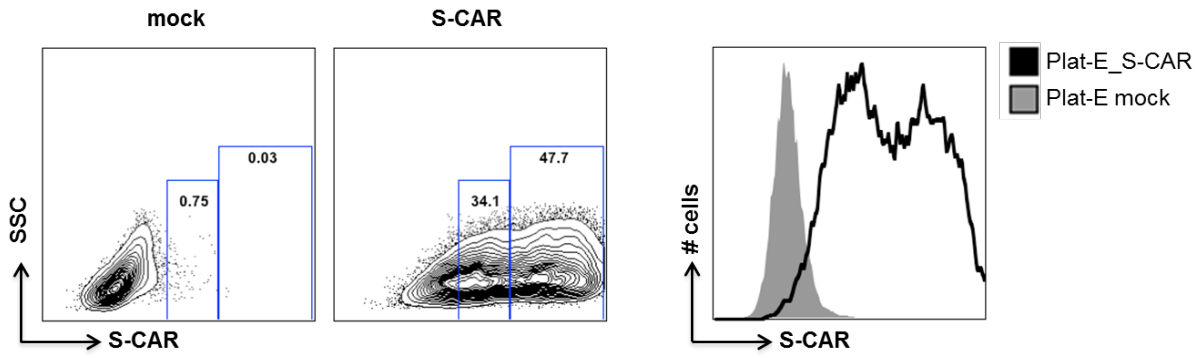


Figure 2.3.2 Transduction of Plat-E cells. Shown are FACS blots of mock-transduced or S-CAR Retrovirus-transduced Plat-E cells stained for S-CAR surface expression. According to the appearance of two populations with different CAR-expression levels, cells were further named as S-CAR$_{low}$ and S-CAR$_{high}$.

The supernatant of S-CAR$_{low}$ as well as S-CAR$_{high}$ cells was finally used for transduction of the murine T cell line B3Z. As shown in Figure 2.3.3 B, supernatant derived from S-CAR$_{low}$ Plat-E cells did not contain retrovirus capable of transduction. On the other hand, Plat-E S-CAR$_{high}$ cells stably secreted retroviral particles into the supernatant that lead to efficient B3Z transduction. These cells were kept in culture as Plat-E_S-CAR cell line, and supernatants were used for efficient transduction of primary murine T cells.

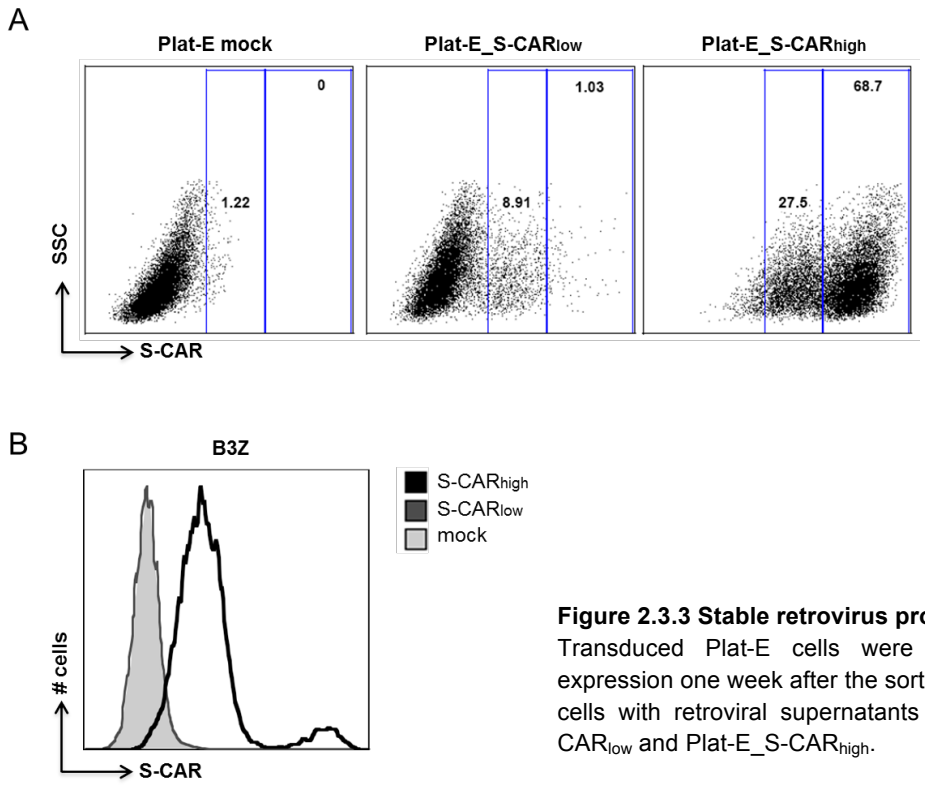


Figure 2.3.3 Stable retrovirus production. **A)** Transduced Plat-E cells were re-stained for S-CAR expression one week after the sort. **B)** Transduction of B3Z cells with retroviral supernatants produced by Plat-E_S-CAR$_{low}$ and Plat-E_S-CAR$_{high}$.

2.4 Long-term survival and phenotypic changes of S-CAR T cells after adoptive transfer

2.4.1 S-CAR T cells can survive in the long term *in vivo*

Figure 2.4.1 depicts the general experimental layout for adoptive transfer experiments to investigate S-CAR T cell functions in HBVtg mice. S-CAR T cells were generated by retroviral transduction of $CD8^+$ T cells derived from mice carrying the congenic marker CD45.1. Since activation and proliferation is required for successful transduction, T cells were stimulated *in vitro* with antibodies against CD3 and CD28 as well as with murine IL-12 that has been found to greatly improve T cell survival *in vivo* (Krebs, Böttinger et al., 2013). FACS analysis was then used to determine the transduction efficiency, which varies between different experiments. Finally, the desired number of S-CAR T cells was calculated and the respective total cell amount was given intraperitoneally to the recipient mice. The congenic marker CD45.1 allowed for monitoring of the transferred T cells *in vivo* (Figure 2.4.1 B).

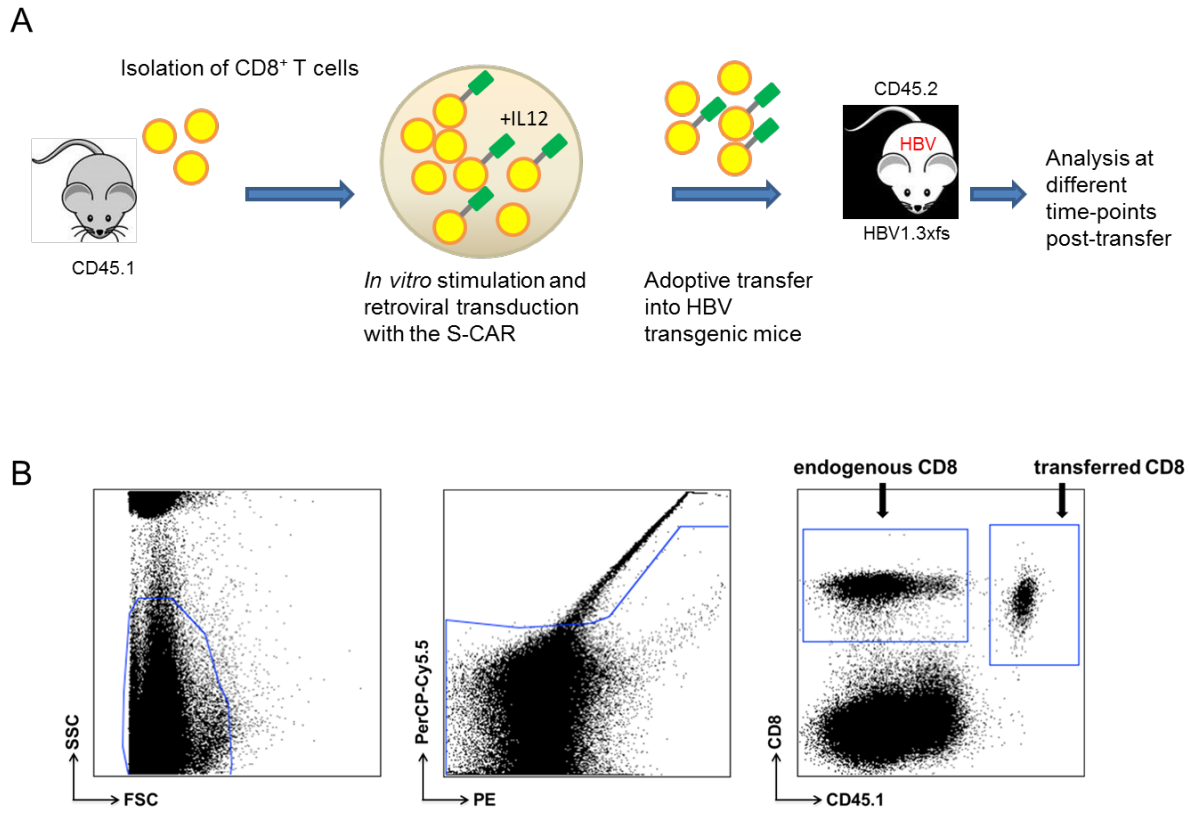


Figure 2.4.1 General procedure for adoptive transfer experiments. A) Experimental setting. CD45.1/CD45.2: congenic marker on leukocytes. IL-12: Interleukin-12. HBV1.3xfs: HBV-transgenic mouse line without functional HBx expression. **B**) Gating strategy after re-isolation. After exclusion of the PerCP-Cy5.5+/PE+ population representing dead cells, transferred T cells are identified by co-expression of CD8 and CD45.1.

In previously conducted adoptive transfer experiments, mice were sacrificed latest at d12 post transfer. Thus, there was no information about long-term survival and properties of transferred cells. To assess this, mice treated with $3x10^6$ S-CAR T cells or CEA-CAR T cells as control (equivalent to $8.6x10^6$ and $1.2x10^7$ total cells; see transduction efficiencies in Figure 2.4.2 A) were monitored up to 13 weeks. Animals were bled at different time points and frequencies of remaining transferred cells were determined as exemplified in Figure 2.4.1 B. Of note, *ex vivo* staining of transferred T cells over time was only possible via the congenic marker CD45.1, whereas S-CAR surface staining was not stable *(see also 2.4.5)*.

As shown in Figure 2.4.2 B, transferred cells were detectable up to 13 weeks post transfer, with high inter-individual differences within the groups. T cells engrafted with the CEA-CAR were also maintained in the circulation, although frequencies were on average lower than those of S-CAR T cells especially at the first time points. The CEA-CAR recognizes carcinoembryogenic antigen, which is absent in HBVtg mice, and thereby provided a control for antigen-unspecific effects on CAR T cells *in vivo*.

Transferred T cells accounted for up to 80% of total CD8 T cells and up to 20% of total PBMC (Figure 2.4.2 C). Frequencies of transferred T cells decreased over time, but most of the S-CAR T cell treated mice maintained a clearly detectable population of $CD45.1^+$ cells up to 12 weeks post transfer. CEA-CAR T cells were monitored up to 9 weeks post transfer, and, in comparison to the continuous decrease of S-CAR T cells, frequencies remained stable from approximately week 4 on at around 8% of total PBMC. Those results suggested that S-CAR T cells initially proliferated *in vivo* upon antigen stimulation, followed by clearance from the circulation that might have still been ongoing at the endpoint of analysis. CEA-CAR T cells, on the other hand, remained at rather stable frequencies independent of stimulation with antigen.

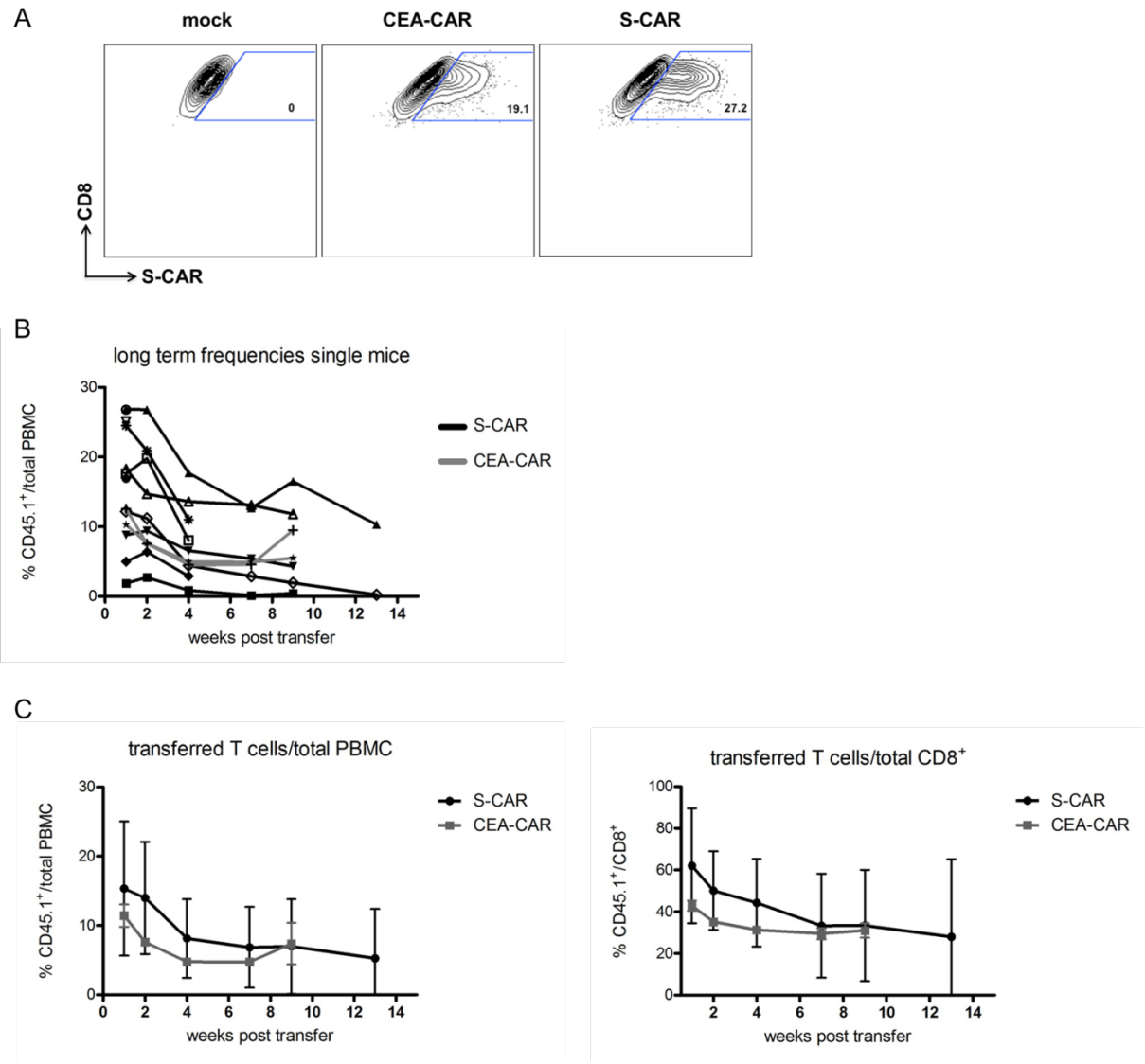


Figure 2.4.2 Monitoring of transferred T cells. A) Transduction efficiencies determined before adoptive transfer. **B)** Long-term frequencies of transferred T cells in individual mice. Black curves: S-CAR treated mice (n=8). Grey curves: CEA-treated mice (n=2). **C)** Mean frequencies of transferred T cells for each group. Left: as percentage of total PBMC; Right: as percentage of total $CD8^+$. Values for the S-CAR were multiplied by factor 1.4 to adapt to the different amounts of total CD45.1 splenocytes transferred (S-CAR: $8.6x10^6$; CEA-CAR: $1.2x10^7$ total cells).

In a second experiment, $4x10^6$ S-CAR T cells were transferred into HBVtg mice and monitored until d34 (~5 weeks). Compared to the experiment described above, T cell transduction performed better with an efficiency of 70%, allowing for transfer of only $5.71x10^6$ total cells per mouse (Figure 2.4.3 A). When monitored *in vivo*, frequencies of transferred T cells accounted for up to 8% of total PBMC followed by continuous decrease, indicating a subsequent clearance from the circulation over time (Figure 2.4.3 B). In accordance with the clearance of transferred T cells from the blood, the frequencies in

different body compartments had decreased as well after 5 weeks post transfer (Figure 2.4.3 C). Analysis of liver associated lymphocytes revealed that transferred T cells had efficiently homed to the liver at d12 post transfer, followed by a strong contraction of the population detected at d34.

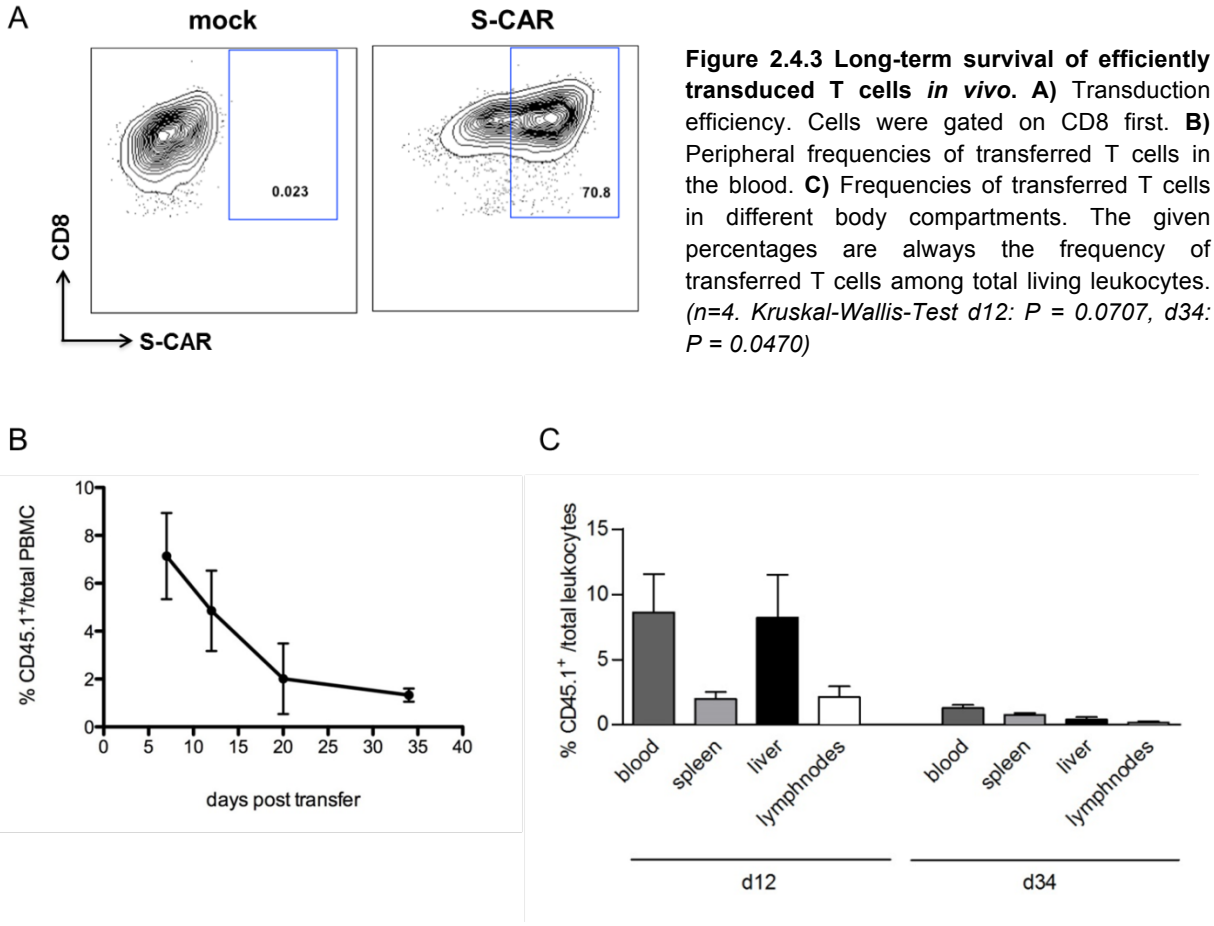


Figure 2.4.3 Long-term survival of efficiently transduced T cells *in vivo*. A) Transduction efficiency. Cells were gated on CD8 first. **B)** Peripheral frequencies of transferred T cells in the blood. **C)** Frequencies of transferred T cells in different body compartments. The given percentages are always the frequency of transferred T cells among total living leukocytes. *(n=4. Kruskal-Wallis-Test d12: P = 0.0707, d34: P = 0.0470)*

2.4.2 At late time-points, S-CAR T cells fail to induce antiviral effects *in vivo*

S-CAR T cells have already been shown to exert specific antiviral effects *in vivo* by reducing the amount of HBV DNA in the liver and in the serum of HBV transgenic mice until d12 (doctoral thesis Karin Krebs). When relative amounts of HBV rcDNA in the liver were analyzed in long-term experiments, the values for mice that received S-CAR T cells were reduced around 50% at week 1 post transfer compared to DNA levels after 4 and 9 weeks. At the later time points, HBV DNA levels of S-CAR treated mice were comparable to levels of mice that received control (CEA-CAR) T cells, suggesting a functional silencing of S-CAR T cell function *in vivo* over time (Figure 2.4.4 A). A more in-depth monitoring of liver HBV DNA levels at earlier time points post transfer confirmed a time-dependent antiviral effect,

with the strongest reduction after 1 week (Figure 2.4.4 B). The fact that a decrease of viral DNA was already measurable at d4 post transfer, but hardly at d20 post transfer with an increase of around 3 fold, indicated a rapid performance of effector functions that lasted only for a limited time period.

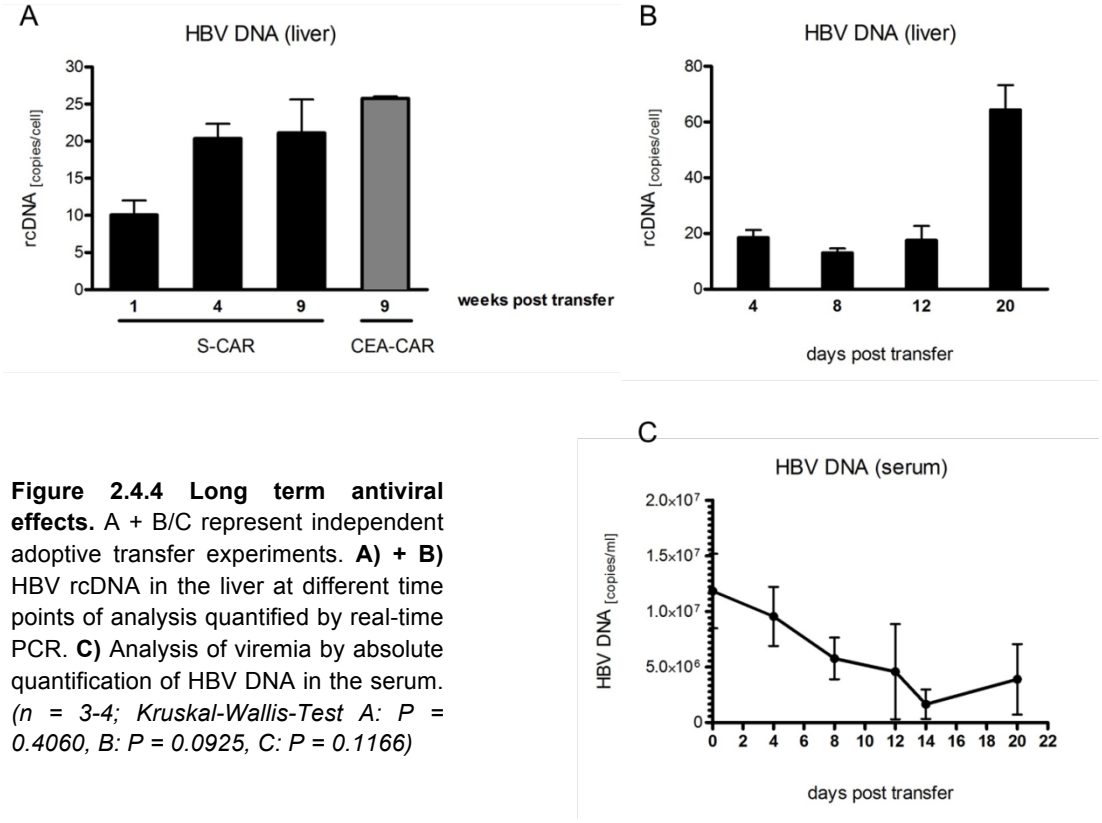


Figure 2.4.4 Long term antiviral effects. A + B/C represent independent adoptive transfer experiments. **A) + B)** HBV rcDNA in the liver at different time points of analysis quantified by real-time PCR. **C)** Analysis of viremia by absolute quantification of HBV DNA in the serum. *(n = 3-4; Kruskal-Wallis-Test A: P = 0.4060, B: P = 0.0925, C: P = 0.1166)*

This was in line with the detected effect on viremia: serum HBV levels were found to drop from d4 onwards; followed by subsequent increase (Figure 2.4.4 C). The maximum decrease of serum DNA of around one log appeared delayed compared to the maximum reduction of HBV DNA in the liver. The increase of HBV markers upon loss of S-CAR T cell effector functions was expected, since virus expression in the HBVtg mice is driven by the transgene, which cannot be eliminated.

2.4.3 *Ex vivo* restimulation of transferred S-CAR T cells

To check whether transferred T cells maintained their ability to specifically secrete cytokines after recognition of HBsAg, lymphocytes were isolated from spleen and liver at different time-points after transfer and stimulated with plate-bound recombinant HBsAg or left unstimulated. After 30h of incubation, cells were stained for CD45.1, S-CAR and intracellular cytokines IFNγ and TNFα. The gating strategy is shown in Figure 2.4.5.

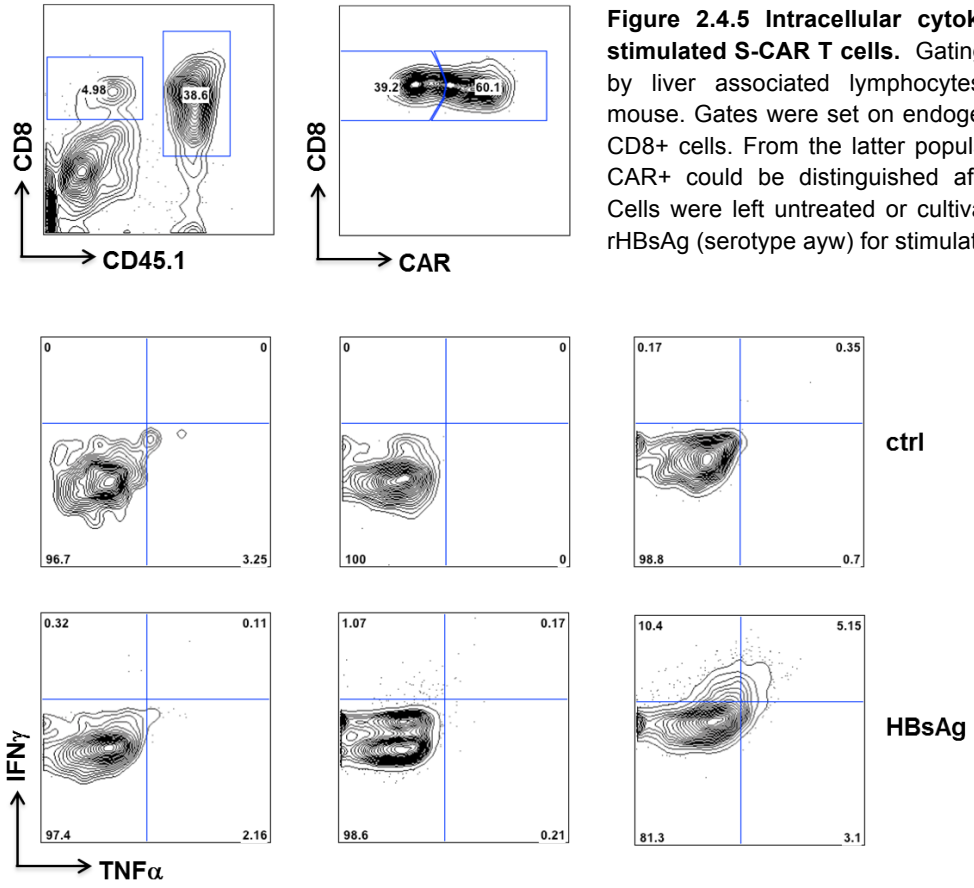


Figure 2.4.5 Intracellular cytokine staining of re-stimulated S-CAR T cells. Gating strategy exemplified by liver associated lymphocytes derived from one mouse. Gates were set on endogenous and transferred CD8+ cells. From the latter population, S-CAR- and S-CAR+ could be distinguished after overnight culture. Cells were left untreated or cultivated with plate-bound rHBsAg (serotype ayw) for stimulation.

As shown in Figure 2.4.6 A, S-CAR T cells isolated from both liver and spleen at d8 specifically produced effector cytokines when re-stimulated with rHBsAg. Most of those cells stained positive for IFNγ, with a proportion of 13% of LALs and 21% of splenocytes, and 5% respectively that produced TNFα in addition. Cytokine production upon antigen stimulation was higher in splenocytes, but was accompanied by a higher background cytokine production of unstimulated cells than in liver associated lymphocytes. Specific cytokine secretion was also detected when splenocytes and liver associated lymphocytes were re-isolated and stimulated 9 weeks post transfer (Figure 2.4.6 B). Compared to d8, frequencies

of cytokine positive cells were lower, and cells were mostly monofunctional with secretion of IFNγ only. Control cells expressing CEA-CAR did not secrete cytokines upon HBsAg contact as expected (Figure 2.4.6 right). Of note, S-CAR T cells showed a specific response to the ayw serotype of the HBsAg (genotype D), which is present in HBVtg mice, but even a stronger response to the adw serotype against which the single chain antibody of the S-CAR was generated (Figure 2.4.6). Those results suggested that a proportion of transferred S-CAR T cells, although apparently dysfunctional *in vivo*, had kept their effector potential and could be re-stimulated even at late time-points.

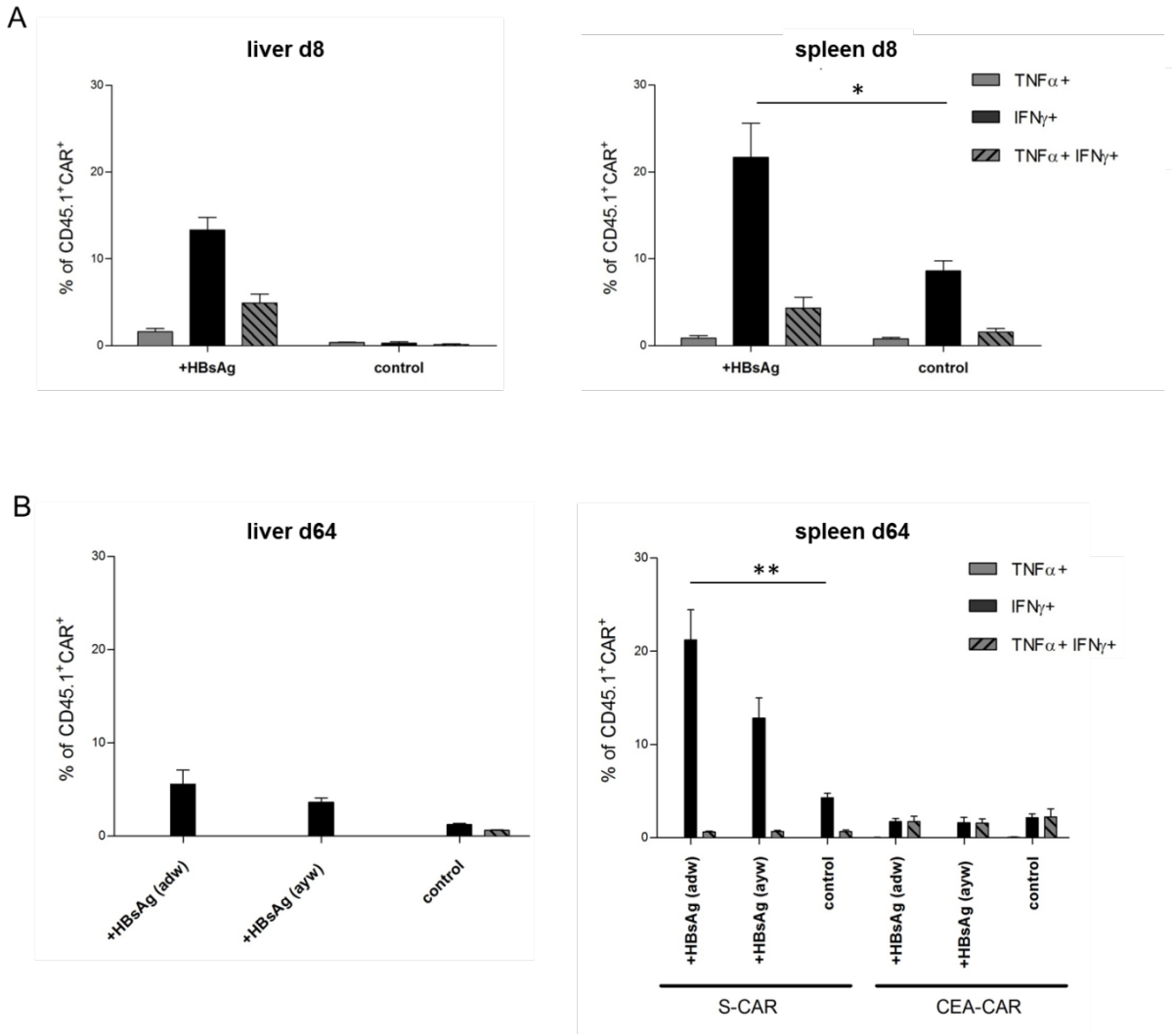


Figure 2.4.6 Intracellular cytokine staining d8 and d64 post transfer. Shown are S-CAR+ CD8 T cells from liver and spleen cultured with or without HBsAg. **A)** ICS d8 post transfer. *(n=2-3; Mann-Whitney-Test, * = P<0.05.)* **B)** ICS d64 post transfer. adw/ayw represent different serotypes of the HBsAg. (*n=2-6; Kruskal-Wallis-Test, ** = P<0.005)*

2.4.4 Maintenance of effector potential in spite of upregulation of the exhaustion marker PD-1

Since the antiviral properties of S-CAR T cells were found to be silenced in the host, transferred T cells were checked and monitored for phenotypic changes. To assess whether S-CAR T cells undergo a functional inactivation *in vivo*, staining of the exhaustion marker Programmed Death-1 (PD-1) was performed. Cells were stimulated with HBsAg in addition, as described above, to allow for parallel detection of intracellular cytokines upon stimulation. The same procedure was done with S-CAR T cells before transfer, revealing that around 90% of T cells that underwent the stimulation and transduction procedure stained positive for PD-1 on their surface, and 40% of PD-1$^+$ cells were still able to secrete IFNγ upon antigen stimulation (Figure 2.4.7 B left). When cells were re-isolated from liver and spleen at week 3, 6 and 9 post transfer, especially S-CAR$^+$ cells also expressed PD-1 to a high proportion (55-65%), and clearly lower than S-CAR$^-$ cells and endogenous CD8$^+$ T cells (Figure 2.4.7 A + C). However, 30 to 50% of PD-1$^+$ cells could be specifically re-activated *ex vivo* (Figure 2.4.7 B right), indicating that PD-1$^+$ S-CAR T cells exhibited a rather activated than exhausted state.

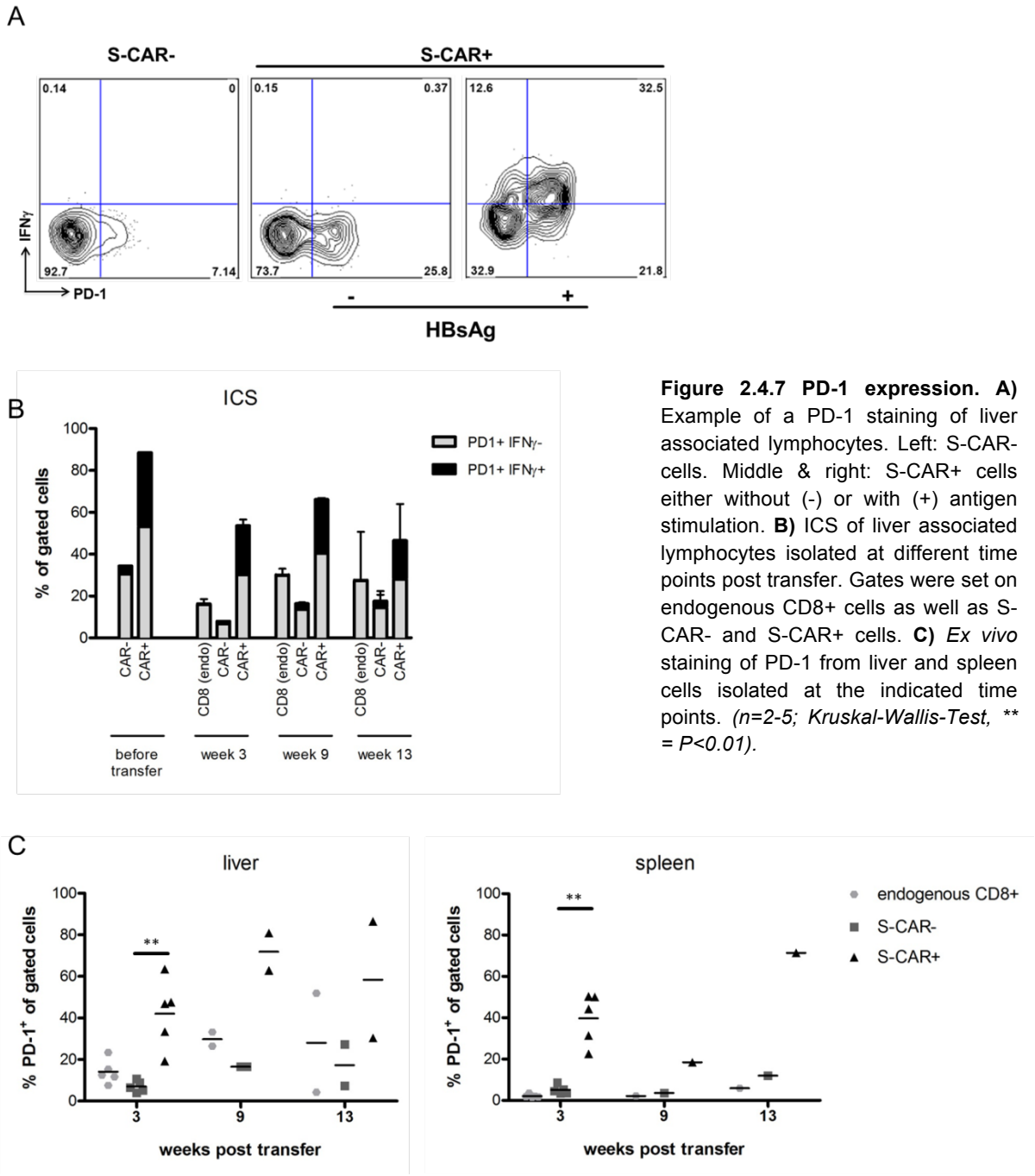


Figure 2.4.7 PD-1 expression. A) Example of a PD-1 staining of liver associated lymphocytes. Left: S-CAR- cells. Middle & right: S-CAR+ cells either without (-) or with (+) antigen stimulation. **B)** ICS of liver associated lymphocytes isolated at different time points post transfer. Gates were set on endogenous CD8+ cells as well as S-CAR- and S-CAR+ cells. **C)** *Ex vivo* staining of PD-1 from liver and spleen cells isolated at the indicated time points. *(n=2-5; Kruskal-Wallis-Test, ** = P<0.01).*

In a second experiment, S-CAR T cells were transferred into HBVtg mice as well as wild type C57 BL/6 mice to check for specific activation and/or exhaustion in the context of HBV replication. Directly after re-isolation at different time-points, $CD45.1^+$ cells derived from livers and spleens of HBVtg mice, but not from BL/6 mice were high in PD-1 surface expression (Figure 2.4.8 A + B). When cells were *ex vivo* re-stimulated at d12 post transfer, between 40 and 50% of $PD\text{-}1^+$ S-CAR T cells isolated from livers of HBVtg mice were able to

produce IFNγ, as already shown before (Figure 2.4.8 C left). Of note, *ex vivo* antigen stimulation markedly increased PD-1 expression also on S-CAR T cells derived from BL/6 mice, with 60% of PD-1$^+$ cells producing IFNγ upon stimulation. Those results suggested that PD-1 upregulation on S-CAR T cells was mostly a consequence of activation – which took place *in vivo* in the HBVtg mice, but only *ex vivo* for cells derived from BL/6 mice. The finding that re-stimulation was somewhat more efficient for BL/6-derived cells could point to a small proportion of S-CAR T cells that had effectively acquired an exhausted phenotype after adoptive transfer into HBVtg mice.

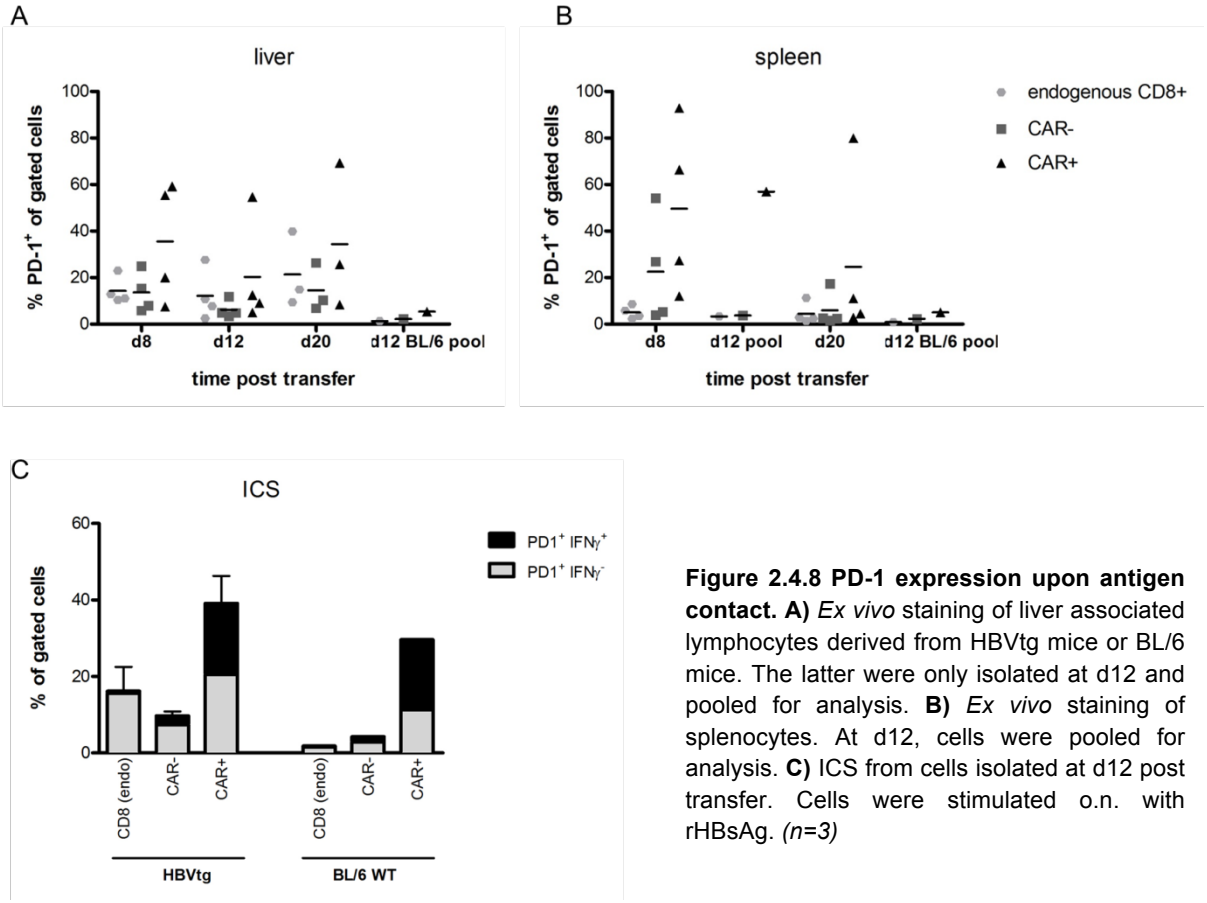


Figure 2.4.8 PD-1 expression upon antigen contact. A) *Ex vivo* staining of liver associated lymphocytes derived from HBVtg mice or BL/6 mice. The latter were only isolated at d12 and pooled for analysis. **B)** *Ex vivo* staining of splenocytes. At d12, cells were pooled for analysis. **C)** ICS from cells isolated at d12 post transfer. Cells were stimulated o.n. with rHBsAg. *(n=3)*

2.4.5 S-CAR surface expression is unstable *in vivo* and regulated by different mechanisms

2.4.5.1 *In vivo* loss of S-CAR surface expression and rescue after *ex vivo* resting

To check which further mechanism could have led to the observed silencing of S-CAR T cells *in vivo*, a detailed analysis of S-CAR surface expression was performed. As already mentioned, surface-located S-CAR on CD45.1$^+$ directly after re-isolation was indeed reduced or even completely absent, although S-CAR surface expression was clearly detectable before transfer.

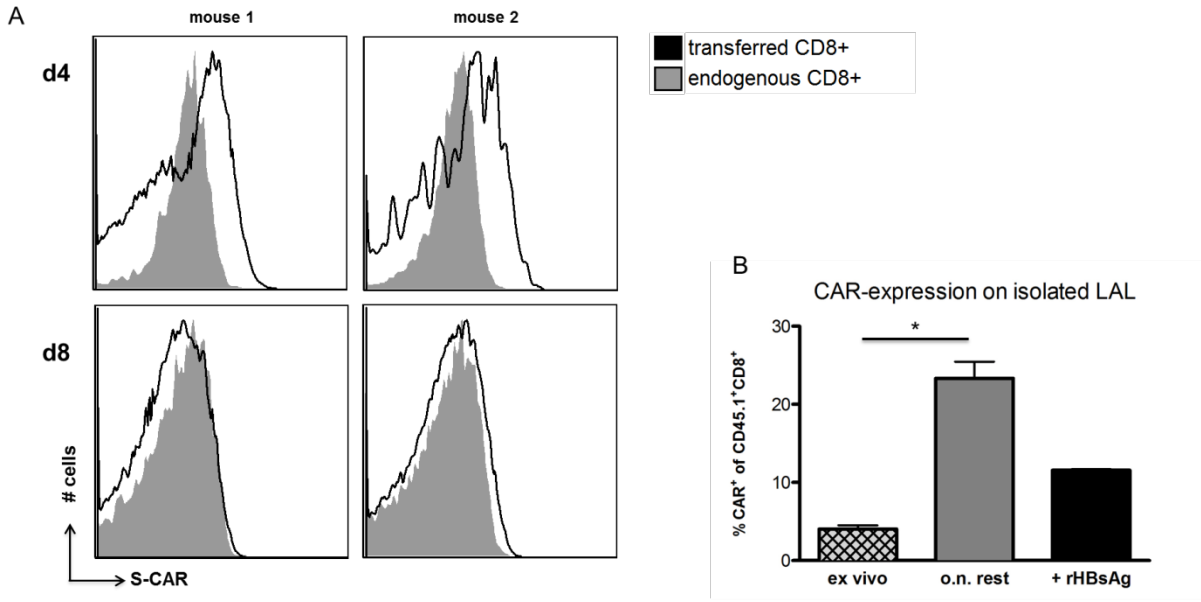


Figure 2.4.9 S-CAR surface expression *in vivo*. A) Representative S-CAR staining on blood cells of two independent mice per time point. Gates were set on endogenous or transferred CD8+ cells. **B)** Representative S-CAR staining on re-isolated LALs d12 post transfer (n=3; Kruskal-Wallis-Test, * = P<0.05).

As presented in the example in Figure 2.4.9 A, the receptor was detectable on blood cells isolated at d4, but almost undetectable at d7 on cells derived from the same group of recipient mice. By resting the re-isolated cells overnight, S-CAR expression could be partly restored especially on liver associated lymphocytes (Figure 2.4.9 B), which goes in line with the finding that cells could be reactivated *ex vivo* (*see part 2.5.3*). However, when cells were stimulated with rHBsAg, specific cytokine secretion was accompanied by a largely diminished S-CAR rescue to the surface (Figure 2.4.9 B). This led to the question whether continuous antigen stimulation *in vivo* might have led to S-CAR down-regulation and thereby functional inactivation of transferred T cells.

2.4.5.2 Influence of HBsAg on S-CAR surface expression *in vitro*

To test whether antigen stimulation has an effect on S-CAR surface expression in murine T cells, *in vitro* stimulation kinetics were performed. Complete splenocytes were transduced with a newly generated retroviral construct (Figure 2.4.10 A) (generated by Karin Krebs/Antje Malo; original construct provided by Paulina Paszkiewicz/Stanley Riddell), which led to parallel expression of the S-CAR with a truncated epidermal growth factor receptor (tEGFR). Since antigen stimulation should not have an impact on tEGFR expression, surface-expressed tEGFR served as a control for non-antigen-specific effects. Cells carrying both receptors were stimulated with plate-bound rHBsAg and S-CAR expression as well as tEGFR expression was monitored over time. As shown in Figure 2.4.10 B, tEGFR expression stayed stable over 24h of incubation time, whereas there was a distinct antigen-specific decrease of S-CAR surface expression, which was partly rescued after 24h. Relative expression levels were calculated and are shown in Figure 2.4.10 C, confirming loss of S-CAR surface expression in an antigen-dependent manner. This mechanism was also dependent on the antigen concentration used for stimulation (Figure 2.4.10 D left). Concentrations starting from 1.25µg/ml rHBsAg for the coating procedure led to S-CAR-down regulation, whereas lower concentrations did not. The concentration of secreted IFNγ corresponded with the degree of S-CAR down-regulation: only rHBsAg concentrations starting from 1.25µg/ml led to profound IFNγ secretion; indicating that activation of the T cells was directly linked to the S-CAR surface expression (Figure 2.4.10 D right).

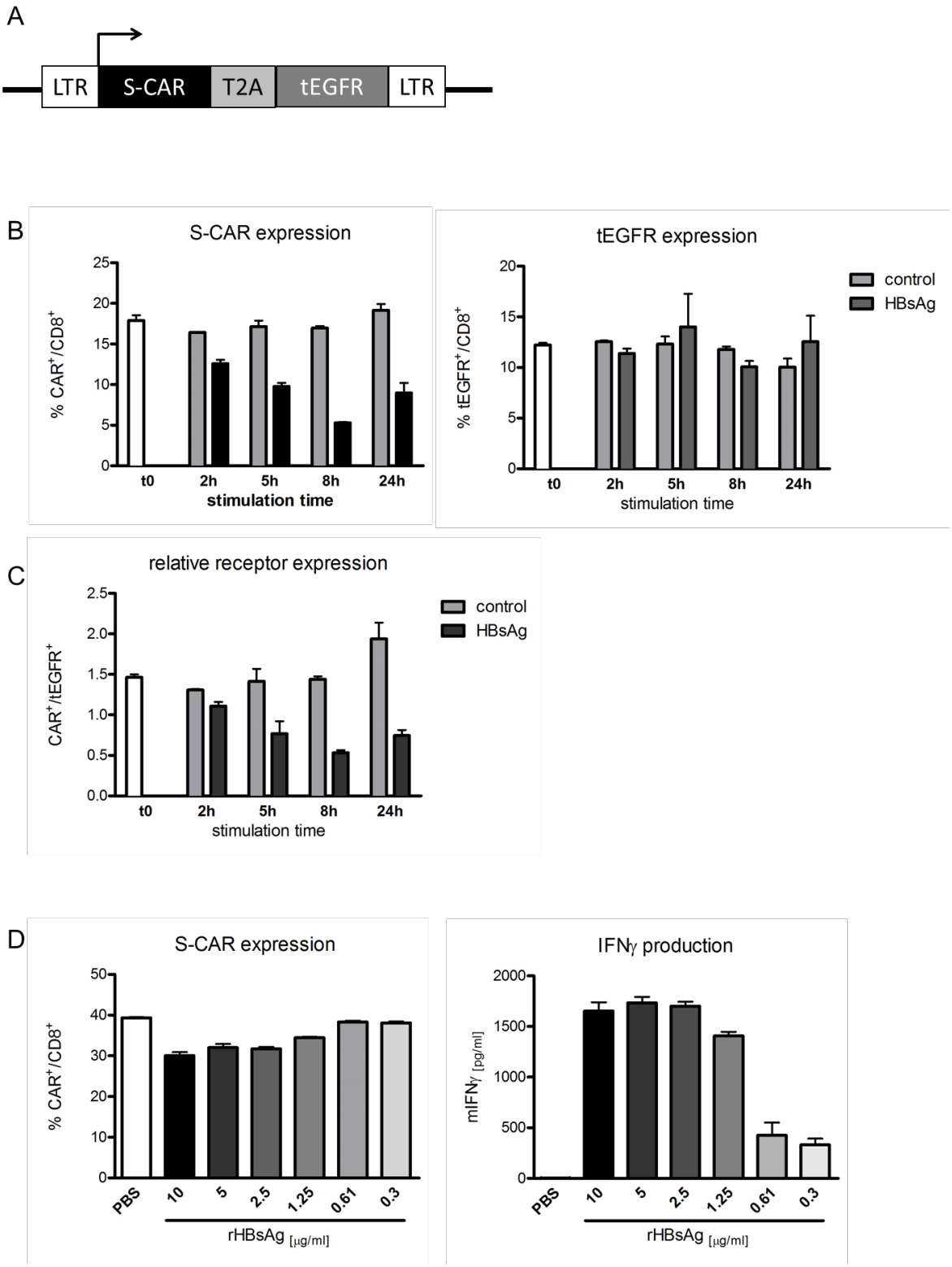


Figure 2.4.10 S-CAR surface expression *in vitro*. A) Vector construct for parallel expression of S-CAR and tEGFR. LTR: Long Terminal Repeat. T2A: virus-derived sequence that allows for co-expression of two transgenes. **B)** Stimulation kinetics with plate-bound recombinant HBsAg (5µg/ml). t0: staining before stimulation. **C)** Relative expression levels calculated by % S-CAR+ / % tEGFR+. Control: unstimulated cells. **D)** S-CAR expression after 5h stimulation with different concentrations of plate-bound rHBsAg (left). Right: secreted IFNγ decected in the supernatant by ELISA after 5h stimulation with different coating antigen concentrations.

2.4.5.3 S-CAR surface expression *in vivo* is not only dependent on antigen stimulation

Since stimulation with rHBsAg might not be fully comparable to the *in vivo* stimulation by infectious virus, T cells engrafted with S-CAR and tEGFR were adoptively transferred into HBVtg mice. Figure 2.4.11 A (left) shows staining of the T cells before transfer (d0), confirming an equal expression of both markers. Mice were bled at d4 and d7 post transfer and analyzed at d8. Parallel monitoring of S-CAR and tEGFR revealed a shift in staining intensity of the population within this time frame (Figure 2.4.11 A right); with a more rapid loss of S-CAR surface expression than tEGFR expression. This was detectable by frequencies of receptor-positive cells and by mean fluorescence intensity that both significantly decreased over time for both receptors, but more profoundly for the S-CAR (Figure 2.4.11 B). Except one animal, which had very high frequencies of transferred S-CAR T cells in blood, spleen and liver (80% or more), cells from all tested animals and body compartments showed clearly reduced S-CAR surface expression at d8 of analysis (Figure 2.4.11 C).

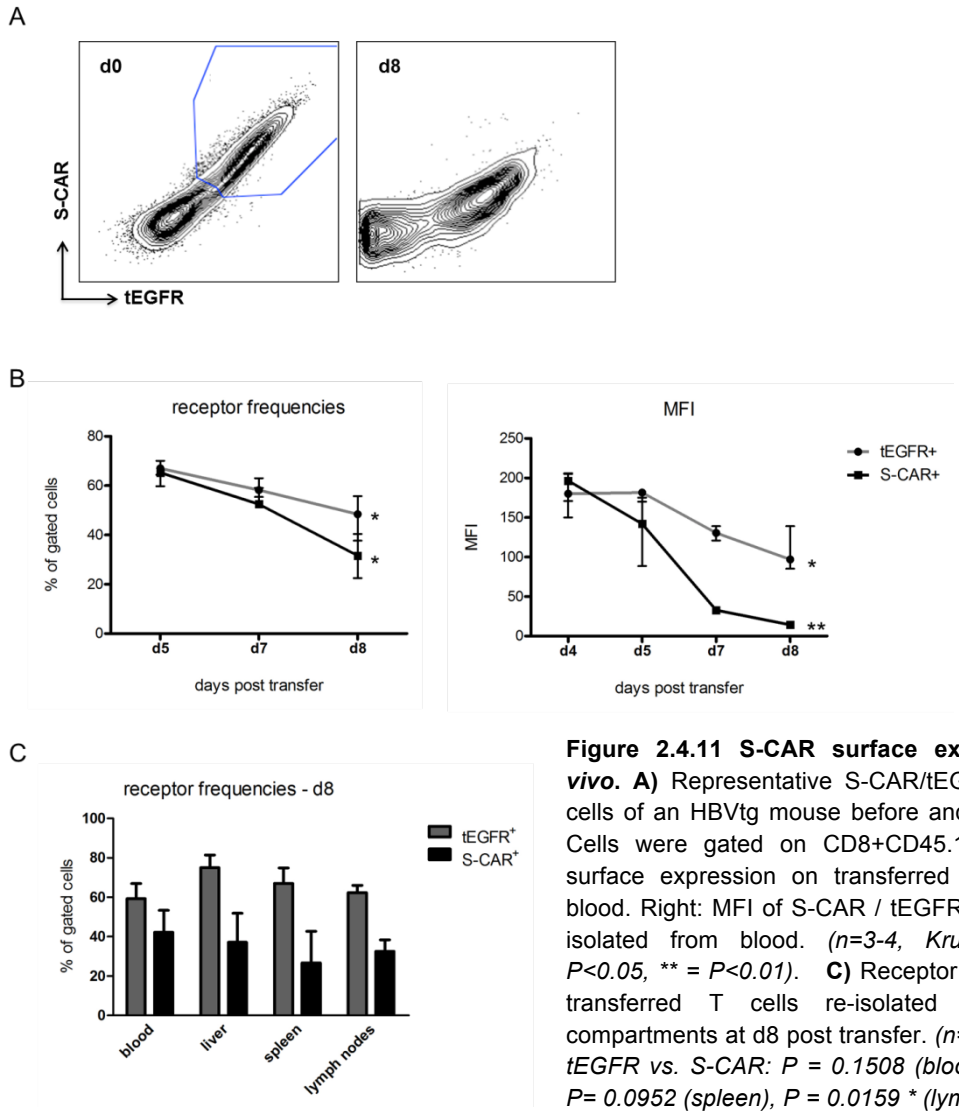


Figure 2.4.11 S-CAR surface expression kinetics *in vivo*. A) Representative S-CAR/tEGFR staining on blood cells of an HBVtg mouse before and 8 days post transfer. Cells were gated on CD8+CD45.1+. **B)** Left: Receptor surface expression on transferred T cells isolated from blood. Right: MFI of S-CAR / tEGFR on transferred T cells isolated from blood. *(n=3-4, Kruskal-Wallis-Test, * = P<0.05, ** = P<0.01)*. **C)** Receptor surface expression on transferred T cells re-isolated from different body compartments at d8 post transfer. *(n=4, Mann Whitney Test tEGFR vs. S-CAR: P = 0.1508 (blood), P = 0.0952 (liver), P= 0.0952 (spleen), P = 0.0159 * (lymph nodes))*

To be able to distinguish between antigen-dependent and -independent effects on S-CAR surface expression, S-CAR$^+$/tEGFR$^+$ T cells were transferred in parallel into HBVtg mice as well as wildtype BL/6 mice. When blood cells were stained at d4 post transfer, the frequency of S-CAR$^+$ T cells derived from HBVtg mice was reduced about 9% compared to tEGFR$^+$ T cells, whereas receptor frequencies differed only about 3% for cells derived from BL/6 mice (Figure 2.4.12 A), indicating antigen-specific S-CAR down-regulation. This situation changed at d8 post transfer: Although percentages of tEGFR$^+$ cells did not change significantly, S-CAR surface expression was reduced to about 50% of the initially positive cells in BL/6 as well as in HBVtg mice (Figure 2.4.12 B). These results indicated that, within the first days post transfer, antigen-stimulation influenced S-CAR surface expression. However, at later time points, S-CAR surface expression seemed to be influenced by antigen-unspecific factors, suggesting that several independent mechanisms played a role in the observed dynamics of S-CAR expression.

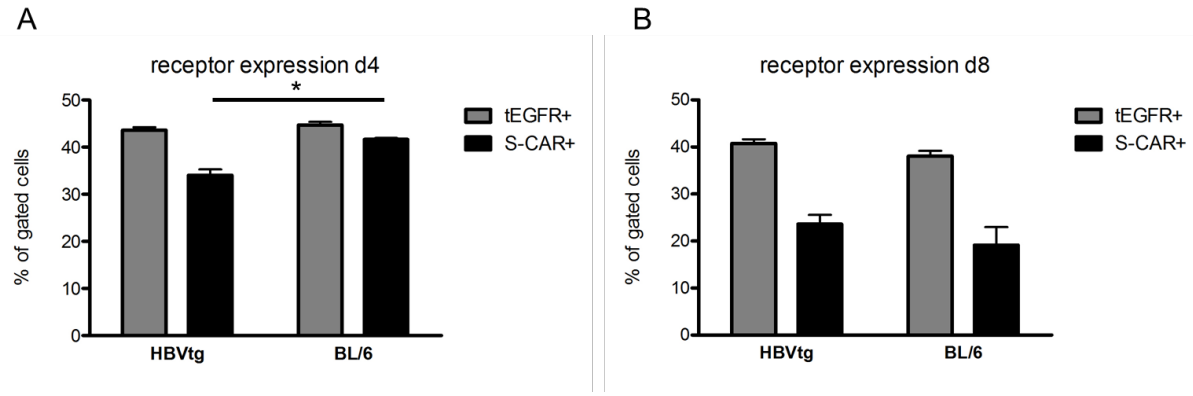


Figure 2.4.12 *In vivo* S-CAR surface expression with and without antigen stimulation. A) Receptor surface expression on transferred cells isolated from blood of HBVtg or Bl6 mice at d4 post transfer, shown as respective frequencies. Cells were pre-gated on CD45.1$^+$CD8$^+$ cells. **B)** Repetition of the staining at d8 post transfer. *(n= 8/3, Mann-Whitney-Test, S-CAR+; * P<0.05)*

2.4.6 S-CAR T cell inactivation *in vivo* does not occur due to methylation of the transgene

2.4.6.1 Establishment of an LTR-specific PCR

The finding that S-CAR surface expression was not stable even in S-CAR T cells that were transferred into HBV-negative mice led to the question whether there could be an impact of methylation on the transgene expression. Although it was shown that S-CAR expression could be rapidly restored by overnight resting, which would argue against a stable inactivation, the possibility remained that transgene expression was shut off epigenetically in the cells that did not show this S-CAR rescue. The S-CAR in transduced T cells is transcribed from the transgene integrated in the cellular genome after retroviral transduction. The promoter driving expression of the transgene is located in the retroviral-derived Long Terminal Repeat (LTR) upstream of the coding region. Within the LTR, several CpG dinucleotides can be found which is typical for promoter regions, allowing for transcriptional silencing of the respective gene. Figure 2.4.13 A shows a schematic picture of the Mo-MLV (Moloney murine leukemia virus)-derived LTR with the CpG islands as putative methylation sites.

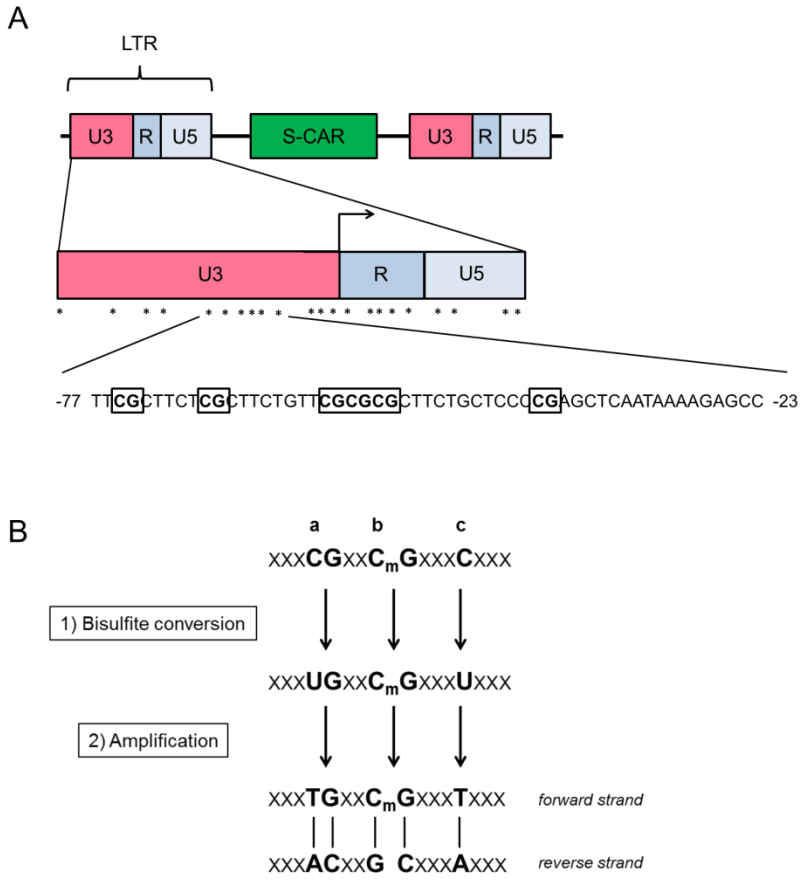


Figure 2.4.13 Detection of methylation patterns in the transgene-promotor. A) Schematic representation of the promoter located in the long terminal repeat of the MoMLV-derived vector. U3: unique 3' region; R: redundant region; U5: unique 5' region. * mark CpG dinucleotides. The black frames show the CpG islands that were analyzed for methylation. **B)** Detection of methylated CpGs using bisulfite conversion and subsequent PCR. a: unmethylated CpG; b: methylated CpG; c: cytosine residue. See text for more explanations. *Modified from: Swindle et al., 2004.*

To assess the question of potential methylation, a PCR was designed, allowing for specific amplification of the Long Terminal Repeat upstream of the S-CAR transgene. Detection of methylated CpGs can be achieved by bisulfite conversion of the DNA prior to the PCR: treatment with bisulfite leads to a conversion of all cytosines present in the DNA (Figure 2.4.13 B, a + c), whereas methylated cytosines are protected from this process (Figure 2.4.13 B, b). By amplification and sequencing of the DNA after the conversion process, it is therefore possible to distinguish between methylated and unmethylated CpGs. When establishing the PCR, it became clear that there has to be an endogenous LTR sequence present in the mouse genome, since PCR with a primer pair only binding within the LTR also amplified the targeted sequence in mice that had not received S-CAR T cells (Figure 2.4.14 A). To circumvent this problem, a nested PCR was performed, using in the first step a reverse primer binding in the S-CAR coding region. By this, specific amplification of the LTR driving the transgene was achieved (Figure 2.4.14 B).

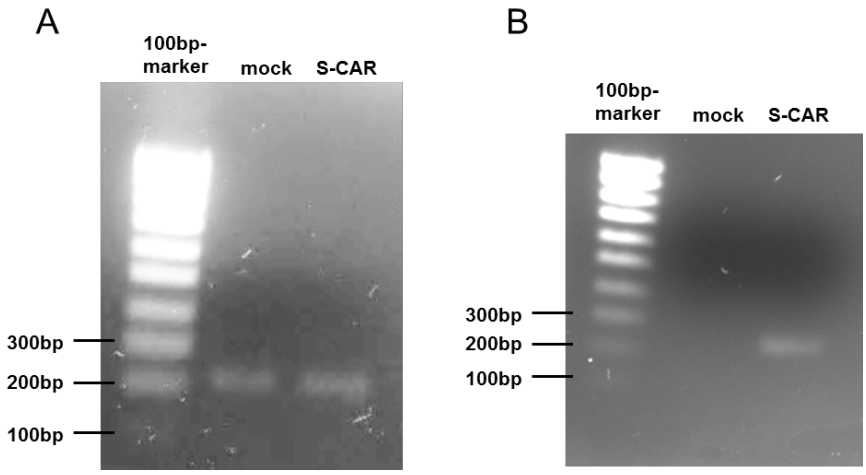


Figure 2.4.14 PCR of bisulfite-converted DNA. A) PCR products generated with LTR-specific primers from bisulfite-converted DNA of non-transduced (mock) or S-CAR transduced murine splenocytes. The expected fragment size was 230 bp. **B)** Products of nested PCR (after the second step). The first primer pair was designed to bind within the S-CAR coding region; the second primer pair was identical to the one used in A.

2.4.6.2 The Long Terminal Repeat driving transgene expression remains unmethylated

Liver DNA of HBVtg or BL/6 mice that had received S-CAR T cells was obtained at d12, d20 and d34 post transfer and the LTR was amplified from the DNA of one mouse per time point using the protocol described above. Since the methylation profile within one population is likely to vary from cell to cell, PCR products were cloned into a vector and sequenced on a

single cell level. Figure 2.4.15 shows the theoretical sequence amplified by nested PCR of completely bisulfite-converted DNA, including 17 CpGs in total. Tested were 6 single clones per mouse; the result is presented in Table 2.4.1. The maximum amount of methylated CpGs per clone was 2, with most of the promoter regions showing complete C→T conversion indicating a completely unmethylated state. Since a transgene shut-off would require a certain methylation density (Swindle et al., 2004), transgene silencing by methylation of the promoter regions did not play a role for transferred S-CAR T cells.

Theoretical sequence after complete bisulfite conversion:

aaggatttgaaatgatttgtgttttatttgaattaattaattagttgttttttgttttgttgtgtgtttttt
gttttttgagtttaataaaagagtttataatttttattggtgtgttagttttttgatagattgtgttgtttgg
gtatttgtatttttaataaagttttttgttgtttgtattgaatgtggattgttgatttttgggagggttttt
ttagat

Underlined: primer sequences

Yellow: Bisulfite-converted C→T
Green: Bisulfite-converted CpG

Total number of CpGs: 17

Figure 2.4.15 LTR sequence containing relevant CpG islands. See description for details.

	Number of methylated CpGs					
	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5	Clone 6
HBVtg d12	1	0	0	1	0	2
HBVtg d34	1	0	0	0	0	0
BL/6 d12	1	0	0	0	0	0
BL/6 d20	0	0	0	0	0	0

Table 2.4.1 Summary of detected methylation sites. Analyzed were 6 PCR clones derived from HBVtg mice (d12/d34 post transfer) and BL/6 mice (d12/d20 post transfer), respectively. Shown is the number of methylated CpGs among the total amount of 17 CpGs tested.

2.4.7 S-CAR T cells change their memory phenotype after adoptive transfer

Since it had been shown by several groups that the memory phenotype determines the fate of adoptively transferred T cells *in vivo* (Berger et al., 2008; Louis et al., 2011), detection of the memory T cell markers CD62L (L-Selectin) and CD127 (IL-7 receptor α) was included in the analysis of S-CAR T cells (Figure 2.4.16 left). Whereas T cells of untreated splenocytes clearly distributed into 4 different populations with a high percentage of double positive cells (Figure 2.4.16 middle), transduced and stimulated CD45.1$^+$ T cells before transfer exhibited a "synchronized" phenotype with high expression of both markers, resembling central memory T cells (Figure 2.4.16 right). This was observed for the stimulation protocols involving IL-12 as well as IL-2, indicating that it was a general consequence of strong T cell activation.

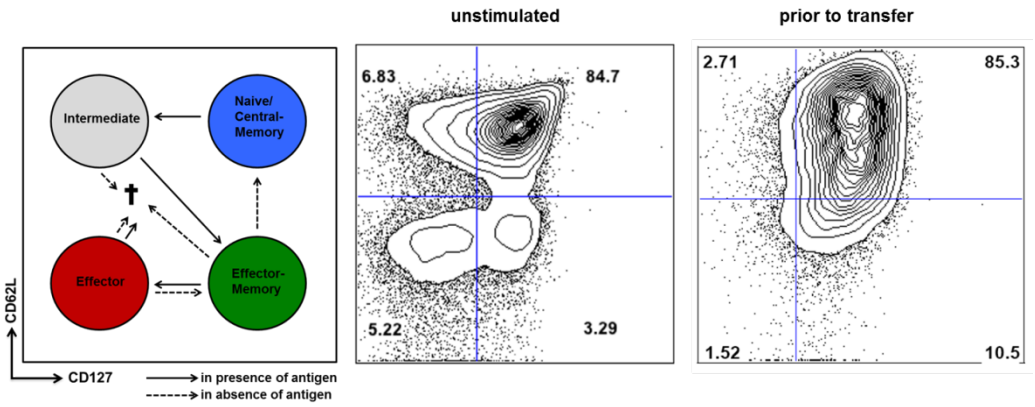


Figure 3.4.16 Memory phenotype of transferred T cells. Left: relation of memory markers CD62L and CD127. *Modified from Bachmann et al., 2005*. Middle: staining of murine CD8+ splenocytes after isolation. Right: staining of stimulated and transduced CD8+ cells.

This phenotype, however, changed *in vivo* (Figure 2.4.17 and Table 2.4.2 and 2.4.3): when cells were re-isolated from different body compartments, they were found to differentially express the tested markers, similar to endogenous CD8$^+$ T cells. Among liver associated lymphocytes, the highest proportion of CD45.1$^+$ cells at d12 and d34 post transfer had acquired an effector memory cell-like phenotype, which was also most abundant among endogenous liver lymphocytes. For blood lymphocytes, an initial frequency of 32% effector-like transferred cells could be detected at d12, whereas remaining cells at d34 mostly resembled effector memory cells. Those *in vivo* changes would support the finding that transferred T cells are cleared from the organism over time, also in the liver as the site of virus replication. Interestingly, remaining transferred T cells in the lymph nodes were mostly cells with a central memory like-phenotype, indicating the establishment of a small, but long-

lived memory cell population in the lymphatic compartment. However, a conclusive interpretation of *in vivo* changes is difficult due to the probably artificial upregulation of both markers after *in vitro* stimulation and transduction.

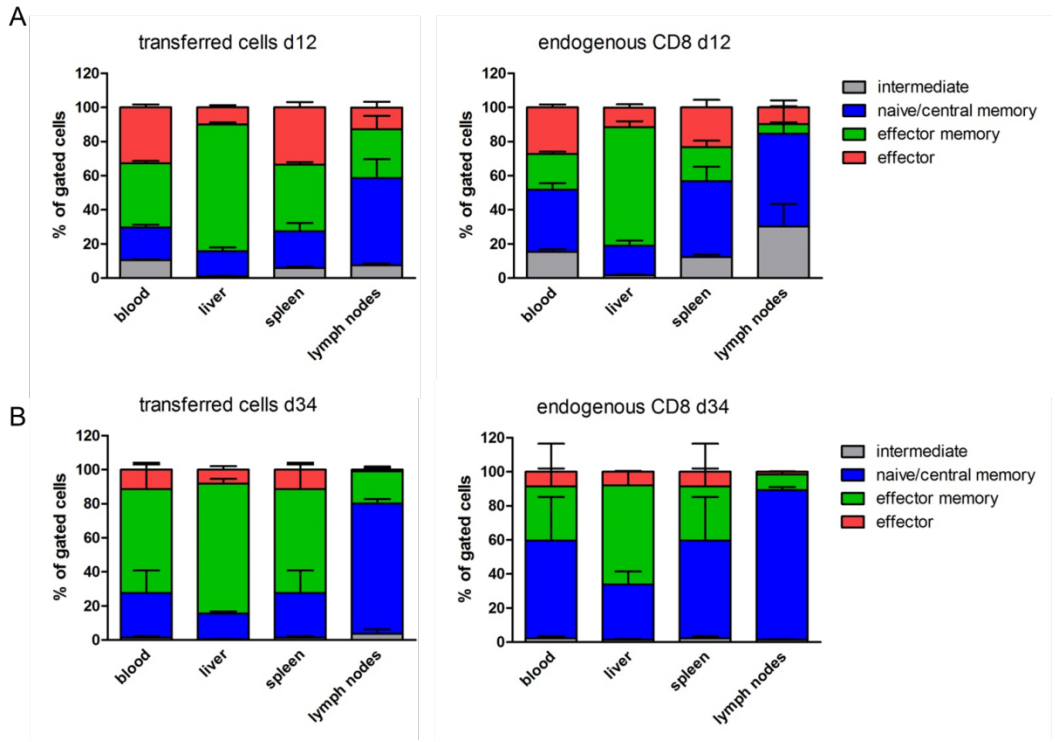


Figure 3.4.17 Memory phenotype of transferred T cells. Staining of transferred and endogenous CD8+ cells at d12 (upper part) and d34 (lower part) post transfer. Shown are the relative frequencies of the 4 different populations. Cells were always gated on CD8 first.

d12

	%	IM	CM	EM	E
transferred	blood	10,5	19,1	37,6	32,8
	liver	1,1	14,8	74,2	10,1
	spleen	5,9	21,7	38,9	33,6
	lymph nodes	7,6	51,1	28,5	12,8
endogenous	blood	15,4	36,4	20,9	27,3
	liver	1,7	17,4	69,3	11,7
	spleen	12,4	44,4	20,0	23,2
	lymph nodes	30,4	54,3	5,5	9,8

d34

	%	IM	CM	EM	E
transferred	blood	1,6	26,0	60,9	11,5
	liver	0,5	15,1	76,2	8,2
	spleen	1,6	26,0	60,9	11,5
	lymph nodes	3,7	76,6	18,9	0,8
endogenous	blood	2,3	57,3	31,9	8,5
	liver	1,4	32,5	58,3	7,8
	spleen	2,3	57,3	31,9	8,5
	lymph nodes	1,4	87,8	9,3	1,5

Table 2.4.2 & 2.4.3 Memory frequencies d12 & d34 post transfer. Shown are the relative frequencies within different body compartments as mean values (n=3-4). Gating was done on CD8+CD45.1- (endogenous) or CD8+CD45.1+ (transferred), respectively. IM: intermediate; CM: central memory; EM: effector memory; E: effector.

2.4.8 Activation-induced cell death as an important mechanism for loss of S-CAR T cells *in vivo*

Another aspect so far unknown was the question whether activation of S-CAR T cells can also lead to subsequent activation-induced cell death. To check for this, first experiments were performed *in vitro*: S-CAR T cells stimulated with rHBsAg were stained in addition with an Annexin V-antibody binding to apoptotic cells. Dead cells were, as always, excluded from the analysis. As shown in Figure 2.4.18 A, S-CAR$^+$ T cells stained stronger positive for Annexin V compared to S-CAR$^-$ T cells already before antigen stimulation. The MFI for Annexin V increased steadily on S-CAR positive T cells between 2 and 24 hours upon HBsAg encounter, whereas it remained stable without antigen stimulation as well as for S-CAR negative T cells. This strongly indicated that S-CAR T cells had acquired an apoptotic phenotype upon activation.

Since stimulation with plate-bound antigen is somewhat artificial and might lead to stronger T cell activation than under physiological conditions, Annexin V staining was used to stain *ex vivo* re-isolated cells derived from blood, spleen, liver and lymph nodes from mice that had received S-CAR/tEGFR$^+$ T cells. Indeed, S-CAR T cells also seemed to be driven into apoptosis upon transfer into HBVtg mice, as presented in Figure 2.4.18 B + C. Gating was done on endogenous, as well as tEGFR$^+$ and tEGFR$^-$ T cells to distinguish between the different populations. Clearly, transferred T cells carrying the transgene (tEGFR+) were significantly more prone to apoptosis than non-transduced and endogenous T cells. This held true for isolated cells from all 4 body compartments tested, namely blood, liver, spleen and lymph nodes, indicating that activation-induced cell death was following an initial expansion and activation of S-CAR T cells.

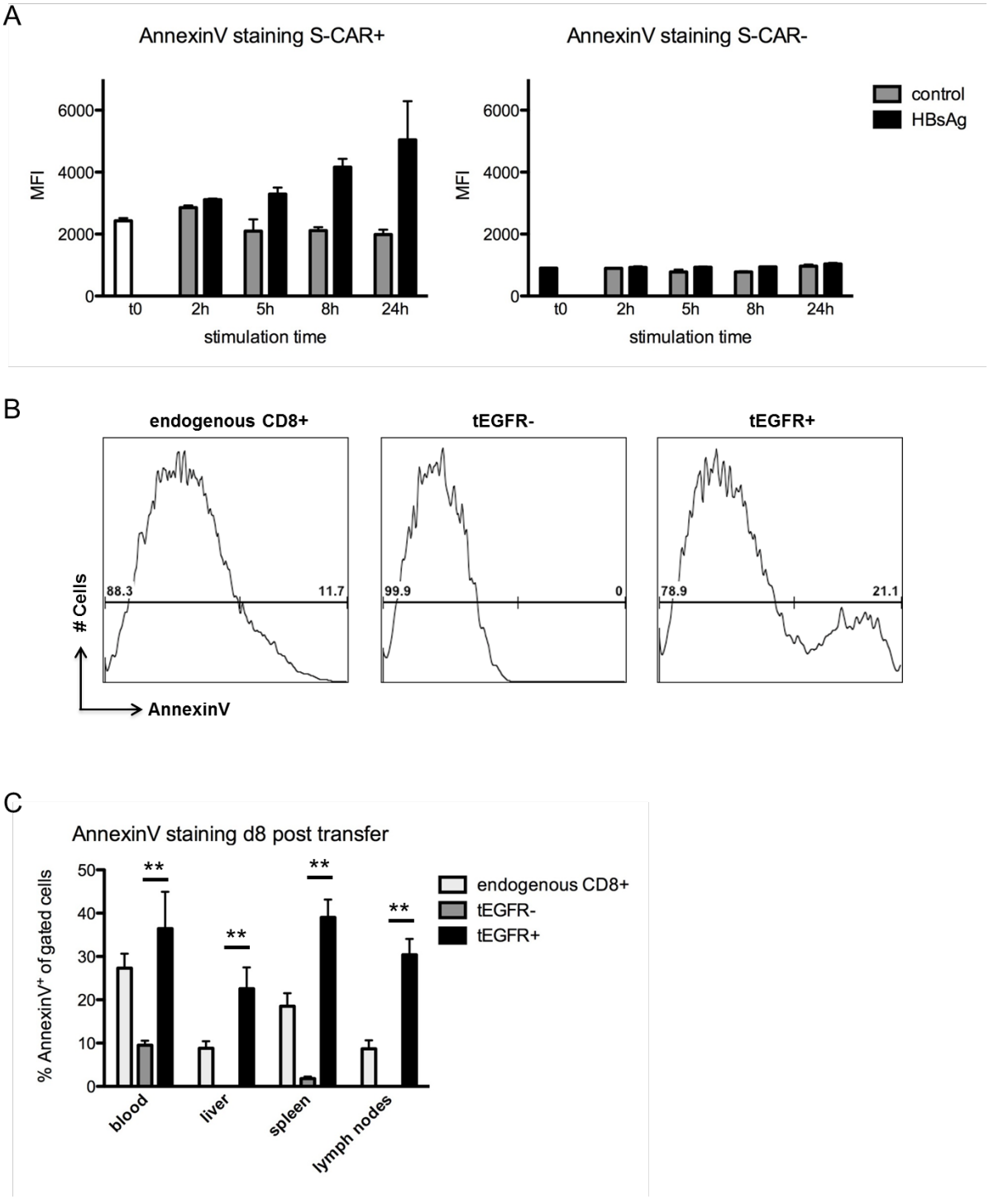


Figure 2.4.18 Detection of apoptotic cells by AnnexinV staining. A) AnnexinV staining of *in vitro* stimulated or unstimulated murine S-CAR T cells. Gating was done on CD8+S-CAR+ (left) or S-CAR- (right). **B)** Example for AnnexinV-stained liver associated lymphocytes isolated at d8 post transfer. tEGFR-expression allowed for distinguishment between receptor-negative and –positive transferred T cells. **C)** AnnexinV staining of CD8+ T cells isolated at d8 from different body compartments *(n=4; Kruskal-Wallis-Test, ** = P<0.01)*

2.5 Characterization of donor cells derived from HBV-replicating mice

So far, the T cells used for adoptive transfer experiments had been isolated from CD45.1$^+$ BL/6 mice, which did not replicate HBV. The adoptive transfer setting in a chronically infected patient, however, would imply the isolation, transduction and transfer of the patient´s T cells. To test whether T cells derived from an HBV-positive background show suppressed effector functions, HBVtg mice were crossed with CD45.1$^+$ mice, and cells from the F1 generation were isolated and used for further experiments. CD8$^+$ T cells of HBVtg (HBV+) and HBV naïve (HBV-) mice were transduced with the S-CAR in parallel. Transduction rates were comparable between the two donor cell populations (Figure 2.5.1).

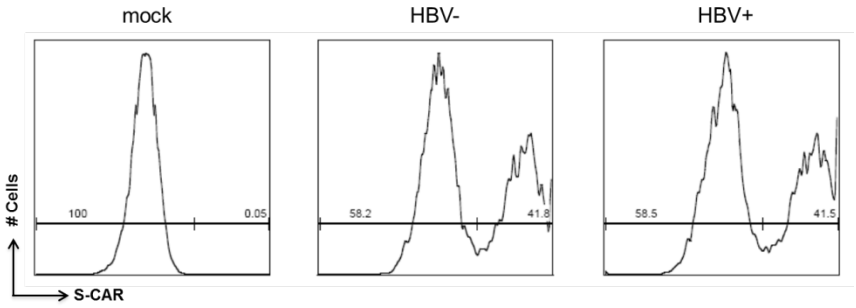


Figure 2.5.1 S-CAR transduction of HBV- and HBV+ cells. Cells were gated on CD8.

2.5.1 S-CAR T cells derived from HBV$^+$ donors do not show suppressed effector functions *in vitro*

To check for effector functions *in vitro*, S-CAR T cells were stimulated with plate-bound recombinant HBsAg. Figure 2.5.2 A shows that antigen stimulation led to a comparable activation of S-CAR T cells derived from HBV- and HBV+ mice as determined by secreted IFNγ. When IFNγ was stained intracellularly, about 25% of S-CAR T cells from both populations specifically produced IFNγ upon stimulation (Figure 2.5.2 B). Parallel staining of the marker LAMP-1 on the surface revealed LAMP-1 mobilization of around 4 fold compared to the control, indicating that both donor cell populations exerted the same killing capacity (Figure 2.5.2 C).

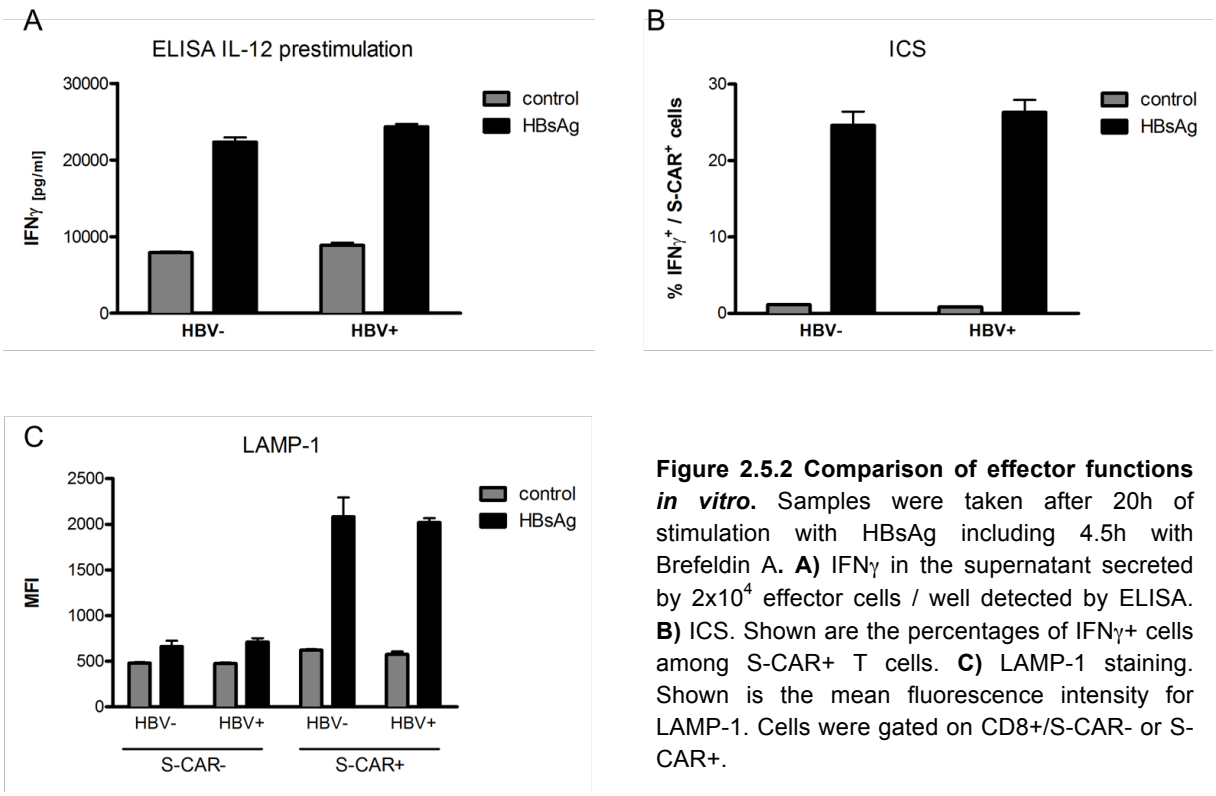


Figure 2.5.2 Comparison of effector functions *in vitro*. Samples were taken after 20h of stimulation with HBsAg including 4.5h with Brefeldin A**. A)** IFNγ in the supernatant secreted by $2x10^4$ effector cells / well detected by ELISA. **B)** ICS. Shown are the percentages of IFNγ+ cells among S-CAR+ T cells. **C)** LAMP-1 staining. Shown is the mean fluorescence intensity for LAMP-1. Cells were gated on CD8+/S-CAR- or S-CAR+.

Since also non-stimulated T cells were found to secrete high amounts of IFNγ (Figure 2.5.2 A), it was hypothesized that treatment with IL-12 led to high background cytokine secretion. To prove this, cells were transduced in the presence of IL-2 instead of IL-12, and either stimulated with rHBsAg or co-cultivated with HepG2/HepG2.2.15 cells. Cytokine levels were detected by ELISA, showing that IL-2 pre-treated cells secreted lower amounts of IFNγ than IL-12 pre-treated cells, and background levels were almost undetectable, indicating that the pre-stimulation protocol had a clear impact on effector functions (Figure 2.5.3). Stimulation with IL-12, though, has been shown to greatly improve *in vivo* survival of transferred T cells (Krebs, Böttinger et al., 2013) and was therefore required for the generation of S-CAR T cells for adoptive transfer.

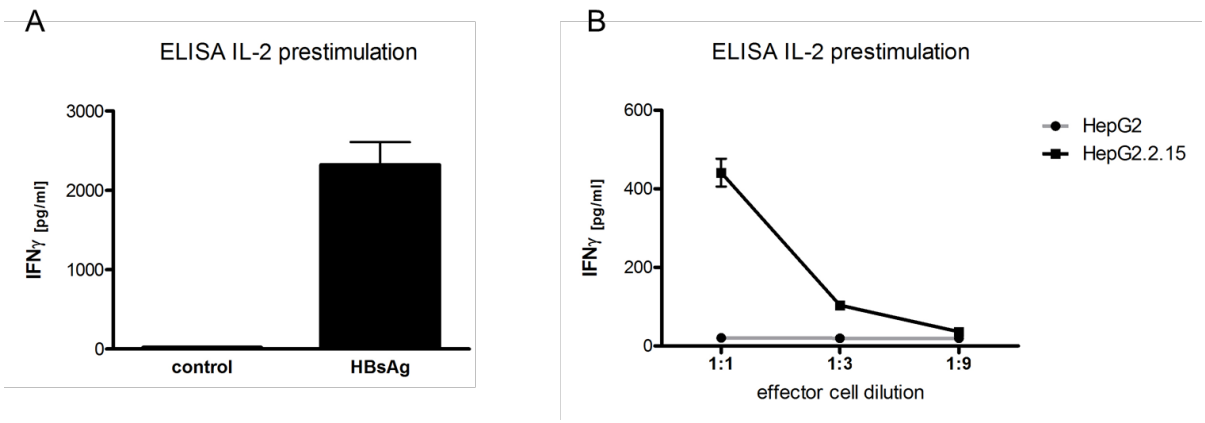


Figure 2.5.3 IL-2 pre-stimulation. IFNγ secretion of splenocytes derived from HBVtg mice upon 15.5h stimulation. **A)** Stimulation with rHBsAg ($3.5x10^4$ effector cells/well). **B)** Stimulation by co-culture with HepG2.2.15 cells. Effector cells were diluted 1:3 and 1:9 with a starting concentration of $3.5x10^4$ effector cells/well.

2.5.2 *In vivo*, HBV$^+$ donor cells perform as well as cells derived from HBV$^-$ mice

To compare *in vivo* effects, CD45.1$^+$ S-CAR T cells derived from either HBV naïve or HBVtg mice were adoptively transferred into HBVtg mice. Frequencies of CD45.1 cells were monitored over time and are shown in Figure 2.5.4 A. Within the first week post transfer, HBV+ T cells represented up to 15% of total PBMC, whereas HBV- T cells reached levels up to 25%; both however with high variability. Decrease of HBV+ cells over time was not as strong as for HBV- cells, resulting in more or less comparable levels up to 13 weeks post transfer. The high standard deviations were due to the highly different inter-individual frequencies. When cells derived from HBV+ mice were re-isolated from liver and re-stimulated with recombinant HBsAg, a fraction of around 40% secreted IFNγ as detected by intracellular cytokine staining (Figure 2.5.4 B), as already shown before for HBV- donor cells. Thus, the *ex vivo* recovery of effector functions did not seem to be impaired due to the HBV+ background of the cells. To investigate the antiviral effect, viremia was monitored over time. This revealed a decrease of about one to two log for both treatment groups within the first week post transfer (Figure 2.5.4 C upper part), and an increase to levels compared to d0 at later time points, further strengthening the fact that persisting T cells became functionally silenced *in vivo*. Interestingly, in this experiment, T cells derived from HBVtg mice showed even a stronger and more consistent antiviral effect than HBV-naïve T cells (see also DNA levels single mice; Figure 2.5.4 C lower part). Of note, the group that had received HBV-naïve cells contained two outliers that did not feature reduction of viremia, whereas the other mice were comparable to the HBV+ recipient group. Taken together, a defect in effector functions due to the HBV+ background of the donor cells could be ruled out for this model. Since *in vitro* as well as *in vivo* results suggested no impact of an HBV+ donor cell

background on effector cell functions, all further experiments were carried out with HBV-naïve donor cells for practical reasons.

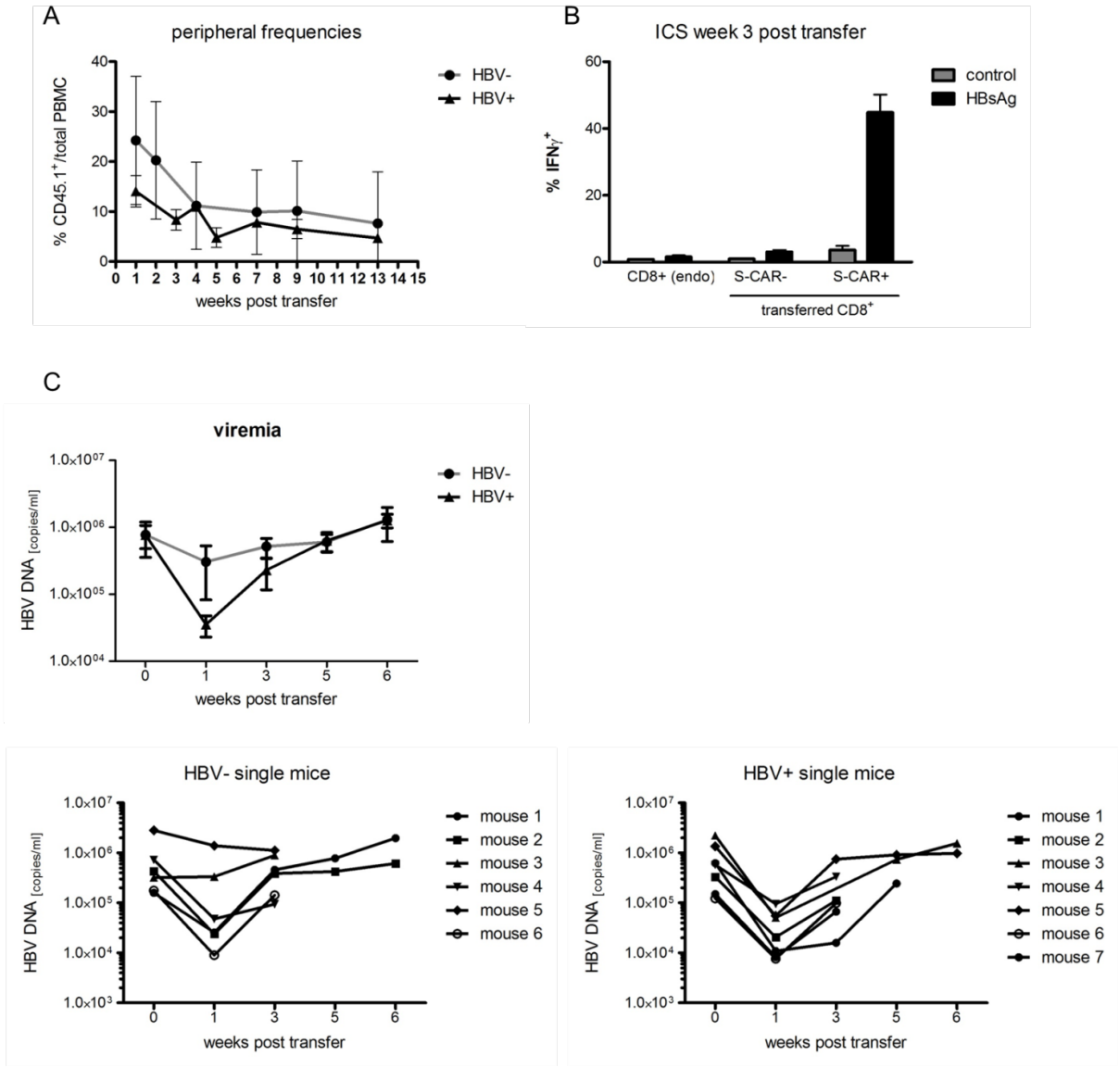


Figure 2.5.4 ***In vivo*** **survival and effector functions of HBV+ cells. A)** Long-term survival of transferred T cells derived from HBVtg or HBV naïve mice. **B)** ICS of HBVtg-derived cells isolated 3 weeks post transfer from the liver and re-stimulated with rHBsAg. n=3. **C)** Viremia of HBVtg mice that had received S-CAR T cells derived from naïve (HBV-) or HBVtg mice (HBV+). Upper part: mean values. Lower part: individual mice. *(n=2-7; Kruskal-Wallis-Test, HBV- P = 0.202; HBV+ P = 0.0020 **).*

2.6 Exclusion of severe side effects induced by S-CAR T cells upon adoptive transfer

One important question concerning a potential future application of S-CAR T cells in HBV-infected patients was whether S-CAR T cells might induce adverse effects in the recipient upon adoptive transfer. As already shown in previously conducted experiments (doctoral thesis Karin Krebs), S-CAR T cells exerted profound antiviral effects in HBVtg mice that were partly due to direct killing of HBV-replicating hepatocytes. Such liver damage is beneficial on the one hand, but bears also the risk of unwanted immunopathology. Besides, S-CAR T cells were shown to secrete cytokines upon antigen contact that counteract HBV replication but could in turn also influence endogenous immune cells, and could potentially induce non-controllable immune reactions. Furthermore, the high concentrations of circulating HBsAg in form of subviral particles, which can be found in chronically infected patients as well as HBVtg mice, could potentially activate S-CAR T cells and lead to unwanted off-target effects.

To check for such unwanted side effects, HBVtg mice were treated with $4x10^6$ S-CART cells per mouse and examined with regard to several parameters. Liver damage was monitored by measurement of serum ALT levels as a marker for liver cell destruction. As shown in Figure 2.6.1 A, S-CAR T cells induced a moderate, but significant ALT elevation peaking one week post transfer at a value of around 800U/ml. Importantly, ALT levels only increased transiently, with rapidly normalizing levels over time. In parallel, treated mice were checked for changes in body weight, since weight loss would have implied a general negative effect induced by S-CAR T cells. However, none of the recipient mice showed noticeable changes in body weight (Figure 2.6.1 B), suggesting that the occurring liver damage did not lead to general distress in the recipient mice. To investigate whether S-CAR T cells induced strong elevation of cytokines in the serum and thereby a “cytokine storm”, the concentrations of TNF-α, IFN-γ, MCP-1, IL-10, IL-12 and IL-6 present in the serum of treated mice were measured using a cytokine bead array (CBA; done by Karin Krebs). As presented in Figure 2.6.1 C, serum concentrations of cytokines TNF-α, IFN-γ, IL-10, IL-6 and the chemokine MCP-1 were indeed significantly elevated at d12 post transfer. However, those effects were mainly transient, and cytokine levels at d34 post transfer were comparable to those of untreated mice again.

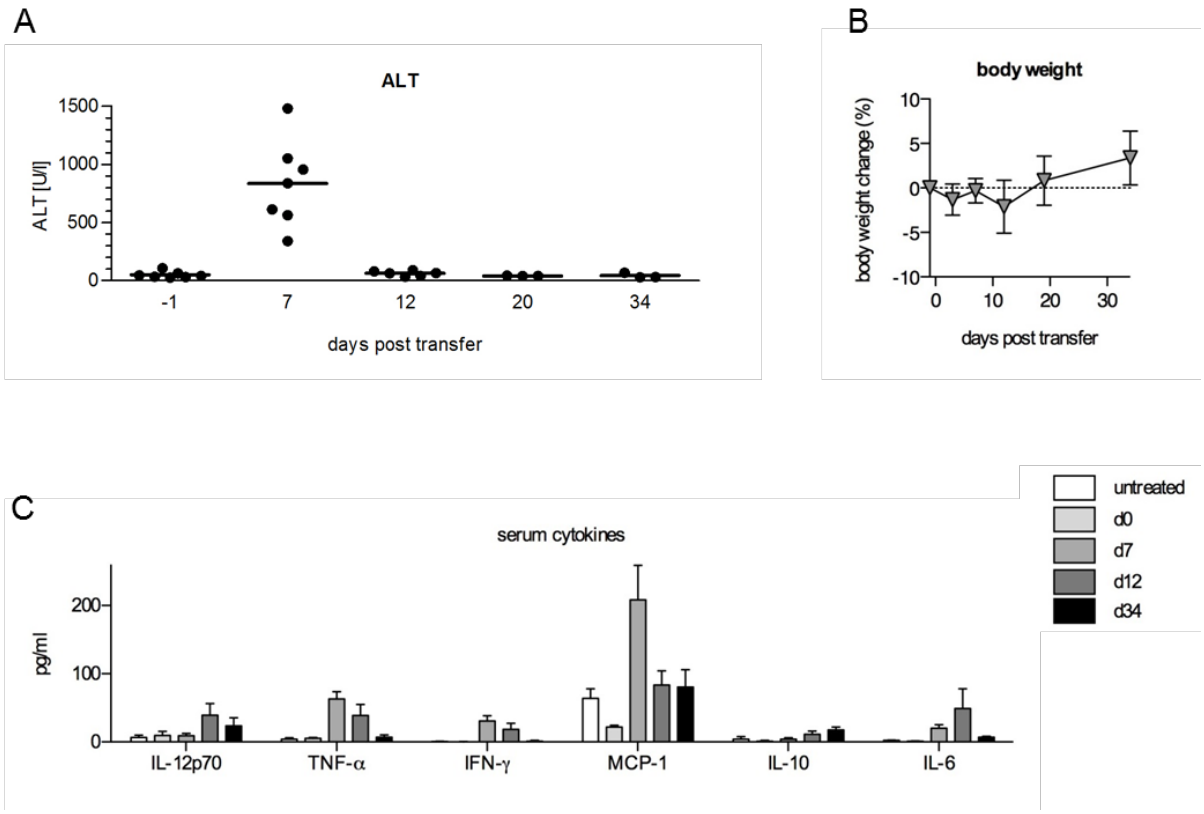


Figure 2.6.1 Investigation of side effects. A) ALT levels as marker for liver damage measured in the serum of treated mice *(Kruskal-Wallis-Test d-1 vs d7: **, d7 vs. d34: *).* **B)** Monitoring of body weight. Shown are changes of body weight in %. **C)** Cytokine bead array from serum taken at different time points post transfer as well as from untreated controls. *(n = 3–7: Kruskal Wallis Test: IL-12 (P = 0.1163), TNF-α (P = 0.0049), IFN-γ (P = 0.0032), MCP-1 (P = 0.0061), IL-10 (P = 0.0238), IL-6 (P = 0.0047)*

Likewise, compositions of endogenous immune cells belonging to the lymphoid (Figure 2.6.2 A) as well as the myeloid lineage (Figure 2.6.2 B) were transiently altered by the transfer of S-CAR T cells. The relative frequencies of CD4 T cells and B cells decreased in spleen and liver, whereas an increase of B cells and NK cells was detected in the blood. The relative amount of myeloid immune cells, comprised of inflammatory monocytes, dendritic cells (DCs) and neutrophils, had especially increased in the liver. Importantly, the described changes were detected at d12 post transfer, and again levels went back to levels of control mice at d34. Those findings further supported the idea of a contraction of the S-CAR T cell population following initial activation and expansion, with the remaining cells displaying a functionally silenced phenotype unable to exert effector functions *in vivo*.

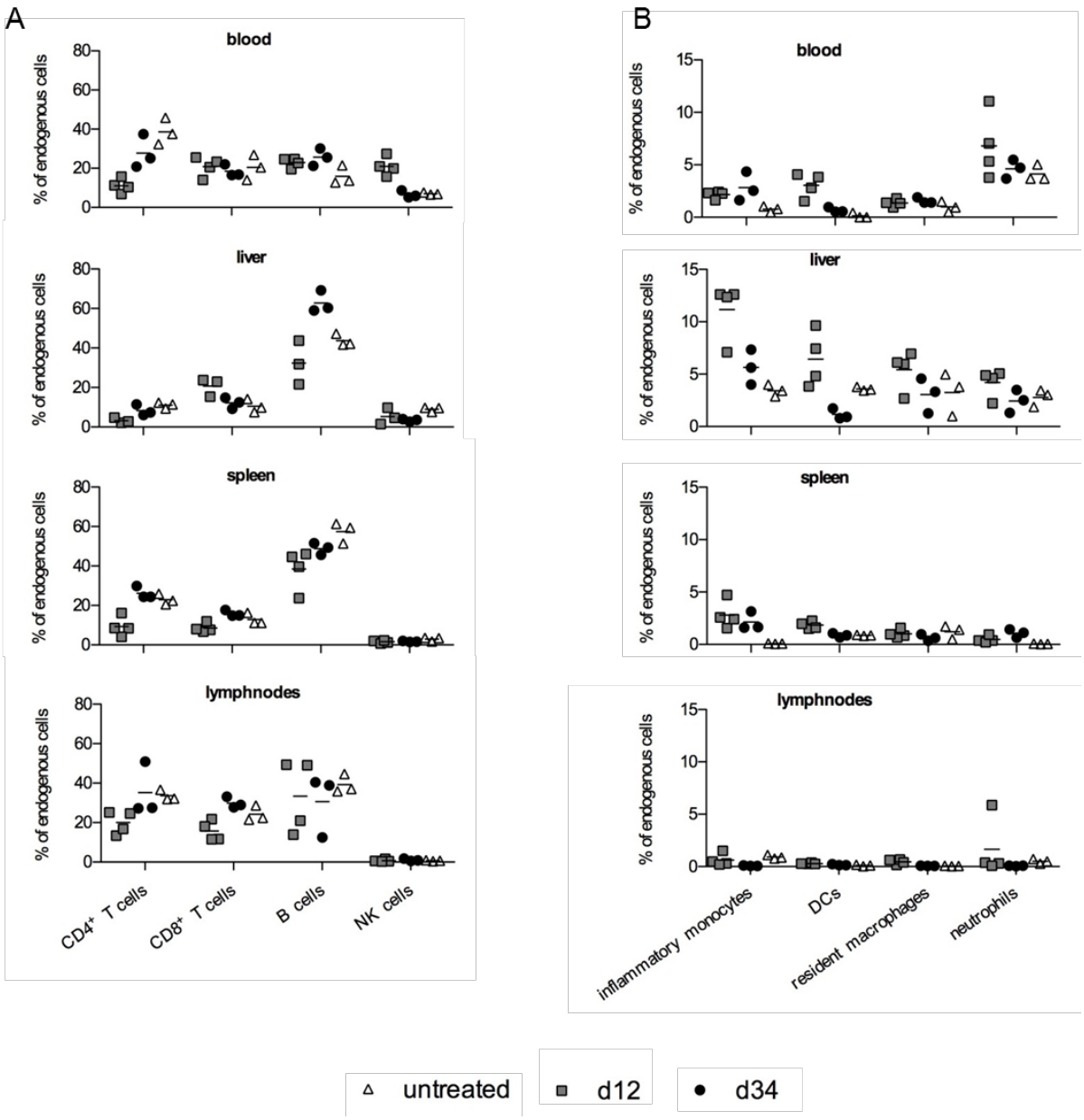


Figure 2.6.2 Endogenous immune cell compositions. A) Staining for lymphoid markers. **B)** Staining for myeloid markers. Cells were isolated from different body compartments at d12 and d34 post transfer and from untreated control mice (n=3-7). The different cell populations were distinguished by the expression of specific markers (*see Material and Methods*). *n=3.*

2.7 Comparison of effector functions exerted by S-CAR T cells and vaccine-induced S-specific T cells

The remaining question with regard to the use of S-CAR T cells in the adoptive therapy setting was whether S-CAR T cells can perform as well as or even better than natural T cells specific for HBsAg. To investigate this, S-specific T cells were generated in CD45.1$^+$ mice by application of an optimized vaccination protocol (Figure 2.7.1): Prime vaccination with rHBsAg together with the adjuvants CpG and PCEP followed by a boost with an MVA (Modified Vaccinia Virus Ankara) vector led to a frequency of around 3.5% T cells among CD8$^+$ cells specific for S-peptides as detected by intracellular cytokine staining (done by Clemens Jäger / Karin Krebs). CD8$^+$ T cells were isolated from spleens of vaccinated mice and 1x10^6 S-specific T cells were adoptively transferred into HBVtg mice. In parallel, adoptive transfer of 1 or 4x10^6 S-CAR T cells generated with the already described protocol was performed, and treated mice were monitored for 5 weeks.

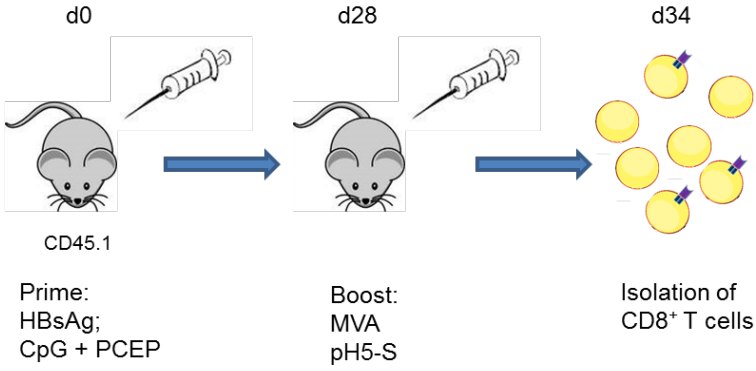


Figure 2.7.1 Generation of S-specific T cells by prime-boost vaccination. Prime: CpG, PCEP (adjuvants) + 32µg rHBsAg. Boost: MVApH5-S: HBsAg-expressing Modified Vaccinia Virus Ankara vector (1x10^8).

As shown in Figure 2.7.2 A, transfer of 1x10^6 S-specific T cells did not lead to a change in serum ALT levels in the recipient mice. On the other hand, transfer of S-CAR T cells induced an elevation of ALT levels, which was profound in mice receiving 4x10^6 S-CAR T cells and clearly lower when 1x10^6 S-CAR T cells had been transferred. Importantly, ALT increase was only transient, peaking at d7 as already shown before. When the frequencies of transferred T cells were detected in different body compartments via the congenic marker CD45.1, analysis revealed that S-CAR T cells had effectively homed to the liver at d12 post transfer, representing around 8% of total liver associated lymphocytes. In contrast, the highest frequency of S-specific T cells (4.5% of total leukocytes) was found to be located in the lymph nodes (Figure 2.7.2 B). At d34 post transfer, frequencies of transferred T cells

were low to undetectable for all three treatment groups (see also 2.4.1), which was likely to be the result of a contraction of the transferred T cell population.

To check for antiviral effects, viremia was determined as well as the presence of HBV DNA and HBcAg in the liver (Figure 2.7.2 C+D). As expected, an initial drop of viremia and reduction of viral load in the liver at d12 was followed by the recovery of transgene expression at d34 when transferred T cells had mostly vanished from the circulation. In general, $4x10^6$ S-CAR T cells elicited the most distinct antiviral effect, especially with regard to reduction of cytoplasmic core protein in hepatocytes d12 post transfer (2.7.2 E). Interestingly, however, antiviral effects induced by S-specific T cells and $1x10^6$ S-CAR T cells were comparable to those of $4x10^6$ S-CAR T cells, in spite of the fact that no or little hepatocyte killing could be detected in those mice: reduction of viremia around 1 log compared to d0, and reduction of rcDNA in the liver around 3 fold compared to d34 were achieved in all three treatment groups. Most likely, the influence of non-cytopathic mechanisms regulating HBV replication was especially relevant for S-specific T cells carrying a natural TCR, whereas S-CAR T cells acted as cytotoxic T cells in addition. With regard to the application in a clinical setting, the hepatocyte killing by transferred T cells could cause the risk of immunopathogenesis, but might on the other hand be required for virus elimination. In this respect, S-CAR T cells showed superior effector functions compared to S-specific vaccine-induced T cells in HBVtg mice.

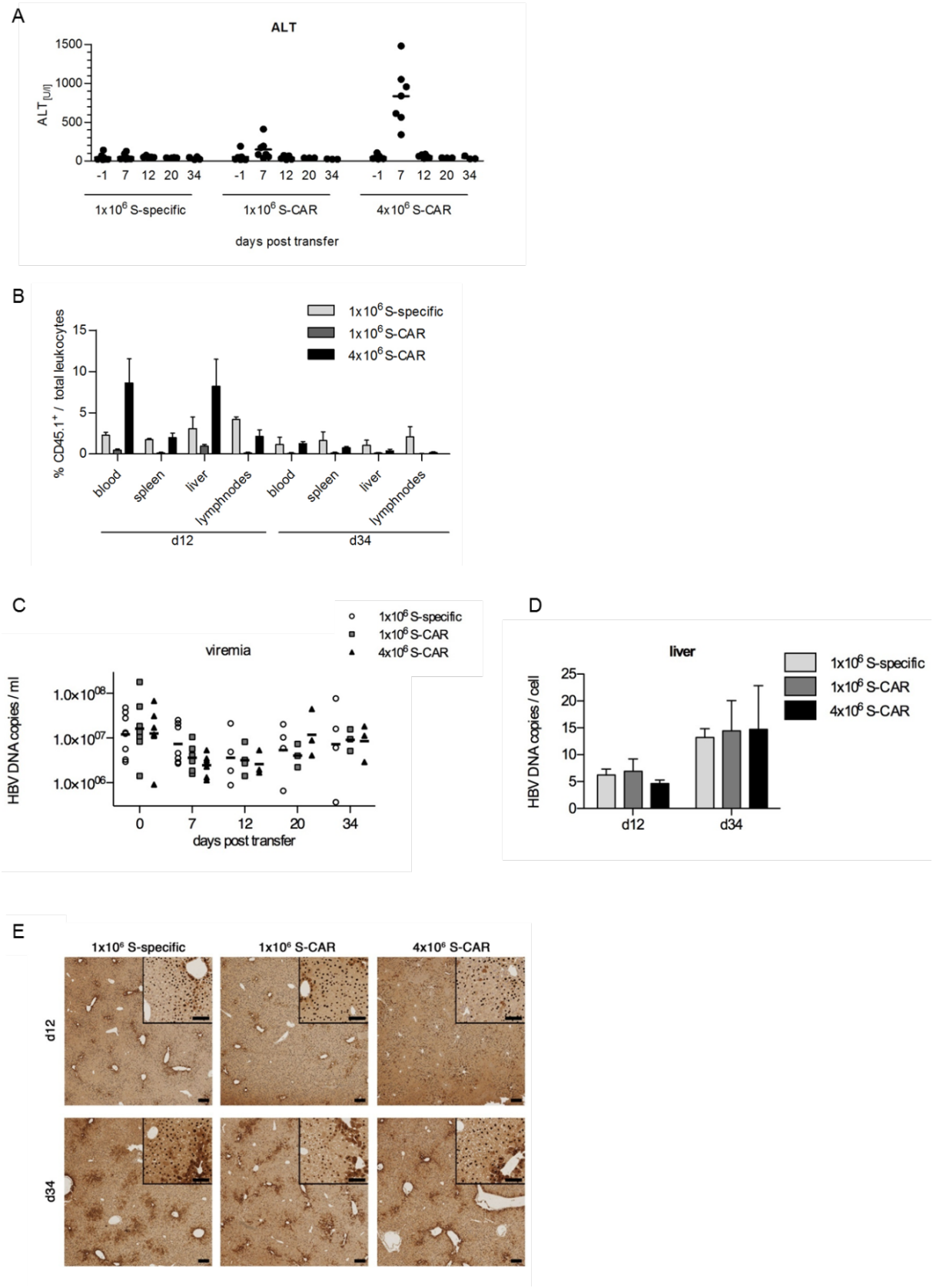
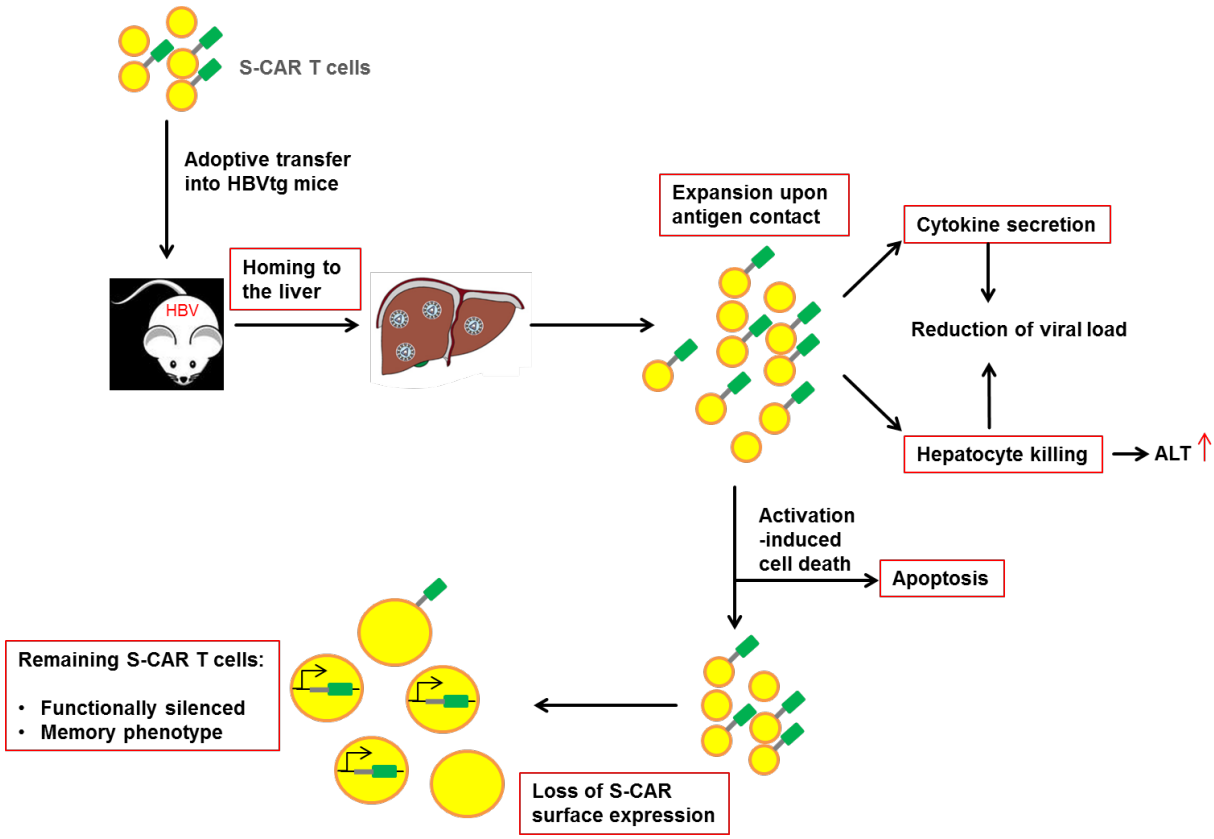


Figure 2.7.2 *In vivo* comparison of S-specific and S-CAR T cells. A) Serum ALT levels. **B)** Frequencies of transferred T cells among total viable leukocytes in different body compartments d12 and d34 post transfer. **C)** Viremia detected by quantification of viral DNA in the serum. **D)** HBV DNA in the liver detected by qPCR. **E)** Staining of HBcAg from liver sections taken at d12 and d34 post transfer. Scale bar: 200µm.

2.8 Summary: Fate of S-CAR T cells *in vivo*

According to the results presented in this work, the following scheme summarizes the fate of S-CAR T cells after adoptive transfer into HBVtg mice:

S-CAR T cells have been shown to migrate to the liver, where they initially expanded and were activated upon contact with the antigen. Activation led to the execution of antiviral effects that could be differentiated between i) the direct elimination of $HBsAg^+$ hepatocytes, leading to a transient liver damage, and the ii) non-cytopathic reduction of viral DNA by cytokine secretion. Antiviral effects, though, were timely limited due to the fact that most S-CAR T cells underwent apoptosis, leading to a contraction of the T cell population. The remaining S-CAR T cells displayed a memory T cell-like phenotype, but acquired a non-functional state, which was due to (partly antigen-dependent) loss of S-CAR surface expression. Although S-CAR T cells did not acquire a generally exhausted phenotype, the liver microenvironment might have silenced T cell functions in addition.



3 Discussion

3.1 HBV-specific TCR-like antibodies do not function in the CAR context

In the first part of this thesis, HBV-specific TCR-like antibodies were cloned as single chain antibodies for further usage in the context of chimeric antigen receptors. Due to their special structure, TCR-like antibodies combine the fine specificity of a T cell receptor with the biological and pharmacological properties of soluble antibodies. Initially, TCR-like antibodies were developed as tools for following intracellular generation, trafficking and surface expression of peptide-MHC complexes, thereby representing a valuable research tool (Cohen et al., 2003, Dao et al., 2013). Besides employment as diagnostic reagents, they show great potential as therapeutic agents. Following the successful targeting of MAGE-A1 expressing cells with a human TCR-like Fab fragment (Chames et al., 2000), a growing repertoire of TCR-like antibodies has been developed; either by the classical hybridoma technology or by using phage display (Dahan et al., 2012). The majority of published TCR-like antibodies is directed against tumor antigens, but also virus-infected cells can serve as targets as proven by specific recognition of HTLV-1 Tax (Yamano et al., 2004), Influenza M1 (Biddison et al., 2003), CMV pp65 (Makler et al., 2010) and HIV-1 Nef (Nunoya et al., 2009) peptides presented on MHC-I by TCR-like molecules.

The Bertoletti group has described the successful production of antibodies recognizing the immunodominant HBV peptides Env183-91 or C18-27 in complex with HLA-A2 (Figure 2.1.1; Sastry et al., 2011, Ji et al., 2012) by immunizing mice with purified MHC:peptide complexes and subsequent generation of hybridoma cell lines. By employing the Env182A2 antibody, liver biopsy specimens from chronic hepatitis B patients could be specifically stained, and a fluorescently-labelled cargo was efficiently transported to and internalized by HLA-A2-Env expressing cells. Moreover, in comparison to CD8 T cells expressing a receptor with the same specificity, the TCR-like antibody showed a superior sensitivity (Sastry et al., 2011). In a second study, the Env182A2 as well as the C18A2 antibody were successfully used for targeted delivery of IFNα to HBV infected cells (Ji et al., 2012). Those results provided the background for the project of this thesis, with the goal of constructing chimeric antigen receptors based on the TCR-like antibodies. Other studies have already shown that TCR-like constructs can redirect T cells to cancer cells in the context of chimeric receptors (Willemsen et al., 2001; Stewart-Jones et al., 2009; Tassev et al., 2012) or bispecific antibodies (Liddy et al., 2012).

When we used Env183A2 and C18A2 hybridoma supernatants to stain endogenously presented peptides on the surface of the HBV^+ hepatoma cell lines HepG2-H1.3 (Figure 2.1.2) and HepG2.2.15 (not shown), however, the specificities of the respective antibodies

could not be shown conclusively. Since hepatocytes constitutively express low levels of MHC-I molecules (Gehring et al., 2007) and their antigen-processing machinery is inefficient for the generation of MHC:peptide complexes (Shin et al., 2006), treatment with IFNγ was perfomed to increase the amount of surface-located MHC complexes. This was also done regularly by the Bertoletti group before staining (Sastry et al., 2011; Low et al., 2012), however with another HBV-replicating cell line (HepG2-117; Sastry 2011), which was not available for our study. In contrast to their findings, IFNγ treatment led to an increased unspecific antibody binding on HBV-negative cells, especially for Env183A2, which could hardly be distinguished from the staining of HBV-H1.3 cells expressing the targeted complex (Figure 2.1.2). It is likely that the employment of the same HBV-replicating target cell line would have been crucial to confirm the staining on endogenously presented peptides. Yet, HLA-A2 positive cells that were exogenously loaded with the respective peptides were specifically stained with both TCR-like antibodies even without prior IFNγ treatment (Figure 2.1.3), finally confirming antibody specificity.

In the next step, the TCR-like antibodies were cloned as single chain fragments to be able to construct chimeric antigen receptors similar to the S-CAR (Figure 2.1.1). The cloning procedure involved the amplification of the respective variable domains (Figure 2.1.4) and the fusion by a linker sequence, forming the scFv. The single chain fragments with supposed TCR-like specificities were subsequently cloned into the context of chimeric antigen receptors. The newly constructed CARs could be expressed on HEK 293T cells, however by far not as strong as the S-CAR (Figure 2.1.5 A), suggesting that the receptors were not expressed in an efficient way. In line with that, it was not possible to produce retroviral supernatants capable of transducing human or murine cells (Figure 2.1.6). To finally check whether the TCR-like chimeric antigen receptors had at least retained their specificity, Env183A2-CAR and C18A2-CAR expressing 293T cells were directly stained with streptamers mimicking the respective MHC complexes. Since also those stainings turned out to be negative (Figure 2.1.5 B), the results indicated that re-cloning of the TCR-like antibodies as scFvs has led to inefficient protein expression and loss of antigen specificity or impaired binding affinity.

To increase protein stability, the respective antibodies could be cloned as Fab fragments and as such employed as chimeric constructs, like described by Willemsen and colleagues with the redirection of T cells carrying a Fab-based chimeric receptor to MAGE-A1 expressing cancer cells (Willemsen et al., 2001). In line with that, most of the above-mentioned studies did not employ single chain fragments, but either Fab fragments or complete IgG antibodies for their experiments (Willemsen et al., 2001; Stewart-Jones et al., 2009; Liddy et al., 2012; Yamano et al., 2004; Biddison et al., 2003; Makler et al., 2010;

Nunoya et al., 2009). Moreover, a screening approach using phage display (Nunoya et al., 2009) could be helpful to generate new TCR-like single chain antibodies with a good stability and high affinity to the MHC:peptide complex.

However, several aspects should be considered before starting such time-consuming experiments: Although the affinity of TCR-like antibodies is in general higher than for T cell receptors with the same specificity (Willemsen et al., 2008; Weidanz et al., 2011), the approach of using peptide-MHC specific CARs is dependent on a sufficient amount of peptide-MHC complexes on the infected cell. Although Bertoletti and colleagues have shown specific staining of liver biopsies with their TCR-like antibody, the amount of positively stained cells was highly variable (Sastry et al., 2011), suggesting that the density would be too low for efficient redirection in certain cases of chronic HBV infection. In a different study, Grotenbreg and colleagues showed that Env183A2 exhibits increased sensitivity compared to TCR tetramers with the same specificity, but was, in contrast to the tetramer, not able to recognize different natural variants of the Env183-191 epitope (Low et al., 2012). Furthermore, a general disadvantage of TCRs or TCR-like antibodies is the MHC restriction, which precludes their application to HLA-A2 negative patients – a problem that does not occur with the S-CAR targeting native viral surface proteins. Regarding those points, further studies focused on therapy approaches involving the S-CAR rather than modifying the TCR-like antibody constructs.

3.2 S-CAR grafted NK cells are non-applicable for adoptive immunotherapy

Natural killer cells (NK cells) are an important component of the innate immune system and account for up to 10-15% of human blood lymphocytes (Trinchieri et al., 1989). They are characterized by a $CD3^-CD56^+$ phenotype and by the expression of a variety of activating and inhibitory receptors (Lanier 2005; Yokoyama et al., 2006). NK cells usually acquire a state of unresponsiveness to self by several tolerance mechanisms (Lanier 2005), which can convert into an activated state under certain circumstances. According to the missing self-model suggested by Kärre and colleagues, NK cells sense the absence of MHCI on other cells and react with target cell lysis (Karre et al., 1986). Target cell killing is tightly regulated and suppressed by inhibitory receptors recognizing MHCI molecules. Only in the absence of the dominant inhibitory signals, activating receptors trigger the release of cytotoxic granules and the secretion of various cytokines like IFNγ and TNFα (Trinchieri et al., 1989, Lanier 2005). In contrast to cytotoxic T cells, cytolysis is mediated without the need of prior sensitization (Trinchieri et al., 1989).

Based on the findings that, instead of T cells, NK cells equipped with a HER-2 specific CAR can specifically lyse HER-2 positive target cells (Kruschinski et al., 2008), the second part of

this thesis dealt with the grafting of primary human NK cells with the S-CAR, a chimeric antigen receptor directed against the HBV surface proteins. Human and murine CD8 T cells were already proven to exert specific effector functions when equipped with the S-CAR (Bohne et al., 2008; Krebs, Böttinger et al., 2013), raising the question whether the adoptive T cell therapy approach could be complemented by parallel use of S-CAR expressing NK cells.

NK cells were expanded from human peripheral blood using an optimized protocol, which resulted in the outgrowth of NK cells with a $CD3^{-}CD56^{+}$ phenotype (Figure 2.2.1 A). S-CAR NK cells were generated by two subsequent rounds of transduction with retroviral vectors, which led to moderate but distinct S-CAR expression with a maximum efficiency of around 15% (Figure 2.2.1 B). Remaining $CD3^{+}$ cells, representing T cells as well as NKT cells, were removed from the population; leading to an almost 100% pure NK cell population (Figure 3.2.1 C). This represented an important step, since the impact of other cell types on target cell lysis had to be excluded. Hepatoma cell lines HepG2 and HepG2.2.15 cells served as target cell lines, with the latter one having been constructed by stable integration of the HBV genome into HepG2 cells (Sells et al., 1988). For first testings, S-CAR grafted NK cells were cultivated with HBV-negative HepG2 or HBV-positive HepG2.2.15 cells and cell lysis was monitored. Clearly, NK cells induced a profound target cell lysis (Figure 2.2.2) during 24h of co-culture; however lysis was occurring also on HepG2 cells and induced by S-CAR negative NK cells. Those results indicated that NK cells had retained their cytolytic potential during the expansion and transduction procedure, but that target cell killing was unspecific. As already described in the last part, hepatoma cell lines, as other tumor cell lines, are low at MHCI expression, which represents an important activation signal for NK cells (Lanier 2005). Treatment with IFNγ upregulates MHC-I expression (Zhou et al., 2009) and was therefore applied for HepG2 and HepG2.2.15 cells (Figure 2.2.3 A). After treatment with IFNγ, unspecific hepatoma cell lysis by NK cells was indeed almost completely blocked. However, S-CAR NK cells did not induce clear additional, antigen-specific lysis of HepG2.2.15 cells (Figure 2.2.3 B), which would suggest that S-CAR NK cells are not functional in an antigen-specific manner.

In contrast to those findings, HER-2 specific NK cells exerted antigen-specific effects when co-cultivated with HER-2 expressing target cell lines and primary tumor cells (Kruschinski et al., 2008). In the case of the HER-2 specific CAR, CAR signaling was apparently able to override the inhibitory signals provided by the corresponding ligands on the target cells. Hepatoma cell lines, though, might differ in the pattern and amount of inhibitory ligands expressed, thereby making it difficult to compare different target cell lines. Besides the target cell line, the CAR-construct itself also determines the strength and quality of the interaction

with the target cell (Chmielewski et al., 2004). The affinity of the HER-2 CAR binding domain with a K_D (dissociation constant) of 10^{-8} M (Kruschinski et al., 2008) is much higher than the binding affinities between known inhibitory NK receptors and their ligands (Vales-Gomez et al., 2000), which is probably contributing to NK cell activation after CAR ligation. The affinity of the S-CAR is not certainly known so far. There have been attempts to determine the affinity of the C8 scFv using surface plasmon resonance (diploma thesis Eva Gückel). Calculation with a special software gave a K_D of $6.56x10^{-5}$ M, which would suggest an affinity of around factor 6000 lower than for the HER-2 CAR and could explain the different killing abilities of grafted NK cells. However, the K_D calculation might not be trustable, since problems had occurred during the measurement (diploma thesis Eva Gückel). The correct determination of the S-CAR affinity will be subject of future experiments.

Compared to various studies involving the adoptive transfer of genetically engineered T cells, NK cell engineering is not as common. In the tumor setting, NK cells are often unresponsive to malignant cells, providing the rational for NK cell redirection. Besides the HER-2 approach, Imai and colleagues described the successful engraftment of primary NK cells with a CD19 specific CAR, allowing for specific killing of leukemic cells *in vitro* (Imai et al., 2005). Other studies aimed at the augmentation of antitumor effects induced by NK cells via blockade of inhibitory receptors (Koh et al., 2001) or expression of activating ligands (Cerwenka et al., 2001). In the setting of chronic HBV infection, however, transfer of S-CAR NK cells might not be beneficial, given the risk of unspecific hepatocyte killing that might aggravate and potentiate pre-existing inflammation. Of note, also HER-2-CAR NK cells induced profound IFNγ secretion upon co-culture with HER-2 negative cells; and degranulation of mock NK cells and lysis of some breast cancer cell lines occurred independent of HER-2 (Kruschinski et al., 2008). Although the specific effects of HER2 NK cells were much stronger, the unspecific effects suggest that clinical application of modified NK cells might cause severe side effects *in vivo*. According to those results, this study further concentrated on the employment of T cells for adoptive therapy involving the S-CAR.

3.3 Long-term fate and phenotypic characterization of S-CAR T cells *in vivo*

3.3.1 S-CAR T cells can survive over long term *in vivo*

The adoptive transfer experiments employing S-CAR T cells in HBVtg mice had so far been conducted with d12 post transfer as the endpoint of analysis. It could be shown that at this time-point, S-CAR T cells pretreated with IL-12 had efficiently homed to the liver, correlating with profound antiviral effects as seen by decrease of viremia as well as of viral DNA in the liver (Krebs, Böttinger et al., 2013). The optimal situation for the treatment of a patient

chronically infected with HBV would be the rapid eradication of all HBV-replicating cells upon a single transfer of S-CAR T cells. However, if this would not be the case, the establishment of a long-lived S-CAR T cell population could eventually control residual or recurrent viral replication.

In the tumor setting, *in vivo* persistence of transferred T cells strongly correlates with the outcome of the disease, as demonstrated inclinical trials for treatment of neuroblastoma with a GD2-specific CAR (Louis 2011), for treatment of CLL with a CD19 specific CAR (Kalos et al., 2011), and for treatment of melanoma using tumor infiltrating lymphocytes (Rosenberg et al., 2011). In both of the latter trials, lymphodepletion was performed prior to adoptive transfer, which was shown to facilitate T cell engraftment and survival (Berger et al., 2009). Suppression of the endogenous immune response creates space in the lymphoid compartment and reduces the competition for homeostatic cytokines like IL-7 and IL-15 (Gattinoni et al., 2005); thereby rendering adoptively transferred T cells more efficient.

T cell depletion or immunosuppression, however, is obsolete in chronic HBV infection, since several studies have reported a relapse of HBV reactivation upon lymphodepletion (Lok et al., 1991; Yeo et al., 2000; Nakamura et al., 1996; Cheng et al., 2004). This enforces the need of stable S-CAR T cell expansion and functionality in an immunocompetent host. To check for the longevity of S-CAR T cells *in vivo* in HBVtg mice, which feature a fully functional immune system, adoptively transferred S-CAR T cells were monitored over long term. Although peripheral frequencies were constantly decreasing, transferred T cells could be detected up to 13 weeks post transfer (Figure 2.4.2 C). The high standard deviations were due to high inter-individual differences between the recipient mice (Figure 2.4.2 B). Interestingly, also transferred T cells grafted with a CEA-CAR as control receptor survived for up to 9 weeks; indicating that also antigen-independent effects can cause prolonged survival *in vivo*. Milone and colleagues have described a strong antigen-independent expansion and persistence of cells grafted with a 3rd generation CAR *in vivo*, which was explained by the cytoplasmic co-stimulatory domain that might induce spontaneous aggregation into oligomers (Milone et al., 2009). In addition to CD28, the cytoplasmic part included a domain derived from the TNF receptor family member CD137 (4-1BB). Inclusion of 4-1BB has also been shown to augment CAR-mediated T cell responses, although different studies comparing CD28 and 4-1BB have given conflictive results (Curran et al., 2012). With regard to the S-CAR, constructs employing the 4-1BB costimulatory domain are currently compared to the original CD28-construct *in vitro* in our laboratory.

In a different experiment of the present study, S-CAR T cells were monitored up to 5 weeks post transfer. Compared to the experiment described above, peripheral frequencies decreased faster, with frequencies below 1% of total lymphocytes at d34 (Figure 2.4.3 B). As shown by analysis of different body compartments, clearance of S-CAR T cells from the

peripheral blood was also accompanied by clearance from lymph nodes, spleen as well as from the liver, where they had initially homed to (Figure 2.4.3 C). Although both experiments described were conducted in the same way, the T cell populations were differing in vitality and proliferation capacity, leading to different transduction efficiencies. Whereas the transduction efficiency in the first experiment was rather low (around 27%; Figure 2.4.2 A) and therefore required the transfer of $8.6x10^6$ cells in total, the efficient transduction (around 70%) in the second experiment allowed for the transfer of only $5.71x10^6$ total CD45.1$^+$ cells (Figure 2.4.3 A). Since detection in the periphery was done by CD45.1 staining, overall frequencies of transferred T cells in the first experiment were, as expected, much higher. However, since comparable amounts of S-CAR T cells had been transferred, this population must have been decreasing faster in the second experiment, leading to the assumption that the quality of the transferred T cell population influences *in vivo* survival. A suboptimal activation and therefore transduction rate might lead to rather unresponsive cells that can survive longer *in vivo,* whereas proper activation before transfer is likely to lead to faster exertion of effector functions and extinction of the cells. In line with that, ALT levels indicating specific liver damage were profoundly elevated in the second (see Figure 2.6.1), but not the first experiment (data not shown). Clearly, the conditions during transduction should aim for optimal transduction efficiency to maximize *in vivo* effector functions. Although frequencies of transferred T cells around 5 weeks post transfer were found to be below 1%, this could still be sufficient to establish long-term protection, similar to the low-level persistence and anti-tumor response described for GD2-specific CAR T cells (Louis et al., 2011).

3.3.2 Antiviral effects *in vivo* are timely limited, although S-CAR T cells retain their effector potential

Although transferred T cells were, with variable frequencies, detectable over long term, the remaining cells were apparently not able to induce antiviral effects at late time-points: Quantification of viral DNA in the liver revealed that the initial reduction 1 week post transfer was followed by increased levels 4 and 9 weeks post transfer that were comparable to mice treated with a control CEA-CAR T cell population (Figure 2.4.4 A). To monitor effects at early time points more closely, mice were sacrificed at d4, 8, 12 and 20 post transfer in a separate experiment. Quantitative analysis of viral DNA in liver (Figure 2.4.4 B) and serum (Figure 2.4.4 C) revealed a time course of antiviral effects, with a maximum decrease of liver and serum HBV DNA 8-14 days post transfer and an increase at d20. Because HBV replication in transgenic mice is driven by the transgene, recurrent virus expression is inevitable.

With regard to a clinical application, however, the maintenance of effector functions *in vivo* would be desirable. For hematological malignancies, a prolonged anti-tumor effect of CAR T

cells has been demonstrated (Brentjens et al., 2011, Kalos et al., 2011). Solid tumors, though, are considered to be much harder to treat, with a hostile tumor environment that might inactivate transferred T cells (Curran 2012). Since also the liver is known for its tolerogenic environment (Protzer et al., 2012), similar problems might occur for treatment of chronic hepatitis B or hepatocellular carcinoma by adoptive T cell therapy.

Some groups have demonstrated that human CAR T cells can retain their effector cell potential upon *ex vivo* stimulation: when re-isolated cells were co-incubated with the respective antigen, they responded with proliferation and degranulation (Brentjens et al., 2011, Kalos et al., 2011). In our experiments, S-CAR T cells could be reactivated *ex vivo* in a similar way: splenocytes as well as liver associated lymphocytes (LAL) were re-isolated at day 8 and day 64 post transfer and subsequently stimulated with HBsAg. S-CAR T cells isolated at d8 from both liver and spleen specifically produced IFNγ upon antigen stimulation (Figure 2.4.6 A), with a frequency of 13% for LAL and 21% for splenocytes. An additional frequency of 5% was found to produce TNFα as a second effector cytokine. Day 8 post transfer correlated with the maximal antiviral effect; therefore S-CAR T cells were expected to exert effector functions. Interestingly, though, also transferred T cells re-isolated at d64 post transfer were shown to specifically react to *ex vivo* antigen stimulation (Figure 2.4.6 B). The overall frequencies of cytokine-secreting S-CAR T cells were lower than on day 8 and cells were mostly monofunctional with only IFNγ secreted. Nevertheless, those results suggest that S-CAR T cells do not attain a completely unresponsive state *in vivo*, but partially retain their effector potential in the tolerogenic intrahepatic environment even at late time points. The question why antiviral effects *in vivo* were nevertheless limited will be discussed in the next parts.

Importantly, S-CAR T cells could be stimulated with the ayw serotype of HBsAg (genotype D), which is expressed in the HBVtg mice (Guidotti et al., 1995), but reacted even stronger to the adw serotype. This can be explained by the fact that the C8 scFv was isolated in a phage display using recombinant HBsAg (adw). The fact that different serotypes can be recognized represents an important advantage for the usage of the S-CAR in the clinical setting.

3.3.3 Exhaustion does not play a major role for S-CAR T cell dysfunction *in vivo*

Even though effector functions could be partly rescued *ex vivo*, the question remained whether *in vivo* antiviral effects were timely limited, with transferred T cells still being detectable at the same time. A likely explanation for the loss of effector functions *in vivo* is the acquisition of an exhausted state, which is known to occur for endogenous, virus-specific T cells during chronic infection in the LCMV mouse model (Zajac et al., 1998), but also

during chronic infection with human pathogenic viruses including HBV (Shin et al., 2007, Blackburn et al., 2009).

Cytotoxic T cell exhaustion is defined as a state of dysfunction that is characterized by the progressive loss of key components of effector functions (Hofmeyer et al., 2011), namely the inability to produce cytokines, lyse target cells and proliferate (Wherry et al., 2004). Several markers have been shown to be up-regulated on exhausted T cells, with Programmed Death-1 (PD-1) as the probably most investigated one. PD-1 belongs to the CD28 superfamily (Sharpe et al., 2007) and was first discovered on a T cell hybridoma cell line undergoing activation-induced programmed cell death (Ishida et al., 1992). Expression was subsequently found to be more likely due to T cell activation, and is now well described for activated B cells, monocytes and T cells (Agata et al., 1996; Vibhakar et al., 1997). However, PD-1 and its ligands have also important functions for T cell exhaustion during chronic viral infection. Binding of PD-1 to its ligands transmits a negative regulatory signal to the T cells, which reduces effector functions (Keir et al., 2008). PD-1 has been shown to be highly expressed by exhausted T cells from chronically LCMV infected mice, but not by functional LCMV-specific CD8 cells from mice that had cleared the infection (Barber et al., 2006). Subsequently, this phenotype was also found on virus-specific CD8 T cells in chronic human viral infections, namely HIV (Petrovas et al., 2006), HCV (Urbani et al., 2006) and HBV (Boni et al., 2007).

Since the acquisition of an exhausted state is thought to be mediated by continuous antigen stimulation (Hofmeyer et al., 2011), this might also occur for transferred T cells that are exposed to high amounts of antigen *in vivo*. CD19 CAR T cells have indeed been shown to be high in PD-1 expression post transfer in patients (Kalos et al., 2011). To check whether S-CAR T cells might exhaust in the HBV-positive environment, re-isolated cells from spleen and liver were stained for PD-1 directly after re-isolation and in addition to the intracellular cytokine staining. Importantly, S-CAR T cells were high at PD-1 expression already before transfer (Figure 2.4.7 B), and higher than non-transduced (S-CAR negative) cells, indicating that the stimulation protocol induces an activated state that might also render the cells more prone to subsequent exhaustion. *In vitro* stimulation of the freshly transduced T cells revealed that around 40% were able to secrete IFNγ at this time point, representing a rather activated than exhausted state (Figure 2.4.7 B). Re-isolated S-CAR T cells, but not S-CAR negative transferred T cells, also expressed high levels of PD-1 when stained directly *ex vivo*, as seen up to 13 weeks post transfer (Figure 2.4.7 C). This indicates that the maintenance of PD-1 surface expression *in vivo* is dependent on stimulation with the cognate antigen. Importantly, overnight re-stimulation led to reactivation of up to 50% of PD-1 positive cells, which suggests that long term *in vivo* stimulation does not generally induce S-CAR T cell exhaustion. However, since re-isolated S-CAR T cells partly secreted IFNγ as

well as TNFα at d8 post transfer, but were found to be monofunctional at d64 post transfer (Figure 2.4.6), a loss of function is evident and partial exhaustion cannot be excluded. That is to say, the progress of exhaustion is described as the hierarchical loss of effector functions, with an early loss of proliferative potential and IL-2 production that is followed by the inability to produce TNFα, and the loss of IFNγ production only at extreme stages of exhaustion (Shin 2007). IFNγ, though, has been demonstrated to be one of the most important cytokines for counteracting HBV replication (Cavanaugh et al., 1997; Guidotti et al., 1999; Yang et al., 2010); therefore PD-1$^+$IFNγ$^+$ T cells should still be sufficiently functional to exert antiviral effects.

Further information with regard to specific exhaustion was obtained by transferring S-CAR T cells into C57 BL/6 mice, representing an HBV negative background. Strikingly, re-isolated S-CAR T cells from BL/6 mice were rather low at PD-1 expression compared to cells derived from HBVtg mice and to the phenotype before transfer, confirming an antigen-specific impact on PD-1 expression *in vivo* (Figure 2.4.8 A+B). When BL/6 derived S-CAR T cells were restimulated *ex vivo*, PD-1 was again upregulated, and activated PD-1$^+$ cells secreted around 10% more IFNγ compared to HBVtg-derived PD-1$^+$cells. Those results point towards a fraction of “truly” exhausted S-CAR T cells *in vivo* in HBVtg mice, although a big proportion of cells can potentially be reactivated also at late time points.

Exhausted T cells are not only characterized by PD-1, but by the co-expression of several inhibitory receptors (Wherry et al., 2007). The severity of chronic infection has been associated with the pattern and intensity of inhibitory receptors expressed (Blackburn et al., 2009). T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3), which was originally described as being expressed on T helper 1 cells (Monney et al., 2002), is another inhibitory receptor of special interest in chronic infections: PD-1 and Tim-3 co-expression has been associated with a more severely exhausted CTL phenotype in mice with chronic LCMV (Jin 2010) or Friend leukemia virus infection (Takamura et al., 2010) as well as for HCV-specific T cells in patients (McMahan et al., 2010). Also in the case of chronic HBV infection, Tim-3 has been described as being expressed by exhausted T cells isolated from patients (Raziourrouh et al., 2010; Wu et al., 2011; Nebbia et al., 2012).

Co-staining of transferred S-CAR T cells for Tim-3 as a second exhaustion marker revealed a small population of around 1% probably fully exhausted cells, being positive for PD-1 and Tim-3, as well as IFNγ-negative upon re-stimulation. This was found for LALs, with an even lower frequency of 0.3% for splenocytes, indicating that exhaustion takes place specifically in the liver, but only in a small fraction of T cells. Those data are in line with the previous findings, suggesting the exhaustion of an only minor part of the S-CAR T cell population. They are not shown in this study since they should be validated in further experiments.

Besides PD-1 and Tim-3, LAG-3 and CTLA-4 would also be candidate inhibitory receptors that could be tested in addition (Wherry et al., 2011).

Although a profound frequency of S-CAR T cells remained functional for IFNγ secretion, the treatment of S-CAR-treated HBVtg mice with an antibody blocking PD-1/PD-L1 interaction could provide an insight into possible negative regulatory functions of PD-1 on S-CAR T cells *in vivo.* Blockade of PD-1/PD-L1 interaction has been shown to partially restore virus-specific responses in the chronic LCMV model (Barber et al., 2006) and also for HCV (McMahan et al., 2010) and HBV specific T cells derived from chronically infected individuals (Boni et al., 2007; Fisicaro et al., 2010).

Besides the suppression of effector functions via ligation of inhibitory receptors, immunosuppressive cytokines like IL-10 can also induce T cell anergy (Li et al., 2010). In several models of immune-mediated liver damage, IL-10 was shown to restrict liver damage (Swain et al., 1999; von Freyend et al., 2011). Importantly, IL-10 expression was significantly up-regulated at d12 post transfer in the livers of S-CAR T cell treated HBVtg mice (Krebs, Böttinger et al., 2013). The cellular source is not known so far, but could be liver-resident regulatory T cells (Mosser et al., 2008), Kupffer cells (Knolle et al., 1997) or even transferred, IL-12 primed T cells themselves (Lee et al., 2007). The antibody-mediated blockade of IL-10 (Ha et al., 2008) could further help to dissect different mechanisms leading to the suppression of S-CAR T cell effector functions *in vivo*.

3.3.4 S-CAR surface expression in unstable *in vitro* and *in vivo*

Since the apparent loss of effector functions of remaining S-CAR T cells *in vivo* could not be explained by a general exhausted phenotype, S-CAR surface expression, which is crucial for effector cell functions, was analyzed in detail. Importantly, surface-located S-CAR was clearly detectable prior to transfer, as seen by FACS analysis of transduced CD8 T cells (Figure 2.4.2 A, 2.4.3 A). However, S-CAR surface expression of re-isolated T cells was rapidly lost over time: whereas transferred T cells stained positive for the S-CAR at day 4 post transfer, surface staining was low or undetectable at later time points (Figure 2.4.9 A). Interestingly, overnight resting of transferred T cells could partially restore S-CAR surface expression (Figure 2.4.9 B), suggesting that the receptor was still present, but might have been internalized. The fact that S-CAR rescue to the surface was largely diminished when cells were stimulated with rHBsAg (Figure 2.4.9 B) indicated an impact of antigen-stimulation on S-CAR surface expression.

To further investigate this, S-CAR surface expression was analyzed in an *in vitro* setup: T cells were transduced with a construct allowing for parallel expression of the S-CAR with a truncated EGF-receptor (tEGFR) as a control (Figure 2.4.10 A). When S-CAR/tEGFR cells

were subjected to HBsAg stimulation, S-CAR surface expression decreased in a time dependent manner, whereas tEGFR surface expression stayed stable. In the absence of antigen, both receptors were expressed at stable levels for up to 24 hours of incubation time (Figure 2.4.10 B + C). The impact of HBsAg on S-CAR surface expression was further confirmed by stimulation of S-CAR T cells with different antigen concentrations, leading to an antigen-dependent decrease of receptor surface expression, which was correlating with the amount of secreted IFNγ (Figure 2.4.10 D).

Kalos et al. have described similar effects with re-isolated T cells carrying a CD19 CAR: surface-located CAR was internalized within two hours of re-stimulation with CD19-expressing cells; however surface expression *in vivo* seemed to be stable (Kalos et al., 2011). Apart from this study, though, not much information is available dealing with CAR internalization. With regard to natural T cell receptors, however, dynamic surface expression is a commonly described phenomenon: A cycling pool of TCRs is constitutively internalized and re-expressed (Minami et al., 1987; Liu et al., 2000). Besides, TCR engagement induces down-regulation of surface TCR levels, activation of protein kinases, and targeting of activated receptors to lysosomes (Valitutti et al., 1997). Since CAR molecules contain an intracellular signaling domain derived from the CD3ζ chain, chimeric antigen receptors might exhibit similar kinetics of down regulation upon activation. However, it would have to be shown if the CD3ζ part of a CAR underlies the same regulatory mechanisms as the CD3ζ chain of a TCR, which seems to be degraded in lysosomes rapidly upon internalization (Valitutti et al., 1997). Furthermore, D'Oro and colleagues have demonstrated that the CD3ζ part of the TCR is rather important for the stabilization of surface-located receptor complexes (D'Oro et al., 2002), preventing rapid internalization.

When cells expressing both S-CAR and tEGFR were adoptively transferred into HBVtg mice, kinetics of receptor surface expression on peripheral blood cells showed that S-CAR expression decreased much faster than tEGFR expression over time, which was also in line with the changes in mean fluorescence intensity of both receptors (Figure 2.4.11 B). Cells re-isolated from spleen, lymph nodes and liver confirmed this phenotype, and further supported the theory of S-CAR down-regulation *in vivo* (Figure 2.4.11 C). However, also on S-CAR/tEGFR positive cells transferred into HBV negative BL/6 mice, frequencies of surface-located S-CAR decreased more rapidly than for the control receptor tEGFR: Whereas at day 4 post transfer, S-CAR down-regulation only occurred on cells in HBVtg recipient mice (Figure 2.4.12 A), both receptors were expressed at comparable levels at day 8 post transfer (Figure 2.4.12 B) in HBV+ and HBV- recipient mice. Together with the presented *in vitro* results, this indicates that there is indeed an antigen-specific impact on S-

CAR surface expression *in vivo*, which is probably rapidly overridden by other, antigen-independent mechanisms that result from a low stability of the S-CAR.

Of note, also human T cells seem to lose S-CAR surface expression in an antigen-independent way over time when cultured *in vitro* (personal communication Antje Malo), which suggests that the molecule itself might be rather unstable. If this effect is due to lysosomal degradation, blocking of the lysosomal pathway with specific agents (Valitutti 1997) would give more information about the mechanism(s) involved. In addition, a metabolic labeling (Beynon et al., 2005) of S-CAR molecules could give information about protein stability, which might then be improved by the mutation of single amino acids of the intracellular domain. Interestingly, reduced S-CAR surface expression on human T cells could be avoided by stimulation with IL-7 and IL-15 (personal communication Antje Malo), which would in turn strengthen the importance of appropriate culture conditions for S-CAR T cell generation. Furthermore, labeling of the S-CAR with a fluorescent dye could allow for direct analysis of the S-CAR fate upon stimulation via live cell imaging; and also for the performance of FACS-based internalization assays (D'Oro et al., 2002).

3.3.5 Transgene methylation is unlikely to occur in S-CAR T cells

The progressive loss of S-CAR expression, but also less pronounced of tEGFR expression over time could hint to the more rapid loss of transduced T cells compared to non-transduced transferred cells, but might also be result of transgene methylation. Methylation represents an important mechanism of gene regulation in mammalian cells (Momparler et al., 2000), and also plays a major role for transcriptional silencing of retroviruses like murine oncoretroviruses, and retroviral-derived vectors (Challita et al., 1994; Lorincz et al., 2001). The long-terminal repeat, which drives transgene expression, typically contains several CpG dinucleotides that are the target of DNA methyltransferases, with a profound methylation leading to a shut-off of transgene expression (Hoeben et al., 1991; Swindle et al., 2004). The retroviral vector used for transgene expression in this study was derived from the murine stem cell virus (MSCV), which has been shown to lack many repressive cis-acting DNA elements that could induce methylation; however it is not completely protected from this mechanism (Swindle et al., 2004). Swindle and colleagues have described two major clusters of six and seven CpG dinucleotides located within proximity of each other in the MSCV long terminal repeat, and demonstrated that methylation of CpGs in both clusters led to complete silencing of proviral genomes (Swindle et al., 2004).

To test whether there might be such an effect on S-CAR expression, a PCR for specific detection of methylated CpGs, covering the crucial sequences of the LTR, was established (Figure 2.4.12, 2.4.14). When DNA obtained from the livers of HBVtg and BL/6 recipient

mice at different time points was analyzed with this method, a maximum amount of 2 methylated CpGs could be detected among the tested clones (Figure 2.4.15). Transgene silencing, however, would require a much more profound methylation of the promoter (Swindle et al., 2004), therefore suggesting that this mechanism is unlikely to occur in S-CAR T cells.

3.3.6 S-CAR T cells change their memory phenotype *in vivo* over time

As discussed earlier, long term *in vivo* persistence and functionality of transferred T cells can vary with the stimulation protocol applied prior to transfer and with the composition of the CAR-intrinsic signaling domains (Sadelain et al., 2009; Berger et al., 2009). Furthermore, longevity and efficiency of transferred T cells have been shown to be influenced by the memory phenotype of transferred T cells, which will be discussed below.

A typical T cell response to an acute pathogen usually starts with priming and activation of naïve T cells by APCs in secondary lymphoid organs, which leads to vigorous proliferation and expansion and thereby the formation of a large population of effector T cells (T_E) that exert their effector functions at the site of antigen expression. This is followed by a contraction phase, during which most of the effector cells undergo activation-induced cell death. However, around 5-10% of T cells survive as long-lived memory cells, which seems to be programmed already during the initial window of activation (Masopust et al., 2004; Sallusto et al., 2004; Prlic et al., 2005). Compared to effector cells derived from naïve T cells, immune responses elicited by memory T cells are qualitatively and quantitatively distinct, with an improved magnitude, a faster response and an improved sensitivity to low doses of antigen (Williams et al., 2007; Jameson et al., 2009). The T cell memory pool contains diverse populations that are phenotypically characterized by the expression of certain memory markers. There can be two broad categories distinguished, that show distinct homing potentials and effector functions: effector memory (T_E) and central memory T cells (T_{EM}), which were initially proposed to be discriminable by the absence (T_{EM}) or presence (T_{CM}) of the lymph node homing receptors CD62L (L-Selectin) and CCR7 (Sallusto et al., 1999). Of note, marker expression is much more diverse, which makes the classification of sharply distinct subsets hardly feasible (Jameson et al., 2009). T_{EM} cells, which are mostly localized in peripheral tissues, are generally described to have a more limited life span and a weaker proliferation potential compared to T_{CM}, which are prevalent in lymph nodes and are able to replenish the other memory populations (Bachmann et al., 2005; Jameson et al., 2009). In line with that, several studies have shown that transferred T cells derived from the T_{CM} subset have superior functions with regard to longevity and effector functions *in vivo*; in preclinical models (Klebanoff et al., 2005; Berger et al., 2008) as

well as clinical trials (Louis et al., 2011). In addition, Kalos and colleagues have shown that long-term persisting transferred CD19 CAR T cells had acquired a central memory phenotype *in vivo* over time (Kalos et al., 2011).

In the present study, the memory phenotype of S-CAR T cells and its changes *in vivo* were determined by co-staining for the markers CD62L (L-Selectin) and CD127 (IL-7R), as proposed (Bachmann 2005) to be adequate markers for discrimination of memory cell subsets (Figure 2.4.16). Untreated $CD8^+$ splenocytes stained directly after isolation from the donor mice clearly distributed into 4 different populations (Figure 2.4.16), with double-positive cells as the most abundant one. Cells expressing both markers are described to be either naïve or central memory T cells (Bachmann et al., 2005), with the first one probably representing the highest frequency in this case. Strikingly, the procedure of activation, expansion and transduction led to the expression of a highly synchronized phenotype of S-CAR T cells with both markers strongly expressed, which hampered the characterization of distinct populations. Importantly, transferred T cells changed their phenotype over time *in vivo* (Figure 2.4.17, Tables 2.4.2 and 2.4.3). Cells that were re-isolated from blood, liver, spleen and lymph nodes could be clearly differentiated between different memory T cell populations, with varying frequencies depending on the body compartment. Interestingly, more than 70% of transferred T cells that were found in the liver at day 12 had lost CD62L expression, showing an effector memory cell like phenotype comparable to endogenous $CD8^+$ liver associated lymphocytes. This held also true for LALs isolated at d34 post transfer, which was consistent with a prolonged and robust exposure to antigen (Kalos 2011). Among transferred T cells found in the blood, the relatively high frequency of 32% effector T cells on day 12 was reduced to 11% on day 34, which seems to reflect the timely limited antiviral effects detected. Of note, also the endogenous CD8 T cell population featured a higher frequency of effector T cells at d12 post transfer compared to d34, suggesting that also endogenous T cells might have been activated and exerted bystander effector functions. Like for LALs, the effector memory cell like population was the most abundant one among transferred blood cells and splenocytes at the late time points.

Importantly, memory formation during chronic infection is different than for acute, resolving infection: the level of antigen persistence strongly influences the relative composition of memory T cell subsets, and continuous antigen stimulation has been shown to suppress the development of long-lived memory populations in the chronic LCMV model (Bachmann et al., 2005). HBV replication in the HBVtg mice is continuously driven by the transgene, thereby rendering the model more similar to a chronic infection, during which a persistent antigen stimulus is present. The high profile of memory cell like populations that are considered to be rather short-lived could be the consequence of this continuous antigen stimulation, and would suggest that, under those conditions, the formation of a long-lived

memory population with the potential of *in vivo* reactivation is rather unlikely. Compared to the chronic infection setting, the phenotype of antigen-specific T cells after a resolved LCMV infection (Bachmann et al., 2005) and HBV infection (Boettler et al., 2006) is marked by co-expression of CD62L and CD127 and thereby representing central memory T cells.

Interestingly, only transferred T cells isolated from lymph nodes were still highly expressing both markers on d12 and, even more sustained, on day 34 post transfer. It would therefore be interesting to investigate whether a long-lived, true memory population had been established and homed to the lymph nodes. A possible experiment to check for the characteristics of remaining S-CAR T cells in the different body compartments would be the specific sorting according to memory marker expression and subsequent transfer in a new group of HBVtg recipient mice, which could then be checked for effects on viral replication, persistence and phenotype of transferred T cells. To evaluate the fate of naïve or memory S-CAR T cells *in vivo*, an S-CAR transgenic mouse line would have to be generated, which could serve as donor for adoptive transfer experiments. A similar approach was used amongst others by Isogawa and colleagues, who have generated a TCR-transgenic mouse line that allowed for the analysis of intrahepatic priming of naïve T cells (Isogawa et al., 2013). With regard to an application in the clinics, however, a comparable procedure would not be possible due to the essential engraftment of the patient´s T cells. Therefore, the protocol of activation and transduction should be designed in a way to allow for efficient T cell repopulation *in vivo*. This could comprise the isolation of patients T cells with a central memory phenotype prior to transduction (Berger et al., 2008), thereby providing a cell population with greater intrinsic capacity to persist *in vivo*.

In addition, the cultivation conditions should be adapted to achieve optimal expansion of the desired T cell population. Antibody-mediated stimulation of CD28 has been described to preserve central memory T cells (Bondanza et al., 2006) and to induce telomerase activity (Weng et al., 1996). Adoptively transferred T cells with longer telomeres have been shown to correlate with enhanced anti-tumor efficacy in clinical trials for malignant melanoma (Shen et al., 2007, Rosenberg et al., 2011). In line with that, Schreiber and colleagues have demonstrated that the age of donor mice strongly impacts the anti-tumor response of adoptively transferred splenocytes upon vaccination (Schreiber et al., 2012). Recently, Cieri and colleagues have described a cultivation protocol for human T cells involving IL-7 and IL-15, which instructs the generation of stem-cell like cells from naïve precursors that show great potential for adoptive T cell therapy (Cieri et al., 2013). IL-7/IL-15 stimulation is currently tested for S-CAR engraftment of human T cells (Karin Krebs / Antje Malo). For the experiments presented here, S-CAR T cells were stimulated in the presence of IL-12. Since it was shown that IL-12 might be involved in the regulation of memory formation (Arens et

al., 2010), an unspecific effect of the pretreatment, which initially led to the described synchronized marker expression, on phenotypic changes can therefore not be excluded.
Besides those considerations, in contrast to the HBVtg mouse model, S-CAR T cells could indeed lead to complete elimination of the virus in a chronically infected patient, which would change the environmental circumstances for *in vivo* persisting cells. To test whether long-lived memory populations would rather be established after such a resolved infection, which can be presumed, the evaluation of S-CAR T cells in a model of natural chronic infection would be desirable. Chimpanzees chronically infected with HBV would represent the ideal model; however such experiments underlie several constraints due to the limited availability and ethical considerations (Dandri et al., 2013). Two HBV-infected chimpanzees have been treated with S-CAR T cells so far, but the complete analysis has not been done yet (cooperation with Barbara Rehermann, NIH). Alternatively, the infection of wildtype mice with low doses of Adeno-HBV represents an appropriate model for persistent, but “curable” HBV infection (Huang et al., 2012), and corresponding experiments of S-CAR T cell transfer will be done in the future.

3.3.7 Contraction of the S-CAR T cell population due to induction of apoptosis

As mentioned earlier, vigorous T cell expansion upon activation is usually followed by massive contraction of the T cell population by apoptosis (Williams et al., 2007). Since similar kinetics of T cell expansion and contraction could also be seen for S-CAR T cells *in vivo*, activation-induced cell death seemed to be a likely explanation for this phenomenon. To check whether S-CAR T cells undergo apoptosis upon antigen stimulation, cells were stained with AnnexinV, which allows for detection of early apoptotic cells. When this was done in the *in vitro* stimulation setting, exclusively S-CAR positive cells that were co-incubated with HBsAg showed a time-dependent increase of AnnexinV binding, whereas the level of unstimulated and S-CAR negative cells stayed stable (Figure 2.4.18 A).
Similar results were obtained *in vivo*, with transduced T cells being recognized via tEGFR staining: When cells were re-isolated from different body compartments at d8 post transfer, receptor positive cells were clearly stronger positive for AnnexinV than endogenous and especially receptor negative cells (Figure 2.4.18 B + C). Conclusively, S-CAR T cells are more prone to undergo apoptosis upon antigen stimulation *in vitro* and *in vivo*, which makes activation-induced cell death a likely reason for contraction of the transferred T cell population. The fact that the frequency of receptor positive apoptotic cells in the liver was lower than in blood, spleen and lymph nodes could hint to a more profound induction of apoptosis, thereby leading to a more rapid clearance by cell death in the liver as the site of virus replication. Transferred S-CAR T cells might therefore undergo similar kinetics like

endogenous, antigen-specific T cells. Of note, also S-CAR T cells transferred into BL/6 mice featured AnnexinV-positive cells at d20 post transfer (data not shown). Since apoptosis can also be induced as a consequence of lacking activating signals, different effects might play a role for the survival of S-CAR T cells *in vivo*, with activation-induced cell death representing one of them.

3.3.8 S-CAR T cells derived from HBVtg mice do not show impaired effector functions

As already described, the application of the S-CAR in a clinical setting would imply the engraftment and re-infusion of the patient´s own T cells.

Many studies have shown that T cell responses are weak in chronically infected patients, with the abundance of an exhausted phenotype of HBV-specific T cells (Schurich et al., 2011). In addition, chronic HBV infection might also affect the functionality of the global CD8 population (Das et al., 2008). Therefore, the question arose, whether this tolerance might negatively affect the functionality of transferred S-CAR T cells. For the adoptive transfer experiments described so far, however, HBV naïve mice had served as donors for the T cell transfer. To mimic the situation of future clinical treatment settings, S-CAR T cells derived from HBVtg mice, expressing CD45.1 in addition, were tested for effector functions *in vitro* and *in vivo*. Parallel expansion and transduction of CD8 T cells from either naïve (HBV-) or HBVtg mice (HBV+) achieved comparable transduction efficiencies (Figure 2.5.1). Subsequent *in vitro* stimulation with rHBsAg revealed that S-CAR T cells efficiently secreted IFNγ and released cytotoxic granules upon antigen contact, which was independent of the donor cell background (Figure 2.5.2). Most importantly, S-CAR T cells derived from HBVtg mice did not exhibit inferior persistence and effector functions *in vivo*: they were detectable up to 13 weeks post transfer (Figure 2.5.4 A), could be efficiently reactivated *ex vivo* (Figure 2.5.4 B) and showed an even more pronounced reduction of viremia compared to HBV naïve donor cells (Figure 2.5.4 C). Therefore it can be concluded that, at least in the HBVtg mouse model, the HBV positive donor cell background did not lead to suppressed effector functions *in vivo*.

The intrinsic tolerance of HBV-specific T cells in the HBVtg mice can certainly not be directly compared to the tolerant state of HBV-specific T cells in the chronically infected patient. T cells in the chronic human infection setting exhibit a state of peripheral tolerance, whereas the tolerance in HBVtg mice probably also has a central component and might therefore be harder to break. Since there was no detectable impact of possible tolerance on effector functions in HBVtg mice, those results suggest that adoptive therapy with autologous S-CAR T cells is unlikely to fail due to an HBV-associated impaired T cell function. In line with that goes a study performed by Gehring and colleagues: PBMCs derived from either healthy or

chronically HBV-infected patients were transduced with a T cell receptor directed against HBV core (C18-27) and compared for effector functions *in vitro*. Engraftment with the TCR led to the reconstitution of a multifunctional T cell response upon specific stimulation, without functional differences between TCR-transduced cells from healthy or HBV-positive donors (Gehring et al., 2011). Those results further imply that the tolerogenic mechanisms involved in global T cell exhaustion in chronic HBV patients do not influence *in vitro* engineered T cells.

3.4 S-CAR T cells do not induce severe side effects in HBVtg mice

Another aspect of great importance with regard to a clinical application of S-CAR T cells is the question of side effects that could be caused in the patient. One major risk of adoptive T cell therapy in the tumor setting is the so-called on-target off-tumor effect, which describes the unwanted targeting of healthy cells that also express the respective antigen (Sadelain et al., 2013). In clinical studies employing chimeric antigen receptors, severe adverse effects caused by this phenomenon have already been described: A patient that had received lymphodepleting therapy died after transfer of T cells modified to express a 3rd generation CAR directed against ERBB2 (Morgan et al., 2010). The incidence was correlated to activation of the CAR T cells by low levels of ERBB2 on lung epithelium, which led to massive overstimulation and a lethal cytokine storm. Furthermore, liver toxicity was noticed for patients with renal cell carcinoma treated with CAIX-specific CAR T cells. In this case, the antigen was present in low levels on bile duct epithelial cells (Lamers et al., 2006).

The aspect of the targeting of healthy cells is, probably, of lower importance in the setting of chronic hepatitis B treatment, since HBsAg is not a self-protein and should only be expressed by infected hepatocytes. Nevertheless, the massive amounts of soluble HBsAg present in the serum of chronically infected patients pose the risk of unwanted activation of S-CAR T cells outside of the liver. Although it has been demonstrated that binding of subviral particles does not induce unspecific killing of non-infected hepatyctes *in vitro*, it might lead to extrahepatic cytokine secretion (Bohne et al., 2008), with the risk of an *in vivo* cytokine storm.

Mice treated with S-CAR T cells were therefore carefully observed for the induction of adverse effects. The adoptive transfer of $4x10^6$ S-CAR T cells did not lead to changes in body weight, which would have otherwise hinted at a general illness of the recipient mice. The liver damage induced by S-CAR T cells was moderate and most importantly only transient; peaking at day 8 post transfer and thereby also timely correlating with the maximal antiviral affect (Figure 2.6.1 A). When the sera of treated mice were checked for several

cytokines related to ongoing inflammation, INFγ, TNFα, IL-6 and the chemokine MCP-1 were significantly elevated at d12 post transfer compared to control animals (Figure 2.6.1 C).
The elevation of proinflammatory cytokines can be regarded as surrogate marker for T cell function (Curran et al., 2012), and in a clinical trial employing CD19 S-CAR T cells, the transient increase of IL-6, IFNγ and MCP-1 in blood and bone marrow correlated with the peak of CAR T cell expansion and clinical symptoms (Kalos et al., 2011). Similarly, cytokine levels in HBVtg mice were elevated concomitantly with the peak of antiviral effects (Figure 2.7.2 C) and high amounts of transferred T cells in the liver (Figure 2.7.2 B). Importantly, the elevation of serum cytokines did not lead to any obvious signs of distress in the mice and was also transient, with levels comparable to control mice at d34 post transfer (Figure 2.6.1 C). Interestingly, also the immunosuppressive cytokine IL-10 was found to be transiently elevated in the serum, which could point towards a regulatory mechanism dampening S-CAR T cell responses. As discussed earlier, IL-10 can be involved in the prevention of excessive liver damage (Swain et al., 1999; von Freyend et al., 2011); and also virus-specific T cells themselves are theoretically able to secrete IL-10, generating an autocrine feedback loop that attenuates antiviral immunity (Abel et al., 2006, Chang et al., 2007). In the HBV transgenic mice, the cell type secreting IL-10 upon S-CAR T cell transfer still has to be determined.
In line with the changes of serum cytokines, also the compositions of endogenous immune cells changed transiently at d12 after S-CAR T cells had been transferred. In the liver, a relative decrease of CD4 and B cell frequencies was accompanied by an increase of myeloid immune cells, especially of inflammatory monocytes and dendritic cells (Figure 2.6.2). Whether those cells might have a regulatory impact on S-CAR T cell function, maybe via IL-10 secretion, might be clarified in future experiments. Importantly, all changes were only transient, and levels went back to normal at d34, when also most of the transferred T cells had been cleared from the circulation.
Those *in vivo* results are encouraging with regard to the safety of S-CAR T cell transfer in the clinics. Some aspects, though, can hardly be investigated in this preclinical model and should therefore be considered in advance. For instance, the amount of circulating HBsAg in patients might differ strongly from the situation in HBVtg mice: the levels in HBVtg mice are, with 1000-1200U/ml, similar to the amounts detected in the low replicative phase of chronic HBV infection (Jaroszewicz et al., 2010). However, patients might feature high levels of virus replication combined with pre-existing liver inflammation, which could over-activate S-CAR T cells and cause severe liver damage. To minimize this risk, patients should be treated with antiviral agents prior to T cell transfer to reduce the amount of virus-replicating hepatocytes and the grade of inflammation. Fortunately, HBV surface proteins are still expressed on the

surface of infected hepatocytes in case of antiviral treatment (Wursthorn et al., 2006), which is crucial for recognition by the S-CAR.

Should S-CAR T cells, despite all necessary precautions, expand in the patient in a non-controllable way, safeguard mechanisms would allow for the specific depletion of transferred T cells. Accordingly, the S-CAR construct was supplemented with a truncated version of the epidermal growth factor receptor (tEGFR; see also 2.4.5.2). If needed, S-CAR T cells could then theoretically be depleted with the clinically approved antibody cetuximab (Wang et al., 2011). As shown in 2.4.5.3, tEGFR expression seems to be more stable than S-CAR surface expression, which would ensure S-CAR T cell depletion also in the case of S-CAR down-regulation.

With regard to enhanced safety of CAR T cell transfer, one could also envision the engraftment of T cells by mRNA electroporation (Zhao et al., 2006, Birkholz et al., 2009). By this, the risk of insertional mutagenesis induced by retroviral transduction of T cells, which is an important point with regard to clinical application, would be circumvented. However, this method would only allow for transient CAR expression, which would presumably require multiple infusions to provide meaningful *in vivo* responses (Sadelain et al., 2013). Besides, the usage of so-called self-inactivating (SIN) retroviral vectors (Yi et al., 2005) or lentiviral vectors (Papayannakos et al., 2013) represent further strategies for gene therapy that are considered to be more safe than gamma-retroviral vectors.

Importantly, in contrast to hematopoietic progenitors (Kustikova et al., 2009; Rivière et al., 2012), T cells seem to be far less susceptible to retroviral vector–induced transformation (Recchia et al., 2006; Newrzela et al., 2008; Scholler et al., 2012), which legitimates the use of retroviral vectors for S-CAR T cell generation.

3.5 S-CAR T cell effector functions are superior to those of vaccine-induced HBV-specific T cells

Finally, with regard to finding the optimal therapeutic strategy for treatment of chronic hepatitis B infection, S-CAR T cells were directly compared with natural HBV-specific T cells in terms of effector functions in HBVtg mice. S-specific T cells were generated by vaccination of CD45.1 mice (Figure 2.7.1), and $1x10^6$ S-specific as well as 1 or $4x10^6$ S-CAR T cells were adoptively transferred into different groups of recipient mice. Analysis at d12 post transfer revealed that S-CAR T cells had effectively homed to the liver, whereas the majority of S-specific T cells was found in the lymph nodes (Figure 2.7.2 B). In line with that, hepatocyte killing was only detected for S-CAR, but not for S-specific T cells (Figure 2.7.2 A) with ALT elevation as surrogate marker. Furthermore, cytoplasmic HBcAg was most efficiently reduced by S-CAR T cells (Figure 2.7.2 E). Interestingly, however, reduction of

viremia as well as HBV DNA in the liver was comparable between all three treatment groups (Figures 2.7.2 C + D). This strongly indicates that non-cytopathic antiviral effects are of special importance in this setting, especially for the mode of action of natural HBV specific T cells. A major contribution of non-cytolytic down-regulation of viral replication was described in studies with experimentally infected chimpanzees (Guidotti et al., 1999) and also HBVtg mice (Guidotti et al., 1996). Nonetheless, the direct killing of hepatocytes might be necessary for complete virus elimination. The fact that clearance of HBV after bone marrow transplantation from anti-HBsAg positive donors was correlated with pronounced ALT elevation (Lau et al., 1997) supports this hypothesis. With regard to this point, S-CAR T cells seem to fulfill the optimal requirements for efficient targeting of HBV *in vivo*.

Although hepatocyte killing was only detected upon transfer of S-CAR T cells in the presented experiments, it has to be mentioned that also vaccine-induced HBV-specific T cells have been shown to be capable of killing target cells *in vivo*, although this has not been demonstrated in the liver (Clemens Jäger, unpublished data). Since the stimulation protocols applied on S-CAR T cells and vaccine-induced T cells in our experiments were not the same, conclusions of different effector functions should be drawn with caution. Interestingly, a recent *in vivo* study employing the adoptive transfer of T cells grafted with an HBV-specific TCR led to elevated ALT levels in the HLA-matched, HBVtg recipient mice at d12 post transfer (Krebs et al., in preparation). This indicates that the kinetics of intrahepatic T cell activation and the exertion of effector functions might differ between adoptively transferred S-CAR and TCR T cells, with the latter ones exhibiting a delayed peak of activation. The fact that, in our setting, the effector functions of S-CAR T cells were superior to those of vaccine-induced S-specific T cells provides a further rationale for the promotion of the S-CAR into the clinics, besides the already mentioned advantages of chimeric antigen receptors over natural T cell receptors.

3.6 Summary and final evaluation: Adoptive immunotherapy for treatment of chronic hepatitis B

The present study dealt with the evaluation of different strategies for treatment of chronic HBV infection using adoptive cell transfer, with the background of a strong need for new treatment approaches that could ensure virus elimination.

In the first part, chimeric antigen receptors derived from T cell receptor-like antibodies were constructed to combine the features of a chimeric T cell receptor with the antigen-binding properties of a natural T cell receptor. However, the newly cloned constructs had lost their

antigen specificity, which illustrated the vulnerability of antibodies to structural changes and led to the exclusion of TCR-like CARs from further evaluations.

The S-CAR represents a chimeric antigen receptor directed against the HBV surface proteins that had already been constructed and evaluated in our group. In the second part of this study, natural killer cells were tested for their ability to specifically eliminate HBV-replicating hepatocytes when equipped with the S-CAR. However, S-CAR grafted NK cells exhibited a strong unspecific killing and S-CAR expression did not lead to redirected lysis of HBV+ target cells, rendering NK cells inapplicable for this treatment approach.

The ability of S-CAR grafted human and murine T cells to exert specific antiviral effector functions provided the rational of the major part of this study, representing a thorough analysis of S-CAR T cells and their properties. It could be shown that S-CAR T cells can survive over long term *in vivo*, accompanied by the acquisition of a memory T cell like phenotype. S-CAR T cells do not generally exhaust upon continuous antigen stimulation and can be reactivated *ex vivo* even at late time points, despite the tolerogenic influence of the hepatic environment. Importantly, S-CAR T cells specifically eliminate HBV replicating hepatocytes, which is probably crucial for virus eradication. Hepatocyte lysis and reduction of viral replication by non-cytopathic pathways, yet, seem to be strictly temporary. Underlying reasons were found to be a contraction of the transferred T cell population due to activation-induced cell death, as well as loss of S-CAR surface expression on the remaining cells.

This silencing of transferred T cells *in vivo* might limit the efficiency of immunotherapy, but could on the other hand have beneficial effects for the patient due to limited pathogenesis and maintenance of liver integrity.

Taken together, and complementing previous work, adoptive therapy with S-CAR T cells was proven to be safe and efficient in our preclinical model, thereby fulfilling important criteria for the further development towards a clinical application.

4 Material and Methods

4.1 Material

4.1.1 Consumables

Product	Supplier
96well plates for qPCR (FrameStar)	4titude
Cell culture flasks, dishes, plates	TPP
Cell strainer 100µm	BD Biosciences
Cryo vials	Greiner Bio One
Cuvettes	Brand
ELISA 96well plates Nunc MaxiSorb	Thermo Scientific
FACS 96well V-bottom plates	Roth
FACS tubes Titertube Micro Test	Bio-Rad
Falcon tubes 15ml/50ml	BD Biosciences; Greiner Bio One
Filter tips	StarLab
Filters 0.45µm and 0.2µm	Sarstedt
Freezing device	Nalgene
MACS separation columns	Miltenyi Biotech
Needles	Braun
Non-tissue culture treated plates (24well)	Greiner Bio One
PCR tubes	Thermo Scientific
Pipette tips 10ul – 1ml	Biozym / Greiner Bio One / Gilson
Pipettes (disposable) 2, 5, 10, 25, 50ml	Greiner Bio One
PVDF membrane	GE Healthcare
Reaction tubes 1.5ml, 2ml	Greiner Bio One, Eppendorf
Reagent reservoirs	Corning
Reflotron ALT stripes	Roche Diagnostics
Syringes	Braun
Whatman filter paper	GE Healthcare

4.1.2 Chemicals / Reagents / Additives

Product	Supplier
Agarose	PeqLab
Ampicillin	Roth
Bovine serum albumin, BSA	Sigma
Brefeldin A 1mg/ml	Sigma
Bromphenol blue	Roth
Collagen R	Serva
Complete Protease Inhibitor	Roche
DMSO	Sigma
DNA ladder 1kb / 100bp	Eurogentec
dNTPs 25mM	Thermo Scientific
EMA	Life Technologies
FCS	Gibco
Ficoll LSM 1077	PAA
Glucose	Roth
Glutamin 200mM	Gibco
Glycerol	Roth
HBV peptides	JPT Peptide Technologies
Heparin-Natrium 25000	Ratiopharm
Hepes 1M	Gibco
Human serum	own production (AG Protzer)
IFNγ IMUKIN	Boehringer Ingelheim (via pharmacy)
IL-12	Provided by Edgar Schmitt, Mainz
IL-2 Proleukin	Novartis (via pharmacy)
IPTG (Isopropyl-β-D-thiogalactopyranosid)	Thermo Scientific
Ketamine	Sigma (via pharmacy)
Lipofectamin2000	Life Technologies
Milk powder	Roth
NaCl for injection	Braun
Non-essential amino acids 100x	Gibco
PageRuler Prestained protein ladder plus	Thermo Scientific
Paraformaldehyde (PFA) 4%	Medite
PBS 10x	Gibco
Pen/Strep 10^4 mg/ml	Gibco
Percoll	GE Healthcare

Polybrene	Millipore
Poly-L-lysine	Sigma
Propidiumiodide	Roth
Protaminsulfate	Sigma
Recombinant HBsAg (Genotype D, adw/ayw)	Rhein Biotech
Retronectin 1µg/µl	Takara
Rompun 2 %	Bayer
SDS	Roth
Sodium pyruvate 100mM	Gibco
MHC molecules	AG Busch, Microbiology
Tissue-Tek O.C.T.	Sakura
TRI-Reagent (Trizol)	Life Technologies
Tris	Roth
Trypan blue	Gibco
Versene EDTA	Gibco
Yeast extract	Roth
β-Mercaptoethanol 50mM	Gibco

4.1.3 Buffers and solutions

Buffer / Solution	Ingredients
TAC buffer	Solution A: NH_4Cl 0.16M Solution B: Tris pH 7.65 0.17M Before use, 9/10 of solution A were freshly mixed with 1/10 solution B
50x TAE buffer	Tris 2M Acetic acid 2M EDTA pH 8.0 50mM H_2O
AP buffer (bacterial lysis buffer)	TrisHCl 100mM Saccharose 500mM EDTA 2mM H_2O
4x SDS loading buffer (WB)	Glycerol 10ml 0,5M Tris pH 6.8 (HCl) 12ml 10% SDS 20ml Bromphenol blue 0.1g addition of fresh β-ME 100µl/ml; storage at 4°C

10x TBS-T buffer pH7.4 (WB)	for 1l: Tris 200mM NaCl 1,4M Tween 20 10ml ad H_2O, adjust pH with HCl
10x Running buffer (WB)	for 1l: Tris 250mM Glycin 2M SDS 1% w/v (10g) ad H_2O
Buffer A1 (WB)	for 1l: Tris 0,3M MetOH 200ml ad H_2O
Buffer A2 (WB)	for 1l: Tris 25 mM MetOH 200ml ad H_2O
Cathode buffer (WB)	for 1l: Tris 25 mM Aminocapron acid 40mM MetOH 200ml ad H_2O
Blocking buffer (WB)	TBS-T Milk powder 5%
Ketamine/Rompun anaesthetic	for 5ml: Ketamine 800µl Rompun 125µl NaCl 4075µl
FACS buffer	PBS BSA 0.1%
10x AnnexinV binding buffer	Hepes/NaOH (pH 7.4), 0.1M NaCl 1.4M $CaCl_2$ 25mM H_2O

4.1.4 Media

Medium	Ingredients
DMEM complete	DMEM 500ml FCS 10% Pen/Strep, 5000U/ml 1% L-Glutamine, 200mM 1% NEAA, 100x 1% Sodium pyruvate, 100mM 1%
RPMI complete	RPMI 1640 500ml FCS 10% Pen/Strep, 5000U/ml 1% L-Glutamine, 200mM 1% NEAA, 100x 1% Sodium pyruvate, 100mM 1%
DMEM transfection medium without antibiotics	DMEM 500ml FCS 10% L-Glutamine, 200mM 1% NEAA 100x 1% Sodium pyruvate, 100mM 1%
Wash medium	RPMI 1640 500ml Pen/Strep, 5000U/ml 1%
Freezing medium	FCS DMSO 10%
Differentiation medium for hepatoma cells	Williams-E 500ml FCS 5% Pen/Strep, 5000U/ml 1% NEAA, 100x 1% Sodium pyruvate, 100mM 1% DMSO 0.5%
Murine T cell medium (mTCM)	RPMI Dutch modified 500ml FCS 10% Pen/Strep, 5000U/ml 1% L-Glutamine, 200mM 1% NEAA, 100x 1% Sodium pyruvate, 100mM 1% β-ME 50µM
Human T cell medium (hTCM)	RPMI 1640 500 ml Human serum 10% Pen/Strep, 5000U/ml 1% L-Glutamine, 200mM 1% NEAA, 100x 1% Sodium pyruvate, 100mM 1% HEPES 1% Gentamicin 208µl
RPMI ALL (NK cell culture)	RPMI 1640 GlutaMAX 500ml FCS 10% β-ME 50µM Gentamicin 10µg/ml

LB-Amp	LB Medium Ampicillin 50µg/ml
SOC medium	for 500ml: Yeast extract 2.5g Trypton 10g NaCl 0.29g KCl 0.093g MgCl2x6H2O 1.02g MgSO4x7H2O 1.23g Glucose 1.802g ad H_2O
Basis media from Gibco:	
DMEM	
DMEM GlutaMAX	
RPMI 1640	
RPMI 1640Dutch modified	
AIM-V	
OptiMEM	

4.1.5 Antibodies

Product	Dilution	Supplier
Cell culture		
anti-murine CD3	Depending on protocol	Provided by E. Kremmer, Helmholtz Zentrum München
anti-murine CD28	Depending on protocol	Provided by E. Kremmer, Helmholtz Zentrum München
Flow cytometry		
Human antigens		
anti-hCD3 PE	1:200	Molecular Probes
anti-hCD8 Pacific Blue	1:50	Dako
anti-hCD56 PE	1:100	Immunotools
anti-hCD56 APC	1:100	Immunotools
anti-hIgG FITC	1:250	Sigma

anti-hIgG DyLight649	1:200	Abcam
anti-hIgG PE	1:200	eBiosciences
anti-HLA-A2 FITC	1:200	Labgen
C18A2 (hybridoma supernatant; 10µg/ml)	50µl	provided by E. Kremmer, Helmholtz Zentrum München
Env183A2 (hybridoma supernatant; 10µg/ml)	50µl	provided by E. Kremmer, Helmholtz Zentrum München
Murine antigens		
anti-mCD3 FITC	1:150	BD Biosciences
anti-mCD3 Pacific Blue	1:200	eBioscience
anti-mCD4 APC-Cy7	1:200	eBioscience
anti-mCD8 APC	1:200	Life Technologies
anti-mCD8 Pacific Blue	1:250	eBioscience
anti-mCD11b FITC	1:300	Life Technologies
anti-mCD11c PE-Cy7	1:150	BD Biosciences
anti-mCD19 PE	1:200	eBioscience
anti-mCD19 AlexaFluor594	1.300	BD Biosciences
anti-mCD45.1-FITC	1:250	eBioscience
anti-mCD45.1 APC-eFluor780	1:200	eBioscience
anti-mCD45.2 PE	1:200	eBioscience
anti-mCD62L PE-Cy7	1:200	eBioscience
anti-mCD107a (LAMP-1) Alexa-eFluor647	1:200	eBioscience
anti-mCD127 APC	1:100	eBioscience
anti-mF480 APC	1:150	eBioscience
anti-mLy6C PerCP-Cy5.5		BD Biosciences
anti-mLy6G APC-Cy7		BD Biosciences
anti-mLy6C/G PE	1:200	BD Biosciences
anti-mNK1.1 PerCP-Cy5.5	1:200	eBioscience
anti-mPD1 FITC	1:100	eBioscience
anti-mPD1 Pacific Blue	1:100	eBioscience
anti-mTim3 Alexa-Fluor647	1:100	Biolegend
anti-mIFNγ PE	1:200	eBioscience
anti-mIFNγ PE-Cy7	1:200	eBioscience
anti-mTNFα PE-Cy7	1:200	eBioscience
goat anti-mIgG Alexa647	1:100	Invitrogen

Other		
AnnexinV FITC	2.5µl/sample	Life Technologies
Streptavidin-PE	1:200	eBioscience
Streptactin-PE	1µl/sample	IBA
Immunohistochemistry		
rabbit-anti-HBcAg		Diagnostic Biosystems
Western Blot		
mouse-anti Histidine-Tag	1:10000	ABD Serotec
anti-mouse HRP	1:10000	Sigma

4.1.6 Cell lines and bacteria

Cell line	Description	Source
HEK 293T	Human embryonic kidney cells	AG Protzer
Platinum-E (Plat-E)	293T, stably transfected with MoMuLV *gag, pol* and *env* driven by the EF1α promoter	AG Protzer
HepG2	Human hepatoma cell line	AG Protzer
HepG2.2.15	Human HBV-replicating hepatoma cell line (obtained by stable transfection of HepG2; containing four copies of a 1.1-fold HBV genome; HBV serotype ayw, genotype D) (Sells 1987)	AG Protzer
HepG2-H1.3	Human HBV-replicating hepatoma cell line (obtained by stable transfection of HepG2; containing one copy of a 1.3-fold overlength HBV genome; genotype D)	AG Protzer
B3Z	**murine** (H2Kb) T cell hybridoma	AG Protzer
Jurkat	human T cell line	AG Krackhardt, MedIII, MRI München
T2	T-B-cell hybridoma, TAP-deficient	AG Protzer
C18A2.11	murine hybridoma producing C18A2 antibody	AG Bertoletti, Singapore
Env183A2.11	murine hybridoma producing C18A2 antibody	AG Bertoletti, Singapore

Bacteria	Description	Source
Stbl3	E. coli, chemical competent	Invitrogen
XL-1 Blue	E. coli, chemical competent	AG Protzer
BL21 (DE3)	E. coli, chemical competent	provided by AG Miethke (Microbiology)

4.1.7 Mouse lines

Mouse line	Description	Source
C57BL/6	WT BL/6 mice	Harlan Laboratories
C57BL/6 CD45.1	WT BL/6 mouse expressing the congenic marker CD45.1	AG Busch, Microbiology
HBV1.3 xfs	HBV transgenic mouse line; contains a frame shift in the X-protein	AG Protzer
HBV1.3xfs/CD45.1	HBV xfs transgenic mouse line expressing the congenic marker CD45.1	AG Protzer (obtained by cross-breeding; F1 generation used for experiments)

4.1.8 Enzymes

All restriction enzymes used were purchased from Fermentas and used with the respective buffers provided.

Product	Supplier
Collagenase	Worthington Biochemical Corporation
0.5% Trypsin/EDTA 10x	Gibco
Benzonase	Novagen
Proteinase K	Roth
RNase H	Macherey-Nagel
Taq polymerase + Buffer	Qiagen
T4 DNA Ligase	Thermo Scientific
EpiMark Hot Start Taq DNA Polymerase	New England Biolabs

4.1.9 Kits

Product	Supplier
CD8a (Ly2) MicroBeads, mouse	Miltenyi Biotec
CloneJET PCR Cloning Kit	Thermo Scientific
Cytofix-Cytoperm	BD Biosciences
Cytometric Bead Array Mouse Inflammation Kit	BD Biosciences
Dynabeads CD3	Life Technologies
ECL Western Blotting Detection	Pierce
ELISA MAX Standard Set Mouse-IFNγ	Biolegend
EpiMark Bisulfite Conversion Kit	New England Biolabs
GeneJet Gel Extraction Kit	Thermo Scientific
GeneJet Plasmid Miniprep Kit	Thermo Scientific
GenElute Endotoxin-free Plasmid Midiprep Kit	Sigma
High Pure PCR Purification Kit	Roche
High Pure Viral Nucleic Acid Kit	Roche
NucleoBond Xtra Maxi Kit	Macherey & Nagel
NucleoSpin Tissue Kit	Macherey & Nagel
Phusion Hot Start Flex Kit	New England Biolabs
Rapid Dephosphorylation and Ligation Kit	Roche
Superscript III Kit	Life Technologies
SYBR Green I Master Mix	Roche
XTT - Cell Proliferation Kit II	Roche

4.1.10 Technical equipment

Product	Supplier
ALT measurement, Reflotron® Reflovet Plus	Roche Diagnostics
Axsym	Abbott
Semi-Dry Blotting Chamber	Bio-Rad
Centrifuge 4K15	Sigma
ELISA-Reader infinite F200	Tecan
Flow cytometer FACSCantoII™	BD Biosciences
Fluorescence microscope CKX41	Olympus
Freezing device	Nalgene

Gel chambers Agarose gelelectrophoresis SDS-PAGE	 Peqlab Biometra
Heating block	Eppendorf
Incubator	Heraeus Holding GmbH
LightCycler® 480 II	Roche Diagnostics
MACS separator	Miltenyi
MoFlo cell sorting	Beckman Coulter
Nanophotometer OD_{600}	IMPLEN GmbH
Photo system for agarose gels & Western blots Fusion Fx7	Peqlab
Photometer NanoVue	GE Healthcare
Pipetboy	Brand
Pipettes	Eppendorf
Shaker and incubator for bacteria	INFORS AG Heraeus Holding GmbH
Sterile hood (cell culture)	Heraeus Holding GmbH
Table-top centrifuge 54173	Eppendorf
Thermocycler T300	Biometra

4.1.11 Plasmids

Plasmid name	Application	Source
ppExpr_C8	Expression of C8 scFv in bacteria	provided by Amgene (formerly Micromet)
ppExpr_C18A2	Expression of C18A2 scFv in bacteria	own construction
ppExpr_Env183A2	Expression of Env183A2 scFv in bacteria	own construction
pMSCV_C8	Retroviral expression of the S-CAR in NK cells	provided by Karin Krebs
pCL10A1	Encoding gag-pol-env for production of retroviral supernatants	AG Blankenstein, MDC Berlin
pMP71_hC8	Retroviral expression of the S-CAR in T cells	provided by Karin Krebs
pMP71_CEA	Retroviral expression of the CEA-CAR in T cells	provided by Karin Krebs
pALF10A1	Encoding GALV env for production of retroviral supernatants	AG Uckert, MDC Berlin
pcDNA3.1MLVgag/pol	Encoding MV gag-pol for production of retroviral supernatants	AG Uckert, MDC Berlin

pJET1.2/blunt cloning vector	cloning of PCR fragments for sequencing	Thermo Scientific

4.1.12 Primer

All primers were synthesized by Life Technologies.

Primer name	Sequence	Application
p-fwHBV	TACTAGGAGGCTGTAGGCATA	Amplification of HBV rcDNA (qPCR)
p-revHBV	GGAGACTCTAAGGCTTCCC	Amplification of HBV rcDNA (qPCR)
mNid2Fw	ATCCTACCGGGAAACAG	Amplification of Nid2 DNA (qPCR)
mNid2Rev	CCCAAGGTCTTCGTTGA	Amplification of Nid2 DNA (qPCR)
S-CAR LTR nested fwd 4	AGTAGAATATGGGTTAAATAGG	Amplification of bisulfite-converted DNA (1st round)
S-CAR LTR nested rev 4	TGTATTAGTTGGTTAATTAGAT	Amplification of bisulfite-converted DNA (1st round)
S-CAR LTR nested fwd 2	AAGGATTTGAAATGATTTTGTGTTT	Amplification of bisulfite-converted DNA (2nd round)
S-CAR LTR nested rev 2	ATCTAAAAAAACCCTCCCAAAAAT	Amplification of bisulfite-converted DNA (2nd round)

4.1.13 Software

Software & Application	Supplier
Microsoft Office (data processing)	Microsoft
FlowJo (analysis of FACS data)	Tree Star Inc.
Serial Cloner (molecular cloning)	serialbasics
LightCycler480 (analysis of qPCR data)	Roche Diagnostics
Magellan Software (ELISA read-out)	Tecan
Methlab (primer design for methylation analysis)	Methlab
GraphPad Prism (graphic presentation and statistical analysis)	GraphPad Software
FCAP Array Software	BD Biosciences

4.2 Methods

4.2.1 Cell culture methods

4.2.1.1 Cultivation and passaging of cell lines and primary cells

All cells were incubated at 37°C, 5% CO_2 and 95% humidity. 293T and Plat-E cells were trypsinized and split 1:5 every 3 to 4 days. HepG2, HepG2.2.15 and HepG2H1.3 cells were kept in collagen-coated flasks (coating with collagen (1:10 in H_2O) for 1h at 37° C) and split 1:10 once a week. For differentiation, hepatoma cell lines were seeded into collagen-coated 96-well flat-bottom plates and grown until confluent; then the medium was changed to differentiation medium and replaced with fresh medium every 3 to 5 days. Cells were differentiated for at least 4 days to achieve appropriate virus production.

The suspension cells Jurkat, T2 and B3Z were divided 1:5 to 1:10 every 2 to 4 days.

Murine splenocytes and human lymphocytes were split to appropriate density according to the observed growth rate.

4.2.1.2 Counting of cells

10µl of the cell suspension were mixed with 10µl Trypan blue and applied to the cell counting device. Either 2 or 4 quadrants were counted, and the cell count per ml was calculated (with the amount of counted cells in one quadrant multiplied by $1x10^4$).

4.2.1.3 Freezing and thawing of cells

For freezing, cells were pelleted by centrifugation at 4°C, resuspended in 1ml cold freezing medium and transferred into a 2ml cryo vial. Tubes were placed into a freezing device and immediately stored at -80°C. When cells were taken out from liquid nitrogen or -80°C, 1ml pre-warmed medium was directly added to the cells for quick thawing. The cells were resuspended by pipetting and transferred into a 15ml tube containing 9ml warm medium, spun down at 1200-1500rpm for 5min and resuspended in an appropriate amount of the respective culture medium.

4.2.1.4 Peptide loading

To exogenously load cells (T2/HepG2) with peptides, $2x10^6$ cells were resupended in 1ml AIM-V medium and transferred into a 48well plate. The respective peptide was added in a concentration of 1µg/ml and the cells were incubated at 37°C for 1h. After the peptide pulse, cells were transferred into 1.5ml tubes, spun down at 1200rpm for 2min and washed twice. They could subsequently be used for staining experiments.

4.2.1.5 Treatment with IFNγ

For upregulation of MHCI expression, hepatoma cell lines were treated with IFNγ in a concentration of 1000U/ml for 48h.

4.2.1.6 XTT Assay

To test if target cell killing had occurred in a co-culture, an XTT assay was carried out to measure target cell viability. The assay was conducted according to the manufacturer's instructions with some modifications. The target cell medium was replaced by 100µl DMEM without phenol red, 25µl XTT reagent and 0.5µl electron coupling reagent per 96well. The plate was incubated at 37°C for 2h or until an intensive color development could be detected and subsequently read with the Tecan plate reader at 450nm.

4.2.1.7 Transfection of cell lines and production of retroviral supernatants

293T or Plat-E cells were seeded into 6-well plates coated with poly-L-lysine (1:20 in PBS, 1h at 37°) to obtain a confluency of 95% at the day of transfection. Before transfection, the medium was changed to 2ml DMEM transfection medium. Cells were transfected with 4µg DNA per well using Lipofectamine2000 according to the manufacturer´s instructions. For production of retroviral supernatants in 293T cells, 2µg of the expression plasmid were co-transfected with 1µg of each of the packaging plasmids.

For the production of retroviral supernatants used for NK cell transduction, 293T cells were seeded into 75cm^2 cell culture flasks and transfected using Lipofectamine2000 with 18µg DNA in total, combining 9µg of the expression vector pMSCV C8 with 9µg packaging plasmid pCL10A1.

Retroviral supernatants were harvested 48h post-transfection and filtered through a 0.45µm filter before transduction. If a second transduction was required, fresh medium was added to the transfected cell line and the harvest was repeated after 24h.

4.2.2 Preparation of primary cells for transduction

4.2.2.1 Isolation of primary human PBMC from peripheral blood

Peripheral blood was drawn from healthy individuals and collected in syringes containing heparin. After 1:1 dilution with RPMI medium or PBS, up to 30ml of blood were carefully pipetted on top of a layer of 15ml cell separation solution in a 50ml tube. Density centrifugation was performed for 20min at 1200xg with the brake switched off. The lymphocyte layer, which was visible as a white ring, was collected carefully, transferred into a new tube and washed twice with 20ml Medium and centrifuging for 300xg. Non-used PBMC were aliquoted and frozen for later applications.

4.2.2.2 Setup of primary human NK cells

Human PBMC (freshly isolated or thawed) were resuspended in RPMI ALL medium in a concentration of $1x10^6$ cells/ml. 20ml cell suspension ($2x10^7$ cells) were seeded into a large (150 cm^2) tissue flask and incubated 30' at 37°C to allow monocytes and dendritic cells to adhere. After incubation, the supernatant containing the PBMC was carefully collected by decanting and cells were counted again. For NK cell setup, $1.5x10^6$ PBMC per well were seeded together with $5x10^5$ T2 feeder cells/well in a total volume of 5ml RPMI ALL in 6-well plates. Feeder cells were obtained by irradiation of T2 cells in AIM-V medium with a dose of 35Gy. After irradiation in a 50ml tube, T2 cells were spun down, washed and counted.
After 5 days of incubation, NK cells were harvested and used for transduction (see 3.2.3.3).
To get rid of remaining T cells in the NK cell culture, $CD3^+$ cells were depleted using human CD3 Dynabeads according to the manufacturer´s instructions.

4.2.2.3 Isolation of primary murine splenocytes

Spleens were removed from sacrificed mice, immediately transferred into tubes containing RPMI wash medium and stored on ice. To isolate lymphocytes, single spleens were put on a 100µm cell strainer and meshed with the plunger of a 2ml syringe into a 50ml tube. This was repeated 3 times, adding 3ml of wash medium per step. Collected cells were transferred to a new tube through the same filter to get rid of large cell clumps. After centrifugation for 5min at 1500rpm, the supernatant was removed; cells were resuspended in 2ml TAC buffer and incubated for 2min at room temperature. To stop erythrocyte lysis, 30ml medium were added and the suspension was spun down again. The splenocytes were resuspended in mTCM, counted and stored on ice until further treatment. When whole splenocytes were required, the volume of cell suspension was adjusted to $3x10^6$ cells/ml and cells were incubated overnight in a cell culture flask in the presence of 300 IU/ml IL-2, 2µg/ml anti-mCD3 and 0.1 µg/ml anti-mCD28 antibodies.

4.2.2.4 CD8 purification with magnetic beads

For adoptive transfer experiments, $CD8^+$ T cells were isolated from splenocytes using CD8a magnetic beads and MACS separation columns according to the manufacturer´s protocol. Purified $CD8^+$ T cells were counted and adapted to a concentration of 1 – $1.5x10^6$ cells/ml in mTCM. Further steps are described in 4.2.3.5.

4.2.3 Retroviral transduction

4.2.3.1 Retronectin coating

Some transduction protocols required the pre-coating of the plate with Retronectin to allow virus adherence. Retronectin was diluted 1:50 in PBS to achieve a concentration of 20µg/ml, and wells of a non-tissue culture treated 24well plate were coated with 250µl Retronectin solution for 2h at RT. Before cells or virus were added, the coated wells were incubated with 500µl blocking buffer (PBS/2% BSA) for 30min at 37°C and washed twice with PBS.

4.2.3.2 Transduction of suspension cell lines

For transduction of B3Z cells, 24well plates were coated with Retronectin as described above. B3Z cells were seeded on coated plates in a density of $1x10^6$ cells/well in 2ml of the respective retroviral supernatant containing 4µg/ml protaminsulfate. Spinoculation was performed for 90min at 800xg and 32°C. After centrifugation, cells were cultivated at 37°C.
Jurkat cells were transduced as described in the NK cell transduction protocol (4.2.3.3).

4.2.3.3 NK cell transduction

Primary human NK cells were obtained by cultivation of complete PBMC with T2 feeder cells for 5 days (see 3.2.2.2). Cells were harvested, spun down and resuspended in RPMI ALL medium containing 16µg/ml freshly thawed polybrene and 400 IU/ml IL-2 in a cell density of $5x10^5$ cells/ml. 1ml cell suspension/well were seeded into a 24well plate and 1ml filtered retrovirus supernatant (see 3.2.1.7) was added per well. Transduction was performed via spinoculation for 1.5 - 2h at 2000rpm and 32°C. Cells were incubated at 32°C and 7.5% CO_2. 1 day later, approximately 1.5ml of supernatant were carefully removed from each well and replaced by 1.5ml fresh retrovirus supernatant mixed 1:1 with RPMI ALL medium and containing 12µg/ml polybrene and 400 IU/ml IL-2. Spinoculation was performed a second time and cells were kept for 1 day more at 32°C and 7.5% CO_2. Subsequently, 1.5ml of medium were discarded and the same amount of fresh RPMI ALL medium containing 200 IU IL2/ml was added to the cells. Cells in each well were resuspended with a 1ml pipette and further kept at 37°C and 7.5% CO_2. NK cells were analyzed for transduction efficiency earliest 2 days post transduction.

4.2.3.4 Transduction of human PBMC

For transduction of human PBMC, retroviral supernatants were coated onto Retronectin-coated plates by centrifuging for 2h at 2000xg and 32°C. PBMC were added after complete or partial removal of viral supernatants in a density of $1x10^6$ cells/ml in human T cell medium

supplemented with 300U/ml IL-2 and spun down for 10min at 1000xg and 32°C. The transduction was repeated after 24h.

4.2.3.5 Transduction of murine cells

Complete murine splenocytes for *in vitro* assays were transduced with the Retronectin protocol as described above for human T cells.

Isolated $CD8^+$ murine T cells for adoptive transfer were seeded into 24well plates coated with anti-mCD3 and CD28 antibodies (10µg/ml in PBS; 250µl/well; 2h at 37°C followed by 2x wash) in a density of $1x10^6$ cells/well in murine T cells medium containing 5ng/ml IL-12 one day before transduction. Before transduction, 1ml of IL-12 containing supernatant was removed and stored at 37°C. Retroviral supernatants were added with protamine sulfate (4µg/ml) and spinoculated onto $CD8^+$ T cells for 2h at 2000rpm and 32°C. After centrifugation, 0.5ml of the supernatant were exchanged by 1ml IL-12 containing supernatant from before. Transduction was repeated 24h later. The cells were checked for transduction efficiency earliest 1d after the second transduction.

4.2.4 Adoptive transfer experiments

4.2.4.1 T cell transfer

For T cell transfer into mice, the cells were resuspended in cold PBS and stored on ice until injection. The cell count was adjusted to contain the right effector cell concentration per mouse ($1-4x10^6$ CAR^+ cells) in 200µl and injected i.p..

4.2.4.2 Bleeding of mice

To obtain blood from mice during an adoptive transfer experiment, the mice were either bled from the eye after anesthesia with Ketamin/Rompune (10µl per g mouse weight) or bled from the cheek. ~100µl blood were collected in 1.5ml tubes for serum recovery; around 30µl blood were transferred into 1.5ml tubes containing heparin.

4.2.4.3 Blood cell isolation and serum preparation

The blood was collected with a syringe after opening of the thorax and cutting the aorta. To isolate blood cells, approximately 50µl blood were immediately mixed with 10µl heparine to avoid coagulation. Red blood cells were lysed by addition of 10ml TAC buffer and incubation in a 15ml tube at 37°C in the water bath with occasional shaking. The cell suspension was spun down at 350xg for 10min and washed with 10ml PBS before cells were transferred into FACS plates for further analysis.

To obtain murine serum, blood without heparin was centrifuged at 5000xg for 10min at RT in a table-top centrifuge. Serum was transferred into fresh 1.5ml tubes and the centrifugation step was eventually repeated to get rid of remaining blood cells. The serum was used for direct measurement of ALT levels using the Reflotron system and further stored at -20°C.

4.2.4.4 Liver preparation and isolation of liver associated lymphocytes (LAL)

The livers were perfused with cold PBS via the portal vein before excision. Liver pieces for several analyses were cut off with a clean scalpel, and stored according to the different conditions required. The liver parts for LAL isolation were stored on ice in murine T cell medium. As the first step, the liver was put on a 100µm cell strainer in a 4cm cell culture dish and 3ml cold medium were added. The liver tissue was homogenized using the plunger of a 2ml syringe. The cell suspension that had passed the filter was transferred into a 50ml tube with a 20-gauge needle and the homogenization procedure was repeated two times. Collected cells were spun down at 350xg for 10 min and resuspended in sterile-filtered 50ml pre-warmed Williams E medium containing 0.1g collagenase and 70µl 2.5M $CaCl_2$ per liver. When parts of the liver were used for other analyses, only half of the amount of medium, collagenase and $CaCl_2$ were added. Collagenase digestion was done at 37°C in the water bath for 20min with occasional shaking. Cells were spun down again, resuspended in 4ml freshly prepared 40% Percoll solution and carefully pipetted onto a layer of 4 ml 80% Percoll solution in a 15ml tube. Density gradient centrifugation was performed at 1400xg for 20min at room temperature with the brakes shut off. The upper layer containing the hepatocytes was carefully removed with a 1ml pipette and discarded, before the white lymphocyte layer was transferred into a fresh tube. If erythrocytes were still present in the lymphocyte layer, 3ml of Ery lysis buffer were added and mixed with the cell suspension. After incubation at RT for 1min, the suspension was spun down for 10min at 1500rpm and RT, followed by resuspension of the cell pellet in 1-2ml mTCM according to the pellet size. LALs could then be counted and used for further analysis.

4.2.4.7 Cultivation of isolated splenocytes and liver associated lymphocytes

When splenocytes or LAL were stimulated or rested overnight, cell concentrations were adjusted to 1-2x10^6 cells per ml for splenocytes and 5x10^5-1x10^6 cells per ml for LAL with mTCM. Cells were seeded in 96well plates with 200µl per well.

4.2.4.8 Stimulation with plate-bound rHBsAg

Recombinant HBsAg was diluted in sterile PBS to obtain a concentration of 5µg/ml. 96well flat-bottom plates were coated with 100µl antigen solution for 2h at 37°C or overnight at 4°C. Before cells were added to the plate, the wells were washed two times with 200µl PBS.

4.2.4.9 Isolation of liver DNA

For isolation of liver DNA, a small piece of the fresh, perfused liver tissue was transferred into 180µl buffer T1 of the NucleoSpin Tissue Kit and immediately stored at -20°C. DNA isolation was carried out after the manufacturer's instructions.

4.2.4.10 Isolation of viral serum DNA

To purify viral DNA from the serum, serum samples were diluted 1:20 or 1:30 with PBS after thawing and further processed with the High Pure Nucleic Acid Kit according to the manufacturer´s instructions.

4.2.4.11 Histopathology

Liver tissue was either embedded in Tissue-Tek and frozen or fixed with 4% PFA and embedded in paraffin. Paraffin sections (2 µm) were either stained with haematoxylin / eosin or automated immunohistochemistry staining was performed using rabbit-anti-HBcAg (done by AG Heikenwalder). Image acquisition was performed on the DotSlide BX51 (Olympus).

4.2.5 Flow cytometry

4.2.5.1 General procedure

1-5x10^5 cells per staining were resuspended in 50µl FACS buffer containing the respective antibody/ies in a 96-well V-bottom polystyrene plate. After incubation for 30 min on ice, cells were washed three times with FACS buffer, transferred into FACS tubes in 150µl FACS buffer and analyzed with the FACS Canto II at the same day. To stain for dead cells, 10µl propidiumiodide (1µg/ml) were added directly before the measurement. The obtained FACS data were evaluated with FlowJo Software.

4.2.5.2 Intracellular cytokine staining

For intracellular staining, Brefeldin A was added to the cultured cells in a concentration of 1µg/ml for the last 4-5 hours of stimulation to inhibit cytokine release. Cells were stained for live-dead discrimination by resuspension in 100µl FACS Buffer with EMA (1µg/ml) and incubation for 20min on ice with exposure to light. Cells were washed subsequently 2 times by with FACS buffer before surface stainings were performed. Permeabilization and fixation was achieved by resuspending the cells in 100µl Cytofix/Cytoperm and incubating on ice for 15min. Cells were then washed again and intracellular stainings were performed. General staining procedure was the same, but the staining and all washings were performed using

Perm/Wash buffer. For flow cytometric analysis, the samples were resuspended in 150-200µl FACS buffer.

4.2.5.3 CD107a (LAMP-1) staining

When LAMP-1 staining was performed, the APC-labeled antibody was added directly to the cell culture (1:200) for the last 5 hours of stimulation time. If parallel cytokine staining was done in parallel, BFA was added 1 hour later. To avoid long exposure to light and resulting damage of the LAMP-1 antibody, EMA staining was done by incubation 20min in the dark and additional 10min with light exposure.

4.2.5.4 AnnexinV staining

Since the AnnexinV antibody binding requires the presence of calcium in the staining buffer, staining was performed following the regular staining according to the manufacturer´s protocol. Staining was done with 2.5µl antibody and 10µl propidium iodide (50ng/ml) in 100µl AnnexinV-Binding Buffer at RT for 15min; afterwards 100µl binding buffer were added and cells were analyzed immediately.

4.2.5.5 Staining with hybridoma supernatants

To visualize peptide-MHC complexes, the cells were resuspended in 50µl of the respective hybridoma supernatant (C18A2/Env183A2) and incubated for 1h at 4°C. After washing 3 times with FACS buffer, staining with a labeled secondary antibody (anti-murine IgG-APC) was performed and cells were subsequently analyzed by flow cytometry.

4.2.5.6 Streptamer staining

Cells were stained with 0.4µg of the respective MHC:peptide complex mixed with 1µl Streptactin-PE in 50µl FACS buffer for 25min on ice. Staining with additional antibodies was performed subsequently (without a washing step in between).

4.2.5.7 Cytometric bead array

Serum cytokines were measured using the Cytometric Bead Array Mouse Inflammation Kit according to the manufacturer´s instructions. Either undiluted or 1:4 diluted serum samples were incubated with 15µl mixed capture beads and 15µl PE detector for 2h at RT. 5000 beads were acquired; analysis was done with the FCAP Array Software 3.0 (BD Biosciences).

4.2.5.8 Staining cells of the lymphoid and myeloid lineage

To be able to distinguish different cell types belonging to the myeloid lineage, cells from different body compartments were stained against CD45.1, CD3, CD11b, CD11c, F4/80 and Ly6C/G. After the exclusion of CD3+ and CD45.1+ cells, the population was gated on $CD11c_{high}$ cells representing dendritic cells (DCs). Subsequently, cells were gated on the CD11b positive population, and expression of Ly6C/G and F4/80 was used to discriminate between resident macrophages (Ly6C/G negative / F4/80 +), inflammatory monocytes (Ly6C/G intermediate / F4/80 +) and neutrophils (Ly6C/G high / F4/80 -). The panel for staining of the lymphoid lineage contained antibodies against CD45.1 CD3, CD4, CD8, CD19 (B cells), and NK1.1 (NK cells).

4.2.6 Molecular biology

4.2.6.1 Heat-shock transformation of competent bacteria

Chemical competent E.coli were thawed on ice and mixed with approximately 1ng of plasmid DNA by flipping the tube 2-3 times. Cells were incubated for 30min on ice before a heat shock (90sec, 42°C) was performed. The bacteria were put on ice again and either directly plated on LB agar plates containing the respective antibiotic.

For transformation of BL-21 (DE3) bacteria the protocol was modified as following: competent bacteria were added to a tube containing 60ng of plasmid DNA and mixed by pipetting up and down. After 30min incubation on ice, a heat shock was done for 2min at 42°C. Bacteria were then incubated with 900µl soc-medium for 30min at 300rpm. The bacterial suspension was centrifuged for 5min at 350xg and the pellet was resuspended in 100µl LB medium for plating.

Plates were incubated overnight at 37°C in the incubator. Clones were harvested the following day.

4.2.6.2 Isolation of plasmid DNA

For small scale DNA preparation, bacterial clones were grown over night in 5ml LB medium with antibiotic at 37°C and 200rpm in a shaker. 2ml of bacterial suspension were used for plasmid preparation with the GeneJet Plasmid Miniprep Kit according to the manufacturer´s instructions.

To obtain larger amounts of plasmid DNA, a starting culture of the respective bacterial clone was diluted 1:2000 in 50 or 100ml fresh LB medium with antibiotics and cultivated overnight in the shaker. The OD_{260} was measured before preparation, and an appropriate amount of bacterial suspension was spun down and prepared with either a Midiprep or Maxiprep Kit for plasmid preparation.

4.2.6.3 Digestions with restriction enzymes

For analytic purposes, 200 to 500ng DNA were digested in a total reaction volume of 10µl. Preparative digestions were performed with 1-10µg DNA or the complete PCR product in a total volume of 20 to 50 µl. The respective enzymes were added in concentrations not exceeding 10% (v/v) to samples supplemented with the recommended buffers. Digestion duration was 2 hours for normal enzymes and 30min for FastDigest enzymes. The resulting fragments were controlled via gel electrophoresis.

4.2.6.4 Agarose gel electrophoresis

1% agarose gels were prepared with TEA buffer. The gels were run with a voltage between 60 and 110mV.

4.2.6.5 DNA extraction from agarose gels

Staining of the DNA with ethidium bromide (EtBr) allowed for detecting the gel bands with the Fusion system. Long exposure with mutagenic UV light was avoided by applying the safety mode. The relevant bands were excised with a clean scalpel and DNA was extracted using the gel extraction kit according to the manufacturer`s instructions.

4.2.6.6 Polymerase chain reaction (PCR)

Approximately 1ng of plasmid DNA were assembled with each 1 µl of forward and reverse primer (20 pmol/µl), 2-5 µl Taq Reaction Buffer, 0.25-0.4 µl dNTPs, 0.5 µl Taq-Polymerase and H_2O in a total volume of 20 to 50 µl. A PCR programme covered a denaturation phase (95°C, 10 s), primer annealing (55-65°C, according to the respective primers, 20 s) and elongation phase (72°C, duration according to fragment size). The cycle was repeated 35 times; PCR products were controlled by agarose gel electrophoresis.

High-Fidelity PCR reactions were performed with the Phusion PCR kit according to the manufacturers' instructions. If the requested PCR product could be verified via gel electrophoresis, the whole sample was either directly purified with a PCR extraction kit or extracted from a preparative agarose gel (see above).

4.2.6.7 Dephosphorylation and ligation

Vector dephosphorylation and ligation of two fragments in the course of a cloning were performed with the Rapid Dephosphorylation and Ligation Kit according to the manufacturer´s instructions. The dephosphorylated vector was usually mixed with a 10 fold molar excess of the respective insert. Ligation reaction was stored on ice until transformation of competent bacteria was carried out.

4.2.6.8 RNA isolation and cDNA synthesis

RNA from hybridoma cell lines was isolated from cell pellets using TRI-Reagent according to the manufacturer's instructions. To generate cDNA, 3µl RNA were mixed with 1µl H_2O, 5µl Buffer and 1µl Polymerase from the Superscript III Kit and subjected to the following protocol on the PCR cycler:

1) 10min 25°C
2) 30min 50°C
3) 5min 85°C
4) 5min 4°C
5) Addition of 0.5µl RNase H
6) 20min 37°C
7) 4°C

4.2.6.9 Amplification of variable domains using degenerated primers

To clone the variable domains of the antibodies produced by hybridoma cell lines, the generated cDNA was amplified using sets of degenerated primers (provided by Amgene, formerly Micromet) according to the following protocol:

1µl cDNA (diluted 1:3)
4µl 10x Taq buffer (+Mg^{2+})
0.6µl fwd primer 20µM
0.6µl rev primer 20µM
0.25µl dNTPs 25mM
0.2µl Taq polymerase (w/o proof-reading capacity)
33.28 µl H_2O

Programm PCR cycler:

1) 3min 94°C
2) 50sec 94°C
3) 50sec 52°C
4) 60sec 72°C → repeat from 2 (35x)
5) 10min 72°C
6) 4°C

4.2.6.10 Quantitative PCR (qPCR)

For quantification of HBV DNA in the liver, purified DNA (see 3.2.4.9) was amplified with primers specific for rcDNA (provided by Ke Zhang/Christian Bach). DNA levels per cell were calculated relative to levels of the single-copy gene Nid2, which was amplified in parallel. A serial dilution of liver DNA served as internal calibrator. To obtain absolute concentrations of viral DNA in the serum, purified serum DNA (see 3.2.4.10) was amplified with the HBV-specific primers in parallel to a serial dilution of a virus standard with known concentration (provided by Christian Bach). The template DNA was amplified with the Light Cycler 480 according to the following protocol:

5µl SYBR Green Master Mix 2x
0.5µl fwd primer (20µM)
0.5µl rev primer (20µM)
2-4 µl template
H_2O to 10µl
Light Cycler Program (rcDNA selective qPCR for HOPE):

1) Initial denaturation 95°C, 300sec
2) Cyclic denaturation 95°C, 15sec
3) Annealing 60°C, 15sec
4) Elongation 72°C, 20sec → repeat from 2) 45 times
5) Melting curve 65°C – 95°C 0.1°C/sec
6) Cooling

Data were analyzed with the LightCycler480 software from Roche.

4.2.6.11 Sequencing

For sequencing, DNA plasmids or PCR fragments were adjusted to a concentration of 50ng/µl and sent to GATC Biotech together with the respective sequencing primer (10µM). Obtained sequencing results could be downloaded from the company homepage and further analyzed.

4.2.7 ELISA

Supernatants tested in an ELISA were usually stored at -20°C and freshly diluted with Assay diluent (PBS, 1% BSA) if necessary. Murine IFNγ was detected using the Biolegend ELISA

kit according to the manufacturer`s instructions. Plates were analyzed with the Tecan Plate reader.

4.2.8 Analysis of methylation patterns

4.2.8.1 Bisulfite conversion and bisulfite-specific PCR

Bisulfite conversion of purified DNA was performed with the EpiMark Bisulfite Conversion Kit according to the supplier´s instructions. 6µl of converted DNA were used for PCR with the EpiMark Polymerase using primers specific for bisulfite-converted templates designed with the online available software from MethLab. The PCR protocol was designed in principal as described in 3.2.5.6, with some modifications:

1st round (primer pair S-CAR nested 4):

- Annealing temperature 53°C
- Elongation: 68°C, 33sec

1st round (primer pair S-CAR nested 2):

- Annealing temperature 59°C
- Elongation: 68°C, 14sec

4.2.8.2 Detection of methylation patterns

PCR products were cloned into the pJET1.2/blunt cloning vector using the sticky-end cloning protocol provided by the supplier. 1µl of non-purified PCR product allowed for efficient cloning. The resulting clones were tested for presence of the PCR fragments via digestion with NotI/XbaI and subsequently sequenced using a T7 primer.

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7 Publications and meetings

7.1 Publications

Krebs, K *., N. Böttinger *, L. R. Huang, M. Chmielewski, S. Arzberger, G. Gasteiger, C. Jager, et al. "T Cells Expressing a Chimeric Antigen Receptor That Binds Hepatitis B Virus Envelope Proteins Control Virus Replication in Mice." Gastroenterology 145, no. 2 (Aug 2013): 456-65. * shared first authorship

7.2 Meetings

2013 International Meeting on Molecular Biology of Hepatitis B Viruses
October 20-23, 2013; Shanghai, China
Oral presentation: “CAR-redirected T cells for treatment of chronic hepatitis B: analysis of T-cell function and fate”

2013 Workshop "Viral hepatitis: viral and host genomics at the omics era"
October 17-20, 2013; Beijing, China
Oral presentation: “CAR-redirected T cells for treatment of chronic hepatitis B: analysis of T-cell function and fate.”

2013 Retreat of the Helmholtz Alliance of Immunotherapy
July 7-9, 2013; Asselheim, Germany
Poster presentation: “T cells redirected by a chimeric antigen receptor recognizing HBsAg efficiently control HBV in vivo in transgenic mice”

2012 International Meeting on Molecular Biology of Hepatitis B Viruses
September 22-25, 2012; Oxford, England
Oral presentation: “Cytotoxic T cells derived from an HBV+ background are fully functional when engrafted with a chimeric antigen receptor directed against HBV”
Travel grant award

2012 Berlin Symposium on Adoptive T Cell Therapy
May 3-4, 2012; Berlin, Germany
Poster presentation: “Cytotoxic T cells derived from an HBV+ background are fully functional when engrafted with a chimeric antigen receptor directed against HBV”

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