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IMMUNE RESPONSE DURING HEPATITIS B VIRUS INFECTION AND RECONSTITUTION OF HBV-SPECIFIC IMMUNITY USING T CELLS REDIRECTED AGAINST HBV

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“He that can have patience can have what he will”
Benjamin Franklin

ABSTRACT

Hepatitis B is one of the most prevalent viral diseases worldwide and is a major public health concern, particularly in Asia. Of the 350 million people suffering from chronic hepatitis B virus (HBV) infection worldwide, approximately 75% are found in Asia. HBV is a noncytopathic, hepatotropic virus that causes acute and chronic hepatitis and hepatocellular carcinoma (HCC). Virus-specific T cells have been found to be associated with the control of HBV infection, but have also been implicated as the principal effectors of liver damage. Although the mechanisms responsible for liver inflammation have been nicely demonstrated in animal models, we still lack evidence for these events in humans. We therefore studied the role of HBV-specific CD8 T cells in different phases of HBV infection. Analysis of the kinetics of innate and adaptive immune activation during hepatic flares in chronic hepatitis B revealed that the rebound of HBV replication following therapy withdrawal occurred without triggering any innate immune activation, with the exception of increased serum CXCL-8. Hepatic flares were temporally associated with high serum levels of the IFN-γ inducible chemokines CXCL-9 and CXCL-10. Both were differentially produced in liver injury present in acute or chronic patients and displayed different *in vitro* requirements for activation. The inflammatory potential of virus-specific T cells was characterized by analysing their ability to produce IL-17 and CXCL-8 during different phases of HBV infection. We also determined whether cytokines present in the liver during inflammation (IL-7 and IL-15) could license virus-specific T cells with additional cytokine profiles that contribute to tissue inflammation. Our results showed that HBV-specific T cells produced CXCL-8, but not IL-17, during periods of liver inflammation in acute or chronic patients. HBV-specific T cells producing CXCL-8 could be expanded from acute/resolved patients in the presence of IL-7 and IL-15, suggesting that virus-specific T cells can acquire through exposure to environmental factors, a cytokine/chemokine profile capable of contributing to parenchymal inflammation.

Despite that today's antiviral drugs efficiently decrease HBV viral load to undetectable levels, they fail to eradicate infection due to the persistence of HBV covalently closed circular DNA in hepatocytes. Long-term treatment is also expensive, and may result in problems of toxicity and emergence of resistant viruses. Attempts to restore HBV-specific immunity in chronic patients with therapeutic vaccines have had little success. We therefore developed a new strategy based on T cell receptor (TCR) gene transfer to reconstitute the defective antiviral immunity of chronic patients. DNA encoding the HBV-specific TCR alpha/beta chain was cloned from immunodominant HBV-specific CD8 T cells from acute/resolved patients. The TCR genes were transferred to primary human T cells using retroviral transduction or mRNA electroporation. Transgenic TCRs were efficiently expressed on T cells of chronic HBV/HCC patients and reconstituted fully functional HBV-specific T cells. Furthermore, these TCR-redirected T cells recognized and lysed natural HCC lines with HBV DNA integration. Despite a transient functionality, the TCR mRNA electroporated T cells efficiently prevented tumor seeding and suppressed the growth of established tumors in a xenograft model of HCC. Overall, we developed a method that represents a practical approach to cell therapy of HCC and its inherently self-limiting toxicity suggests potential for application in other HBV-related pathologies.

LIST OF PUBLICATIONS

I. Anthony T. Tan, SARENE KOH, Winnie Goh, Heng Yee Zhe, Adam J. Gehring, Seng Gee Lim, Antonio Bertoletti. A longitudinal analysis of innate and adaptive immune profile during hepatic flares in chronic hepatitis B. *Journal of Hepatology*, 2010 Mar, 52(3): 330-339.

II. Adam J. Gehring, SARENE KOH, Adeline Chia, Komathi Paramasivam, Valerie Suk Peng Chew, Zi Zong Ho, Kang Hoe Lee, Mala K.Maini, Krishnakumar Madhavan, Seng Gee Lim, Antonio Bertoletti. Licensing virus-specific T cells to secrete the neutrophil attracting chemokine CXCL-8 during hepatitis B virus infection. *PLoS ONE*, 2011 Aug, 6(8): e23330.

III. Adam J. Gehring, Shao-An Xue, Zi Zong Ho, Denise Teoh, Christiane Ruedl, Adeline Chia, SARENE KOH, Seng Gee Lim, Mala K. Maini, Hans Stauss, Antonio Bertoletti. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. *Journal of Hepatology*, 2011 Jul, 55(1): 103-110.

IV. SARENE KOH, Noriko Shimasaki, Rossarin Suwanarusk, Zi Zong Ho, Adeline Chia, Nasirah Banu, Shanshan Wu Howland, Alice Soh Meoy Ong, Adam J. Gehring, Hans Stauss, Laurent Renia, Matti Sällberg, Dario Campana and Antonio Bertoletti. A practical approach to immunotherapy of hepatocellular carcinoma using T cells redirected against hepatitis B virus. *Molecular Therapy Nucleic Acids*, 2013 Aug, 2: e114.

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LIST OF ABBREVIATIONS

ACT	adoptive cell transfer
AFP	alpha fetoprotein
ALT	alanine aminotransferase
APC	antigen presenting cell
CAR	chimeric antigen receptor
CBA	cytometric bead array
cccDNA	covalently closed circular DNA
cGMP	current good manufacturing practice
CHB	chronic hepatitis B
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen-4
DCs	dendritic cells
DHBV	duck hepatitis B virus
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
ER	endoplasmic reticulum
HBcAg	hepatitis B core antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBx	hepatitis B X
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HF	hepatic flares
HSCs	hematopoietic stem cells
IFN	interferon
MHC	major histocompatibility complex
mRNA	messenger RNA
NK	natural killer
NSG	NOD-SCID-IL2RG^{null}
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PD1	programmed cell death 1

pgRNA	pregenomic RNA
SB	Sleeping Beauty
sgRNA	subgenomic RNA
T_{CM}	central memory T
TCR	T cell receptor
T_{EM}	effector memory T
TLR	toll-like receptor
Treg	regulatory CD4 T
T_{SCM}	T memory stem cells
WHBV	woodchuck hepatitis B virus
ZFN	zinc finger nuclease

1 THESIS SUMMARY

The aim of these studies was to study the role of T cells in HBV pathogenesis. It has been shown that virus-specific T cells are associated with the control of HBV infection. The frequency and function of antigen-specific T cells in patients who resolve HBV infection is far superior to that in subjects with chronic infection. The ability of these cells to produce antiviral cytokines (IFN-γ and TNF-α) results in HBV clearance from infected hepatocytes without extensive direct killing. In contrast to their protective role, the activation of intrahepatic HBV-specific T cells can also trigger an influx of inflammatory mononuclear infiltrate (consisting granulocytes, monocytes and T cells) to the liver, which is the principal cause of hepatic injury. Although the mechanisms responsible for liver inflammation have been nicely demonstrated in animal models, we still lack evidence that these events are occurring in humans. Thus, we were particularly interested in studying the role of HBV-specific CD8 T cells in different phases of HBV infection, and their contribution to hepatic immunopathology.

We had a unique opportunity to study a group of chronic hepatitis B (CHB) patients that voluntarily ceased antiviral treatment (n=5) that allow us to perform a longitudinal analysis of virological and immunological events before and during hepatic flares (HF) (Paper I). We found that the rebound of HBV replication following antiviral therapy withdrawal did not trigger innate immune activation, with the exception of increased serum CXCL-8 detected. Our study also confirmed the involvement of CXCL-9 and CXCL-10 in liver inflammation (as has been shown in animal models) in humans, and their differential production in acute and chronic patients during liver injury suggests that different mechanisms could trigger their secretion.

Additionally, at that time, a new subset of CD4 T helper cells, Th17, characterized by their production of IL-17 has been described. Th17 cells have been reported to be involved in mediating pathological inflammation and tissue injury in autoimmune diseases; however, they also serve a protective role in host defence against bacterial infections. The role of Th17 cells in viral infections is less clear, and seems to be pathogenic. The discovery of Th17 cells sparked our interest in investigating their potential role in mediating hepatic inflammation during chronic HBV infection, as IL-17 is known to recruit neutrophils (Paper II). We did not detect any HBV-specific IL-17 producing T cells in PBMCs of acute and CHB patients. Moreover, we did not detect any increase in non-specific IL-17 producing T cells in peripheral blood or intrahepatic lymphocytes. By contrast, we found antigen-specific CXCL-8 producing T cells from intrahepatic lymphocytes of CHB patients and from peripheral blood T cells in acute/resolved patients. Using IL-7 and IL-15 (cytokines that are present in the inflammatory liver environment), we were able to induce HBV-specific CXCL-8 producing T cells from PBMCs of acute/resolved patients. Our study demonstrates the functional plasticity of virus-specific T cells during HBV infection, and that factors within the liver microenvironment could perhaps license HBV-specific T cells with an inflammatory function.

We were also very interested in the development of immunotherapy for the treatment of CHB/hepatocellular carcinoma (HCC). Although currently available antiviral

drugs efficiently decrease HBV viral load to undetectable levels, they fail to eradicate infection due to the persistence of HBV covalently closed circular DNA (cccDNA) in hepatocytes, leaving patients at risk of developing cirrhosis and HBV-related HCC. Moreover, long-term treatment is expensive, may result in problems of toxicity and emergence of resistant viruses. Functional impairment of T cell responses that occurs during persistent HBV infection, leading to exhausted T cells and ultimately deletion of HBV-specific T cells could explain the failure to achieve viral clearance. An efficient adaptive immune response seems to be the key for sustained control of HBV replication. Therefore, we developed an alternative strategy based on T cell receptor gene transfer to reconstitute the defective antiviral immunity of CHB patients (Paper III). We selected immunodominant HBV-specific CD8 T cells from acute/resolved patients, cloned the DNA encoding the HBV-specific TCR alpha/beta chain and inserted into a retroviral vector for gene transfer into primary human T cells. The introduced TCRs can be efficiently expressed on T cells of chronic HBV and HBV-related HCC patients and reconstituted fully functional HBV-specific T cells. Furthermore, these TCR-redirected T cells recognized and lysed natural HCC lines with HBV DNA integration.

Having shown that it is possible to generate functional TCR-redirected T cells through retroviral gene transfer, we next evaluated the therapeutic efficacy of adoptive transfer of tumor-specific TCR-redirected T cells for HCC therapy in mouse models. The use of retro-/lenti-viral vectors to introduce exogenous HBV-specific TCRs on T cells to redirect their specificity is complex and expensive to implement in clinical trials. Therefore, we aimed to develop a safer and easier alternative strategy to redirect the specificity of T cells using mRNA electroporation (Paper IV). Using this technology, approximately 80% of CD8+ T cells expressed functional HBV TCR 24 hours post-electroporation, an expression efficiency much higher than that obtained by retroviral transduction (~18%). Antigen-specific cytokine production of electroporated T cells was efficient within 72 hours period, after which the redirected T cells lost their HBV-specific function. Despite this transient functionality, the TCR-electroporated T cells efficiently prevented tumor seeding and suppressed the growth of established tumors in a xenograft model of HCC. We also established a method for large-scale TCR mRNA electroporation that yielded large numbers of highly functional clinical-grade anti-HBV T cells. Results from this study are encouraging and we aim to use TCR mRNA electroporated T cells for clinical trials in patients with HBV-related HCC in the future.[1,2]

2 HEPATITIS B AND HEPATOCELLULAR CARCINOMA

2.1 THE HISTORY OF HEPATITIS B

"Hepatitis" means inflammation of the liver, with manifestations of jaundice and fever. The history of viral hepatitis goes back thousands of years. As early as the 5th century BC, epidemic jaundice was described by Hippocrates. The first recorded case of serum hepatitis, or hepatitis B, was made by Lurman in 1885. An outbreak of smallpox occurred in Bremen when shipyard employees were vaccinated with lymph from other people. After several weeks, and up to months later, hundreds of the vaccinated workers became ill with jaundice and were diagnosed as suffering from serum hepatitis. Other employees who had been inoculated with different batches of lymph remained healthy. In the early and middle parts of the 20th century, serum hepatitis was repeatedly observed following the use of contaminated needles and syringes. Blood was identified as a vehicle for virus transmission when Beeson described jaundice that had occurred in seven recipients of blood transfusions. In 1947, MacCallum classified viral hepatitis into two types: Viral Hepatitis A, or short incubation infectious hepatitis, and Viral Hepatitis B, or long incubation serum hepatitis.[3] In 1965, Baruch Blumberg discovered Australia antigen (later known to be hepatitis B surface antigen, or HBsAg) in the blood of Australian aboriginal people, which led to the identification of the Dane particle (the complete hepatitis B virion) by electron microscopy in 1970.[4] The Dane particle consisted of a lipid envelope with HBsAg on the surface and a core made up of the hepatitis B core antigen (HBcAg). This was followed by the discovery of the HBeAg and its antibody (anti-HBe).[5] Treatment of the Dane particle with detergent revealed that the viral nucleocapsid contained the viral DNA genome and an endogenous DNA polymerase.[6] The identification of serologic markers for HBV infection followed, which helped clarify the natural history of the disease.

2.2 EPIDEMIOLOGY OF HEPATITIS B

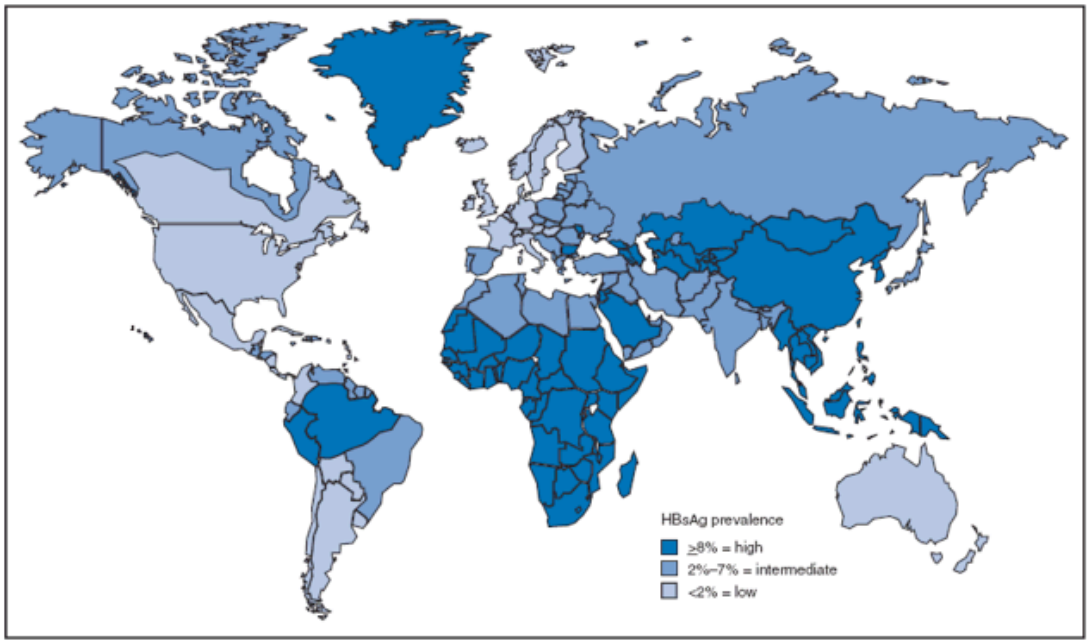
Hepatitis B is a major global health problem. According to the World Health Organization, an estimated two billion people have been infected with the hepatitis B virus and more than 240 million have chronic liver infections. In areas where hepatitis B is endemic, such as Africa and Asia, strongly predisposes to the development of chronic liver disease and subsequent development of hepatocellular carcinoma (HCC). HCC is the third leading cause of cancer deaths worldwide,[7] and more than 600,000 people die every year from HBV-related chronic liver disease, including cirrhosis and HCC.

The world can be divided into three areas where the prevalence of chronic HBV infection is: high (>8%), intermediate (2 – 8%), and low (<2%) (Figure 1). Hepatitis B is endemic in parts of Asia (particularly China), the central Asia, parts of the Middle

East, sub-Saharan Africa, the Amazon Basin, and some countries in eastern Europe. Most people in this region become infected with the hepatitis B virus during childhood and up to 20% of the population can be chronically infected. Low endemic areas include North America, Western and Northern Europe, Australia, and parts of South America, with a carrier rate of less than 2%. The rest of the world falls into the intermediate range of HBV prevalence, with 2 – 8% of a given population being HBV carriers.

Eight genotypes of HBV (A – H) have been identified.[8] A possible new genotype "I" has been described,[9] but acceptance of this notation is not universal.[10] The genotypes differ by more than 8% divergence of the full nucleotide sequence and have distinct geographical distributions.[11] For example, genotypes A and D are prevalent in Western Europe and North America, while genotypes B and C are commonly found in Asia.

Hepatitis B immunization is an effective way to interrupt HBV transmission. Since the introduction of universal immunization of neonates and vaccination of high-risk populations in the 1980s, together with the screening of blood donors, reported cases of HBV have declined tremendously.[12] Nonetheless, there are still problems that hamper the ultimate eradication of HBV infection and its sequelae. Data from Taiwan show that despite universal hepatitis B vaccination of neonates, approximately 1.5 – 2.1% of the vaccinated population were still seropositive for HBsAg and had chronic HBV infection, possibly due to failure to protect high-risk infants from intrauterine HBV infection.[13,14]



* For multiple countries, estimates of prevalence of hepatitis B surface antigen (HBsAg), a marker of chronic HBV infection, are based on limited data and might not reflect current prevalence in countries that have implemented childhood hepatitis B vaccination. In addition, HBsAg prevalence might vary within countries by subpopulation and locality.
Source: CDC. Travelers' health; yellow book. Atlanta, GA: US Department of Health and Human Services, CDC; 2008. Available at http://wwwn.cdc.gov/travel/yellowbookch4-HepB.aspx.

Figure 1. Geographic distribution of chronic hepatitis B virus infection worldwide.

2.3 VIRAL STRUCTURE AND GENOME

HBV is a member of the *Hepadnaviridae* family. The virion is 42 nm in diameter, with an outer lipoprotein envelope that contains three related envelope glycoproteins (or surface antigens, HBsAg) – large (L), middle (M) and small (S). Within the envelope is an icosahedral viral nucleocapsid, or core. The core contains the viral genome, a relaxed-circular, partially double-stranded DNA of 3.2 kb, and a DNA polymerase with reverse transcriptase activity that is responsible for the synthesis of viral DNA in infected cells. In addition to virions, HBV-infected cells produce two distinct subviral lipoprotein particles: 20 nm spheres and filamentous forms. These particles composed of only HBsAg are present in 10^3 – 10^6 fold excess over whole virions.[15] They are not infectious, are produced in excess during the life cycle of the virus, and were used in the first-generation hepatitis B vaccine, an inactive plasma-derived vaccine (Figure 2).

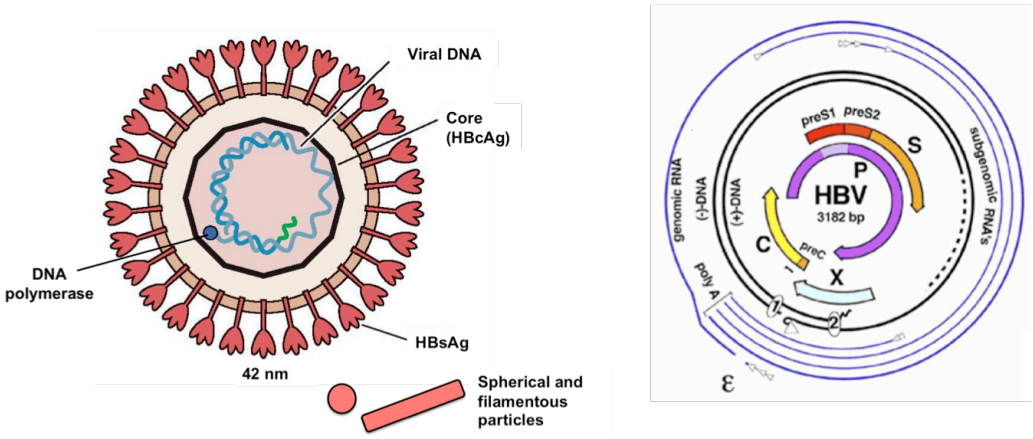


Figure 2. The structure of hepatitis B virion, subviral particles and the genome organisation of HBV. Reproduced with permission from Elsevier. Michael Nassal. Hepatitis B viruses: Reverse transcription a different way. *Virus Res.* 2008 Jun 134:235-249.

The HBV genome consists of a partially double-stranded DNA (a complete minus-strand and an incomplete plus-strand) that encodes for four overlapping open reading frames (ORF) (Figure). The preS-S region of the genome is a long ORF that encodes the three envelope proteins by differential translation at three in frame start codons. The most abundant protein is the 24 kDa small (S) protein (also known as HBsAg). The middle (M) protein is coded by the preS2 + S region while initiation at the most upstream codon generates the large (L) protein coded by the preS1 + preS2 + S region. The preS1 protein plays a role in binding of the virus to host cell receptors[16] and in the assembly of the virion and its release from the cell.[17] The C ORF encodes for the viral capsid (HBcAg) and the HBeAg initiated from an upstream start codon at the precore region. The preC region encodes a signal sequence, which directs the precore protein into the secretory pathway. Cellular proteases cleave the precore in the endoplasmic reticulum to generate HBeAg, a 16 kDa fragment that is secreted into the blood. The P ORF encodes for the viral polymerase that is involved in DNA synthesis and RNA encapsidation. The X ORF encodes for the X protein, the function of which is not completely understood, but it is required for *in vivo* replication of the virus.[18]

2.4 HBV LIFE CYCLE

HBV virions first attach reversibly to cell-associated heparan sulfate proteoglycans, after which the viral particle binds specifically to a hepatocyte-specific preS1-receptor.[19] A recent study has identified this receptor to be sodium taurocholate cotransporting polypeptide (NTCP).[20] This protein is normally involved in the circulation of bile acids in the body. The precise mechanism by which HBV enters into the hepatocyte is still unknown. Endocytosis and direct fusion of the viral envelope with the plasma membrane have been proposed as potential pathways. After uncoating of the virus in the cytoplasm and transport of the nucleocapsid into the nucleus, the relaxed circular partially double-stranded DNA (rcDNA) is repaired by both viral and cellular enzymes. The incomplete plus-strand of the rcDNA is completed by the viral polymerase, and then the viral polymerase on the 5' end of the minus-strand and RNA primers used for DNA plus-strand synthesis are removed by cellular enzymes. The covalently closed circular DNA (cccDNA) is formed by covalent ligation of both DNA strands. The viral cccDNA (encodes for four overlapping ORFs as described above) serves as a template for RNA synthesis and the cellular transcriptional machinery is utilized to produce all viral RNAs necessary for protein production and viral replication. Processing of viral RNAs, nuclear export as well as stabilization of the viral RNAs appears to be exclusively mediated by host factors. The cccDNA is transcribed into subgenomic RNA (sgRNA) and pregenomic RNA (pgRNA) and then transported to the cytoplasm. The core protein and the viral polymerase are translated from the pgRNA while the X-protein and the three envelope proteins are translated from the sgRNAs. The pgRNA forms a complex with the core protein and the polymerase and self-assemble into an RNA-containing nucleocapsid. The RNA-containing nucleocapsids undergo maturation to DNA-containing nucleocapsids by reverse transcription of the pgRNA followed by plus-strand DNA synthesis within the nucleocapsid. DNA-containing nucleocapsids can be either re-imported into the nucleus to form additional cccDNA molecules or can be enveloped for secretion via the endoplasmic reticulum (ER). The envelope proteins are co-translationally inserted into the ER membrane, where they bud into the ER lumen, and are secreted by the cell, either as 22 nm non-infectious subviral spherical or filamentous particles or as 42 nm infectious virions (Dane particles).

2.5 NATURAL HISTORY

HBV is transmitted predominantly parenterally or through sexual contact. In Asia, the most common route of transmission is vertical transmission from mother-to-child during childbirth[21] while horizontal transmission during childhood seems more prevalent in Africa[22] and Europe.

Primary infection (or acute hepatitis) has an incubation period of 4 to 10 weeks, followed by the detection of circulating HBsAg and HBeAg and high viremia of ~10^9 to 10^{10} virions/ml (Figure 3). This is followed shortly by the appearance of anti-HBc antibodies, mainly of IgM isotype early in the infection. Acute hepatitis is mainly

asymptomatic. Symptoms such as tiredness, anorexia, abdominal discomfort, nausea and vomiting, often progressing to jaundice, generally appear at the time the liver is being cleared of infection. The hallmark of acute hepatitis is the striking elevation of serum alanine aminotransferase (ALT) that occurs with clearance of infected hepatocytes. Titers of virus in the liver and blood begin to drop. With clearance of the infection, the viral antigens HBsAg and HBeAg disappear from the circulation, followed by seroconversion to anti-HBs and anti-HBe. Anti-HBc IgM antibodies convert to IgG with convalescence and recovery. However, low levels of HBV DNA in the blood may persist for many years.[23] Most primary infections in adults are self-limited, with clearance of virus from blood and liver and the development of lasting immunity to reinfection, only less than 5% do not resolve and develop persistent infection.[24]

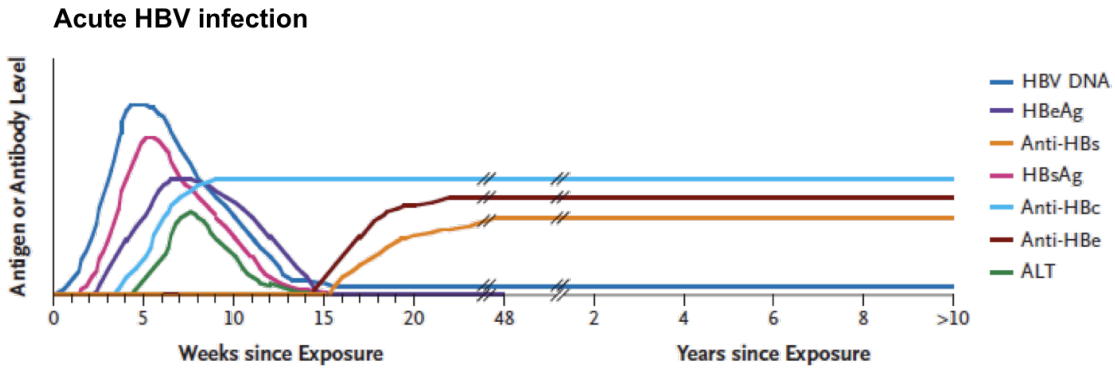


Figure 3. Typical patterns of serologic and molecular markers in acute HBV infection. ALT, HBV DNA, HBsAg, HBeAg, anti-HBc, anti-HBe, and anti-HBs antibodies are shown. Reproduced with permission from Ganem D and Prince AM. Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med.* 2004 Mar 11;350(11):1118-29. Copyright Massachusetts Medical Society.

The risk of developing chronic infection, or the carrier state, defined as the persistence of HBsAg in the blood for more than 6 months, is dependent on the age and immune function of the patient at the time of initial infection. More than 90% of infected newborns and 30% of children younger than 5 years are generally unable to resolve a primary infection and develop chronic infection. In persistent infection, viral replication continues in the liver and there is continual viremia, although the titers of virus in the liver and blood are variable. Figure 4 shows typical patterns of serologic and molecular markers in chronic HBV infection.

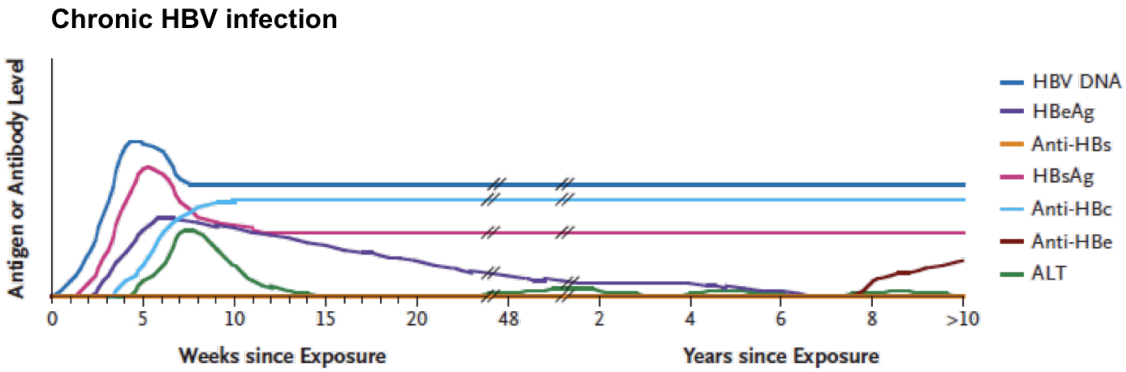


Figure 4. Typical patterns of serologic and molecular markers in chronic HBV infection. ALT, HBV DNA, HBsAg, HBeAg, anti-HBc, anti-HBe, and anti-HBs antibodies are shown. Reproduced with permission from Ganem D and Prince AM. Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med.* 2004 Mar 11;350(11):1118-29. Copyright Massachusetts Medical Society.

Three stages of chronic HBV infection are recognised: the immune tolerant phase, the chronic hepatitis B phase, and the inactive chronic carrier phase (Table 1).

Stage	HBeAg/Anti-HBe status	ALT	HBV DNA (copies/ml)	Liver histology
Immune tolerant	HBeAg	Normal	>100,000	Normal or minimal inflammation
Chronic hepatitis B	HBeAg or anti-HBe	Elevated	>100,000	Chronic inflammation
Inactive hepatitis B	Anti-HBe	Normal	<100,000	Normal or minimal inflammation

Table 1. Stages of chronic HBV infection. From the National Institutes of Health Workshop on Management of Chronic Hepatitis B 2000.

The immune tolerant phase most commonly occurs after perinatal transmission and is characterized by the presence of HBeAg, high serum HBV DNA levels, normal ALT, and minimal or no liver inflammation on biopsy. The term "immune tolerant" do not really have any immunological meaning except for defining the presence of a low ALT level as have been reported in a recent work that showed that children and young adult patients with CHB, contrary to current opinion, are not more tolerant to HBV antigens than their adult counterparts; they possess HBV-specific T cells with the ability to expand and produce distinct antiviral cytokines,[25] illustrating the complexity of defining disease activity in CHB and the limitations of disease classification based on serology or biochemical markers alone. It also highlights the inadequacy of ALT levels in reflecting the presence/absence of an antiviral T cell response in these patients.

The chronic hepatitis B phase is seen after horizontal transmission of HBV during early childhood or adulthood, and usually occurs later in life in persons who acquired HBV infection from vertical transmission. It is characterized by elevated ALT levels, high levels of HBV DNA, active liver disease on biopsy and is a dynamic phase. In persons who are HBeAg+, spontaneous seroconversion from HBeAg to anti-HBe may be preceded or accompanied by a transient rise in ALT, also know as a flare, and this process reflects immune-mediated destruction of infected hepatocytes. Reductions in the level of viremia as great as five orders of magnitude may accompany seroconversion to anti-HBe antibodies.

The inactive chronic carrier phase, also known as the "asymptomatic chronic carrier" state, occurs after HBeAg to anti-HBe seroconversion, most persons have normal ALT levels and lower levels of HBV DNA. 70 – 80% of carriers remain in the inactive carrier phase indefinitely. About 10 – 20% may have one or more reversions back to the HBeAg+ state, usually accompanied by elevations in ALT levels, during which liver inflammation reactivates. Others develop chronic anti-HBe+ hepatitis, which is characterized by elevated ALT levels, HBV DNA levels $>10^5$ copies/ml and active liver disease.

2.6 HEPATOCELLULAR CARCINOMA

The primary adverse outcomes of chronic HBV infection are cirrhosis and hepatocellular carcinoma (HCC). HBV is a leading risk factor for HCC, with over 80% of HCC cases occurring in the regions where HBV is endemic (i.e. China, Southeast Asia and sub-Saharan Africa)[26] and chronic HBV infection strongly predisposes to the development of HCC. Other risk factors include male gender, age > 45 years, having a first-degree relative with HCC, the presence of cirrhosis, HBeAg+ status in adults, and reversion from anti-HBe to HBeAg status.[27,28] HBV promotes tumorigenesis by integrating into the genome of HCC cells[29] and a high frequency of HBV integrations has been observed in HBV-related HCC tumor[30] and non-tumor[31] liver tissues. The accumulation of genetic damage due to chronic immune-mediated hepatic injury during chronic HBV infection further drives the development of HCC.[32]

2.7 IMMUNE RESPONSE DURING HBV INFECTION

HBV is a hepatotropic, non-cytopathic virus, and it is widely accepted that both viral control and liver pathology is mediated by the host immune response. In contrast to most viruses, HBV has a peculiar kinetic of replication. While most viruses (for example, HCV, HIV, HCMV, influenza and dengue) enter a logarithmic phase of propagation immediately after infection, resulting in febrile symptoms; HBV infection is characterized by a delayed amplification of HBV replication and spread, and acute HBV infection is mainly asymptomatic. Moreover, while viral persistence is frequently associated with low viral load and protein expression (eg, HCV, HCMV), chronic HBV infection is typically characterized by the production of extremely high quantities of

HBsAg and HBeAg. An efficient control of viral infections requires the coordinate action of both the innate and adaptive immune responses. Here, we will discuss the immunological events that occur during HBV infection.

2.7.1 Innate immunity against HBV

The innate immunity plays a role immediately after infection and can rapidly recognise pathogen-associated molecular patterns (PAMP) such as viral nucleic acids and viral proteins or tissue damage through pattern-recognition receptors. It induces an antiviral state on infected cells through three major mechanisms, namely (i) by producing type I interferons (IFN), (ii) directing natural killer (NK) cell-mediated killing of viral infected cells, and (iii) stimulating the production of pro-inflammatory cytokines (TNF-α, IL-6, IL-12) which might then induce an efficient triggering of the adaptive immunity.[33]

A limited or even absent activation of innate immunity seems to be the hallmark of acute natural HBV infection. Pro-inflammatory cytokines are often undetectable during the early phases of infection (within the first 30 days) in humans infected with HBV and, when present, their production is of lower magnitude and delayed kinetics compared with HCV and HIV infected patients.[34] A lack of induction of type I interferons was also seen in a cohort of 21 acute HBV patients, eight of whom were sampled from the start of the viral expansion phase.[35] These observations support seminal results obtained in HBV infected chimpanzees, showing lack of induction of IFN-related genes during the phase of viral entry and expansion.[36] A lack of IFN-I induction might reflect the capacity of HBV to escape innate recognition. This could be the result of the replication strategy of HBV which uses a transcriptional template (cccDNA) that is sequestered within the nucleus of infected cells, where it might not be detected by the innate DNA sensing cellular machinery, and produces polyadenylated viral mRNA that resembles the normal cellular transcripts. Moreover, newly transcribed genomes are protected within viral capsids in the cytoplasm.

On the other hand, an opposing scenario has been reported by others and this is supported by different lines of evidence that HBV can be sensed by the innate immunity.[37–39] For example, HBV replication in HepaRG (a cell line that is physiologically closer to normal hepatocytes and is permissive of HBV infection) activates IFN-β and other interferon-stimulated genes.[38] In addition, acute infection with high doses of woodchuck hepatic virus (WHV) can induce immune genes immediately after infection;[39] and Kupffer cells, despite not supporting active HBV replication, seem to be able to sense HBV with up-regulation of IL-6 production.[40] A transient though slight activation of IFN-α genes was also detected in human hepatocytes infected by HBV in chimeric mice.[41]

The above conflicting results are likely due to the experimental limitations associated with the study of early immunological events during HBV infection, and that they are derived from very different experimental systems. Data obtained from patients after natural infection with HBV are limited by the difficulty in recruiting patients at the

earliest pre-symptomatic stages of acute infection. Additionally, an *in vitro* infection system for HBV in non-transformed hepatocytes is not widely available, its infection efficiency is around 10% of cultured hepatoma cell line and the level of HBV replication is low.[42,43] Animal models, although able to provide good physiological data, are hampered by ethical issues and high costs (chimpanzees), by a scarcity of reagents to analyse immunological events (woodchucks),[44] and by technical challenges to standardise the number of human hepatocytes grafted in chimeric mice.[45]

It is also possible that HBV can actively suppress instead of escaping the innate immunity through the action of different viral proteins. For example, it has been shown that HBV polymerase has the potential to inhibit IFN-β induction by interfering with interferon regulatory factor signaling;[46,47] while the hepatitis B X protein (HBx) can actively inhibit the innate immunity by interfering with signaling mediated by the cytosolic sensory molecules (RIG-I, helicases) through interaction with the β interferon promoter stimulator 1 (IPS-1) protein which is essential for the induction of type I IFN.[48–50] However, all these studies were performed on HBV transfected cells; intracellular IFN-β production was activated by heterologous inducers (poly dAT:dAT or poly I:C or vesicular stomatitis viruses); RIG-I and IPS-1 were overexpressed in HepG2 cells, while HBx/IPS-1 interaction was detected by over-expressing HBx. The real efficiency of inhibitory mechanisms mediated by HBV proteins in these artificial systems is however difficult to translate to the natural infection.

Innate immunity inhibition in HBV infection can also result from the action of HBV proteins (HBsAg, HBeAg) actively secreted by HBV during its replication cycle. There have been reports that secretory HBV proteins can abrogate the TLR-induced innate response[51] and modulate the surface expression of TLR-2,[52] but these need to be demonstrated in more physiological systems. There is also some experimental evidence on the possibility that the secretory HBV proteins can interact and modulate the inflammatory liver environment directly or through the induction of immunosuppressive cytokines, such as IL-10.[35,52]

Finally, a major cellular component of innate immunity contributing to the initial containment of viral infection is NK cells, which are able to recognize and kill viral infected cells. NK cells are extremely abundant in the normal liver, constituting 30 – 40% of intrahepatic lymphocytes.[53] Moreover, hepatocytes express very low levels of MHC class I[54] such that there would be minimal engagement of inhibitory NK cell receptors, and any up-regulation of cellular stress ligands able to engage NK activatory receptors should be sufficient to induce local NK cell effector function. NK cells can also be directly activated by cytokines, such as type I IFN and IL-12, induced in viral infections. In chimpanzees, the clearance of HBV infected hepatocytes by adaptive immunity was found to be preceded by an increase in intrahepatic IFN-γ and TNF-α, which could be produced by NK cells.[55] However, subsequent experiments showed a critical role for T cells rather than NK cells in HBV control in this model.[56] Studies of patients around the time when HBsAg and

HBV DNA was first detected revealed an increase in the number of circulating NK cells,[57,58] but their activation and effector function was suppressed as viral load increased and only peaked once viraemia had resolved.[35] This inhibition of NK cell activation and effector potential showed an inverse temporal correlation with an induction of IL-10, raising the possibility that HBV can actively evade immune responses. No data are available regarding the involvement of NK cells in humans in the immediate response to infection. However, recent results obtained in woodchucks infected with high WHV dose (10^{11}) show an activation of NKp46, a gene related to NK cell activation immediately after infection (8 – 12 h).[39] It is therefore possible that NK cells might contribute to HBV control in the earliest lag phase of infection, but such a hypothesis still awaits proper demonstration. Additionally, NK cells might contribute to liver damage, as there is an increase in the $CD56^{bright}$ subset coinciding with flares of liver inflammation that can induce hepatocyte death through the TNF-related apoptosis-inducing ligand (TRAIL).[59]

Overall, the majority of data seem to indicate that during natural HBV infection induced by low infectious dose and characterized by the typical kinetics of HBV amplification, innate immune activation of an intracellular antiviral response is absent or weak, possibly due to a combination of inefficient triggering and active HBV-mediated suppression of the innate immunity. A better understanding of the ability of the innate immune response to control HBV infection during natural infection still awaits data generated in more physiological systems.

2.7.2 Adaptive immunity against HBV

The adaptive immune response consists of an intricate web of effector cell types that play important roles in the development of anti-HBV immunity. CD4 T cells, classically referred to as helper T cells are robust producers of cytokines. They are also required for the efficient development of effector cytotoxic CD8 T cells and B cell antibody production. CD8 T cells are able to recognize virus-infected cells and clear HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms,[60] thus reducing the levels of circulating virus. B cells can neutralize free viral particles through antibody production to prevent (re)infection[61] and might modulate helper T cell function through their ability to present HBV antigens.

HBV-specific CD4 and CD8 T cells-mediated responses become generally detectable immediately after the start of the exponential increase in HBV replication, which follows an initial phase of negative or weakly positive HBV-DNA lasting for about 4 to 7 weeks after infection. There are clear differences in the adaptive immunity of patients with resolved HBV or chronic infection. The CTL response to HBV is vigorous, polyclonal, and multi-specific in patients with acute hepatitis who ultimately clear the virus, while it is weak or barely detectable in patients with chronic hepatitis (Figure 5).

CD8 T-cell depletion experiments performed in HBV infected chimpanzees have provided strong evidence that CD8 T cells are the main cellular subset responsible for viral clearance.[56] Viral clearance is mediated by both cytolytic and noncytolytic

effector functions of the CD8 T cell response. CD8 CTLs can induce infected hepatocytes to undergo apoptosis via the Fas-Fas ligand pathway, killing them.[62] However, this one-on-one process by which CTLs kill their targets is relatively inefficient, considering that the number of infected hepatocytes is so large relative to the number of virus-specific CTLs. Instead, HBV-specific CD8 CTLs can inhibit HBV replication in the liver by a noncytolytic pathway that is mediated by IFN-γ and TNF-α secreted following antigen recognition.[63]

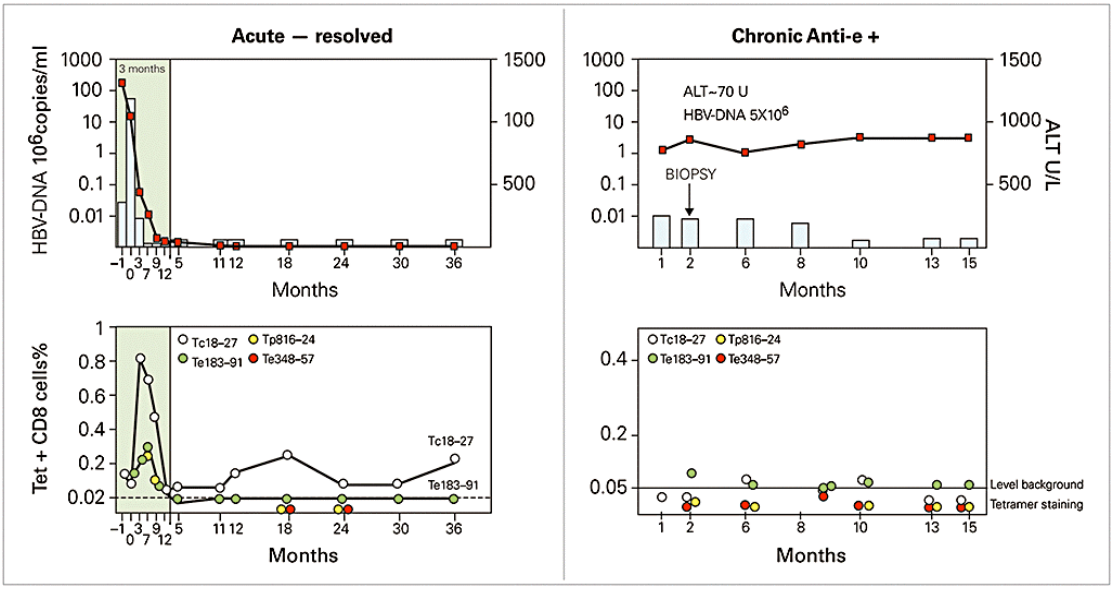


Figure 5. A comparison of HBV-specific CD8 T cell responses in acute-resolved and chronic HBV patients. Longitudinal profile of HBV DNA (line), alanine aminotransferase (ALT, bars, upper panels) HBV-specific cytotoxic T lymphocytes in one acute HBV patient (left) and one anti-hepatitis B e (HBe)-positive HBV chronic patient (right). © 2008 The Authors. Journal compilation © 2008 Journal of Gastroenterology and Hepatology Foundation and Blackwell Publishing Asia Pty Ltd.

In contrast to their protective role, virus-specific CTLs have been implicated as the principal effectors of liver damage. The activation of intrahepatic HBV-specific T cells can trigger an influx of inflammatory mononuclear infiltrate (consisting granulocytes, monocytes and T cells) to the liver, which is the principal cause of hepatic injury. This has been clearly demonstrated in HBV transgenic mice, whereby adoptively transferred HBV-specific T cells trigger the recruitment of neutrophils and mononuclear cells that result in liver damage.[64] Interestingly, depletion of neutrophils prior to T cell transfer abolished the inflammatory infiltrate into the liver without affecting the antiviral efficiency of HBV-specific T cells or reducing CXCL-9 and CXCL-10 production,[65] two chemokines induced by IFN-γ known to recruit inflammatory cells to the liver.[66]

In humans, different levels of HBV replication can coexist with similar numbers of HBV-specific CD8 T cells in the circulation and liver of CHB patients. CHB patients lacking evidence of liver damage but controlling HBV replication possess functionally active HBV-specific CD8 T cells both in the circulation and in the liver.

By contrast, in patients with a high level of HBV replication and evidence of liver inflammation, the frequency of intrahepatic CD8 T cells specific for core 18–27 was much lower due to their dilution in a large infiltrate of antigen non-specific T cells. However, the actual number of intrahepatic HBV-specific CD8 T cells was similar to that seen in patients without liver disease, taking into account the difference in the size of the total CD8 infiltrate. These results in chronic HBV infection show that comparable numbers of intrahepatic virus-specific CD8 T cells could be associated with either protection or pathology.[67] It is likely that CTL responsiveness, rather than absolute numbers, is associated with the ability to control HBV. By contrast, an inadequate CTL response in relation to the infecting virus could derive from the inability to mount a multispecific repertoire[68] or from differences in help from CD4 T cells.[57,69] This chronic stimulation of a relative inefficient CTL response to contain HBV replication could lead to liver damage.

The inability to control HBV infection and the establishment of chronicity leads to a state of relative collapse of virus-specific adaptive immunity. Experiments performed in animal models of viral infections like lymphocytic choriomeningitis virus have clearly demonstrated that the sustained presence of viral antigens leads to a progressive functional decline of virus-specific CD8 T cell responses and ultimately deletion of virus-specific T cells.[70,71] Similarly, in HBV-infected patients, the frequency and function of circulating and intrahepatic HBV-specific CD8 T cells are inversely proportional to the level of HBV DNA;[72–74] suppression of HBV-specific T cell responses is more profound in patients with high HBV viral load and T cells are more dysfunctional within the liver than in the periphery. This defective T cell function is probably maintained primarily by the prolonged exposure of T cells to high quantities of viral antigens,[75] the tolerogenic features of the liver[76–78] and perhaps by the contribution of regulatory cells[79–81] or dendritic cell defects.[82,83] Other mechanisms, such as impairment of T cell receptor signaling by CD3ζ chain down-regulation,[84] increased levels of arginase[84,85] and enhanced T cell apoptosis caused by Bcl2-interacting mediator (Bim) up-regulation[86] can also contribute to alter the T cell function. The functional exhaustion of virus-specific T cells is characterized by an increased expression of negative co-stimulatory molecules and a dysregulation of co-stimulatory pathways,[72,87,88] such as PD-1/PDL1 that affects the quality and intensity of the antiviral T cell response.

Despite the cellular immune response being a major contributor to HBV clearance, humoral responses also play a role in the control of HBV. HBV clearance is associated with the production of anti-envelope antibodies[61] and sera with high levels of antiviral antibodies (specific for the viral envelope) can control HBV infection.[89] Thus, the coordinated activation of both the cellular and humoral arms of the adaptive immune response ultimately allows the host to control the infection.

2.8 IN VIVO MODELS TO STUDY HBV INFECTION AND HCC

The study of HBV infection *in vivo* has been hampered by the limited range of

appropriate animal models that recapitulate human infection. Substantial challenges persist in modeling HCC in animals whose natural history requires continuous cycles of chronic inflammation/injury and fibrosis leading to malignancy. Besides humans, HBV infects chimpanzees and tree shrews. The limited availability, high costs, endangered status, and the lack of chronic liver disease precludes the study of pathogenesis of cirrhosis and HCC in chimpanzees; while the low infectivity and transient nature of HBV infection,[90] in addition to the lack of reagents to analyse the immune response are limitations to studies in tree shrews. Studies performed with other hepadnaviruses, such as woodchuck hepatitis B virus (WHBV) and duck hepatitis B virus (DHBV) have contributed to the understanding of HBV replication. Nevertheless, these models have shortcomings: WHBV and DHBV are similar but not identical to HBV and cause slightly different spectrum of liver diseases; the lack of inbred strains and immunological reagents to study lymphocyte subsets in woodchucks remains an obstacle to investigation of the immunological pathogenesis of HBV infection. Thus, an appropriate, inbreed, laboratory small-animal model is necessary for studying immunopathogenesis during HBV infection or HCC. Some of the commonly used mouse models of HBV infection and HCC are summarized in Table 2. There are limitations to each mouse model, and there is no single animal model that is ideal for studying all features of HBV. Thus, it is up to investigators to select the appropriate model to answer particular questions.

Models	Technical method	Applications	Limitations
HBV infection			
Transgenic mouse	HBV DNA (full genome or specific HBV genes) is microinjected into fertilized eggs, followed by implantation of eggs into pseudo-pregnant females	• Antiviral innate and adaptive immune responses[91,92] • Immunopathogenesis[64–66] • Immune tolerance[93,94] • Testing immunomodulatory therapies[95]	• HBV does not infect mouse hepatocytes (no true infection) • Absence of HBV cccDNA • Mice are immunologically tolerant to HBV antigens
Hydrodynamic HBV-transfected mouse	Tail vein injection of HBV plasmid in a volume ~8-12% of body weight preferentially delivers HBV transgene into hepatocytes and induces acute hepatitis	• Innate and adaptive immune responses[96,97]	• HBV does not infect mouse hepatocytes (no true infection) • Off-target transfection of other organs • Transient transfection precludes generation of persistently transfected mice without

			additional manipulations
Human-mouse liver chimera	Transplantation of human hepatocytes into uPA-SCID or $Fah^{-/-}Rag2^{-/-}IL\text{-}2R\gamma^{-/-}$ mice that are permissive for HBV infection	• Host innate immune response[41] • HBV infection[98] • Antiviral drug testing[99]	• Difficult to standardize number of engrafted human hepatocytes • Maintenance of chimeras is challenging due to high neonatal mortality • Study of HBV immunopathogenesis and development of HCC not possible due to immunodeficient nature of the model
HCC			
Carcinogen-induced	Administration of genotoxic carcinogen (e.g. diethylnitrosamine, CCl_4) over a prolonged period generates a model that mimics the injury-fibrosis-malignancy cycle seen in humans	• Molecular mechanisms of hepatocarcinogenesis[100]	• Age, sex and genetic background of mice influence predictability of HCC development
HBV transgenic	Microinjection of HBV DNA fragment encoding the viral envelope into fertilized eggs, followed by implantation of eggs into pseudo-pregnant females	• HBV-induced hepatocarcinogenesis[101,102]	• Not a model of immunologically induced chronic hepatitis leading to HCC
Xenograft tumor	Human tumor cell lines (e.g. HepG2, Hep3B, Huh7) or tissue fragments are implanted in immunodeficient mice	• Preclinical evaluation of anticancer agents[103]	• Proof-of-principle experiments only, results can seldom be repeated in cancer patients

Table 2. Mouse models of HBV infection and HCC.

2.9 TREATMENT OF CHRONIC HEPATITIS B AND HBV-RELATED HCC

There are seven drugs approved by the FDA for the management of chronic HBV infection: interferon (IFN)-α, pegylated interferon (PEG-IFN) and nucleoside (lamivudine, entecavir, and telbivudine) or nucleotide (adefovir and tenofovir) analogues. An ideal anti-viral agent should lead to complete clearance of HBV and seroconversion of HBsAg to anti-HBs, but this is rarely achieved with current available anti-HBV agents. IFN-α is well known for its side effects and has limited therapeutic effects: loss of HBsAg is achieved in < 8% of patients,[104] while nucleos(t)ide analogues inhibit the viral reverse transcriptase but do not eliminate the HBV cccDNA. Although nucleos(t)ide analogues are effective in suppressing HBV replication, patients rarely establish immunological control over HBV and elimination of the virus is rarely achieved due to the persistence of HBV cccDNA in the host cell nucleus and the cellular integration of HBV DNA into the host genome. Moreover, the response is hardly durable as patients might experience reactivation of disease after therapy interruption, with HBV replication levels rebounding to pretreatment levels, which may be accompanied by hepatitis flares that can be severe. Long-term therapy with these antiviral agents is required in patients with CHB; however, this does not seem to be a successful strategy to improve efficacy as it only modestly increases the rate of sustained responses. Furthermore, patients are exposed to potentially toxic effects of continuous treatments that are not only costly but often lead to the selection of resistant viral variants.[105]

For HCC, liver transplantation and surgical resection are the most effective standard therapies. However, only 30 – 40% of patients are eligible for such therapies, as HCC frequently remains undiagnosed until an advanced stage.[106] Therapeutic options are limited, particularly in patients with advanced disease, who almost invariably succumb to their disease. Thus far, Sorafenib, a multi-targeted tyrosine kinase inhibitor is the only drug that modestly prolonged survival in patients with advanced HCC by nearly 3 months.[107] A further major problem is that HCC recurrence in liver transplanted patients frequently occurs due to the seeding of HCC cells that often carry HBV integrations in the newly transplanted normal liver or in extra-hepatic locations. Therefore, new strategies that target HCC are needed.

2.10 IMMUNE THERAPY FOR THE TREATMENT OF CHRONIC HEPATITIS B AND HBV-RELATED HCC

Immunotherapy provides a more efficient and selective targeting of viral infected or tumor cells by boosting existing or inducing new viral/tumor-specific immune responses. The virus/tumor is eliminated by activation of either non-specific host immune responses or HBV-specific T-helper and cytotoxic T lymphocytes (CTL).

2.10.1 Cytokines

Cytokines with antiviral activity that non-specifically enhance host-immune responses or make the tumor more immunogenic by increasing MHC expression and antigen

presentation, such as IFN-γ, IL-2, IL-12 or IL-18, although able to inhibit HBV replication in mouse models, have little effect alone on HBV replication or HCC in humans. It is likely that many of these cytokines may not be potent as single agents and should be used in combinational regimens. In a trial with 20 advanced stage HCC patients, transarterial chemotherapy combined with IFN-γ and IL-2 resulted in reduced tumor burden and reduced serum α-fetoprotein (AFP) in 14 patients, indicating a positive effect of this combination treatment.[108] The effect of cytokines in combination treatments might prove effective and needs to be further investigated in clinical trials.

2.10.2 Toll-like receptor (TLR) agonists

TLRs play an important role in innate immune recognition and regulation. They recognize pathogens through their pathogen-associated molecular patterns and activate phagocytes and dendritic cells (DC) to mount an immune response against viruses and microbes.[109] TLR agonists have been shown to inhibit HBV replication in hepatocytes through the induction of antiviral cytokines in a HBV transgenic mouse model[110] and more recently, the therapeutic efficacy of a TLR-7 agonist in woodchucks[111] and chimpanzees[112] have been reported. The use of TLR agonists to stimulate the innate immune responses to HBV is a promising immunotherapeutic approach for the treatment of CHB.

2.10.3 Inhibitory receptor blockade and co-stimulatory agonists

HBV persistence is associated with impaired CD8 T cell function. Several mechanisms have been demonstrated to contribute to HBV-specific CD8 T cell failure in chronic HBV infection, such as inhibition via the programmed cell death 1 (PD-1) or cytotoxic T-lymphocyte antigen 4 (CTLA-4) pathways. A possibility to boost endogenous HBV-specific immunity is to block inhibitory interactions or to provide co-stimulatory agonists. Blocking the PD-1/PD-L1 interaction by using anti-PD-L1 antibody has already been shown *in vitro* to increase the frequency and function of HBV-specific T cells in chronic HBV patients.[72] Blockade of the co-inhibitory 2B4[88] and cytotoxic T lymphocyte antigen-4 (CTLA-4)[87] and the co-stimulatory CD137[113] pathways were also tested alone or in combination with PD-1/PD-L1 blockade to optimise functional T cell restoration. The CTLA-4 blocking antibody tremelimumab has recently been tested in a phase II clinical trial (NCT01008358) in patients with advanced HCC and it will be interesting to see if blocking CTLA-4 is able to induce durable antitumor responses. Additionally, it is possible that simultaneous blockade of T-cell inhibitory receptors in combination with therapeutic vaccination might increase the quantity of HBV-specific T cells with effector functions capable of eliminating infected hepatocytes or HCC.

2.10.4 Therapeutic vaccines

A therapeutic vaccine is assumed to overcome T-cell tolerance and induce CTL in an antigen-specific fashion. Antigen-based vaccines are injected to stimulate the individual's antigen presenting cells (APCs) to take up the antigen and present it on MHC class I and II molecules to the T cells to trigger an immune response. Various nucleic acids-based, peptide/proteins-based and cellular-based vaccines have been

developed and are currently being tested in CHB and HCC. Early therapeutic vaccine trials based on the use of HBsAg prophylactic recombinant vaccines had not been successful in CHB patients. Few patients achieved HBsAg clearance and anti-HBs seroconversion. The therapeutic efficacy of vaccines designed to specifically induce HBV-specific CTL responses have also been disappointing. DNA vaccines encoding HBV envelope proteins transiently increased HBV-specific CD8 T-cell frequencies in chronic HBV carriers but without sustained clinical and virological therapeutic efficacy.[114,115] Therapeutic vaccination using a modified HBcAg delivered by a retroviral vector showed a possible effect in one out of three treated chimpanzees.[116] An immunotherapy pilot study using the Theradigm™-HBV vaccine, which includes a single immunodominant HBV core $CD8^+$ T-cell epitope, was not effective in patients with chronic hepatitis B[117] despite being immunogenic in healthy individuals.[118] Furthermore, vaccination strategies using HBsAg-loaded DCs gave conflicting results.[119] Nonetheless, interesting data were obtained in a recent phase III clinical trial with an antigen-antibody complex (HBsAg-HBIG) as a therapeutic vaccine candidate for CHB patients.[120] Immunizations with the antigen-antibody complex with alum as adjuvant resulted in a decrease of the HBeAg seroconversion rate from 21.8% to 14.0%, but an increase from 9% to 21.9% in the alum alone group, suggesting that multiple injections of alum alone could have stimulated potent inflammatory and innate immune responses. This result is also in line with a recent work whereby circulating viral antigens in CHB patients could be cross-presented by monocyte-derived DCs and used to activate autologous virus-specific T cells.[121] The use of adjuvants or cytokines for therapeutic vaccination, relying on viral antigen present in patients during chronic infection seems promising, and should be further explored.

For HCC vaccines development, HCC-specific immune responses can be induced by targeting tumor-associated antigens or viral antigens. Alpha fetoprotein (AFP) is a commonly targeted HCC-specific tumor associated antigen. AFP is the most abundant serum protein in the fetus, but is transcriptionally repressed after birth. However, it is re-expressed in HCC. Immunization with AFP peptides generated transient AFP-specific T cell responses in the peripheral blood, but regrettably no clinical responses in HCC patients.[122] A follow-up trial using AFP-loaded autologous DC resulted only in transient CD8 T-cell responses, possibly due to the lack of CD4 T cell help.[123] Other proteins whose expression is upregulated in the majority of HCC tumors and are studied as potential HCC targets include the cancer-testes antigens (MAGE-A, NY-ESO), Glypican-3, and Aurora-A. A particular concern with targeting these proteins is that they are self-antigens, and antitumor immunity might result in autoimmunity. Viral antigens in patients infected with HBV or HCV are other potential targets. They have the advantage of being foreign proteins that are not recognized as “self” by the immune system. However, it is important to use HBV-specific T cells with caution since both normal and transformed hepatocytes can express HBV viral antigens, and immune responses targeting HBV viral proteins have been implicated in the pathogenesis of liver injury during chronic HBV/HCC.

In the case of unknown or mutated HCC antigens, whole tumor cell or tumor lysate-based immunization strategies can be employed. A subsequent phase II clinical trial

using autologous DC pulsed with a liver tumor cell line HepG2 lysate before infusion in patients with advanced HCC reported functional IFN-γ-producing antigen-specific T-cell responses, disease stabilization or reduction in serum AFP levels in some patients.[124] These encouraging results suggest that further improvement of DC-based vaccines, such as using multiple tumor epitopes and in combinatorial approaches with chemotherapy or radical therapy may translate to better clinical responses.

Overall, therapeutic vaccine strategies have demonstrated the extreme difficulty in restoring HBV-specific immunity in HBV chronically infected or HCC patients. A general problem in many therapeutic vaccine studies is that high antigen doses are already present and do not induce sufficient T-cell activity, likely because of HBV-specific T-cell exhaustion or deletion commonly associated with chronic infection.[125,126]

2.10.5 Antibodies targeting infected hepatocytes or blockade of immunosuppressive cytokines and enzymes

In addition to boosting HBV-specific immune responses, a monoclonal TCR-like antibody that specifically recognizes peptide/MHC class I complex (Env183-191:HLA-A201) on the HBV-infected cell surface but does not bind free virus or the MHC class I molecules alone has been developed.[127] This antibody may allow specific delivery of antiviral drugs or cytotoxic compounds to infected hepatocytes and improve treatment efficacy.

The intrahepatic cytokine milieu within the liver microenvironment, such as the presence of immunosuppressive cytokines and increased levels of arginase might also modulate the function of HBV-specific T cells. For example, an increase in serum IL-10 has been reported during hepatic flares in CHB patients with defective CD8 T cell responses.[128] Interestingly, IL-10 blockade *in vitro* could recover polyfunctional HBV-specific CD8 T-cell responses. Blocking IL-10 as a potential therapeutic strategy to reverse the dampening of adaptive immune responses should be used with caution as IL-10 might play an anti-inflammatory role to limit liver injury during persistent infection. Additionally, replenishment of L-arginine is able to restore CD3ζ expression in CD8 T cells, accompanied by a recovery in proliferation;[84] or the addition of arginase inhibitor is able to restore CD8 T cell function.[85] Further, blocking TGF-β might also be an appealing approach for HBV immunotherapy. TGF-β can be produced by HBV- and HCV-specific T cells, leading to their auto-suppression. Blocking TGF-β might contribute to the functional restoration of CD8 T cells while reducing the pro-fibrogenic activity of this cytokine in the liver microenvironment.

2.10.6 Adoptive T-cell transfer

Besides improving current therapeutic vaccines and the restoration of existing T-cell defects, adoptive T-cell transfer that aims at generation of new virus/tumor-specific T-cell responses is an alternative to reconstitute the dysfunctional HBV-specific immunity. This concept is based on the observation that chronic HBV patients

receiving bone marrow transplantation from immune donors[129,130] or HBV immune recipients receiving a liver from a chronic HBV donor cleared or controlled HBV infection.[131] Therefore, adoptive T-cell transfer strategies are currently under development for the treatment of chronic viral hepatitis and HCC.

Adoptive immunotherapy using the transfer of viral-specific T cells is already a well-established procedure for treatment of post-transplant associated viral infections and rare viral-related malignancies using viral-specific peripheral blood lymphocytes isolated and expanded from bone marrow donors.[132–134] To target human tumors, early adoptive T-cell therapies have used autologous *ex vivo* expanded tumor infiltrating lymphocytes[135] or tumor-specific T cell clones[136] generated from autologous peripheral T cells. However, it is not possible to isolate TILs or enrich for tumor-specific T cells from peripheral blood mononuclear cells in all patients or for all tumor types. One strategy to overcome these limitations is to introduce viral- or tumor-antigen targeting receptors into human T lymphocytes by genetic engineering. Adoptive T-cell therapy with receptor gene-engineered T cells is based on the ability to transfer a viral/tumor-specific receptor into autologous T cells, expand them *ex vivo* and infuse the T cells into the patient.

Two types of recombinant antigen receptors for T cells have been described (Figure 6). In the first approach, expression of the α and β chains of a high avidity T-cell receptor (TCR) cloned from T cells selected *in vitro* can redirect the antigen specificity of a T cell. TCRs introduced in human PBLs are functional *in vitro* and identical to the reactivity of the parental viral/tumor-specific T cell.[137,138] When adoptively transferred in murine models, TCR gene-engineered T cells are antigen-specific and functional *in vivo* as evidenced by the induction of anti-tumor immune responses after antigen encounter.[139,140] Clinical gene therapy trials using TCRs as "off-the-shelf" reagents to confer tumor reactivity to T cells from patients with the appropriate HLA-restricting allele have been initiated. Encouraging results have been obtained and clinical responses were observed for patients with metastatic melanoma, colorectal and synovial carcinoma.[141–144] Most importantly, these clinical trials demonstrated the feasibility of using TCR redirected T cells for the treatment of various tumors. Based on the success in reconstituting antitumor immunity in cancer patients, we and others therefore developed a similar approach based on TCR gene transfer to reconstitute the defective antiviral immunity of chronic HBV,[145] HCV[146] and severe acute respiratory syndrome[147] patients. We successfully engineered fully functional HBV-specific T cells, by retroviral transduction of memory T cells with HBV-specific TCR. These multifunctional TCR-engineered T cells are able to recognize and lyse natural HCC lines with integrated HBV DNA.

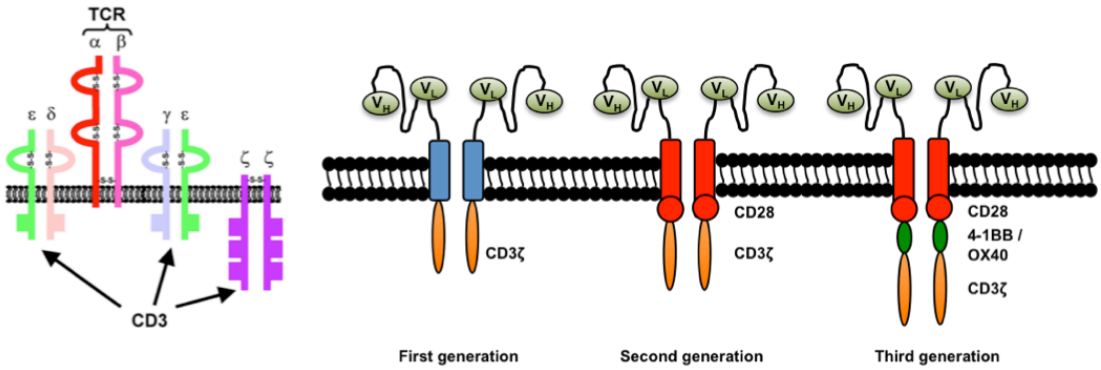


Figure 6. Structure of TCR and CARs. TCRα/β chains form a complex with CD3 components and determine the antigen specificity of a T cell. When the TCR binds its cognate peptide/MHC complex, immunoreceptor tyrosine-based activation motifs of CD3 δε, γε, and ζζ signaling dimers become phosphorylated and initiate T cell activation. CARs are typically composed of a scFv containing the heavy (V_H) and light (V_L) chain variable regions specific to a tumor antigen, fused to a transmembrane region of the CD8, fused to a cytoplasmic signaling domain of the TCR (most commonly CD3ζ). Second generation CARs include a co-stimulatory domain (CD28) while third generation CARs contain tandem cytoplasmic signaling domains (CD28-4-1BB-CD3ζ or CD28-OX40-CD3ζ).

Redirection of T-cell specificity by TCRs is limited by HLA restriction, which restricts the applicability of TCR gene therapy to patients who express the particular HLA type. In addition, tumors can lose their antigen expression by downregulation of HLA.[148] A second approach to redirect T cells towards tumors is using chimeric antigen receptors (CARs).[149] CARs are fusion proteins consisting of a tumor-antigen binding domain of a single chain antibody (scFv) fused to intracellular signaling domains capable of activating T cells upon antigen stimulation. CARs can confer non-HLA restricted specificity to T cells based on antibody recognition, which makes them broadly applicable irrespective of the patient's HLA and enables the recognition of tumor cells that have downregulated HLA expression. The presentation of target antigen to CAR T cells is not dependent on MHC expression levels and antigen processing efficiency, and there is no risk of mispairing with the endogenous TCR. However, CARs are able to target cell surface antigens/proteins only (and not MHC/peptide complexes). A potential pitfall of the use of CARs is their immunogenicity. As many of the antibodies used for CARs are murine monoclonal antibodies, human anti-mouse antibody immune responses have been reported,[150,151] and this could potentially limit the long-term clinical use of CARs. A potential application of CAR-redirected T cells has been demonstrated in HBV infection. CARs that fused the antigen-specific determinants of an anti-HBs antibody to the signaling domain of the TCR enable primary human T cells to recognize HBsAg-positive hepatocytes, release IFN-γ and IL-2, and most importantly, lyse HBV-infected primary human hepatocytes, demonstrating the therapeutic potential of CAR-redirected T cells for treatment of chronic hepatitis B.[152]

3 T CELL RECEPTOR (TCR) GENE THERAPY

T lymphocytes express a heterodimeric αβ receptor on their surface called the TCR. A given T cell recognizes its cognate antigen through the binding of its TCR to an epitope presented by major histocompatibility complex (MHC) molecules on the target cells, hence the TCR dictates the specificity of a T cell. It is possible to endow T cells with new specificities by transferring the genes encoding the alpha and beta chains of a TCR, and TCR gene transfer was first successfully demonstrated in a murine system.[153] This strategy was then applied to redirect human T lymphocytes to recognize tumor-associated antigen using a melanoma-specific TCR *in vitro*[137] and then *in vivo* using a virus-specific TCR.[139] Here, we will describe how to redirect T lymphocytes to a new specificity using TCR gene transfer, the different vector platforms available for genetic modification of T lymphocytes, the various strategies that are employed to optimize the surface expression and affinity of the introduced TCR. We will also discuss on the ways to improve the success of adoptive transfer and the safety issues of the use of TCR-engineered T cells.

3.1 DEVELOPMENT OF TCR-ENGINEERED T CELLS USING TCR GENE TRANSFER

The first step in TCR gene transfer is to isolate a high affinity T cell clone recognizing a defined target antigen. T cell clones can be isolated from tumor infiltrating lymphocytes from cancer patients, virus-specific memory T cells from healthy individuals or resolved patients that recognize an immunodominant viral epitope or from immunized HLA transgenic mice. The TCR α and β chains are isolated from a T cell clone and the cDNA cloned into a gene expression vector.[145,147] These chains are then introduced into T cells, usually by viral or non-viral vector based approaches (discussed below). Engineered T cells can be expanded in culture to numbers sufficient for clinical applications, and then adoptively transferred into preclinical mouse models for evaluation of efficacy or into patients (Figure 7).

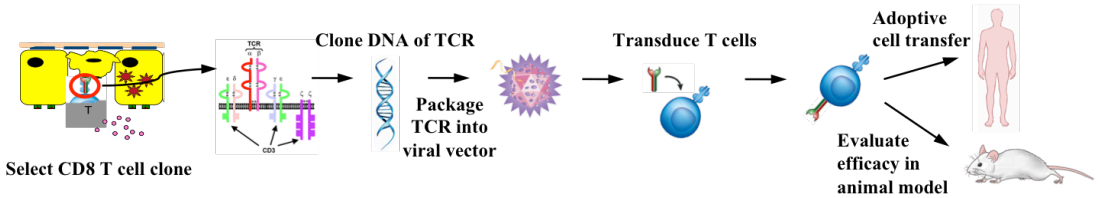


Figure 7. Genetic modification of T lymphocytes for adoptive immunotherapy. A general scheme for the engineering of T cells with TCR recognizing a target antigen.

3.2 TOOLS FOR GENETIC MODIFICATION OF T LYMPHOCYTES

3.2.1 Virus vector-based approaches

Most clinical studies to date have used integrating vectors (γ-retrovirus, lentivirus) for gene transfer in T lymphocytes. Such virus-based systems mediate stable gene transfer due to integration into the host genome, thus providing long term expression of transgenes in the transduced cells. For γ-retrovirus based vector system, the mouse stem cell virus (MSCV) or myeloproliferative sarcoma virus (MPSV) are most frequently used for TCR gene transfer studies and for clinical use to date. As retroviral vectors infect only dividing cells, T lymphocytes need to be stimulated prior to retroviral transduction. The activation of T cells with anti-CD3 monoclonal antibody and high dose IL-2 has been reported to contribute to reduction of functional potential and TCR repertoire diversity of genetically modified T cells.[154,155] Moreover, safety concerns due to possible insertional mutagenesis can lead to malignant transformation of hematopoietic stem cells.[156] Despite the higher resistance of mature T lymphocytes to malignant transformation,[157,158] such risk can certainly not be excluded.

Other studies have used lentiviral vectors due to their ability to integrate into the genome of non-dividing cells, hence avoiding the need for T cell activation prior to transduction. Nonetheless, the use of cytokines is required to achieve desirable levels of TCR expression.[159,160] Higher transduction efficiencies can be achieved with lentiviral transduction and transgenes are expressed for a longer period. Self-inactivating and conditionally replicating lentiviral vectors have been developed to improve the safety profile of using lentiviral vectors.[161,162] In the first human clinical trial using T cells modified with lentivirus vector for treatment of HIV, no adverse events have been reported so far.[163]

Viral vectors derived from human adenovirus serotype 5 and 35 (Ad5-35) have also been used in gene transfer studies, and have been reported to have transduction efficiency of up to 40 – 60% of T cells after CD3/CD28 activation. As adenovirus-based vectors do not integrate into the genome, the transgene does not persist, and hence the use of Ad5-53 vectors is limited to clinical applications where transient expression of a transgene is required. Recently, adenovirus vector is used to deliver zinc finger nuclease (ZFN) to knock out CCR5 (a receptor for HIV infection) on CD4 T cells, hence preventing HIV from killing these CD4 T cells after infection.[164] A phase I clinical trial using ZFN modified CD4 T cells for HIV treatment is currently ongoing. Hopefully such trial will give an indication of the safety profile of using ZFN-modified T cells for clinical application.

3.2.2 Non-virus-based approaches

Non-viral means to engineer lymphocytes has shown increasing promise and such approaches would reduce the production time. Electroporation can efficiently introduce plasmid DNA[1] or *in vitro* transcribed messenger RNA (mRNA)[165–167] into lymphocytes for transient expression of transgenes, and redirected T lymphocytes transduced with mRNA encoding TCRs or CARs have shown potential efficacy in therapeutic settings.

However, a major disadvantage of this method is the rapid disappearance of transgene after a few days as DNA or mRNA is not integrated into the host genome. The first clinical trial testing the adoptive immunotherapy of engineered T lymphocytes using electroporation was recently reported to have suboptimal engraftment and antitumor effects,[1] but nonetheless, demonstrated the safety and feasibility of this approach to engineer T lymphocytes.

Another non-viral approach is based on the use of the transposon system.[168] A transposon is a discrete element of DNA that has the ability to move from one chromosomal location to another. An example of a transposon that has been used in gene therapy is the Sleeping Beauty (SB). This system consists of the transposon encoding the gene of interest and an expression plasmid that encodes the transposase. The transposase catalyzes the excision of the gene of interest and its integration into the host genome. Transposon integration is more efficient than DNA plasmids that do not contain an integrating element and large transgenes (> 10 kb) can be accommodated in SB vector. SB transposons may be a safer alternative to integrating viral vectors as they have a random pattern of integration. Even though SB transposons do not show biased integration into genes, intragenic integrations can still occur which may have genotoxic risks. Further assessment of the genotoxic risks and efficacy of transposons for gene therapy in preclinical models is necessary.

Non-virus-based approaches to engineer T lymphocytes have several advantages over viral vectors. In addition to their safety profiles, clinical grade plasmid DNA- or mRNA-based electroporation are substantially less expensive and laborious than production of clinical grade virus supernatant. Moreover, the regulatory approval process for use of DNA or mRNA electroporated T cells in the clinic is easier as it is not a gene transfer procedure whereas the use of retroviral transduced cells is governed by biosafety regulations.

3.3 STRATEGIES TO OPTIMIZE FUNCTIONAL AVIDITY OF TCR-ENGINEERED T CELLS

An important aspect of TCR gene transfer is to endow T cells with superior functional avidity that can increase T cell sensitivity to target antigen and compensate for sub-optimal TCR expression. The functional avidity of TCR-engineered T cells is dictated mainly by the affinity of the TCR and the number of TCR molecules expressed on the cell surface. Thus, much effort has been focused on improvement of TCR affinity and enhancement of TCR chain pairing that will increase expression of the introduced TCR. Various strategies that have been employed to improve the functional avidity of TCR-engineered T cells will be discussed below.

3.3.1 Strategies to enhance preferential TCR chain pairing

A consequence of introducing exogenous TCR to T lymphocytes using TCR gene transfer is the ability of the introduced TCR α and β chains to mispair with the naturally

expressed endogenous TCR chains, resulting in the formation of mixed dimers. The surface expression of a TCR requires intracellular assembly with CD3 molecules, and because of limited number of CD3 components, the different forms of αβ TCR will compete for CD3, leading to reduced levels of the desired introduced TCR. Moreover, TCR chain mispairing may lead to generation of new TCRs with unknown antigen specificities that can cause autoimmunity and self-reactivity, as demonstrated in *in vitro* and *in vivo* models. To circumvent these problems, various approaches have been developed to enhance preferential pairing and expression of the introduced TCR (Table 3). It will be important to evaluate the *in vivo* consequences of these alterations in TCR gene transfer strategies with respect to the intended function of these TCR-modified cells and also the occurrence of TCR gene transfer induced autoimmune pathology.

Strategy	**Rationale**	**Evidence**
Introduce a second inter-chain disulfide bond between TCR α and β chain constant domains	Promote preferential pairing of the two modified chains and thereby limit mixed dimer formation.	Preferential pairing of cysteine-modified TCR. Increased surface expression of the introduced TCR in human TCR-modified T cells resulted in enhanced peptide-specific T cell cytotoxicity and IFN-γ production.[169,170]
Human-murine hybrid TCRs	Based on the observation that murine TCR chains have reduced propensity to pair with endogenous TCR chains when introduced into human T cells. Thus, mixed TCR dimer formation in human T cells may be reduced by using human-murine hybrid TCRs.	Human TCR chains with murine constant domains have reduced propensity to pair with endogenous TCR chains after transfer to human T cells.[171] However, murinized regions may induce immunogenicity.[172]
Chimeric TCR-CD3ζ chain	TCRα and β chain each fused to a human CD3ζ or both TCR chains fused to a CD3ζ may result in selective assembly of the modified TCR chains and therefore prevent mixed TCR dimer formation in T cells.	Selective pairing of modified human TCR- CD3ζ chains, high surface TCR expression and Ag-specific T cell functions observed in human T cells.[173,174]
Chimeric single chain TCR (scTCR)	Use of chimeric scTCR composed of VαVβCβ domains may prevent pairing with endogenous TCR chains and therefore prevent mixed TCR dimer formation in T cells.	Chimeric three-domain scTCR do not pair with endogenous TCR chains and are functional in human T cells,[174] but require higher thresholds of antigen stimulation to be functional.[175] Chimeric two-domain scTCR

		(VαVβ) fused to intracellular T cell signaling domains also reduced mispairing and mediated efficient T cell activation in mouse and human T cells.[176]
Inversion of residues in CαCβ TCR interface	Inversion of two interacting amino acid residues in the CαCβ TCR interface may result in a selective assembly of the modified TCR chains.	Preferential assembly of modified TCR observed in human T cells.[177]
Silencing of endogenous TCRs	Inhibition of endogenous TCRs and expression of resistant exogenous TCR may prevent formation of mixed TCR dimers.	Using siRNA to knock down endogenous TCRs, increased expression of exogenous TCR and increased cytotoxic function observed in human T cells.[178] Knock down of endogenous TCRs by zinc finger nucleases (ZFN) followed by introduction of exogenous TCR in human T cells avoided mispairing, maintained anti-tumor activity and reduced non-specific alloreactivity.[179]
TCR gene transfer into γδ T cells	Prevent formation of mixed TCR dimers by transfer of αβ TCR genes into γδ T cells.	γδ human T cells co-transduced with αβTCR and CD8 αβ genes acquired cytotoxicity against tumor cells and produced cytokines in both αβ- and γδ-TCR-dependent manners.[180,181]
TCR gene transfer into oligoclonal T cell populations	Oligoclonal T cell populations express a limited repertoire of endogenous TCR chains, hence TCR gene transfer may reduce the risk of formation of self-reactive mixed TCR dimers.	Oligoclonal CMV-specific human T cells with anti-leukemic reactivity can be generated by TCR gene transfer.[182]

Table 3. Strategies to limit or prevent the formation of mixed TCR dimers in TCR-modified T cells.

3.3.2 Strategies to enhance TCR ligand-binding affinity

As most of the tumor antigens are self-antigens, the isolation of high affinity tumor-specific TCRs from human donors or patients is a major challenge, since high avidity CTLs specific for cancer cells may be deleted by negative selection. Increasing TCR affinity can augment T-cell sensitivity to target antigens and compensate for sub-optimal TCR expression. Such high affinity TCR should also function in CD8 negative cells such as Th1 cells, that can provide additional support for the anti-tumor response.[183,184] Several approaches that have been developed to enhance the ligand-binding affinity of the introduced TCR are summarized (Table 4).

Strategy	**Rationale**	**Evidence**
Mutations in TCR complementarity determining regions (CDRs)	Point mutations in selective amino acids in highly polymorphic loops of the TCRα and β chain variable domains (CDRs) may increase TCR affinity.	A Gag-specific TCR mutated in CDR2β and CDR3α with enhanced affinity (pM range) efficiently controlled the spread of HIV *in vitro*.[185] Also, using an alanine scan of CDRs and site-directed mutagenesis of one or two residues, the affinities of suboptimal TCRs directed against NY-ESO-1 and CEA were increased several fold.[186,187]
TCR deglycosylation	Decreased glycosylation of surface proteins may result in a decreased activation threshold.	The removal by point mutation of defined N -glycosylation motifs in the constant domains of TCR chains can increase the functional avidity of T cells transduced with these TCRs, and enhance recognition of tumor cells.[188]
Isolation of TCRs from HLA-mismatched or transgenic donors	As allo-HLA molecules are not expressed within the thymic environment during lymphopoiesis, allo-HLA reactivity of T cells is not subjected to negative selection. Beneficial high-avidity allo-HLA-reactive T cells with antitumor reactivity may thus be identified.	High affinity human TCRs can be isolated from HLA-mismatched donors,[189,190] HLA-transgenic mice[191] or transgenic mice expressing the human TCR repertoire.[192]
Codon optimization	Due to degeneracy of the genetic code, a defined amino	Codon optimized TCRs have higher expression levels and

	acid can be encoded by several codons, which are differentially expressed in the cell. Cryptic splice sites, mRNA secondary structures and instability motifs can also reduce protein expression.	enhanced reactivity both *in vitro* and *in vivo.*[193,194]
Co-expression of CD3	Introduced TCR is likely to compete with endogenous TCR molecules for CD3 components. Endogenous CD3 chains are rate limiting for TCR expression and antigen-specific T cell function.	Co-transfer of TCR genes together with the genes encoding the CD3 complex increased TCR expression, and was associated with increased T cell avidity and enhanced anti-tumor immunity *in vivo.*[195]

Table 4. Strategies to enhance TCR ligand-binding affinity.

3.4 TYPE OR SUBSET OF T CELLS TO BE ENGINEERED

Adoptive cell transfer (ACT) approaches have largely focused on strategies to harness the effector function of CD8 CTLs as the majority of human cancers express MHC class I molecule-associated epitopes. In the majority of TCR gene therapy clinical trials performed so far, T cells from unselected peripheral blood lymphocytes were engineered with TCRs. Emerging data from preclinical and clinical studies have increased our understanding of the complexity of the immune system and the mechanisms that underlie successful immunotherapies and have identified particular T cell subsets that can most effectively promote tumor eradication.

3.4.1 CD4 T cell help

Adoptive transfer of CD4 T helper cells concurrently with CD8 T cells has been shown to result in effective anti-tumor or anti-viral T cell responses, due to the ability of CD4 T cells to promote humoral and cell-mediated immunity. Administering both CD4 T helper cells together with CD8 was able to prevent deletion or exhaustion of the infused CD8 CTLs in both animal models [196,197] and human clinical trials,[133,134] due partly to the ability of CD4 T cells to produce IL-2[198,199] and to recruit and sustain tumor- or virus-specific CD8 T cells. CD4 T cells can also activate professional antigen presenting cells through interactions of CD40 ligand and CD40,[200,201] which leads to priming of antigen-specific CD8 CTL function. This is further supported by evidence from adoptive transfer of antigen-specific CD4 Th1 cells that resulted in *de novo* generation of antigen-specific CD8 T cells and activation of endogenous CD8 T cells.[202] Moreover, CD4 T cells producing Th1-type cytokines (IFN-γ and IL-2) induced CD8 CTLs to eradicate tumors *in vivo*, while Th2 cells induced tumor necrosis through the help of other inflammatory cells,[203,204] suggesting the importance of both CD8 and CD4 T cells for effective tumor

eradication.

Because the generation of tumor-specific MHC class II-restricted CD4+ T lymphocytes is hampered by the lack of well-defined MHC class II-binding peptides and to obtain therapeutic numbers of IFN-γ producing T cells for ACT, CD4 T cells have been engineered with MHC class I-restricted αβTCRs and these engineered CD4 T cells exhibit cytolytic effector functions[205,206] and secrete cytokines in a coreceptor independent[183,207] and dependent manner.[208] Several preclinical studies in animal models[205,209,210] have demonstrated the potential of tumor-specific CD4 T cells for cancer immunotherapy. However, in a recent clinical trial in which nine patients with metastatic melanoma were treated with tumor-specific CD4 T cell clones, the results obtained were modest, with one patient showing complete response,[211] perhaps indicating the importance of combining both cytotoxic and helper T cells for effective therapy.

By contrast, regulatory CD4 T (Treg) cells characterized by expression of the transcription factor FOXP3 and high levels of expression of CD25, can suppress the anti-tumor activity of adoptively transferred tumor-reactive T cells in an *in vivo* model.[198] Intratumoral Treg cells are also over-represented in tumor lesions from patients with cancer, and they have been shown to inhibit the function of infiltrating T cells.[212–214] The immunoregulatory effects of Treg cells might contribute to the poor clinical response rates in cancer patients that receive immunotherapy in non-lymphodepleting settings. Furthermore, exogenous administration of IL-2 in a lymphodepleting setting can increase the proliferation of Treg cells.[215] Thus, the ability to selectively deplete Treg cells is likely to improve the efficacy of ACT.[216]

Taken together, CD4 T cells are able to orchestrate multiple distinct effector immune responses, and harnessing these cells may enable the development of more effective adoptive immunotherapy protocols for treatment of infectious and malignant diseases.

3.4.2 Use of less differentiated TCR-engineered T cell

Data from both preclinical studies and clinical trials indicate that the differentiation state and the degree of persistence of the adoptively transferred T cell populations is crucial for the success of ACT therapy.[217–219] CD8 T cells can be categorized into distinct differentiation states based on their phenotypic and functional characteristics.[220,221] Following activation, naïve T cells follow a progressive pathway of differentiation into effector T cells, central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). The strength of the TCR signal determines the extent of differentiation while the cytokine environment that T cells encounter during antigen-specific activation have qualitative effects on T-cell differentiation and acts in concert with TCR signalling (Figure 8).

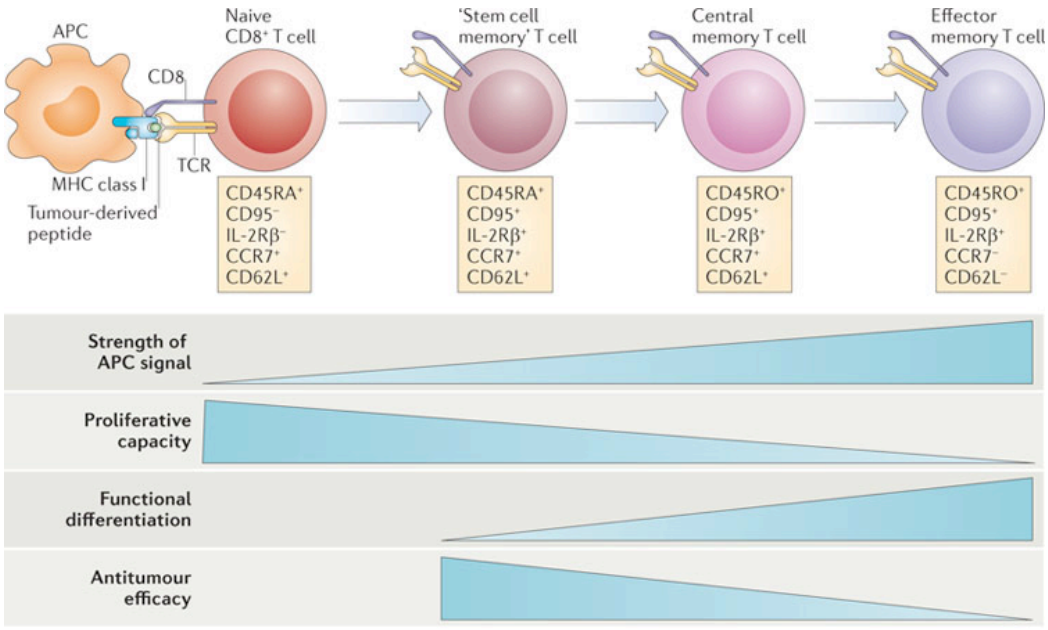


Figure 8. T cell differentiation is inversely correlated with proliferative capacity and antitumor efficacy. T cells experience progressive phenotypic and functional changes following antigen-specific activation. Depending on the strength and duration of the signals that they encounter during activation, they proliferate and differentiate into different states. T_{SCM} cells are more effective against tumors than T_{CM} cells, which are more effective than T_{EM} cells.
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The functional and phenotypic qualities associated with T cell differentiation that can affect the ability of tumor-specific T cells to mediate tumor regression in ACT therapy have been elucidated.[217] Interestingly, CD8+ T cells that acquire complete effector properties and exhibit increased anti-tumor reactivity *in vitro* are less effective at triggering tumor regressions *in vivo*. The acquisition of full effector function is associated with increased production of granzymes and reactive oxygen species, but also with the loss of ability to produce IL-2 and to home to lymph nodes, reduced replicative capacity and length of telomeres, and entry into a proapoptotic state. Current ACT therapy performed use *in vitro* expanded T cells.[222] The long culture period *in vitro* can induce progressive T cell differentiation towards a highly differentiated late effector state, resulting in T cells that are less fit to mediate anti-tumor responses *in vivo*. Similarly, highly tumor-reactive T cell clones did not induce objective regressions of cancer[136,223,224] and rapid disappearance of T cell clones was observed after adoptive transfer, possibly because they have exhausted their proliferative and survival capabilities.

On the contrary, from the naïve[225] or T_{CM}[226–228] subsets have been proposed as preferred subsets for T cell transfer-based immunotherapy due to their ability to resist terminal differentiation, maintain high replicative potential and have also shown increased anti-tumor activity compared to T_{EM}-derived ones in animal models. Moreover, CTL clones infused in patients that persisted long term acquired phenotypic

and functional qualities of T_{CM} *in vivo,*[229] suggesting the potential sustained clinical benefit of this subset. More recently, clones derived from melanoma-specific T_{CM} cells with high IL-2:IFN-γ index were shown to engraft favourably and persist after adoptive transfer, despite a modest impact on tumor growth with no objective tumor responses.[230] Adjuvant therapies such as cytokines, host conditioning and antibodies against negative regulatory molecules CTLA-4, PD-1 and TIM-3 are likely to further improve the efficacy of ACT therapy.

Another subset of T cells that is endowed with both naïve and memory properties termed T memory stem cells (T_{SCM}) was found to mediate even more potent anti-tumor efficacy than naïve or T_{CM} cells.[231] In humans, T_{SCM} cells resemble naïve T cells with CD45RA+CD45RO- phenotype. Their expression of the IL-7 receptor α-chain, as well as high levels of lymph node-homing molecules CD62L and CCR7, costimulatory molecules CD27 and CD28 resemble that of naïve T cells, while the expression of high levels of CD95 and IL-2Rβ resemble that of conventional memory T cells. Moreover, T_{SCM} cells are able to self-renew and have multipotent capacity to generate all memory and effector T cell subsets *in vitro*. The T_{SCM} subset is rare in human peripheral blood, representing 2 – 3% of all circulating lymphocytes. However, they can be generated *in vitro* by stimulating naïve T cells in the presence of TWS119, a Wnt pathway activator. The enhanced self-renewal and multipotency of the T_{SCM} cells provide a promising subset for TCR gene engineering for use in ACT for cancer.

3.4.3 Use of virus-specific T cells

Another possibility is to engineer bi-specific T cells; for example, virus-specific T cells (such as CMV, EBV of influenza) can be engineered with an additional TCR targeting tumor cells.[232,233] The use of T cells with a defined specificity may reduce the potential risk of off-target effects and toxicity and they can provide protection from latent viruses during the immunocompromised phase prior to ACT. Moreover, the continuous expression of viral antigens by latent viruses (such as EBV) following initial infection may provide constant stimulation leading to increased persistence of these cells and hence greater anti-tumor efficacy.

3.4.4 Use of hematopoietic stem cells (HSCs)

Precursor cells such as HSCs[234] and induced pluripotent stem cells[235] can be engineered to express a TCR, and then further differentiated into T cells. The plasticity and pluripotent capacity of HSCs to differentiate into any T cell subsets make them an attractive subset for TCR gene transfer. Moreover, it was shown that TCR-transduced HSCs efficiently target tumors in mouse models.[236,237] However, one caveat related to the use of engineered HSCs is the possible transformation induced by retroviral vectors,[238,239] although it may be circumvented using non-viral-based platforms.

3.5 COMBINATION THERAPY

3.5.1 Administration of exogenous cytokines or ex vivo expansion of lymphocytes with cytokines

Early studies of adoptive transfer of tumor-infiltrating lymphocytes in combination with the administration of IL-2 have shown to mediate the regression of tumors in humans.[240–242] IL-2 is often used to support the persistence, expansion, and activation of adoptively transferred T cells in many current clinical studies. However, it has undesirable effects such as decreasing the expression of lymph node homing molecules and promoting the terminal differentiation of T cells, driving them to activation-induced cell death.[243,244] This cytokine also has the potential to expand Treg cells,[215] and severe toxicity is associated with high dose IL-2 therapy.[245]

Alternative γ_c chain cytokines have been explored either for exogenous administration or for *in vitro* culture of T cells. IL-15 is a cytokine highly related to IL-2 that induces the mild expansion of memory CD8 T cells, NK cells, and NKT cells.[246] Administration of antitumor T cells in combination with IL-15 have been demonstrated to increase the persistence and function of the adoptively transferred cells in preclinical animal models.[247,248] Interestingly, administration of a complex of IL-15 and its receptor IL-15Rα enhances the biological activity of IL-15 and promotes rapid tumour regression mediated by T cells.[249] IL-12 combined with IL-15 have also been shown to mediate tumor rejection *in vivo*, possibly through Th1 priming effect of IL-12 and boosting of IFN-γ-producing CD8 T cell responses mediated by IL-15.[250] Due to the encouraging results obtained from preclinical models, a clinical trial with intravenous IL-15 administration following a nonmyeloablative lymphocyte depleting chemotherapy and autologous tumor-infiltrating lymphocytes transfer in patients with metastatic melanoma is already ongoing.

IL-7, a cytokine involved mainly in memory T cell survival, has also shown beneficial effects in ACT. The combination of IL-7 and IL-15 was demonstrated to generate gene-modified human T_{CM} cells that showed potent antigen reactivity and prolonged persistence in a mouse model.[251]

IL-21 is another recently identified cytokine that share the γ_c chain with IL-2. Antigen priming of CD8 T cells in the presence of IL-21 suppresses differentiation of naive T cells into cytolytic effector T cells, which in turn enhances *in vivo* persistence and increases the antitumor efficacy of cells for adoptive transfer.[252] Moreover, unlike IL-2, antigen-expanded cells do not undergo AICD in the presence of IL-21, but they survive while maintaining effector function.[253] *Ex vivo* expansion of primary T cells derived from melanoma patients,[254] or primary mouse T cells that are transduced with human TCR,[255] in the presence of IL-15 and IL-21 results in improved T cell cytotoxicity and production of IL-2 and IFN-γ. In summary, these results suggest the potential of alternative γ_c chain cytokines for use in adoptive T cell therapy in the clinic, as undesirable effects associated with the use of IL-2 can be avoided.

3.5.2 Preconditioning regimens

Besides the use of cytokines, preclinical studies have indicated that immune ablation is an effective preconditioning regimen that can enhance T cell responses after adoptive transfer.[256,257] Early studies using TILs with cyclophosphamide preconditioning resulted in objective response rate of 35%.[241] Improved objective response rates up to 51% and 72% can be achieved with ACT following lymphodepleting non-myeloablative chemotherapy[135] or chemoradiation[258] respectively. Depleting the host immune system prior to ACT can augment the efficacy of tumor-reactive T cells. Immunosuppressive cells such as endogenous Treg cells and myeloid suppressor cells are eliminated. Moreover, endogenous immune cells that compete for activating cytokines (i.e. cytokine sinks) are depleted, and thus cytokines are more readily available for the infused T cells. Infused T cells can then proliferate independently of self-peptide-MHC complexes in the lymphopaenic environment, a process known as homeostatic proliferation.[259] Systemic chemotherapy or total body irradiation before adoptive cell transfer might also modify the tumor-bearing host.[260,261] Necrosis or apoptosis of tumor cells due to these preconditioning treatments may result in APC uptake of tumor antigens and subsequent cross-presentation of these antigens to the adoptively transferred T cells.[262] There might be a net increase in lymphocyte activation because of reduced competition for antigen at the APC surfaces.[263] Concurrently, the release of Toll-like receptor agonists after mucosal damage can activate dendritic cells and increase lymphocyte activation.[264] Through several mechanisms, lymphodepletion enhances the engraftment, proliferation and effector functions of transferred T cells and will ultimately increase their antitumor reactivity.

3.6 SAFETY ISSUES

Beyond improvement of antitumor responses, the other major challenge of TCR gene therapy is to prevent or limit toxicity. One major advantage of using TCR-engineered T cells for ACT therapy is their autologous origin, which facilitates their engraftment and persistence *in vivo*. However, certain transgenes or part of them (e.g. murinized TCR, virus-derived 2A peptides) might trigger immunogenicity. For example, antibodies directed to the TCR variable regions has been found in patients treated with lymphocytes expressing murine TCRs.[172] Previous studies have also shown that the transfer of tumor antigen-specific T cells can cause autoimmune manifestations[265,266] due to reactivity to normal tissues expressing the targeted antigen (on-target toxicity). Choosing a target antigen of which the expression is restricted to the tumor tissue might prevent such toxicity. However, this is not always possible for all tumors. For example, in the context of HBV-related HCC, HBV antigen expression is not exclusive to transformed hepatocytes; non-tumor hepatocytes might also express HBV antigens. Since HBV-specific CD8 T cells have the potential to initiate liver damage through the killing of infected hepatocytes, a specific concern regarding the use of anti-HBV TCR-redirected T cells in HBV-related HCC is that adoptive T-cell therapy could trigger severe liver damage.[64,67,267] Other possible risks associated with TCR gene therapy are the possible newly generated specificities associated with TCR mispairing (off-target

toxicity),[268,269] and insertional mutagenesis due to viral integration. Despite the various safety concerns, thus far, γ-retrovirus- and lentivirus-transduced T cells have a proven safety record for use in human T-cell therapy.[158,270]

Several approaches have been developed to eliminate engineered T cells in case of adverse events. The introduced vector may include a suicide gene such as Herpes Simplex Virus I – thymidine kinase (HSV-TK)[271] or an inducible Caspase 9 molecular switch (iCasp9).[272] Cell death can be triggered when desired by Ganciclovir or a chemical inducer of dimerization respectively. Peptide tags (such as c-myc or HA) can be added to the N-terminal part of the TCR, and antibodies directed against the tag can be injected to deplete TCR-expressing T cells *in vivo*.[273] An alternative approach to avoid long-term toxicity of transferred T cells is to use non-virus-based approaches. T cells transiently expressing the introduced TCR can be generated using non-virus-based approaches such as mRNA electroporation, and we have taken this approach to generate anti-HBV T cells. The transient expression of anti-HBV TCR and their self-limiting toxicity is an advantage in the context of HBV-related HCC to minimize liver injury.

Strategies to avoid immunogenicity of xenograft receptor, off-target toxicity, and insertional mutagenesis have been discussed in previous sections.

4 AIMS OF THESIS

This thesis aims to study the immunological events before and during hepatic flares in chronic hepatitis B patients and to develop a new therapeutic strategy to reconstitute T cell immunity in chronic HBV or HBV-related HCC patients. The specific aims of each study are outlined here.

Paper I:

The pathogenesis of hepatic flares in patients chronically infected with HBV is controversial and the events preceding hepatic flares are difficult to study. Therefore, we studied the kinetics of innate and adaptive immune activation before and during hepatic flares in chronic hepatitis B.

Paper II:

Virus-specific T cells are essential for the control of HBV infection but are also implicated in triggering the inflammatory events leading to hepatic injury. The ability of virus-specific T cells to orchestrate such inflammatory phenomenon is not well understood. Thus, we characterized the inflammatory potential of virus-specific T cells, analyzing their ability to produce different effector molecules during different phases of HBV infection.

Paper III:

Virus-specific T cells capable of controlling HBV and eliminating HCC expressing HBV antigens are deleted or dysfunctional in patients with chronic HBV or HBV-related HCC. The aim of this study was to determine if TCR gene transfer can reconstitute HBV-specific T cell immunity in lymphocytes of chronic HBV patients and investigate whether TCR-redirected T cells can recognize HCC cells with natural HBV-DNA integration.

Paper IV:

The use of viral vectors to introduce exogenous HBV-specific TCR on T cells to redirect their specificity is complex and expensive to implement in clinical trials. Moreover, it raises safety concerns related to insertional mutagenesis and potential toxicity of long-lived HBV-specific T cells in patients with persistent infection. We therefore determined if anti-HBV T cells generated by mRNA electroporation can acquire anti-HCC potential, despite their transient TCR expression.

5 COMMENTS ON MATERIALS AND METHODS

5.1 HUMAN SAMPLES

In papers I, II and III, peripheral blood mononuclear cells (PBMC) from chronic HBV patients were collected longitudinally for a year at monthly intervals under informed consent from the National University Hospital of Singapore and from acute patients at different time points at the onset of disease and after disease resolution. Buffy coats from healthy donors obtained from the National University Hospital of Singapore were used as controls in papers I – III and for retroviral transduction and electroporation experiments in paper IV. PBMC were isolated by gradient centrifugation using Ficoll-paque, and resuspended in FBS with 10% DMSO. All samples were stored in liquid nitrogen until analysis. Several samples from one patient were thawed and tested at the same time, using the same FACS acquisition template.

Patient sera were stored at -80°C until analysis, and tested for cytokines or chemokines using commercially available kits and following the manufacturers' protocols.

5.2 CELL LINES

As we are studying HBV, a hepatotropic virus, the targets that we have used in our assays are either primary human hepatocytes or HepG2 cells that stably express the entire HBV genome and produce infectious virus (HepG2.2.15). We have also used lentiviral transduction to stably express HBcAg or HBsAg in HepG2 cells (HepG2-core or HepG2-env). For HBV-related HCC, natural HCC lines expressing HBV antigens from integrated portions of HBV DNA (e.g. Hep3B, PLC-PRF-5, SNU-368 and SNU-387, SNU-475) were used to mimic closely the situation *in vivo*. In paper III, to test whether HLA-A2-restricted TCRs can recognise natural HCC lines, we used a lentiviral vector to stably express HLA-A2 in HCC lines that are HLA-A2 negative (e.g. Hep3B, PLC-PRF-5).

HLA-A2+ T2, a human lymphoblastoid cell line was used as antigen presenting cells to test the functional profile (i.e. cytokines production) of HLA-A2 restricted HBV-specific TCR-redirected T cells (Papers III and IV). T2 cells are profoundly defective in the presentation of endogenously synthesized antigens to CTL due to a deletion of MHC class II-encoded genes for transporters associated with antigen presentation (TAP1/TAP2). Being TAP deficient, T2 cells allow easy loading of exogenous peptide on their MHC class I molecules.

As the density of HLA-A2 molecules on T2 can be very high, other targets that have lower HLA-A2 expression such as HepG2 or SNU-475 loaded with peptide were used to study if TCR-redirected T cells can recognise and lyse targets in a more physiological condition. HepG2 cells that stably express the entire HBV genome and

produce infectious virus (HepG2.2.15) were used to test if TCR-redirected T cells can recognise endogenously processed antigen.

5.3 SYNTHETIC PEPTIDES

In paper I, 15-mer synthetic peptides (above 80% purity) overlapping by 10 amino acids, covering the entire proteome of HBV genotype B were used to test HBV-specific T-cell responses. The reason for choosing genotype B peptides was because the chronic HBV patients in our study are Asians infected by HBV genotype B. Overlapping peptides were pooled according to their protein and included up to 45 individual peptides at a final concentration of 2 μg/ml per peptide; 1 core, 1 X, 2 envelope (Env), and 4 polymerase (Pol) pools were made and used for stimulation of PBMC to reduce the number of individual peptides to be tested.

In paper II, 15-mer peptides, overlapping by 10 amino acids, covering the entire proteome of HBV genotypes C and D were used to test HBV-specific T-cell responses of chronic and acute HBV patients respectively. Overlapping peptides were pooled according tor their protein and included up to 45 individual peptides; 1 core, 1 X, 2 Env, and 4 Pol pools were made as above.

In paper III, defined amino acid epitopes HBV surface (HBs) 183-191 (FLLTRILTI); HBs370-79 (SIVSPFIPLL) and HBV core (HBc) 18-27 (FLPSDFFPSV) were used to evaluate TCR-redirected T-cell reactivity. These are HLA-A2 restricted immunodominant CTL epitopes of CD8 T cell clones isolated from patients that resolved HBV, that is likely to be associated with protection.

In paper IV, defined amino acid epitope HBs183-191 (FLLTRILTI) was used to evaluate s183-TCR-mRNA electroporated T-cell reactivity.

5.4 QUANTIFICATION OF ANTIGEN-SPECIFIC CD8+ T CELLS AND MEASUREMENT OF CTL ACTIVITY

5.4.1 Tetramer/Pentamer staining

The MHC class I-peptide tetramer/pentamer allow detection of antigen-specific CD8+ T cells by binding directly to TCRs of a particular specificity, determined by the MHC allele and peptide combination. The MHC class I-peptide pentamer comprises five MHC-peptide complexes, and all five complexes are available for binding to complementary TCRs. In comparison to MHC tetramer, the MHC-peptide complexes are held in a tetrahedral complex where due to spatial organization no more than three complexes are available for TCR-binding. The MHC pentamer also comprises five fluorescent tags for bright and efficient labeling. We used HBV-specific HLA-A2 tetramers and anti-CD8 mAb to detect and measure the frequency of HBV-specific CD8+ T cells in PBMC from HBV patients (Paper I). We also used the HLA-A201-HBc18-27 and HLA-A201-HBs183-191 pentamer (Proimmune) and anti-CD8 mAb to

monitor the correct pairing and surface expression of TCR after retroviral transduction or TCR-mRNA electroporation of T cells (Paper III and IV).

5.4.2 Cytotoxicity assay

Antigen-specific CD8+ T cells or CTLs can recognize a specific antigen through their TCR. CTLs can kill target cells by releasing lytic granules containing perforin and granzymes, leading to apoptosis and target cell lysis or they can also trigger apoptosis via Fas/Fas ligand interactions. In our cytotoxicity assays, HepG2 expressing HBcAg (HepG2-core) or HBsAg (HepG2-env) and luciferase were used as targets. The targets were plated overnight in a 96-well flat bottom plate to permit adherence. They were cocultured with effector retrovirally transduced T cells or electroporated T cells (CD8+/pentamer+) at various effector : target (E : T) ratios in triplicates in AIM-V supplemented with 2% human AB serum for 24 or 72 hours. Cytotoxicity was measured by quantifying luciferase expression in remaining target cells after coculture. Briefly, culture medium was discarded and 100 µl of Steady-Glo reagent was added to each well and incubated for 5 min to allow cell lysis. Luminescence was measured with a microplate reader. Target cells without effectors were used as a reference for maximum luminescence. Results were expressed as % lysis = 100% - (luminescence remaining after lysis / maximum luminescence)% and calculated as mean of triplicate measurements +/- standard deviation.

5.5 DETECTION OF HBV-SPECIFIC T-CELL RESPONSES

5.5.1 Enzyme-Linked Immunosorbent Spot Assay (ELISPOT)

The ELISPOT assay enables analysis of activated or responding cells at the single cell level. The sensitivity of the ELISPOT assay (limits of detection below 1/100,000) allows for frequency analysis of low-level antigen-specific responses that is not possible using bulk assay methods or when limited numbers of cells are available and has high reproducibility. The ELISPOT assay was used to detect IFN-γ or IL-17 producing HBV-specific T cells after stimulation with peptides covering the entire proteome of HBV. Multiscreen-HTS 96-well plates were coated overnight at 4°C with capture mouse anti-human cytokine antibody. The plates were then washed with PBS, blocked and a total of $1x10^5$ cells were added to each well. HBV peptides from the patients' respective genotype were added to a final concentration of 2 µg/ml and plates were incubated for 18 hours at 37°C. Following incubation, cytokine spot forming units were detected using anti-human biotinylated mAb; followed by incubation with streptavidin-alkaline phosphatase. The spots were visualized by addition of alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium chloride [BCIP-NBT]; and counted using an automated spot reader. Positive responses in the ELISPOT assay were confirmed using intracellular cytokine staining.

5.5.2 Intracellular cytokine staining

Intracellular cytokine staining and detection by flow cytometry can identify the nature and frequency of cells that produce cytokines, chemokines and inflammatory mediators. The advantage of flow cytometry is that individual cells can be analyzed for coexpression of several markers, including cell surface and intracellular molecules, allowing for multiparameter analysis. Thus, it is possible to analyze CD8 and CD4 antigen-specific T-cell responses in the same sample in mixed populations of cells, such as in whole blood or PBMC. In our assays, PBMC were incubated with AIM-V + 2% human AB serum alone (as control), or stimulated with overlapping HBV peptide pools for 5 hours using 10 µg/ml brefeldin A as a protein secretion inhibitor. In some assays, anti-CD107a mAb was added at the beginning of the 5 hours incubation. CD107a (Lysosomal associated membrane glycoprotein-1) is exposed on the cell surface of responding antigen-specific T cells, providing a positive marker of degranulation. Following incubation, cells were stained with anti-CD8 or anti-CD4 and anti-CD3 mAbs for 30 min at 4 °C and then fixed and permeabilized using Cytofix/Cytoperm. Intracellular staining was performed for IFN-γ, TNF-α, IL-2, IL-17 or CXCL-8.

5.6 SERUM/SUPERNATANT CYTOKINE ANALYSIS

5.6.1 Cytometric Bead Array (CBA)

CBA is a flow cytometry application that simultaneously detects and quantifies multiple soluble analytes (Figure 9). A series of antibody-coated beads can specifically capture a particular type of soluble protein present within biological fluids. Each capture bead has a unique fluorescence intensity so that they can be mixed with a sample and a mixture of detection antibodies that are conjugated to a reporter molecule (PE) and run simultaneously in a single tube to significantly reduce sample requirements and time in comparison with traditional ELISA. This is useful when only a small amount of sample is available, maximizing the number of proteins that can be analyzed. We have used CBA to analyze Th1/Th2 cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, IL-17A) and chemokines [CXCL-8 (IL-8), CCL-5 (RANTES), CXCL-9 (MIG), CCL-2 (MCP-1) and CXCL-10 (IP-10)] in patients' sera (Paper I) and cell culture supernatants (Paper II and IV). Results were analyzed using FCAP Array Software (BD).

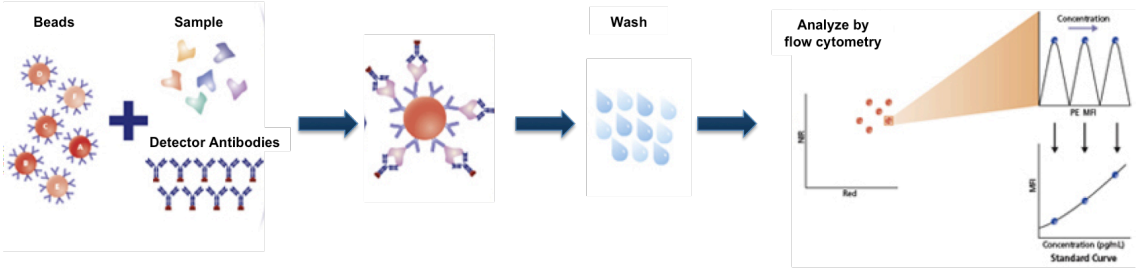

Figure 9. A schematic of the CBA procedure. Modified from BD.

5.6.2 Luminex

Similarly, Luminex can simultaneously detect and quantify multiple soluble analytes, up to 41 human cytokines in one assay. The principle of the assay is similar to ELISA (microplate well) and CBA (capture antibody-coated beads of defined spectral properties). The amount of fluorescence can be quantified on a Luminex analyzer based on the principles of flow cytometry. We used Luminex to obtain a global view of what cytokines are present in cell culture supernatants.

5.7 GENERATION OF HBV-SPECIFIC TCR-REDIRECTED T CELLS BY RETROVIRAL-MEDIATED TCR GENE TRANSFER AND MRNA ELECTROPORATION

5.7.1 Isolation and cloning of TCR alpha and beta chains

We first screened and selected CD8 T cells recognizing immunodominant epitopes by growing short-term lines from resolved HLA-A2+ HBV patients stimulated with a peptide library covering the entire proteome of HBV and then tested using ELISPOT and intracellular cytokine staining with a peptide matrix. HBV-specific T cell clones were generated by limiting dilution. Total RNA was isolated from HBc18-27, HBs183-191, or HBs370-379 specific T cell clones using TRIzol. For the identification of TCR sequence, we used rapid amplification of cDNA ends (RACE) PCR and ligated into Topo2.1 for sequencing. Following functional confirmation of the TCR, the codon optimized TCR alpha and beta chain constructs linked via a viral 2A peptide were cloned into retroviral MP71 vector (from Professor Hans Stauss, UCL). The 2A peptide allows stoichiometric coexpression of the TCR alpha and beta chains.[274] We chose retroviral mediated gene transfer system to stably express TCRs on primary human T lymphocytes as the use of retrovirally transduced T cells has achieved impressive clinical results in melanoma and leukemia. We used the retroviral MP71 vector containing the myeloproliferative sarcoma virus (MPSV) long terminal repeat (LTR) promoter-enhancer sequences and improved 5' untranslated sequences derived from the murine embryonic stem cell virus (MESV) that will allow higher transgene expression in human primary T lymphocytes compared to the standard Moloney murine leukemia virus (Mo-MLV)-based vectors.[275]

5.7.2 Production of retrovirus and transduction of primary human T lymphocytes

We chose the Phoenix amphotropic packaging cell line to produce replication-incompetent retrovirus that can infect human T cells (Figure 10) as amphotropic virus recognizes a receptor found on a broad range of mammalian cell types. We used calcium phosphate transfection to transiently transfect Phoenix packaging cells with the retroviral expression vector. The virus supernatant produced by the packaging cells was harvested at the peak of virus production at 48 hours post-transfection, and used to transduce target cells. Our target cells are primary human T lymphocytes and since for successful retroviral infection, the target cells must be actively dividing at the time of infection, we activated PBMC with 600 U/ml rIL-2 and 50 ng/ml anti-

CD3 (OKT-3) for 48 hours. For transduction, lymphocytes were plated into retronectin-coated wells and mixed with retroviral supernatant. Retronectin, a recombinant human fibronectin fragment CH-296, enhances retroviral mediated gene transduction by co-localizing target cells and virions on the CH-296 molecules. The mixture of T cells and retroviral supernatant was centrifuged at 2000 rpm, 30°C for 1 hour (spinoculation) to increase the transduction efficiency. We monitored TCR surface expression at 72 hours post-transduction by staining T cells with anti-CD8, anti-TCRVβ and HLA-A201-HBV pentamer and analyzed by flow cytometry.

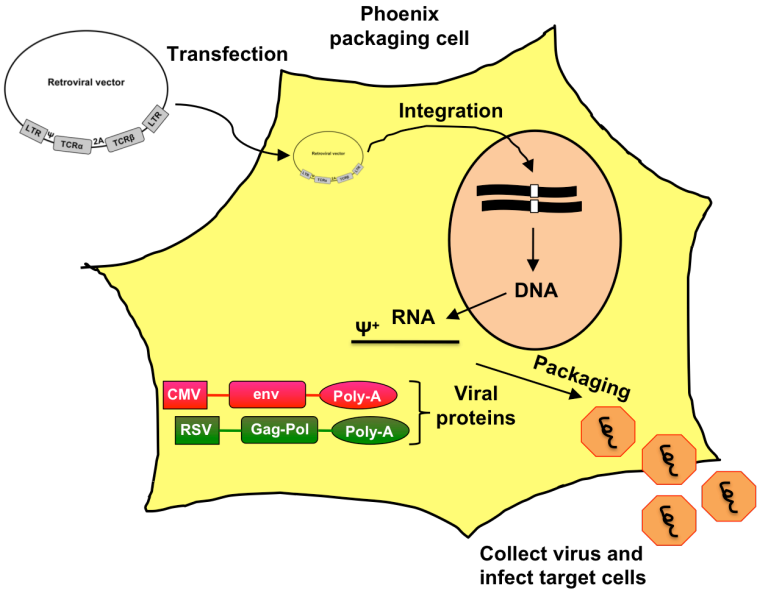

Figure 10. Virus production in Phoenix packaging cell line. The *gag, pol* and *env* genes required for viral production are integrated into the packaging cells genome. The retroviral vector provides the viral packaging signal, commonly denoted Ψ, and the gene of interest. Once the packaging cell line is transfected with a retroviral expression vector that contains a packaging signal, the viral genomic transcript containing the gene of interest are packaged into infectious virus within 48–72 hrs. The virus is secreted into the culture medium and can be harvested and used to transduce target cells and transmit the gene of interest; however, it cannot replicate within target cells because the viral structural genes are absent.

5.7.3 TCR-mRNA electroporation of human T lymphocytes

The use of retroviral vector-based transduction has pros and cons (Table 5). To develop a more practical and safer approach to cell therapy of HBV-related HCC, we used electroporation of mRNA encoding anti-HBV TCR. We subcloned the TCR gene into the pVAX1 vector that contains a T7 RNA polymerase promoter site that is needed for efficient *in vitro* transcription. The plasmid was linearized and the linearized DNA was used as template to produce the TCR mRNA using the mMESSAGE mMACHINE T7 Ultra kit. For electroporation, we used the nucleofector device II (Lonza). PBMC were activated for 8 days with 600 IU/ml rIL-2 and 50 ng/ml OKT-3 in AIM-V 2% human AB serum, and rIL-2 was increased to 1000 IU/ml one day before

electroporation. We found that electroporation with 10 million cells per reaction gave the highest transfection efficiency (70 – 88%) and cell viability (~70%), and followed this protocol for subsequent experiments.

Gene transfer delivery system	Advantages	Disadvantages
Retroviral vector-based transduction	• Stable expression of transgene • Single application of transduced T cells might be sufficient for immunotherapy	• Risk of autoreactive T cells generated • Risk of insertional mutagenesis or oncogene activation • Complex and laborious to produce retrovirus • Expensive to implement in clinical trials
mRNA electroporation	• Self-limiting toxicity due to its reduced half-life • No risk of insertional mutagenesis • Simple and widely applicable technology • Lower costs	• Transient expression of transgene • Repetitive applications of electroporated T cells required for immunotherapy; no long-lasting memory will be established

Table 5. A comparison of advantages and disadvantages of retroviral transduction versus mRNA electroporation.

5.8 GENERATION OF MOUSE XENOGRAFT MODEL OF HCC

We used NOD-SCID-IL2RG^{null} (NSG) mice that lack mature T cells, B cells, or functional NK cells, and are deficient in cytokine signalling. These mice represent a superior, long-lived model suitable for studies employing xenotransplantation strategies. Eight- to ten weeks old NSG mice were inoculated with 1.0 x 10^6 HepG2-env expressing luciferase per mouse by intrasplenic injection following a protocol previously described.[276,277] The spleen was chosen as the recipient organ as it is well known that hepatocytes engrafted into the spleen can survive, multiply and even translocate to the liver.[278,279] Tumor growth was monitored by *in vivo* imaging (IVIS; Xenogen, Alameda, CA). We observed in a pilot experiment that few mice have HepG2-env tumor cells growing in the liver, while the majority of the mice had tumors in the spleen. In contrast, in mice injected with HepG2 intrasplenic, the HepG2 cells were able to migrate to the liver. A possibility could be that HepG2-env cells had decreased migration and invasion ability compared with its parental HepG2 cells due to HBV transfection.[280] Nonetheless, this xenograft mouse model might more closely recapitulates the events that are occurring after liver transplantations in chronic HBV patients with HCC as HCC cells may seed in the newly transplanted normal liver or in extra-hepatic locations after liver transplantation. The most important limitations of our

model are that HCC-like cells seed and expand preferentially in the spleen and not in the liver, and these cells are the only cells that express HBsAg (the model does not have HBsAg-positive normal hepatocytes).

6 RESULTS AND DISCUSSION

6.1 THE KINETICS OF INNATE AND ADAPTIVE IMMUNE ACTIVATION DURING HF IN CHRONIC HEPATITIS B (PAPER I)

The immunological and virological events responsible to set off HF in chronic hepatitis B patients are controversial. We therefore studied a group of chronic hepatitis B patients that voluntarily ceased anti-viral treatment (n = 5) for about 1 year at monthly intervals. This will allow us to study the events preceding HF and associated with viral rebound. Serum cytokines (pro-inflammatory and anti-inflammatory), chemokines and circulating T, NK and Treg cell populations were studied in three distinct phases. (1) Green: during treatment with reduced ALT and low HBV replication (approximately $\leq 10^4$ HBV- DNA copies/ml). (2) Blue: a phase of viral rebound with no change in ALT levels after therapy termination. (3) Red: and the third phase when values of ALT start to rise with 4 out of 5 patients experiencing HF. Two major findings are summarized and discussed here:

6.1.1 The rebound of HBV replication following therapy withdrawal did not trigger innate immune activation

A progressive increase of HBV replication precedes HF (about 5–6 log in the 10–12 weeks before HF) but occurs without detection of innate immune activation. Serum values of pro-inflammatory cytokines (IL-1, TNF-α, IL-6 and IFN-α) were consistently normal (<15 pg/ml), with the exception of increased serum CXCL-8 detected in 4 out of 5 chronic HBV patients (Figure 11). The frequency of total NK cells or TRAIL expression on NK (CD56+ and CD56 bright) in the peripheral compartment did not fluctuate significantly with respect to the occurrence of HF.

The almost complete absence of signs of immune reactivation during the rebound of HBV replication is in agreement with the suggestion that HBV might escape intra-cellular innate immune recognition mechanisms (i.e. TLR3, 7 and 9) and early NK cell recognition.[36] CXCL-8 is a chemokine able to recruit granulocytes, NK cells and T cells to the inflammatory site[281] and might also interfere with the anti-viral effect of IFN-α.[282] In addition, it has been shown to be elevated prior to HFs where it was proposed to synergize with IFN-α to activate NK cells.[53] It is very likely that this chemokine has a role during hepatic infection because we have also detected high CXCL-8 values during acute HBV,[283] but not during other acute viral infections (dengue, influenza, adenovirus) (A. Bertoletti, unpublished data).

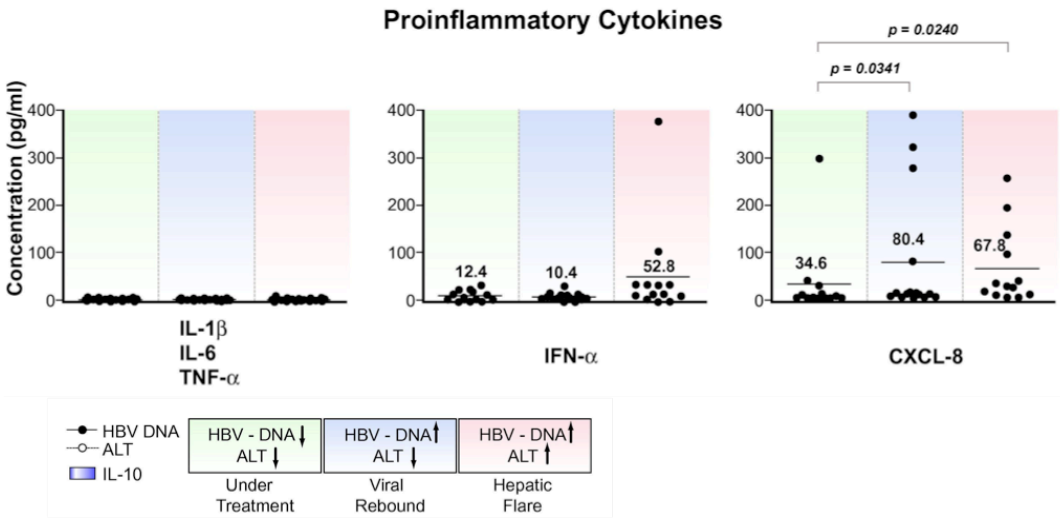

Figure 11. Kinetics of pro-inflammatory/antiviral cytokines. Mean serum levels of pro-inflammatory/antiviral cytokines during different disease phases. Reprinted from the *Journal of Hepatology*, Mar 52(3), Tan et al. A longitudinal analysis of innate and adaptive immune profile during hepatic flares in chronic hepatitis B, 330-339, Copyright 2010, with permission from Elsevier.

6.1.2 HF were temporally associated with high serum levels of CXCL-9 and CXCL-10

High serum levels of IFN-γ induced chemokines CXCL-9 and CXCL-10 were temporally associated with HF in patients studied longitudinally and were also found in chronic hepatitis B patients with high levels of ALT, but not in patients with normal ALT values. In addition, chronic patients with HF have CXCL-9 and CXCL-10 elevated to similar levels (mean CXCL-9/CXCL-10 concentration: spontaneous flares = 179.4/202.4 pg/ml; anti-viral withdrawal induced = 2543/2458 pg/ml), but acute patients have a greater increase in CXCL-9 than CXCL-10 (mean CXCL-9/CXCL-10 concentration: 2631/486.9 pg/ml) (Figure 12). Moreover, CXCL-9 and CXCL-10 displayed different in vitro requirements for activation. IFN-γ was sufficient to induce production of both CXCL-9 and CXCL-10 in normal hepatocytes and in the HCC lines, while IFN-α and TNF-α alone or in combination induced production of CXCL-10.

CXCL-9 and CXCL-10 are potent chemo-attractants of activated T cells[284]and have been shown in several animal models to recruit antigen-specific and inflammatory T cells to the liver.[66,285] In addition to its involvement in T cell recruitment, CXCL-10 has been shown to present hepato-protective properties, inhibiting liver damage and promoting liver regeneration.[286] It is thus possible that CXCL-10, in addition to IL-10 that was slightly elevated at the time of HF, might play a role not only in inducing inflammation but also in preserving liver viability. We were able to show in vitro that IFN-γ is necessary for both CXCL-9/10 production by hepatocytes, which was mimicked more closely by activation of T cells and not by monocytes or NK activation, suggesting a direct involvement of T cells in HF. However, we were unable to detect HBV-specific T cells in the periphery during the occurrence of HF. Thus, we could not

correlate an increase of circulatory HBV-specific T cell response (as well as circulatory activated NK cells) with the presence of HF. On the contrary, an increased HBV-specific T cell response was detectable in the only patient that maintained a lower level of HBV replication and did not develop HF following anti-viral withdrawal, in line with the correlation of HBV-specific T cell response with viral control more than with liver injury.[67,74] We also showed here that the two chemokines are triggered differently. This *in vitro* result is somewhat in line with our *ex vivo* observation. Dysfunctional T cells are a hallmark of chronic HBV infection while patients who resolve the infection are characterized with poly-functional T cell response.[72,287] The presence of functional T cells producing IFN-γ and TNF-α in acutely infected patients could represent the source of signals necessary for CXCL-9 secretion which leads to the observed biased elevation of CXCL-9 versus CXCL-10. In contrast, dysfunctional T cells (and perhaps the involvement of activated monocytes and NK cells) present in chronic patients which is inferior in IFN-γ production[72] might result in the expression of identical levels of CXCL-9/10 observed in chronic patients.

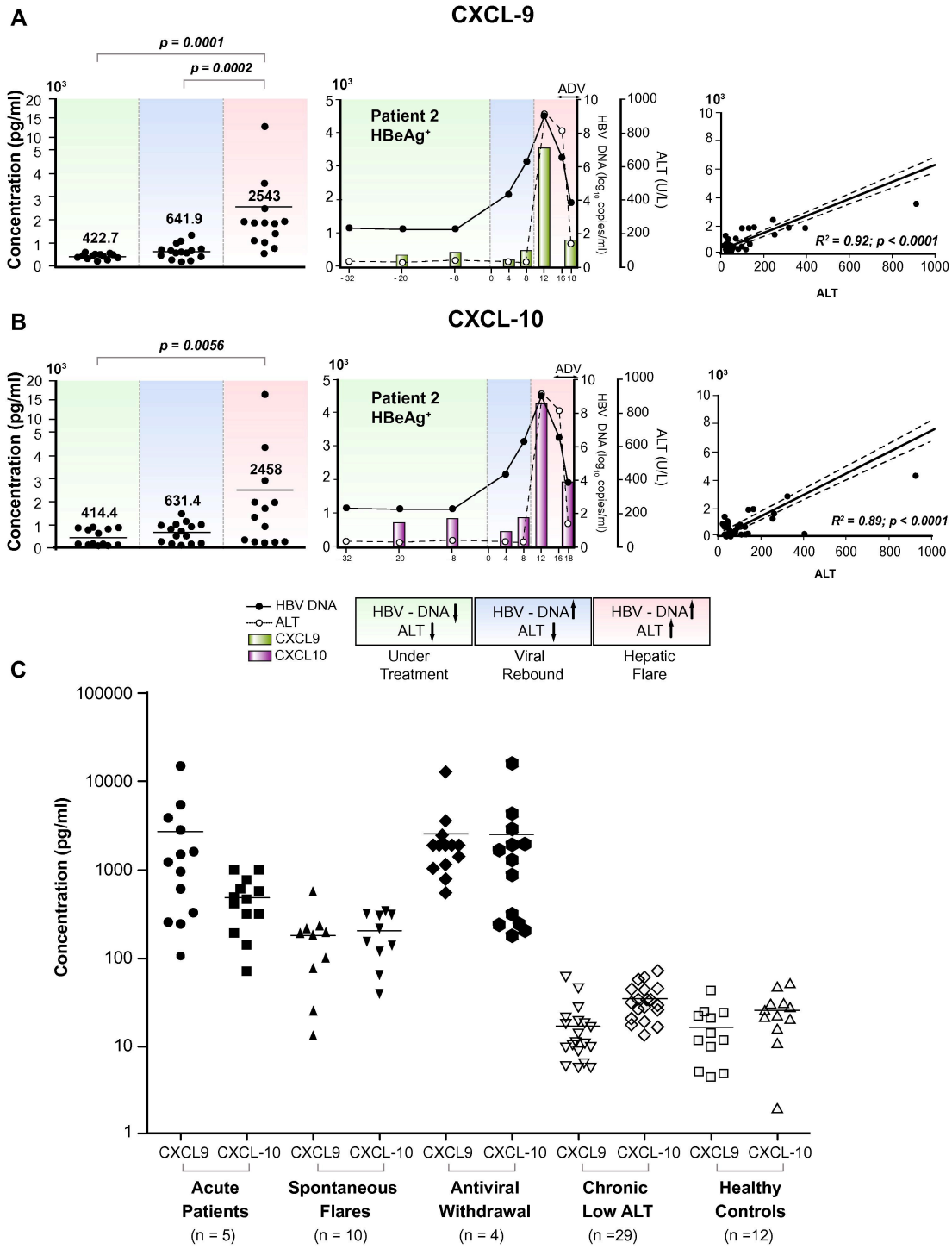

Figure 12. Strong correlation of serum CXCL-9 and CXCL-10 concentrations with hepatic injury. Serum (A) CXCL-9 and (B) CXCL-10 concentrations at the different time points were quantified by the cytometric bead array system. (C) Serum CXCL-9 and CXCL-10 concentrations measured in various patient groups with elevated (acute, spontaneous flares and anti-viral withdrawal induced flares) or normal (chronic patients without HF and healthy subjects) ALT is shown. The mean cytokine concentrations at each disease phase are indicated. Reprinted from the *Journal of Hepatology*, Mar 52(3), Tan et al. A longitudinal analysis of innate and adaptive immune profile during hepatic flares in chronic hepatitis B, 330-339, Copyright 2010, with permission from Elsevier.

6.2 VIRUS-SPECIFIC T CELLS SECRETE THE NEUTROPHIL ATTRACTING CHEMOKINE CXCL-8 DURING HBV INFECTION (PAPER II)

Virus-specific T cells are essential for the control of HBV infection but are also implicated in triggering the inflammatory events leading to hepatic injury. The ability of virus-specific T cells to orchestrate such inflammatory phenomenon is not well defined during infection with HBV, which causes acute and chronic liver inflammation. Thus, we characterized the inflammatory potential of virus-specific T cells, analyzing their ability to produce different effector molecules during different phases of HBV infection. We focused on IL-17 and CXCL-8 due to their inflammatory potential and ability to recruit neutrophils, which represents a key step in animal models of acute viral hepatitis. CXCL-8, a primary chemotactic factor for neutrophils, can be produced in large quantities by T cells[288,289] and elevated levels of CXCL-8 are found in patients with chronic liver disease[290] and chronic HBV patients prior to hepatic flares.[53] Likewise, IL-17 is known to recruit neutrophils[291] and has been associated with inflammatory diseases,[292,293] including hepatic flares in chronic HBV patients[294]. In addition, the inflammatory cytokine milieu present during HBV infection may impact on T cell function. IL-15 is elevated in the liver of patients with active hepatitis,[295,296] has been demonstrated to induce IL-17 production[297,298] and can stimulate CXCL-8 and MCP-1 expression from monocytes.[299] IL-7 can be up-regulated in the liver by inflammation and enhances T cell cytotoxic activity and cytokine production.[300] Therefore, we determined whether these two cytokines, which are present in the liver during inflammation, could license T cells with additional cytokine profiles that contribute to tissue inflammation. Our data demonstrate that:

6.2.1 HBV-specific T cells produce CXCL-8, but not IL-17, during periods of liver inflammation

HBV-specific CXCL-8 production was detectable from intrahepatic lymphocytes of chronic HBV patients. Using immunofluorescence, CXCL-8 producing T cells were detectable in the liver of chronic HBV patients with active hepatitis (Figure 13). Moreover, in acute HBV patients CXCL-8 production by T cells was temporally limited to the acute phase of disease, concomitant with the peak of liver inflammation and disappeared as liver inflammation subsided. On the contrary, HBV-specific IL-17 producing T cells were not detectable in acute or chronic HBV patients *ex vivo* or after *in vitro* expansion by Elispot, intracellular cytokine staining or peptide specific production in the supernatant of intrahepatic lymphocytes. We also did not observe an increase in non-specific IL-17 producing T cells using Elispot after SEB stimulation in acute and chronic HBV patients.

CXCL-8 production by T cells was previously a rare quality. CXCL-8 producing T cells have only been described in immune-mediated inflammatory skin reactions[288,301] and to our knowledge there has been no such description of this function in pathogen-specific T cells. We previously characterized cytokines detectable in the serum of HBV patients and were unable to detect IL-1β or IL-6, two cytokines that play a role in the

development of Th17 cells.[302] Therefore, the inflammatory environment during HBV infection may not lend itself to Th17 differentiation. However, our data on non-specific IL-17 production is in contrast to recent reports suggesting that IL-17 producing T cells were increased in chronic HBV patients with liver inflammation.[294] This discrepancy could be due to the sample size or assays and mitogens used to stimulate IL-17 producing T cells. Additional studies, particularly in the intrahepatic compartment, will be necessary to determine if IL-17 producing cells are involved in HBV pathology but our data suggest that HBV-specific IL-17 producing T cells are not present in acute or chronic HBV patients.

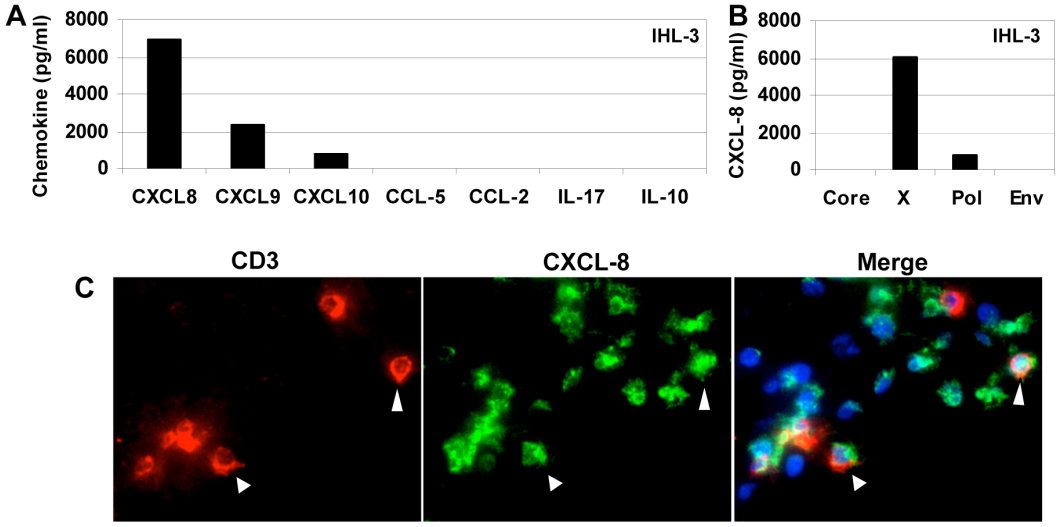

Figure 13. Chemokines produced by intrahepatic mononuclear cells following HBV peptide stimulation. A) Cumulative amount of chemokines produced in response to all HBV peptides in a representative HBV patient. B) Antigen distribution of CXCL-8 production from panel A. All supernatants were harvested after 20 h culture and background from unstimulated wells was subtracted to give peptide specific concentrations. C) Representative immunofluorescence staining of a biopsy from a chronic HBV patient with elevated ALT. Figure shows CD3 (red, left panels), CXCL-8 (green, middle panels) and merged images, including DAPI staining (right panels). Reprinted from the *PLoS One*, 6(8), Gehring et al. Licensing virus-specific T cells to secrete the neutrophil attracting chemokine CXCL-8 during hepatitis B virus infection, e23330, Copyright 2011.

6.2.2 IL-7 and IL-15 can license T cells with the ability to produce CXCL-8

We expanded HBV-specific T cells from 6 acute/ resolved HBV patients in the presence of IL-2 alone or IL-2 plus IL-7 and IL-15 and found that T cells grown in IL-2 alone produced IFN-γ but little or no CXCL-8. In contrast, cells from the same patient, grown in IL-2+IL-7+IL-15 in parallel, showed a significant increase in CXCL-8 producing T cells (Figure 14). Furthermore, CXCL-8 production is not restricted to HBV-specific T cells and could also be induced in other virus-specific T cells, such as CMV in healthy individuals.

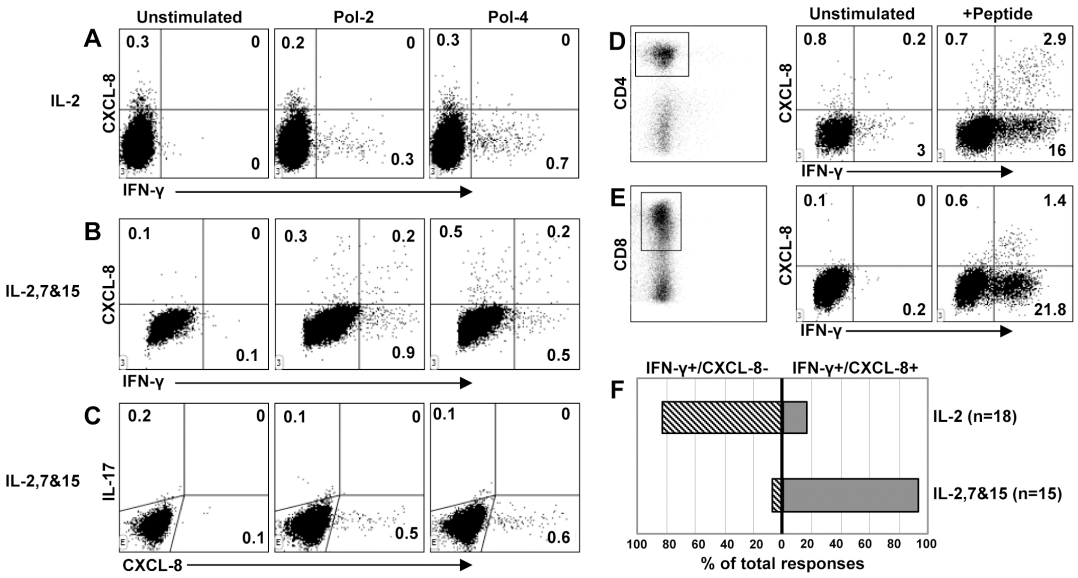

Figure 14. IL-7 and IL-15 induce CXCL-8 production. Acute patient PBMC cultured in A) IL-2 alone or B) IL-2,7&15 were expanded with peptides covering the entire HBV proteome and tested with peptide pools for IFN-c and CXCL-8 production. C) PBMC from the same patient expanded in IL-2,7&15 were tested with peptide pools for IL-17 and CXCL-8 production. D) HBV-specific CD4 T cells and E) HBV-specific CD8 T cells restimulated in IL-2,7&15 expand and maintain CXCL-8 production. F) Distribution of functional phenotypes of all HBV-specific T cell responses detected in acute/resolved HBV patients after in vitro culture in IL-2 or IL-2,7&15. Reprinted from the *PLoS One*, 6(8), Gehring et al. Licensing virus-specific T cells to secrete the neutrophil attracting chemokine CXCL-8 during hepatitis B virus infection, e23330, Copyright 2011.

The fact that IL-7 and IL-15 were required to detect CXCL-8 producing T cells in nearly all HBV-specific responses suggests a particular environment is necessary before virus-specific T cells are licensed with such inflammatory function. The liver is a particularly well suited environment for the induction of CXCL-8 producing virus-specific T cells since IL-15 can be produced by hepatic stellate cells (Ito cells),[303] a specialized liver-resident antigen presenting cell, while IL-7 can be produced by hepatocytes[300] and both cytokines can be upregulated in the liver during inflammation.

In summary, our data demonstrate that human virus-specific T cells have, or can acquire through exposure to environmental factors, a cytokine/chemokine profile capable of contributing to parenchymal inflammation observed in non-cytopathic viral infection like HBV.[64–66]

6.3 ENGINEERING VIRUS-SPECIFIC T CELLS THAT TARGET HBV INFECTED HEPATOCYTES AND HEPATOCELLULAR CARCINOMA CELL LINES (PAPER III)

Virus-specific T cells capable of controlling HBV and eliminating hepatocellular carcinoma (HCC) expressing HBV antigens are deleted or dysfunctional in patients with chronic HBV and/or HBV-related HCC. As a result, attempts to restore virus-specific T cell immunity in chronic HBV patients using antiviral therapy, immunomodulatory cytokines (IFN-α), or therapeutic vaccination have had little success.[304–306] T cell receptor (TCR) gene transfer is an alternative approach to overcome the obstacles of T cell deletion and dysfunction[307] and adoptive T-cell therapy has shown impressive clinical results in melanoma and leukemia.[143,308] Therefore, we determined if T cell receptor (TCR) gene transfer can reconstitute HBV-specific T cell immunity in lymphocytes of chronic HBV and HBV-related HCC patients. We cloned TCRs specific for HLA-A2- restricted core and envelope epitopes and tested the ability of TCR re-directed T cells to recognize HBV infected hepatocytes and HCC cell lines expressing viral antigen from naturally integrated HBV DNA. We demonstrate in this paper that:

6.3.1 HBV-specific TCRs can be expressed on lymphocytes from chronic HBV/HCC patients and were functional

We used retrovirus-mediated gene transfer to introduce HLA-A2-restricted, HBV-specific TCRs (specific for HBc18-27, HBs183-191 and HBs370-379 epitopes) into T cells of chronic HBV and HBV-related HCC patients, and we found that the introduced TCRs were expressed on the cell surface, measured by Vβ and pentamer staining (Figure 15). TCR re-directed T cells from different cohorts of chronic patients (five HBeAg- (HBV DNA $<10^6$ copies/ml) and five HBeAg+ (HBV DNA $>10^7$ copies/ml), as well as five HBV-related HCC patients) expressed similar levels of introduced TCRs and had a functional profile similar to genetically modified T cells from healthy donors. They produced IFN-γ, TNF-α and to a lesser extent IL-2, upon stimulation by peptide loaded, HLA-A2+ T2 cells (Figure 16).

The similar expression of exogenous TCR in all patient groups demonstrate that transduction of lymphocytes was not affected by the presence of HBeAg, HCC, the level of HBV replication, or any other potential tolerogenic mechanisms implicated in global T cell exhaustion or deletion in chronic HBV patients.[82,84,309,310] CD3 zeta down-regulation and arginine depletion are likely reversed due to *in vitro* culture required for TCR transduction and regulatory cells or altered dendritic cell function do not impair T cell proliferation needed for efficient retroviral transduction.

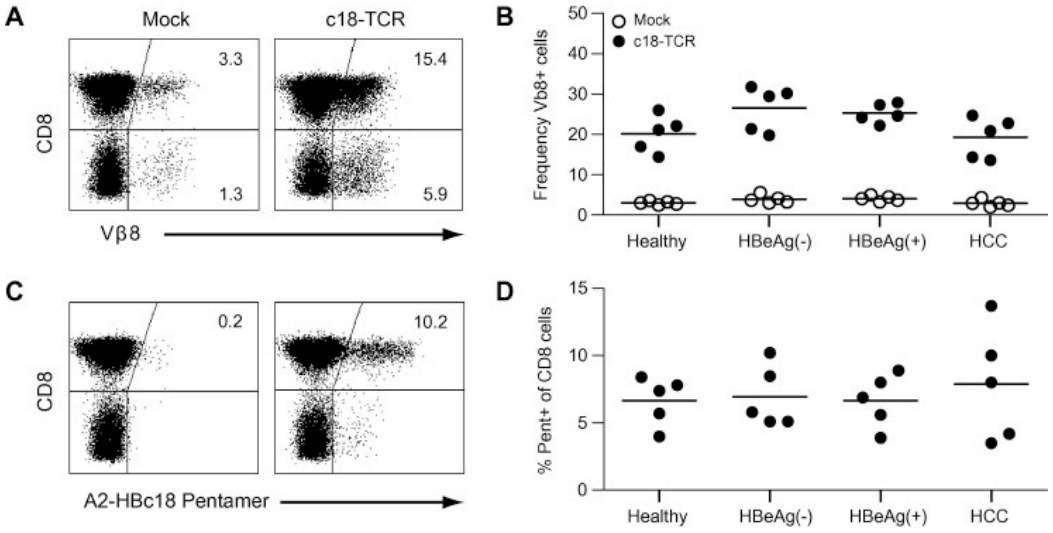

Figure 15. Expression of introduced TCR. (A) Dot plot of Vb8.2 expression in mock or c18-TCR transduced T cells from a representative HBeAg- patient. (B) Mean frequency of CD8+ Vb8.2+ T cells in mock and c18-TCR transduced T cells from five patients in each group. (C) Dot plot of HLA-A2-HBc18-27 pentamer staining in mock or c18-TCR transduced T cells from a representative HBeAg- patient. (D) Mean CD8+ pentamer+ T cells, in mock and c18-TCR transduced T cells from five patients in each group. There was no statistically significant difference between pentamer+ or Vb8+ cells from each patient group using one-way ANOVA analysis ($p > 0.05$). Reprinted from the *Journal of Hepatology*, Jul 55(1), Gehring et al. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines, 103-110, Copyright 2011, with permission from Elsevier.

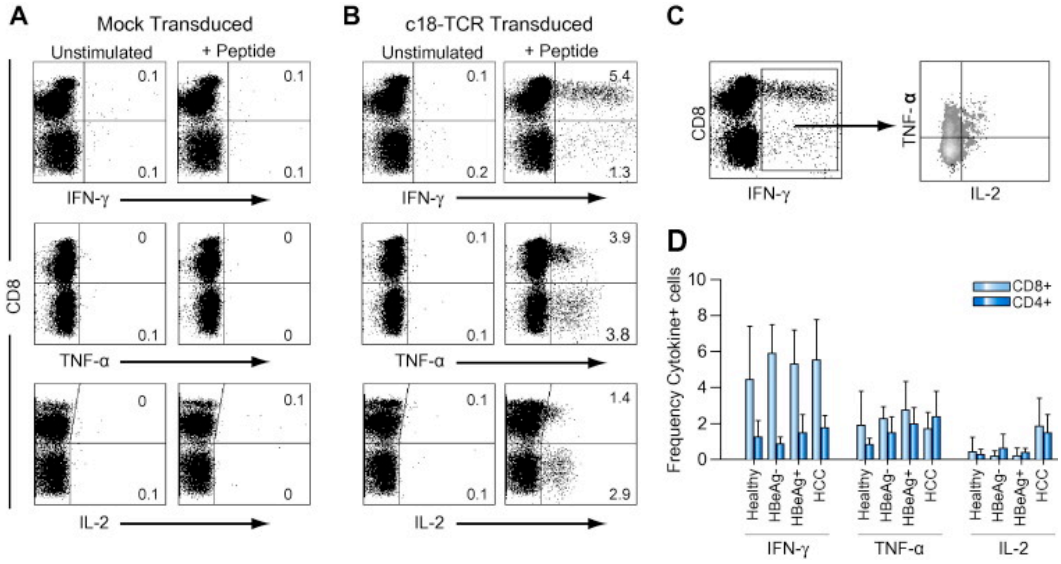

Figure 16. Functional profile of transduced T cells. Dot plots from a representative HBeAg_ (A) mock and (B) c18-TCR transduced T cells, +/- peptide stimulation, stained for CD8 and IFN-γ (top row), TNF-α (middle row) and IL-2 (bottom row). (C) TNF-α and IL-2 production by IFN-γ+ cells to demonstrate multi-functionality of TCR transduced cells. (D) Mean frequency of cytokine positive cells from all patients in each group. Black portion represents mean of CD8+ contribution +/- standard

deviation and open bars represent mean of CD4 T cell contribution. Reprinted from the *Journal of Hepatology*, Jul 55(1), Gehring et al. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines, 103-110, Copyright 2011, with permission from Elsevier.

6.3.2 HBV-specific TCR-redirected T cells recognized and lysed natural HCC lines

Since the goal of engineering HBV-specific TCR-redirected T cells is to use them for immunotherapy for HBV-related HCC, it is important that these genetically modified HBV-specific T cells are able to recognize natural HCC, where tumor cells can express HBV proteins from integrated portions of HBV DNA. To do this, we tested TCR-redirected T cells with HCC lines PLC-PRF-5 and Hep3B that were transfected with HLA-A2. Coculture of HBV surface antigen specific TCR-redirected T cells with HLA-A2 negative PLC-PRF5 and Hep3B cell lines did not result in T cell activation. Significant s183-TCR T cell activation was only observed after co-culture with PLC-PRF5-A2 while s370-TCR T cells were activated by both PLC-PRF5-A2 and Hep3B-A2 (Figure 17). Cytotoxicity assays performed against both HCC lines using s370-TCR transduced cells further confirmed the ability of TCR-redirected T cells to recognize HCC tumor cells with naturally integrated portions of HBV DNA.

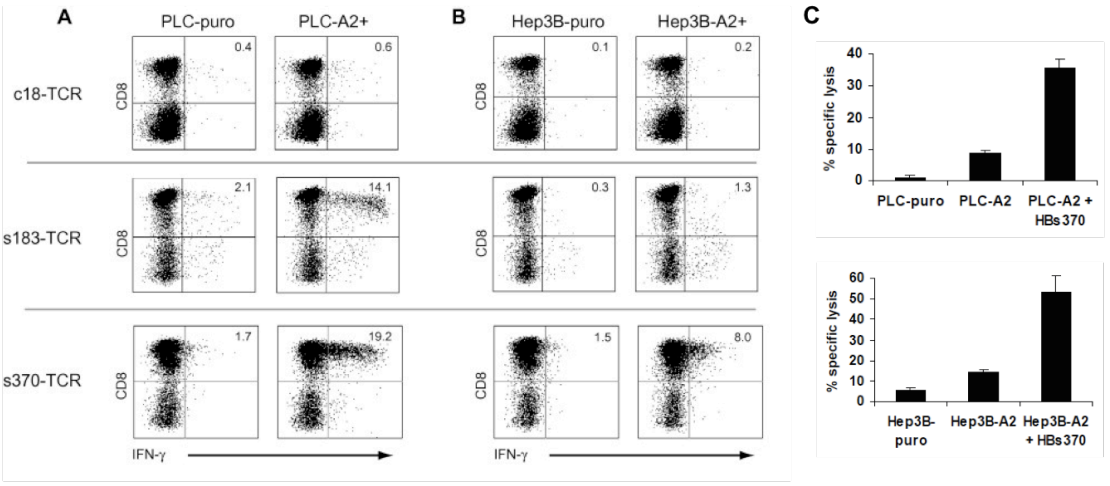

Figure 17. Recognition and lysis of HCC cell lines naturally expressing HBV proteins. C18-TCR (top row), s183-TCR (middle row), and s370-TCR (bottom row) transduced T cell IFN-γ production following overnight co-culture with (A) HLA-A2 negative PLC-puro and HLA-A2+ PLC-A2 or (B) Hep3B-puro and Hep3B-A2 cells. T cells used for this experiment were derived from healthy donors. (C) Cytotoxicity of PLC-A2 (top panel) and Hep3B-A2 cells (bottom panel) by s370-TCR T cells. s370-TCR T cells were incubated overnight with PLC-puro or PLC-A2 cell lines at effector (CD8+/IFN-γ+):target ratio of 1:1 in triplicate and specific lysis was measured. PLC-A2 cells loaded with 1 μg/ml peptide (+HBs370) served as positive control for maximum lysis. Reprinted from the *Journal of Hepatology*, Jul 55(1), Gehring et al. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines, 103-110, Copyright 2011, with permission from Elsevier.

Our data demonstrate that retrovirally transduced TCR-redirected T cells could recognize and lyse HCC cell lines expressing HBV antigens from naturally integrated HBV DNA *in vitro*. This is also supported by the fact that *in vivo*, c18-TCR transduced T cells are able to lyse HepG2 expressing HBV core antigen in a xenograft mouse model. The different profiles of HBs-specific T cell activation that were observed with PLC-A2 and Hep-3B-A2 cells could potentially be explained by the variability in antigen expression in different HCC lines. The different expression of HBV antigens and epitopes between HCC tumors is a rationale for developing TCRs specific for multiple HBV epitopes to broaden the applicability of TCR gene therapy for the treatment of HBV-related HCC. In summary, genetically modified T cells could be used to reconstitute virus-specific T cell immunity in chronic HBV patients and target tumors in patients with HBV-related HCC, and is a promising immune-based therapy to complement current antiviral therapies.

6.4 A PRACTICAL APPROACH TO IMMUNOTHERAPY OF HEPATOCELLULAR CARCINOMA USING T CELLS REDIRECTED AGAINST HEPATITIS B VIRUS (PAPER IV)

As a follow-up to our previous work on the use of viral vectors to introduce exogenous HBV-specific TCRs on T cells to redirect their specificity, we develop a more practical and safer approach to cell therapy of HCC using electroporation of mRNA encoding anti-HBV TCR. A specific concern regarding the use of retrovirally transduced T cells that permanently express anti-HBV specificity in HCC is that HBV antigen expression is not exclusive to transformed hepatocytes; non-tumor hepatocytes might also express HBV antigens and thus, adoptive T cell therapy could potentially trigger severe liver damage.[64,67,267] Moreover, viral vectors carry the risk of oncogene activation.[156,158,238] and finally, the costs and regulatory requirements of implementing viral transduction in clinical trials further add to the complexity of implementing this form of cell therapy. Therefore, we determined whether effective anti-HBV T cells, transiently expressing anti-HBV TCR, could be generated by mRNA electroporation. We demonstrate in this paper that:

6.4.1 mRNA electroporation generated high TCR expression efficiency and electroporated T cells were polyfunctional

We prepared mRNA encoding the alpha and beta chains of the HBV s183-TCR and used electroporation to introduce it into activated T cells from 5 healthy donors. Expression of TCR was measured by pentamer staining and flow cytometry. As early as 6 hours after electroporation, 42% ± 23% of CD8+ T cells expressed the s183-TCR (Figure 18a,b). The highest TCR expression was measured at 24 hours post-electroporation, where 64%-95% (mean 80.0%) of CD8+ T cells expressed the TCR (Figure 18a,b). TCR expression then gradually decreased and was not detectable after 72 hours (Figure 18b). The level of expression at 24 hours was much higher than that typically achieved by retroviral transduction (12%-25% (mean 17.8%); n = 3). Mock electroporated activated T cells did not show any expression of TCR and as a negative

control for functional assays, an irrelevant CMV pp65-TCR was also expressed on activated T cells by mRNA electroporation (Figure 18a).

We tested electroporated T cells for their capacity to produce cytokines in response to s183-191 peptide-loaded T2 cells (a TAP-deficient HLA-A2+ human lymphoblastoid cell line) at regular intervals from 6 to 120 hours. The highest level of IFN-γ was produced at 24 hours post-electroporation, concomitant with peak TCR expression (Figure 18b). At maximal TCR expression, not all pentamer+ CD8+ T cells produced IFN-γ (Figure 18b) in contrast to retrovirally transduced T cells where ≥98% of pentamer+ CD8+ T cells produced IFN-γ. Importantly, while s183-TCR expression in electroporated T cells became undetectable after 72 hours, approximately 20% of CD8+ T cells still produced IFN-γ. Mock- or CMV pp65-TCR mRNA electroporated T cells did not produce any cytokines in response to s183-191 peptide-loaded T2 cells while the s183-TCR mRNA electroporated CD8 and CD4 T cells showed a level of polyfunctionality superior to the s183-TCR retrovirally transduced T cells and are able to efficiently produce IL-2 (Figure 18c,d). About 36% of cytokine-producing electroporated T cells co-expressed all three cytokines (IFN-γ, TNF-α, IL-2) in contrast to only 13% of cytokine-producing retrovirally transduced T cells (Figure 18e).

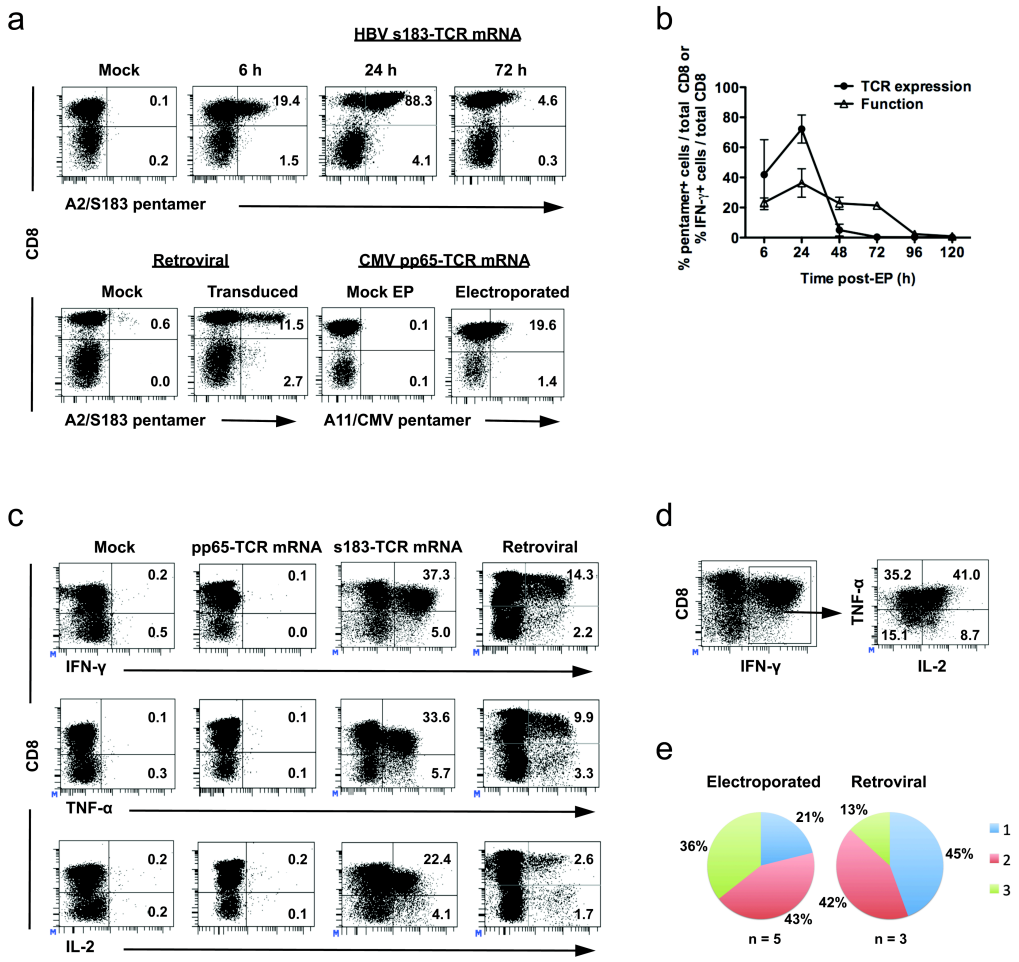

Figure 18. High TCR expression efficiency and polyfunctionality of mRNA electroporated T cells. (a) Dot plots from a representative HLA-A2-HBs183-191 pentamer staining in HBV s183-TCR mRNA electroporated T cells at 6, 24 and 72

hours postelectroporation and retrovirally transduced T cells at 72 hours. T cells that were mock-electroporated or electroporated with an irrelevant CMV pp65-TCR mRNA and mock-transduced served as negative controls. The percentages of pentamer+ cells out of CD8+ or CD8- cells are indicated. (b) Expression of TCR on electroporated CD8+ T cells and frequency of IFN-γ-producing CD8+ T cells after overnight coculture with s183 peptide-loaded T2 cells were determined at several time points as indicated. Results expressed as mean + SD (n = 5). (c) Dot plots from a representative healthy donor's activated T cells electroporated or retrovirally transduced with s183-TCR, after overnight coculture with s183 peptide-loaded T2 cells and stained for CD8 and IFN-γ (top row), TNF-α (middle row) and IL-2 (bottom row). Mock electroporated and CMV pp65-TCR mRNA electroporated T cells cocultured with s183 peptide-loaded T2 cells served as negative controls. (d) TNF-α and IL-2 production by IFN-γ+ T cells demonstrate polyfunctionality of electroporated T cells. (e) Cytokine co-expression subsets expressed as a percentage of total cytokine-producing electroporated or retrovirally transduced T cells. Mean for each group is shown. Single producers, IFN-γ+, TNF-α+ or IL-2+; double producers, IFN-γ+TNF-α+, IFN-γ+IL-2+ or IL-2+TNF-α+; triple producers, IFN-γ+IL-2+TNF-α+.

6.4.2 Anti-HBV TCR mRNA electroporated T cells prevent the growth of human HCC-like cells *in vivo*

To test the anti-tumor effect of T cells redirected against HBV, we established a mouse xenograft tumor model using HepG2 cells constitutively expressing HBV surface antigen and luciferase (HepG2-env) injected in the spleen of NOD-SCID-IL2RG^{null} mice. We then tested the effect of T cells electroporated with anti-HBV TCR mRNA. Tumor-bearing mice were given s183-TCR electroporated T cells, consisting of 3 x 10^6 pentamer+ CD8 plus pentamer+ CD4; one infusion of T cells every three days. Three infusions of electroporated T cells blocked tumor growth and maintained stable disease (Figure 19a). Of note, when therapy was interrupted after three infusions, the tumor grew, further demonstrating the anti-tumor effect of T cell therapy. To further test the *in vivo* antitumor efficacy, we tested whether electroporated T cells could prevent HCC engraftments in NSG mice. Mice were inoculated with 1 million HepG2-env, and graded numbers of s183-TCR electroporated T cells were injected intravenously 4 hours later. In mice receiving 3 million pentamer+ T cells, tumor seeding and growth was completely prevented, while lower numbers of T cells slowed tumor development in comparison to mice receiving similar numbers of mock-electroporated T cells (Figure 19b).

Here, we showed that three infusions of electroporated cells were required to suppress tumor cell growth but were not sufficient to fully eliminate them. These results were consistent with previous work performed with chimeric antigen receptor-electroporated T cells[311] and are likely due to the transient nature of TCR expression after electroporation. It is possible that higher doses of T cells and/or a more intense administration schedule could have been more effective. Nevertheless the potential protective efficacy of our TCR-electroporated T cells was demonstrated by the fact that

one single infusion of them was sufficient to prevent engraftment of HCC-like cells in our mouse model. We think that these data are particularly important since HCC therapy relies mainly on liver transplantation,[106,312] and HCC recurrence in transplant patients frequently occurs due to the seeding of HCC cells often carrying HBV integrations[313] in the newly transplanted normal liver or in extra-hepatic locations.

The most important limitations of our model are that HCC-like cells seed and expand preferentially in the spleen and not in the liver, and these cells are the only cells that express HBsAg (the model does not have HBsAg-positive normal hepatocytes). In this regard, the model might somewhat recapitulate the scenario occurring after liver transplantation in chronic HBV patients with HCC. Conceivably, mRNA electroporated T cells expressing anti-HBV TCR might become a potential postoperative immunotherapy intervention to block the dissemination of HBV-expressing tumor cells.

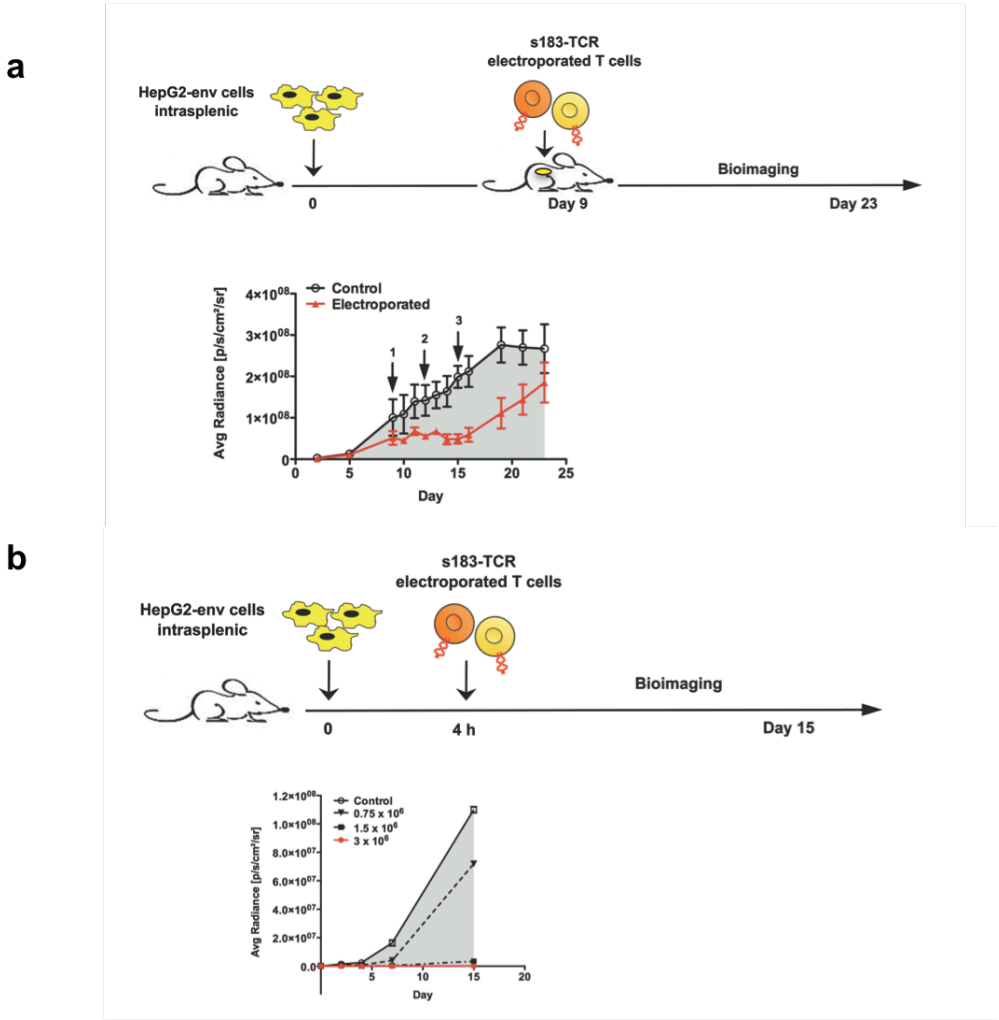

Figure 19. (a) Multiple infusions of activated mRNA electroporated T cells control tumor growth and maintained stable disease. 1 million HepG2-env tumor cells were inoculated by intrasplenic injection in NSG mice (n = 11). 9 days after tumor inoculation, mice were treated with three doses of 3 x 10^6 activated s183-TCR electroporated T cells per dose, (n = 4, red line) injected i.v once every three days. Mice treated with 3 x 10^6 mock-electroporated T cells served as controls (n = 3, grey shaded

area). **(b) Prevention of HCC tumor cells seeding by mRNA electroporated T cells.** 1 million HepG2-env tumor cells inoculated by intrasplenic injection in NSG mice (n = 14). Four hours later, mice (n = 4 or 3 per group) were treated with graded doses (0.75, 1.5, 3 x 10^6 pentamer+ CD8) of s183-TCR electroporated T cells injected i.v. Mice treated with 3 x 10^6 mock-electroporated T cells served as controls (grey shaded area). Tumor size was monitored by bioluminescence imaging and plotted as average radiance (p/s/cm^2/sr) of the mean + SD.

6.4.3 Anti-HBV TCR is successfully expressed on large numbers of cells following cGMP compliant procedures

To adapt this technology to large-scale conditions that can be used in HCC patients, we used a current good manufacturing practice (cGMP)-compliant electroporator. We transfected 2 x 10^8 activated T cells in a cGMP environment. Twenty-four hours after electroporation, 40% of CD8+ T cells were pentamer+. In total, we obtained 7 x 10^7 antigen-specific T cells (Figure 20). These cells produced IFN-γ, TNF-α and IL-2 after HBV-specific stimulation. Overall, functionality was similar to that of T cells electroporated in the small-scale, research laboratory setting. Thus, it is possible to generate large batches of clinical-grade, functional antigen-specific T cells.

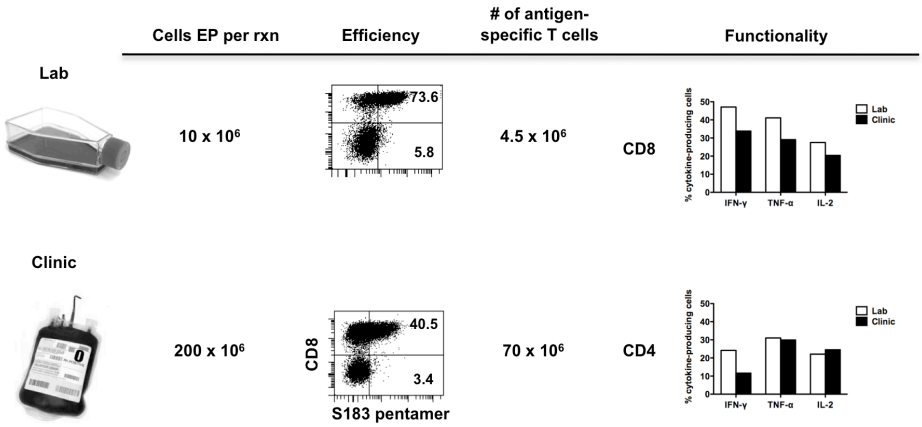

Figure 20. High level of TCR expression and multifunctionality of mRNA electroporated T cells produced in large-scale, clinical-grade conditions. A schematic illustrating cell numbers, efficiency, yield and functionality of laboratory-grade (top row) versus clinical-grade (bottom row) electroporation of T cells. Dot plot of CD8 and HLA-A2-HBs183-191 pentamer staining in s183-TCR electroporated T cells at 24 hours postelectroporation. Bar charts show the frequency of IFN-γ, TNF-α and IL-2 producing cells out of CD8 or CD4 electroporated T cells.

In summary, our results demonstrate the anti-HCC potential of T cells transiently expressing anti-HBV TCR by mRNA electroporation. The number of cells expressing the receptors 24 hours after electroporation was much higher than that achieved by retroviral transduction. Due to the transient receptor expression and antigen-specific

functionality of electroporated T cells, multiple infusions of electroporated T cells may be necessary to achieve significant clinical effects. This requirement should not be a major limitation because the transduction method is straightforward and requires only a few hours even when performed in a large-scale cGMP setting. Because of the transient TCR expression resulting from mRNA electroporation, the concerns of adoptive transfer of antigen-specific CTLs inducing or exacerbating hepatitis should be less worrisome. Importantly, the successful TCR expression in a large number of cells using cGMP-compliant procedures provides proof-of-principle that this approach can be translated into clinical application, and we plan to use these cells for experimental treatment of HBV-related HCC patients in the future. Its practical features and their reduced half-life also suggest potential for other uses, such as attempts to boost antiviral T cell therapy in patients with chronic hepatitis B. Finally, it should be possible to adapt the method to TCR recognizing other viral peptides, like EBV, for treatment of other virally-associated malignancies.

7 FUTURE PERSPECTIVES

In this thesis, we set out to better understand the role of HBV-specific CD8 T cells in different phases of HBV infection, and their contribution to hepatic immunopathology. We also developed a new strategy based on TCR gene transfer to reconstitute the defective antiviral immunity of chronic HBV/HCC patients. Our results showed that CXCL-8, -9 and -10 present during HBV infection could contribute to liver inflammation, and that HBV-specific T cells can be induced to secrete particular cytokine/chemokine through exposure to environment factors, suggesting that the intrahepatic cytokine milieu can possibly modulate the function of immune cells in the liver microenvironment. We also showed the possibility to engineer polyfunctional T cells (by retroviral transduction or mRNA electroporation of anti-HBV TCR) to eliminate HBV-expressing HCC. These encouraging results support the use of TCR-redirected T cells in the clinic, and we plan to initiate further studies in mouse model and experimental treatments in patients in the future. The study of the contribution of HBV-specific T cells to liver immunopathology could perhaps provide insights to the adoptive transfer of such cells and the possibility that they could trigger severe liver injury, and future studies should aim to minimize the degree of liver injury.

7.1 EXPANSION OF HBV TCR LIBRARY

Because the expression of HBV antigens and epitopes will differ between HCC tumors, therefore developing TCRs specific for multiple HBV epitopes will be necessary to broaden the applicability of TCR gene therapy for the treatment of HBV-related HCC. Over the past few years, our laboratory has been actively cloning TCRs restricted to different HLAs and directed towards different antigens of HBV (Table 6). Additionally, we have also cloned TCRs specific for other viral diseases, such as CMV, EBV, SARS and influenza. We plan to streamline the process of TCR cloning by using TCR mRNA electroporation (takes 3 – 4 days) instead of retroviral transduction (2 weeks) to screen for productive and functional TCRs in primary T cells.

HBV epitope	Sequence	HLA restriction	TCR chains
Core 18-27	FLPSDFFPSV	A0201	Vα17 Vβ12.4
Env 171-180	FLGPLLVLQA	Cw0801	Vα5 Vβ20.1
Env 183-191	FLLTRILTI	A0201	Vα34.1 Vβ28
Env 370-379	SIVSPFIPLL	A0201	Vα12 Vβ7.8

Table 6. Library of HBV-specific TCRs.

7.2 FURTHER DEVELOPMENT OF TCR MRNA ELECTROPORATED T CELLS

We have shown that it is possible to express functional HBV TCR on activated T cells by TCR mRNA electroporation, with an expression efficiency of 80% of CD8+ T cells expressing anti-HBV TCR 24 hours postelectroporation. Recently, we have also successfully expressed anti-HBV TCR on resting T cells or total PBMC using mRNA electroporation. Although the expression efficiency on both resting T cells and total PBMC is low, (approximately 20% and 10% of CD8+ T cells respectively expressed the TCR) these cells are polyfunctional (produced IFN-γ, TNF-α and IL-2). In addition to their different cytokine profile from activated T cells, resting electroporated T cells expressed low levels of perforin and granzyme, as measured by mean fluorescence intensity, in comparison to activated electroporated T cells. It will be interesting to further characterize the functional profile of resting electroporated T cells, in particular their ability to mediate noncytolytic clearance of HBV-infected hepatocytes through the production of antiviral cytokines, in comparison to activated electroporated T cells or retroviral transduced ones.

With mRNA electroporation, it will be easier to redirect the specificity of particular subsets of cells and analyze their antiviral/antitumor efficacy. An important aspect of adoptive transfer of HBV-specific T cells is their ability to home specifically to the liver. An interesting finding regarding T cell homing to the liver was reported whereby a higher level of CD161 is expressed on HCV- and HBV-specific CD8+ T cells compared to CD8+ T cells specific for non-hepatotropic viruses.[314] CD161+CD8+ T cells also expressed CXCR6, a chemokine with a major role in liver homing, they could produce proinflammatory cytokines (IFN-γ and TNF-α) but contained low levels of cytolytic molecules (granzyme B and perforin) suggesting that they may have diminished capacity to kill virally infected cells through the cytolytic pathway. It will therefore be interesting to redirect the specificity of CD161+CD8+ T cells and to evaluate their ability to home specifically to the liver. Furthermore, we can study if these cells will minimize the degree of hepatocyte lysis compared to conventional CD8 CTLs.

7.3 IN VIVO STUDY OF TCR MRNA ELECTROPORATED T CELLS

Results from our xenograft model have provided a proof-of-principle that TCR mRNA electroporated T cells can eliminate HCC *in vivo*. However, a limitation of our current model is that the model does not have HBsAg-positive normal hepatocytes while in HBV-related HCC patients, normal hepatocytes expressing HBV antigens and transformed hepatocytes are present in the liver. We will therefore like to further analyse TCR mRNA electroporated T cells in a mouse model that more closely recapitulate HBV-related HCC in patients. A possibility is to study the adoptive transfer of TCR mRNA electroporated T cells in HBsAg transgenic mice that develop hepatocellular carcinoma.[32,102] Additionally, the antiviral effect of

mRNA electroporated T cells can be studied in a chronic HBV infection mouse strain with human HLA-A2/DR1 transgenes and transduced with adeno-associated virus carrying a replication-competent HBV DNA genome.[315] Particular concerns are how efficiently the redirected T cells will be recruited to the liver parenchyma and if these cells will trigger massive liver injury. Hence, it will be important to properly analyse the number of redirected T cells for adoptive transfer that can exert efficient antiviral/antitumor effect and concurrently minimize the degree of liver injury.

Additionally, we are very interested in using TCR mRNA electroporated T cells for experimental treatments of HBV-related HCC patients. At the moment, due to safety concerns, we intend to evaluate the clinical efficacy of mRNA electroporated T cells in HCC patients who have had liver transplantation and the liver is not HBV-infected but have metastases of HCC. Our first experiment in a patient has given some indication of the anti-HCC efficacy of the redirected T cells. This patient was a 62 years old male who 11 years earlier had a HLA-mismatch liver transplantation, and then exhibited HCC metastases one year ago, with spread to the bones and lungs. The HCC metastases were HBsAg+/HLA-A2+ while the liver was uninfected. The patient was out of treatment options, and agreed to be treated using retrovirally tranduced TCR-redirected T cells (10^4 cells/kg) at the University of Pisa, Italy. Four weeks after cell therapy, the serum HBsAg dropped significantly from >3500 IU/ml to <1000 IU/ml, demonstrating the anti-HCC effect of the infused cells (H. Stauss and A. Bertoletti, manuscript in preparation). Such encouraging results support the testing of mRNA electroporated T cells in more patients like this in the future.

Through these studies in mouse models and experimental therapies, we hope to bring the use of TCR-redirected T cells for personalized treatment of chronic HBV/HCC one step closer to clinical practice.

8 ACKNOWLEDGEMENTS

This journey has been one of the longest and toughest process in my education so far. Nonetheless, it was a fulfilling experience of learning about science, friendships, family and love. I have seen myself maturing over the years not only in scientific thoughts, but also in emotional and social intelligence. Many people have contributed to this work and to my personal development, and I would like to take this opportunity to express my appreciation and sincere gratitude to the following:

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