Therapeutic vaccination for chronic hepatitis B in the Trimera mouse model

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1 Introduction

1.1 Epidemiology of Hepatitis B (HBV) Infection

Hepatitis B is one of the major diseases of mankind and is a serious global public health problem. According to World Health Organization (WHO) over 2 billion people have been infected with the hepatitis B virus (HBV), among them, more than 360 million have chronic (lifelong) infection which may lead to liver cirrhosis and hepatocellular carcinoma. Each year, more than 600,000 persons die worldwide with hepatitis B-associated acute and chronic liver diseases (Shepard, Simard et al. 2006). The prevalence of chronic HBV infection continues to be highly variable, ranging from 10% in some Asian and western pacific countries to under 0.5% in the United States and northern European countries.

The routes of transmission include vertical (mother to child), early life horizontal transmission (through bites, lesions), and adult horizontal transmission (through sexual contact, intravenous drug use, and medical procedure exposure). These routes are evident to varying degrees in every country. Perinatal or early horizontal infection in childhood are the main routes of HBV transmission in high endemic area, such as south-east Asia and Africa whereas in low endemic regions, such as western countries, Hepatitis B is transmitted mainly by sexual activity and injection drug use, thus it is considered as adolescent disease. In any region of the world, younger age acquisition of HBV infection continues to be the most important predictor of chronic carriage (Fattovich 2003).

1.2 Structure of Hepatitis B virus

The hepatitis B Virus is a hepatotropic, non-cytopathic, enveloped, double-stranded DNA-Virus (Figure 1). The human HBV virus is the prototype for a family of viruses, referred to as Hepadnaviridae. It is the smallest DNA virus known, having only 3200 bases in

its genome (Ganem and Varmus 1987)**.** The most closely related to human HBV have been found in woodchucks (WHV) and ground squirrels with 70% homology. The host range of human HBV is narrow, to date, productive infection have been established only in human beings and higher primates (Seeger and Mason 2000)**.** In permissive hosts, viral antigens and DNA are found primarily within liver cells. However, recently viral DNA sequences have been detected in lower copy number in cells other than hepatocytes, -most commonly in peripheral blood leukocytes and bone marrow (Ganem and Varmus 1987).

Electron microscopic studies on purified HBV from human serum reveal 43nm double shelled particles called "Dane Particles" which now are known as infectious virions (Figure 3A). These virions contain an outer lipoprotein envelope consisting of three related envelope glycoproteins termed surface antigens. Within the envelope is the viral nucleocapsid, or core. The core is composed of basic phosphoprotein of 21kd, the hepatitis B core antigen (HBcAg). These core particles contain the viral genome, a relaxed-circular, partially duplex DNA of 3.2 kb (plus and minus strand), and a polymerase that is responsible for the synthesis of viral DNA in infected cells (Ganem and Prince 2004). In addition to virions, HBV-infected cells produce two distinct sub-viral lipoprotein particles**:** 20-nm spheres present in 10^3-10^6 fold excess to virions (Figure 3B) and filamentous forms of similar diameter. These HBsAg particles contain only envelope glycoproteins and host-derived lipids (Ganem and Varmus 1987).

The HBV genome contains four open reading frames **(ORFs)** which are encoded by the minus-strand DNA (Figure 2). These ORFs encode the viral envelope, nucleocapsid, polymerase, and X proteins. The nucleocapsid open reading frame (ORF C) encodes Hepatitis B core antigen (HBcAg) and hepatitis e antigen (HBeAg). This ORF C contains two in-phase start codons that define two overlapping polypeptides. The shorter of these polypeptides, 21-kD Core protein (HBcAg) self assembles in the cytoplasm of hepatocytes to

form nucleocapsid particles that package the viral polymerase and pregenomic RNA and thereby facilitate viral replication. These nucleocapsid particles migrate to the nuclear membrane of the host cell where the particles disassemble and release the viral genomes into nucleus then maturing into covalently closed circular viral DNA (cccDNA) (Chisari and Ferrari 1995). A longer part of core protein, a 24-kD precore is translocated via a signal peptide at its extreme amino terminus into the endoplasmic reticulum (ER) and then it undergoes truncation of amino and carboxy terminal residues and is secreted into blood as 16-kD long HBeAg which serve as good serological marker of viral replication in infected patients with wild type virus.

The envelope open reading frame (ORF S, = surface) contains three in-phase start codons and encodes the three viral surface antigens. The most abundant protein is the 24-kD-S protein (which is known as small or major HBsAg). Initiation at the more upstream start codon generates the M (middle or preS2) protein. Initiation at the most upstream start codon yields the L (large or preS1) protein, which is thought to play key roles in the binding of the virus to host-cell receptors and in the assembly of the virion and its release from the cell (Klingmuller and Schaller 1993; Ganem and Prince 2004). All three envelope polypeptides assemble to the viral envelope and are essential components of the infectious viron (Dane particle), that forms by budding into the lumen of the endoplasmic reticulam (ER); the viron is secreted after glycosylation of envelope residues in the Golgi apparatus (Chisari 1992).

The polymerase open reading frame encodes the viral polymerase protein. It contains reverse transcriptase, DNA polymerase and RNAse H domain as well as a 5’ DNA binding protein, which serves as a primer for reverse transcription of the viral pregenome. The polymerase gene products play essential roles in encapsidation and replication of the viral genome (Chisari and Ferrari 1995).

The X open reading frame encodes the 17 kD size viral X protein (pX), the role of which in the viral life cycle is not clearly understood (Kremsdorf, Soussan et al. 2006). Based on *in vitro* data, the pX represents a moderate transcriptional transactivator of the native viral promoters which directly and indirectly affect host and viral gene expression (Colgrove, Simon et al. 1989). Tissue culture experiments as well as transgenic mice experiments showed that the pX gene product is not required for HBV replication and virion secretion (Blum, Zhang et al. 1992; Reifenberg, Nusser et al. 2002), whereas X-protein activity is absolutely required for the *in vivo* replication and spread of the virus in woodchuck hepatitis (Zoulim, Saputelli et al. 1994). In transgenic mouse studies it was shown that high level expression of HBx increases the incidence of hepatocellular carcinoma (HCC) (Koike, Moriya et al. 1994).

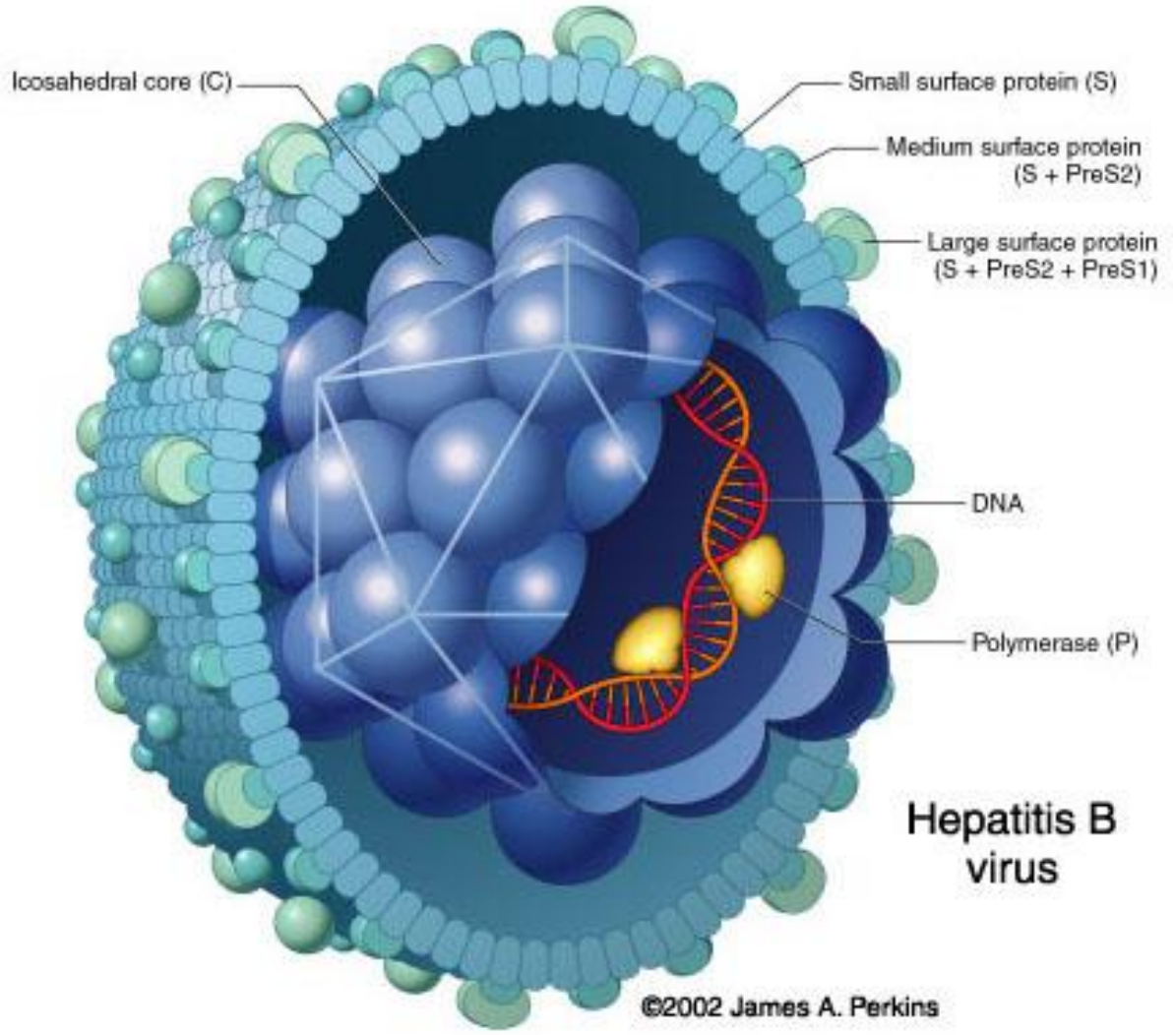


Figure 1: Model of the Hepatitis B Virus.

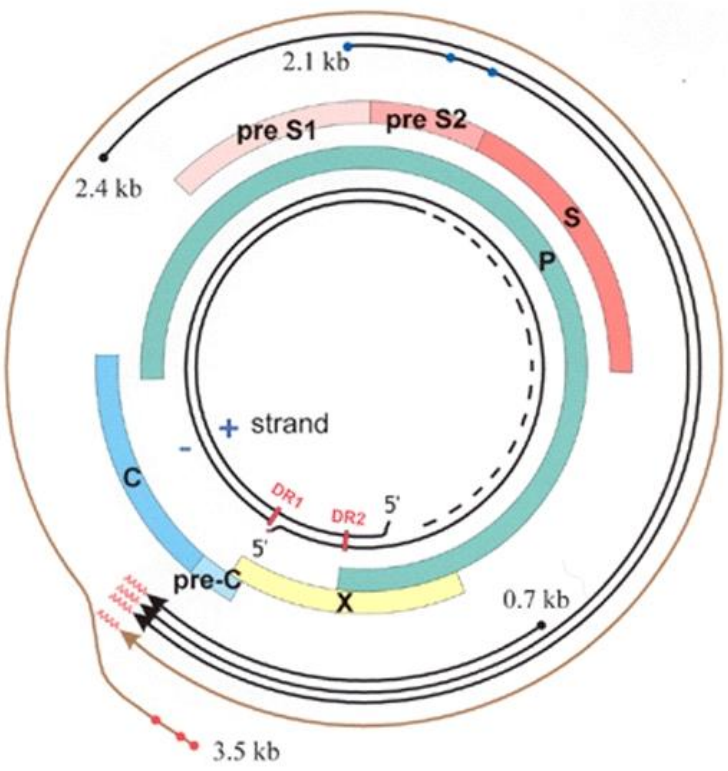


Figure 2: Schematic representation of the HBV genome showing the four main transcripts: Surface (S), Polymerase (P), Core (C) and X gene, arranged in overlapping reading frames. Adopted from (Kidd-Ljunggren, Miyakawa et al. 2002)

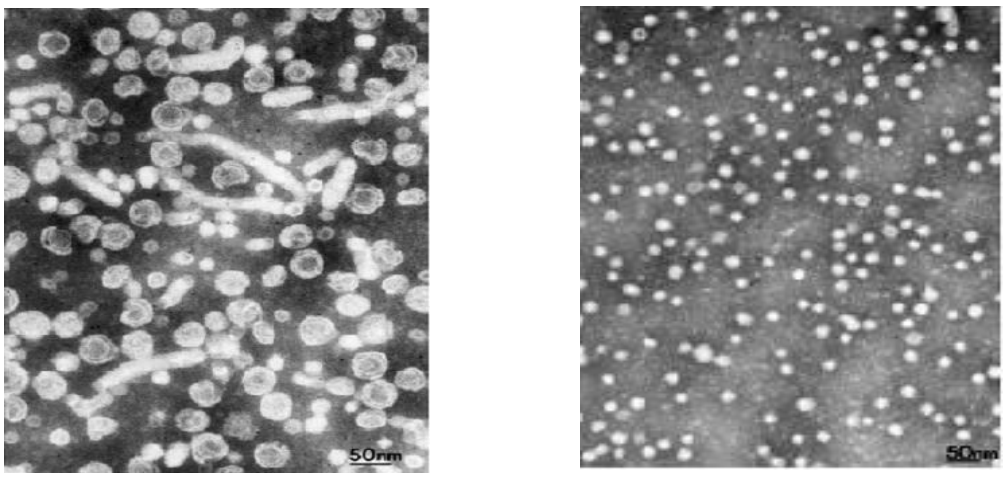


Figure 3: Transmission electron microscopic picture showing structure of HBsAg-Associated Particles. Panel A shows HBV virions or Dane particles and panel B shows 20-nm HBsAg particles. Adopted from (Ganem and Prince 2004). Scale bar = 50 nm. Original magnification X 400 000.

1.3 Clinical consequences of HBV Infection

Hepatitis B infection can run either clinically symptomatic or asymptomatic course, acute self limited hepatitis or fulminant hepatitis and chronic or persistent infection (Figure 4). The likelihood that newly infected persons will develop chronic HBV infection is dependent on their age at the time of infection. More than 90% of infected infants, 25-50 % of children infected between 1 and 5 years of age, and 6-10 % of acutely infected older children develop chronic infection. In contrast, most primary infections in adults, whether symptomatic or not, are self limited with clearance of virus from blood and liver and the development of lasting immunity to reinfection. However, less than 5% healthy adults do not resolve but develop into persistent infection where viral replication continues in the liver and there is persistent viremia (Shepard, Simard et al. 2006).

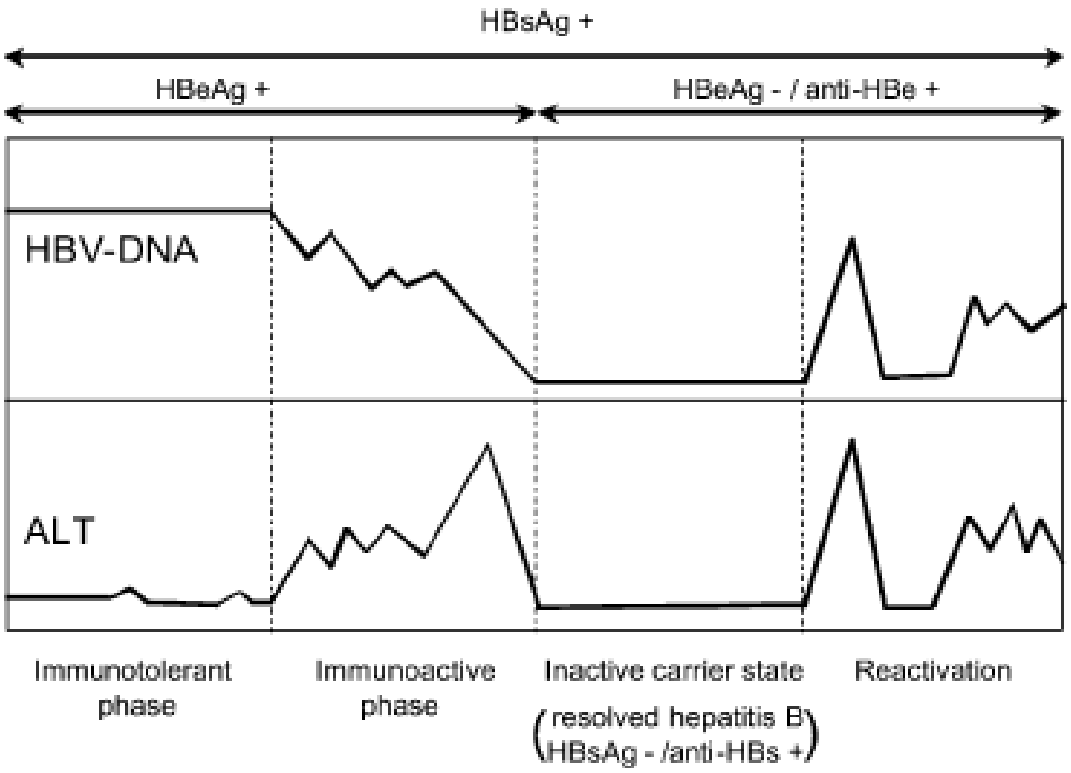


Figure 4: Natural course of HBV infection and schematic representation of the main four different clinical phases. Adopted from (Fattovich 2003)

1.3.1 Acute Infection

Acute hepatitis B can range from sub clinical disease to fulminant hepatic failure. The course and outcome of acute hepatitis B varies greatly by age and sex. About 25-50% cases of adult acute HBV infection are symptomatic; the remainders are asymptomatic or are associated with unspecific symptoms, whereas over 90% of perinatal HBV infections are asymptomatic (Shepard, Simard et al. 2006). Many acutely infected Persons will develop symptoms like abdominal pain, fever, jaundice, and dark urine.

In acute HBV infection, HBsAg becomes detectable in the blood after an incubation period of 4 to 10 weeks, followed shortly by antibodies against the HBV core antigen (anti-HBc), which are mainly of the IgM isotype. Viremia is well established by the time HBsAg is detected, and titers of virus are very high, frequently 10^9 to 10^{10} copies per milliliter (Ribeiro, Lo et al. 2002). Circulating HBeAg becomes detectable in most cases, and animal studies with primary hepadnaviral infection show that 75 to 100 percent of hepatocytes are infected when this antigen is evident (Ganem and Prince 2004).

In early incubation phase patients have normal or slightly increased serum ALT levels and minimal histological activity, which implies that there is a lack or very weak immune response against the infected hepatocytes. In adults, this phase lasts for 2-3 weeks whereas in infants this phase often lasts for decades. Lateron, patients enter into immuno-reactive phase in which the T cell mediated immune response is generated, and liver injury will be triggered. This phase usually lasts for 3-4 weeks with clinical symptoms and jaundice. In this phase, the ALT levels in serum will be elevated and titers of virus in blood and liver begin to drop. With clearance of infection, the viral antigens HBsAg and HBeAg will disappear from the circulation and anti-HBs antibodies become detectable (Fattovich 2003).

1.3.2 Chronic Infection

The chronic Hepatitis B infection is characterised by persistence of HBsAg and HBV DNA for more than six months or by the presence of HBsAg in a person who tests negative for IgM antibodies to Hepatitis B core antigen. (Shepard, Simard et al. 2006). Approximately 10% of all acute HBV infections progress to chronic infection. (Lok and McMahon 2001).

The course of chronic HBV infection may include four phases. **The first phase (immune tolerance)** is characterized by the presence of HBeAg, high levels of serum HBV DNA, normal ALT levels with minimal or no inflammation on liver biopsy. This phase typically occurs after perinatal infection and may last 1 to 4 decades. Spontaneous and treatment induced HBeAg seroconversion is infrequent in this phase (< 5% per year). Follow-up studies for periods of 10.5 years showed, that only 5% of patients progressed to cirrhosis and none to HCC indicating that prognosis is favorable for patients who are in this immune tolerant phase. However, recent studies showed that in the patients with childhood or adult-acquired HBV infection, the “immune tolerant” phase is short-lived or absent.

The second phase (immune clearance/HBeAg positive chronic hepatitis) is characterized by the presence of HBeAg, high or fluctuating serum HBV DNA levels, persistent or intermittent elevation in serum ALT levels and active inflammation on liver biopsy. The flares of ALT levels are believed to be manifestations of immune-mediated lysis of infected hepatocytes and increased T cell responses to HBV core antigen (HBcAg) and HBeAg. An important outcome of this immune clearance phase is seroconversion of HBeAg to anti-HBe. Factors involved in this seroconversion are older age (McMahon, Holck et al. 2001), high ALT levels (Liaw 2003; Yuen, Yuan et al. 2003) and HBV genotypes (Yim and Lok 2006). Several longitudinal studies have shown that in most cases, seroconversion from HBeAg to anti-HBe is associated with marked reduction or elimination of HBV replication

together with biochemical and histologic regression of inflammatory activity. Histologic improvement occurs gradually months to years after HBeAg seroconversion.

The third phase (inactive HBsAg carrier state) is characterized by absence of HBeAg, presence of anti-HBe, persistently normal ALT levels and low or undetectable serum HBV DNA, minimal or no necroinflamation. Liver biopsy usually shows mild hepatitis and minimal fibrosis, but inactive cirrhosis may be observed in patients who had accrued severe liver injury during the preceding "immune clearance" phase. Follow-up studies indicate that these carriers show sustained biochemical remission and very low risk of cirrhosis or hepatocellular carcinoma (HCC) (Fattovich 2003). Approximately, 20-30% of inactive carriers may undergo spontaneous reactivation of HBV replication with reappearance of biochemical and necroinflamatory activity. This reactivation may occur spontaneously or as a result of immunosuppression (Lok, Liang et al. 1991). Some carriers eventually become HBsAg negative and develop anti-HBs. The incidence of delayed HBsAg clearance has been estimated 1-2% per year in western countries where HBV infection is usually acquired in adulthood, but only 0.05- 0.8% per year in endemic areas where HBV infection is mostly acquired perinatally or in the early childhood (Fattovich 2003).

The fourth phase (reactivation of HBV replication/HBeAg-negative chronic HBV) is characterized by negative HBeAg, positive anti-HBe, detectable HBV DNA, elevated ALT levels and continued hepatic necroinflammation (Hadziyannis and Vassilopoulos 2001). Most patients reach this phase after a variable duration of inactive carrier state, but some progress directly from HBeAg-positive chronic hepatitis to HBeAg-negative chronic hepatitis. The atypical serological profile is sustained by HBV variants which are unable to express HBeAg. The most frequent precore mutation is a G to A change at nucleotide 1896 (G1896A), which creates a stop codon in the precore region of the HBV genome with loss of HBeAg synthesis. Other patients may have other changes in core

promoter region which reduces HBeAg production, the most common core promoter mutations are, A-T at nucleotide 1762 and G-A at nucleotide 1764 (Fattovich 2003). HBeAg-negative chronic hepatitis B has been reported in all parts of the world. The geographic variations in prevalence of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants is related to the predominant HBV genotype in that region (Yim and Lok 2006). Patients with HBeAg negative chronic hepatitis are usually older and have more advanced liver disease.

1.3.3 Liver cirrhosis and Hepatocellular carcinoma

The complications of chronic hepatitis B include progression to cirrhosis and liver failure, hepatocellular carcinoma and extra-hepatic diseases (Villeneuve 2005**).** The overall incidence of progression to cirrhosis in chronic HBV infected patients is about 6%, with a cumulative five year incidence of cirrhosis of 20% (Fattovich, Brollo et al. 1991; Buster and Janssen 2006). The rate of cirrhosis has been reported higher in HBeAg negative HBV patients as compared to HBeAg positive HBV patients (Fattovich 2003). In HBeAg positive patients the annual incidence of progression to cirrhosis is 2 to 5 % (de Franchis, Hadengue et al. 2003), whereas in HBeAg negative patients, progression to cirrhosis occurs at annual rates of 8 to 10% (Yim and Lok 2006).

The variability in the rate of progression to cirrhosis may be related to differences in the clinical and serological features of HBV infected patients. The higher rate of cirrhosis among HBeAg-negative patients is related to older age and more advanced liver disease at presentation and among HBeAg positive patients, the rate of cirrhosis development is higher in those who remained HBeAg positive during follow-up (Fattovich 2003). The other factors associated with progression to cirrhosis include habitual alcohol intake, high serum HBV

DNA and coinfection with hepatitis C virus (HCV), hepatitis D virus (HDV) or human immunodeficiency virus (HIV**)** (Buster and Janssen 2006).

Studies in Asian patients indicate that hepatitis virus genotype C might be associated with a higher risk of cirrhosis than genotype B and preliminary data suggest that genotype C, but not core promoter or pre-core mutation correlates with more severe liver disease in HBeAg negative chronic hepatitis (Kao, Chen et al. 2000; Fattovich 2003). Concurrent HBV/HCV infection has been reported in approx. 10-25% of patients and longitudinal studies show that progression to cirrhosis in HBV/HCV coinfection is more common than in those with single infection. (Fattovich, Tagger et al. 1991; Fattovich, et al 2003). Once cirrhosis is established, the yearly incidence of hepatic decompensation is about 3% (Realdi, Fattovich et al. 1994) and the risk of decompensation is much higher in patients with active viral replication than in inactive carriers (de Jongh, Janssen et al. 1992).

Hepatocellular carcinoma (HCC) is cancer that arises from hepatocytes, the major cell type of the liver. The development of HCC is a major global health problem and chronic hepatitis B infection and cirrhosis are the most important risk factors to develop hepatocellular carcinoma (Fattovich 2003). The incidence of HBV related HCC is higher among Asians (800 to 1000 case per 100,000 person-year) compared with Canadians and Alaskans (470, 190 cases per 100,00 person-year), respectively (Chan and Sung 2006). The most relevant oncogenic agent for HCC development is chronic Hepatitis B virus (HBV) or hepatitis C virus infection (HCV) (Bosch, Ribes et al. 1999). This prevalence varies largely among different geographic areas showing a close correlation of the prevalence of viral infection and HCC (Bruix and Llovet 2003). The annual incidence of HCC is < 1% for non-cirrhotic carriers and 2% to 3% for patients with cirrhosis (McMahon, Holck et al. 2001; Hsu, Chien et al. 2002). Host, viral and other factors may contribute to the progression of hepatitis to HCC. Several studies showed that male gender and older age are important determinants of

HCC (McMahon, Alberts et al. 1990; Fattovich 2003). The other risk factors for HCC are co-infection with HCV, habitual alcohol intake, high levels of HBV replication, HBV genotype (C>B) and core promoter mutations (Yim and Lok 2006).

The oncogenic mechanisms of HBV are not fully understood (Okuda 2000). HBV DNA does integrate into the genome of infected hepatocytes, causing chromosomal deletions, increased liver cell proliferation and tumor induction (Shafritz, Shouval et al. 1981; Kremsdorf, Soussan et al. 2006). The viral X gene may have an important role as it is a powerful trans-activator for transcription of oncogenes such as C-myc and C-jun (Bruix and Llovet 2003). Recent human studies suggest that HBV positive HCC are characterized by higher chromosomal instability leading to loss of heterozygosity as compared to tumors related to HCV (Laurent-Puig, Legoix et al. 2001).

Mortality from HCC did decrease due to implementation of preventive strategies. In patients with chronic hepatitis B, interferon-α, lamivudine, adefovir and other antiviral agents reduced the incidence of cirrhosis and also of HCC. However if cirrhosis is well established there is no agent that has been proven to diminish the HCC risk (Bruix and Llovet 2003).

1.4 Therapy of HBV Infection

The indication for treatment of hepatitis B infection depends on the clinical stage of the disease. Rates of progression to cirrhosis and HCC vary according to the state of immune system, age of the patient, the histological stage of infection, geographic and genetic factors (Lee 1997).

Antiviral treatment is, in general, not recommended for patients with acute hepatitis B infection, since the outcome from acute hepatitis B is good in the majority of immuno-competent adult patients (Buster and Janssen 2006). The HBeAg positive chronic patients, with high viral loads (>10^5copies/mL) and persistence of elevated ALT levels have an

increased risk of progression to cirrhosis and HCC thus these patients are recommended for treatment. (Ganem and Prince 2004). An HBV DNA level of 10^4copies/mL is currently used as cut-off for HBeAg negative patients, since this level was found to differentiate patients from having HBeAg-negative chronic hepatitis or being inactive carriers (Manesis, Papatheodoridis et al. 2003). These patients have normal ALT values, tend to have relatively stable course with low rates of clinical or pathological progression (de Franchis, Meucci et al. 1993). At present, therapy is usually not offered to such patients. However, some HBeAg negative patients have liver dysfunction and substantial viremia (>10^5copies/mL)(Ganem and Prince 2004). Results from a recent study suggest that many of these patients would also benefit from effective antiviral therapy (Hadziyannis, Tassopoulos et al. 2003).

The goal of therapy in patients with hepatitis B infection is suppression of viral load leading to reduction of hepatic-necroinflamation and progression of liver fibrosis (Buster and Janssen 2006). The established markers of successful therapy are the loss of HBeAg, seroconversion to anti-HBe antibodies, normalisation of liver enzymes and reduction of circulating viral load. Generally the patients with stable seroconversion to anti-HBe positive status, have improved histologic findings in the liver and this improvement tend to be maintained over the long term (Niederau, Heintges et al. 1996). True cure of infection (loss of HBsAg and complete disappearance of viremia) is achieved only in a small proportion of patients (1to 5%) with currently available antiviral agents. Increasing numbers of new antiviral drugs might increase the success rate of HBV treatment (Ganem and Prince 2004).

In the patients with HBeAg-negative chronic hepatitis, there is no valid surrogate marker of long term treatment response. Thus, serum HBV DNA level has become the most important marker of treatment response in the management of HBeAg-negative chronic hepatitis B (Buster and Janssen 2006).

Two major types of antiviral drugs are currently approved for the treatment of chronic hepatitis B**:** drugs that directly interfere with replication and drugs that modulate the HBV specific immune response. Nucleoside and nucleotide analogues, such as lamivudine, adefovir and entacavir directly inhibit the viral polymerase, while interferon alfa, peginterferon α-2a act directly antiviral and by immune stimulation (Buster and Janssen 2006).

1.4.1 Interferon alfa

Interferon alpha (IFN-α) was approved for the treatment of chronic hepatitis B infection in most countries in the early 1990s (Ganem and Prince 2004). Patients with chronic hepatitis B have deficient responses to endogenous interferon. Recombinant interferons, resemble the naturally occurring cytokines produced in response to viral infections. These recombinant interferons have immunomodulatory, antiproliferative, and antiviral properties (Haria and Benfield 1995). By inducing the display of HLA class I molecules on hepatocyte membranes, they promote lysis of infected cells by CD8+ cytotoxic lymphocytes, thus inhibiting viral protein synthesis (Perrillo, Schiff et al. 1990). In addition, they induce a multitude of intracellular ISG (interferon sensitive genes), that act directly antiviral (Rang, Gunther et al. 1999). However, clinical efficacy of interferon alfa (IFN-α) is limited to a small percentage of highly selected patients. (Lok and McMahon 2001)

In patients with HBeAg-positive hepatitis B, interferon -α given subcutaneously at a dose of 5 MU daily or 10 MU thrice weekly induces loss of HBV DNA and HBeAg in 37% and 33%, respectively, compared with 17% and 12% in controls after 12 to 24 weeks of therapy (Ocama, Opio et al. 2005). The HBe seroconversion and normalization of ALT levels is usually sustained after therapy discontinuation (Wong, Cheung et al. 1993). However, standard IFN-α induced responses are less durable in HBeAg-negative chronic HBV with

sustained response in only 10 to 35% (average 24%) at 12 months after cessation of therapy (Lampertico, Del Ninno et al. 1997; Lok, Heathcote et al. 2001). Long term follow-up studies in response to interferon-α therapy showed better overall survival and lower incidence of hepatic decompensation and HCC (van Zonneveld, Honkoop et al. 2004).

The addition of a polyethylene glycol (PEG) molecule to interferon significantly prolongs half-life and results in more sustained IFN activity. Two pegylated IFNs have been used for treatment of HBV, a large branched 40kDa PEG linked to IFN α-2a (peginterferon α-2a) and a smaller linear 12kDa PEG linked to IFN α-2b (peginterferon α-2b) (Craxi and Cooksley 2003). A 48 weeks course with one of these peginterferons achieved sustained virological response rates of 30-40% in HBe negative and positive patients (Wursthorn, Lutgehetmann et al. 2006). However, only Peginterferon α-2a has been registered for the treatment of chronic HBV in Europe and USA and is given by subcutaneous injection once weekly for 48 weeks in a dose of 180μg in both HBeAg positive and HBeAg negative patients. Peginterferon α-2b will only be registered in specific east-Asian countries (Buster and Janssen 2006).

IFN-α therapy is associated with many adverse effects including influenza like symptoms, fatigue, headache, myalgia, gastrointestinal symptoms (nausea, anorexia, weight loss) depression, and local reaction at the injection site. Most patients develop tolerance to the flu-like symptoms after the first week, but fatigue and depression may persist throughout the course of treatment and for a few weeks after discontinuation of therapy. Moreover, use of PEG-IFN is not recommended in advanced liver disease because it may potentially precipitate immunological flares and liver failure in these patients. (Lok and McMahon 2001; Buster and Janssen 2006)

1.4.2 Lamivudine

Antiviral agents that directly affect viral replication have become standard of care in recent years. All these drugs (to date) are nucleoside or nucleotide analogues that selectively target viral polymerase (Ganem and Prince 2004).Lamivudine is the enantiomer of 2'-3' dideoxy-3' thiacytidine and this is the first drug which was licensed for therapy of chronic hepatitis B. Incorporation of the active triphosphate (3TC-TP) into growing DNA chains results in premature chain termination so that the HBV DNA synthesis is inhibited (Lok and McMahon 2001). Lamivudine is taken orally in a dosage of 100mg daily and is well tolerated with an excellent safety profile.

In general, treatment with Lamivudine results in reduction of 3 to 4 log in circulating levels of HBV DNA in the first three months of therapy. This decline is associated with more rapid loss of HBeAg, seroconversion to anti-HBe-positive status, and improvement in serum ALT levels (Ganem and Prince 2004). In naïve patients, who received Lamivudine for one year, HBeAg seroconversion occurred in 16% to 18% compared to 4% to 6% in untreated controls. Histologic improvement was observed in 49% to 56% in treated patients compared to 23% to 25% in untreated controls (Lok and McMahon 2001). Higher cumulative HBeAg seroconversion rates were observed with increased duration of lamivudine treatment, with 29% at two years, 40% at three years and 47% at four years of therapy (Leung, Lai et al. 2001). Although lamivudine is not an immunomodulator, there is strong evidence that successful treatment relies to some extent on an adequate host immune response. In particular, pretreatment ALT level has been found to be the most important predictor for response. Recent studies showed that HBeAg seroconversion occurred in 65% of patients with pretreatment ALT levels more than 5 times than normal, as compared to only 26% of patients with pretreatment ALT levels 2 times higher than normal (Chien, Liaw et al. 1999; Perrillo, Lai et al. 2002).

Lamivudine therapy is also beneficial for patients with HBeAg-negative chronic hepatitis B. A recent study on HBeAg negative chronic hepatitis B patients showed that virologic and biochemical responses were achieved in 63% (34 of 54) of patients who received 24 weeks of lamivudine therapy, compared with 6% (3 of 53) of those receiving placebo. Of the 54 patients who completed 1 year of lamivudine therapy, serum HBV DNA was undetectable in 65% by bDNA assay and in 39% by PCR assay , and histologic improvement was observed in 60% of patients (Tassopoulos, Volpes et al. 1999). Lamivudine can also safely be used in patients with decompensated cirrhosis. Studies of lamivudine in such patients showed that lamivudine treatment is well tolerated and results in clinical improvement (Villeneuve, Condreay et al. 2000).

The major limitation of lamivudine, which significantly limits its use as first line therapy, is the high rate of viral resistance occurrence, which is mediated largely by point mutations at YMDD motif of the polymerase gene. The most important mutation is a substitution of valine or isoleucine for methionine at position 204 (Tipples, Ma et al. 1996). Lamivudine resistance is usually manifested as breakthrough infection defined as reappearance of HBV DNA in serum. The frequency of resistance increases with the duration treatment, from 24% at one year to 38% at two years, 50% at three years and 67% at four years (Leung, Lai et al. 2001). However, although the level of viremia rises, in many patients it may still remain below pretreatment levels due to the reduced viral replicative fitness of most mutant viruses. In addition, some patients continue to undergo conversion from HBeAg-positive to HBeAg negative status, even after appearance of lamivudine resistance mutants in circulation (Ganem and Prince 2004). In case of resistance appearance, treatment is changed to another drug with different resistance profile, preferably as combination treatment.

1.4.3 Adefovir dipivoxil

Adefovir dipivoxil is a second antiviral drug which was approved by the FDA in 2002 to treat HBV infection. Adefovir is a nucleotide (adenosine monophosphate) analogue and it is a prodrug that undergoes two intracellular phosphorylations to yield the active drug which is an inhibitor of the viral polymerase (Buster and Janssen 2006).Adefovir at the dose of 10mg daily is well tolerated and has a good safety profile. In HBeAg positive patients one year of adefovir therapy the serum HBV DNA ($<10^3$ c/mL) and normalization of ALT levels were observed as 21% and 48% in treated patients, compared with 0% and 16% of untreated controls. Moreover a study with 515 HBeAg positive chronic HBV patients showed that HBeAg seroconversion occurred in 12% of patients who received adefovir compared to 6% in the placebo group (Marcellin, Chang et al. 2003).

A different study of 185 patients with chronic hepatitis B who were negative for hepatitis B e antigen (HBeAg) showed that the treatment with adefovir for one year resulted in serum HBV DNA $<10^3$ c/mL and normalization of ALT levels in 51% and 72% of treated patients, compared with 0% and 29% of untreated controls (Hadziyannis, Tassopoulos et al. 2003). Resistance to adefovir is less common and occurs later in the course of HBV treatment compared with lamivudine. To date, mutations in the polymerase gene conferring resistance to adefovir include rtN236T and rtA181V. The cumulative probability of adefovir resistance is 0% at one year reaching 28% at five years of treatment. Moreover, studies showed that adefovir effectively inhibits the replication of lamivudine-resistant HBV mutants, both *in vitro* and *in vivo* (Perrillo, Schiff et al. 2000; Buster and Janssen 2006)

1.5 Immunopathogenesis of HBV infection

The hepatitis B virus (HBV) is an enveloped DNA virus that can cause acute and chronic liver disease. Most of the *in vitro* and *in vivo* studies proved that HBV is not directly

cytopathic for the infected hepatocyte (Hui and Lau 2005). The outcome of infection and pathogenesis of liver disease are determined by virus and host factors, which have only partially been defined because the host range of HBV is limited to man and chimpanzees (Bertoletti and Gehring 2006). Since HBV is not directly cytopathic, the immune response to HBV encoded viral antigens is responsible for both liver disease and viral clearance fallowing HBV infection (Chisari and Ferrari 1995). In general, the T- cell response during acute self-limited HBV infection is characterized by a vigorous, polyclonal, and multispecific cytotoxic and helper-T-cell response, and in chronic patients who are unable to clear the virus, this immune response is weak or undetectable (Jung and Pape 2002).

The initial protection against HBV infection is mediated by non-specific mechanisms of innate immunity. Among these mechanisms killing of virus infected cells with out HLA restriction, secretion of antiviral cytokines by natural killer (NK) cells and NK-T cells is believed to play an important role in host defense against HBV (Kakimi, Guidotti et al. 2000). However complete eradication and control of infection cannot be accomplished by natural immunity, therefore effector mechanisms capable of accurate recognition of specific viral structures are needed to successful elimination of HBV infection, which is the main function of adaptive immunity.

There are two major arms of the adaptive immune system to deal with free extra cellular virus and virus infected cells, humoral and cellular immunity. The humoral arm, which consists of B lymphocytes producing immunoglobulin to recognize viral antigens in their native form, either soluble secreted proteins or intact molecules expressed on infected cell surfaces (Jung and Pape 2002). The cellular arm is composed of various cell types, including macrophages and T-lymphocytes. Among these, T-lymphocytes play the major role in elimination of virus infected cells by recognition of processed viral antigens presented on the surface HLA molecules of infected host cells (Chisari and Ferrari 1995).

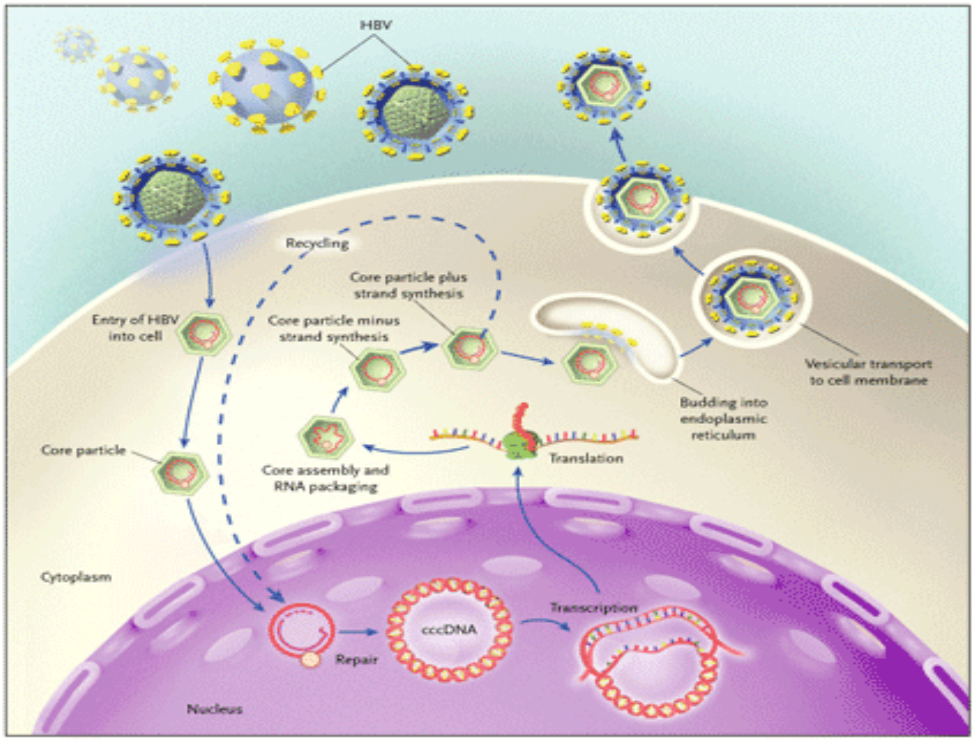


Figure 5: Schematic representation of The Replication cycle of HBV. Adopted from (Ganem and Prince 2004).

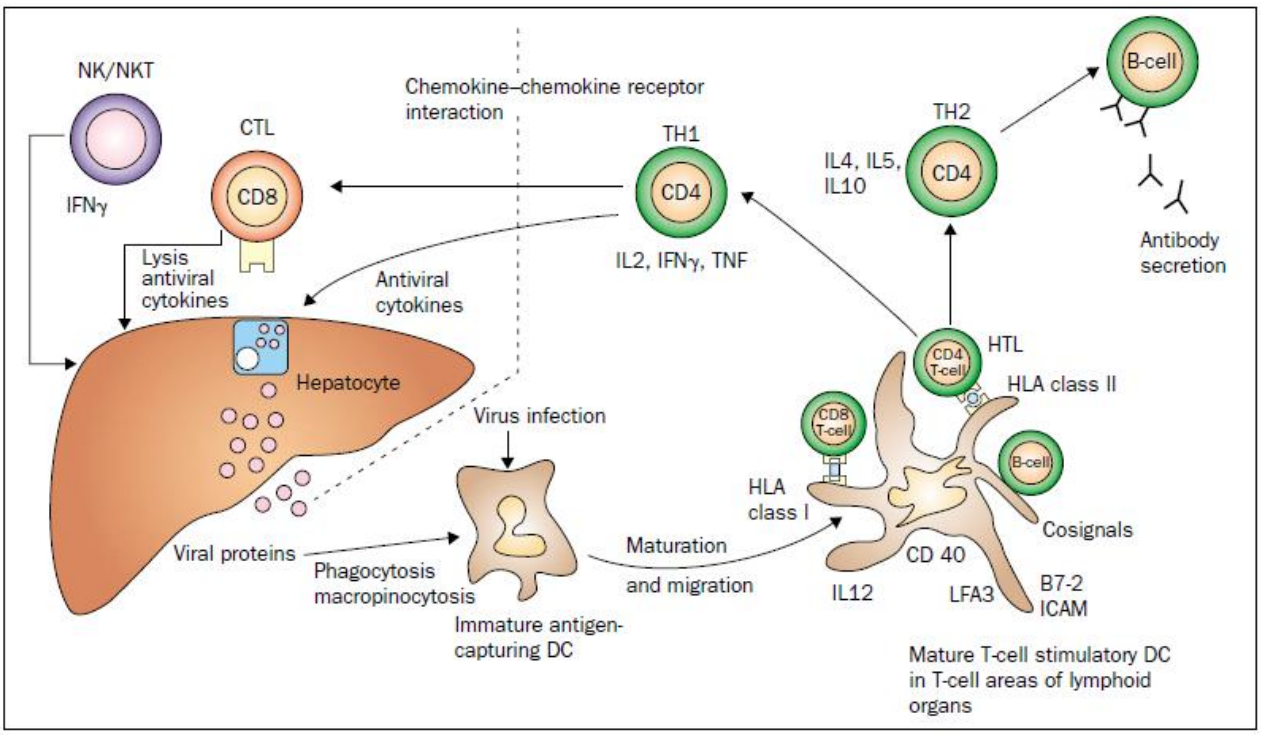


Figure 6: Interaction of different cell system in the immune response against HBV. Adopted from (Jung and Pape 2002).

1.5.1 Unspecific immune response

Innate immunity generally plays a role immediately after infection to limit the spread of the pathogen and initiate efficient development of an adaptive immune response. Innate host responses during the early phases of viral infections are mainly characterized by the production of type 1 interferon (IFN- α/β) cytokines and the activation of natural killer (NK) cells Experimental data mainly from animal models but also from humans show that after inoculation, HBV does not immediately start to replicate efficiently. HBV DNA and antigens are not detectable in serum or the liver until 4-7 weeks post infection. Following this period HBV begins a logarithmic expansion phase where viral copies reach 10^9-10^{10}/mL and can be detected in the liver and serum (Guidotti, Rochford et al. 1999; Thimme, Wieland et al. 2003). Experiments in chimpanzees showed that the initial lag phase of HBV replication does not appear to be a consequence of HBV inhibition by elements of innate and adaptive immunity. The activation of IFN-γ, interleukin (IL2) and tumour necrosis factor (TNF)-α and intrahepatic recruitment of inflammatory cells is delayed until the logarithmic expansion of HBV (Guidotti, Rochford et al. 1999). Moreover longitudinal analysis of activation of cellular genes in chimpanzees showed that no cellular genes were activated within the liver during the lag phase of infection, confirming that intrahepatic activation of innate immunity did not affect initial HBV spread (Wieland, Thimme et al. 2004).

HBV replication can be efficiently limited by IFN α/β, but data on acutely infected chimpanzees suggest that in contrast to other viral infections such antiviral cytokines are not triggered by HBV replication (Wieland, Thimme et al. 2004). HBV might have evolved strategies to escape this initial antiviral defense mechanism. Immediately after the exponential phase of HBV expansion, chimpanzees able to control the virus show a typical acute phase of disease with a robust activation of IFN-γ, TNF-α and many cellular genes

linked to Th1 type of response (Guidotti, Rochford et al. 1999; Wieland, Thimme et al. 2004). It is possible that this initial host response to HBV is primarily sustained by NK and NK-T cells. Although we lack direct evidence for this role of NK and NK-T cells in natural infection, data from different animal models support the possible role of these cells. Activation of NK T cells in the transgenic mouse model of HBV infection can inhibit virus replication through the production of IFN-γ (Kakimi, Guidotti et al. 2000). This is also proved in acutely infected chimpanzees, a rapid drop in virus replication occurs in the presence of intrahepatic IFN-γ production in absence of CD3 cells (Guidotti, Rochford et al. 1999). In humans the analysis of NK cell frequencies in patients showed increased numbers of circulating NK cells during the incubation period of acute hepatitis B and the peak of HBV replication (Webster 2000).

Thus activation of elements of innate immunity able to produce large quantities of IFN-γ seems to be a factor that determines the subsequent efficient induction of adaptive immunity and ultimately the outcome of HBV infection.

1.5.2 HBV specific Antibody response

Humoral responses play an important role in controlling HBV infection, Antibody production is critical for the neutralization of free circulating HBV particles and for the interference with virus entry into the host cells. Thus, antibodies limit cell to cell spread of viral infection (Bertoletti and Gehring 2006, Ferrari, Missale et al. 2003).

The antibody response to HBV- envelope antigens (HBsAg) is a T-cell- dependent process (Milich and McLachlan 1986). These anti-envelope antibodies are readily detectable in patients who clear the virus and recover from acute hepatitis, and they are usually undetectable or in low frequencies in patients with chronic HBV infection (Chisari and Ferrari 1995; Bocher, Herzog-Hauff et al. 1999). Antibodies to HBsAg serve as neutralizing

antibodies. These neutralizing antibodies can prevent spread of viral infection to new cells by forming complex with free viral particles and removing them from circulation and they can also prevent attachment and uptake of viral particles by susceptible cells. (Jung and Pape 2002).

A recent study on acute and chronic HBV patients or HBs vaccinated controls showed, that HBs vaccine recipients have maximal HBs-specific B- and T-cell responses after the first injection. After *in vitro* stimulation with HBsAg, peripheral blood mononuclear cells (PBMC) of only 1 of 5 acute and 1of 6 chronic HBV patients, but of all 6 vaccine recipients, secreted varying amounts of interferon gamma (IFN-γ) and addition of IFN-γ, resulted in strong increases of anti-HBs-secreting B cells in vaccine recipients and chronic carriers (Bocher, Herzog-Hauff et al. 1999). However *in vivo* studies in Human/mouse chimera showed that, PBMC from chronic HBV carriers failed to produce anti-HBs antibodies after adoptive transfer into chimeric mice and vaccination with HBsAg, whereas PBMC from recovered patients produced strong anti-HBs response. These findings suggested that there is a defect of antigen specific Th1 cells which are responsible for production of IFN-γ. This insufficient production of IFN-γ by antigen specific Th1 cells is suggested to be involved in viral persistence in such patients (Bocher, Herzog-Hauff et al. 1999; Bocher, Galun et al. 2000).

The role of the antibody response to the HBV nucleocapsid antigens (HBcAg, and HBeAg) and to the non-structural proteins still remains a debated issue. It is generally accepted that anti-HBc antibodies do not express virus-neutralizing activity because they are present in high titers in both acute and chronic HBV infected patients (Ferrari, Missale et al. 2003). The antibody responses to the viral polymerase and viral transactivator protein (pX) have not been extensively studied. However it has been reported that carboxy terminus of

polymerase, specially its RNase H domain, appears to be immunodominant at antibody level (Weimer, Schodel et al. 1990)

1.5.3 HBV specific T cell response

Cellular immune response to viral antigens play the major role in clearance of HBV infection. T cells recognize processed viral antigens in the form of short peptides associated with HLA molecules. HLA class –I molecules bind to peptide fragments derived from intracellular viral antigens synthesized within the infected cells and present to CTL which lyse virally infected target cells. In contrast, HLA class-II molecules generally bind viral peptides derived from extracellular antigens that are proteolytically processed in an acidified endocytic compartment of the antigen presenting cell (APC). These molecules are recognized by HLA class-II restricted CD4+ Th cells which express a regulatory function by secreting lymphokines that modulate the activity of antigen specific B cells and, CD8+ cytotoxic T cells (Chisari and Ferrari 1995).

In acute self limited hepatitis a vigorous HLA class-II restricted, CD4+ helper T cell response to multiple epitopes in the HBV nucleocapsid antigens (HBcAg/HBeAg) is detectable in the peripheral blood and core specific CD4+ response is temporally associated with the clearance of HBV from the serum, thus it seems to be essential for efficient control of virus. Whereas the envelope specific Th cell response is much less vigorous in the same patients (Jung and Pape 2002). Despite the vigorous response to nucleocapsid antigens, the basis for the absence of a strong HBV envelope specific Th cell response in *acutely* infected patients is not well understood. Among the several HBc/eAg epitopes that have been defined, the epitope located between core residues 50-69 is most commonly recognized by acutely infected patients (Ferrari, Penna et al. 1990; Ferrari, Bertoletti et al. 1991).

During chronic HBV infection, the HLA class-II restricted peripheral-blood Th cell response to all viral antigens, including HBcAg and HBeAg, is much weaker than in patients with acute hepatitis. These T cells appear in the liver at low frequency and functionally a Th-1 like response is found in these intrahepatic infiltrates (Chisari and Ferrari 1995). The nucleocapsid specific T cell response seems to be accentuated during acute exacerbations of disease which can often be preceded by increased serum HBV DNA and HBeAg concentrations that could drop substantially as the flare in disease activity subsides (Tsai, Chen et al. 1992).

Anti-viral CTL's are believed to play a major role in eradication of infection by virtue of their capacity to identify and kill virus infected cells through recognition of viral peptides presented by HLA class-I molecules (Yewdell and Bennink 1992). In acute HBV infection it has been shown that vigorous polyclonal multispecific class-I restricted CTL responses to all HBV antigens can be observed. These studies were based on the combined use of short synthetic peptides that mimic the processed antigen fragments and eukaryotic expression vectors that direct the synthesis of HBV antigens in human cells so that they can be processed and presented in context of HLA class-I molecules (Jung and Pape 2002). Most of the CTL epitopes identified thus far are HLA-A2 restricted and contain the HLA-A2 binding motif with leucine in position 2 and valine at the C-terminus (Bertoletti, Chisari et al. 1993; Nayersina, Fowler et al. 1993).

So far, a single HLA-A2 restricted CTL epitope has been identified in the nucleocapsid, eleven epitopes have been identified in the HBV envelope protein and five epitopes in the polymerase protein (Nayersina, Fowler et al. 1993; Chisari and Ferrari 1995). In addition, exogenous HBV envelope antigens, initially processed by the endosomal pathway can enter the class-I processing pathway and induce CD8+ class-I restricted CTL's. This exogenous envelope antigen is as effective at inducing class-I restricted CTL as

endogenously synthesized antigen. Perhaps this explains why the envelope-specific CTL response is so strong during acute hepatitis (Schirmbeck, Melber et al. 1994). In general, core 18-27, envelope 183-191, envelope 250-258, envelope 335-343, and polymerase 455-463 are most common CTL epitopes recognized by the majority of acutely infected patients.

In contrast to the vigorous CTL response in acute self limited patients, a weak or undetectable virus specific CTL response is observed in chronic HBV patients. Recent experiments indicate that HBV- specific CTL are nonetheless present at very low levels in periphery and in infected liver and they are specific for all of the viral antigens (Bertoletti and Gehring 2006, Rehermann and Nascimbeni 2005). Available data indicate that HBV specific T cell responses in the chronic stage of HBV infection are functionally impaired and much weaker than those detectable in acute self limited infection. Deletion, anergy, and a spectrum of functional impairments can affect virus specific CD8 cells in this chronic HBV stage (Bertoletti, A. et al 1994).

The high level production of viral antigen may lead to the exhaustion of virus specific CD8 cells or to the selection of virus specific T cells with altered functions. Mutations in relevant CTL epitopes or down regulation of MHC-I molecules are likely to play an important role in viral persistence (Chisari, F., and C.Ferrari. et al 1995) However, the main immunological feature that characterizes chronically infected patients is a state of relative hyporesponsiveness of HBV specific T cells compared to acutely infected patients.

In a recent study, MHC/peptide tetramer and intracellular cytokine staining (ICCS) were used to evaluate the biological properties of the HBV specific CD8 cells in chronic HBV patients with high level viral replication. Both *ex vivo* and *in vitro* studies showed that low frequency of CD8 cells are able to escape from peripheral deletion and persist along with high dose of viral antigen, displaying altered reactivity to the specific HLA –tetramer. In addition, *in vitro* data from the same study demonstrates that these CD8 cells were not

anergic and no TCR down regulation is operating, suggesting that the virus specific CD8 cells are not completely deleted and they are functionally active. However, these CD8 cells have reactivity restricted to the envelope antigen, suggesting that this impairment may be regulated differentially according to the dose and presentation of individual antigen (Reignat, S. et al 2002)

In vivo studies in Lymphocytic Choriomeningitis Virus (LCMV) mouse models showed that the persistent exposure to viral antigens can lead to virus specific T cell deletion or functional impairment. These exhausted T cells hyperexpress the programmed death 1 (PD-1) molecule. Blocking the engagement of PD-1 with its ligand (PD-L1) lead to an enhancement of the antiviral function of these T cells (Barber, D. L. et al 2006; Brooks, D. G. et al 2006).

In a recent longitudinal study of anti HBe positive chronic HBV patients vs. acute patients, the impact of fluctuations in HBV DNA levels and disease activity on the profile of the global HBV specific T cell repertoire and potential role of the PD-1/PD-L1 pathway in the modulation of HBV specific T cell function was evaluated. In general, HBV specific T cells were rarely detected directly *ex vivo* in chronic patients in contrast to acute patients and strengths of HBV specific T cell responses by *in vitro* expansion was correlated with the levels of HBV viremia, demonstrating that the viral load can directly influence the HBV specific T cell repertoire. In the same study, it was found that the circulating HBV specific CD8 cells in chronic HBV patients were mainly PD-1 positive. The profile of PD-1 expression differed from the phenotypes of influenza virus specific CD8 cells derived from the same patients with chronic HBV infection, suggesting that PD-1 expression on HBV specific CD8+ cells was likely due to the chronically high levels of HBV antigenic stimulation. The blocking of the PD-1/PD-L1 interaction *in vitro* enhanced various functional

parameters, the capacity of HBV specific T cell populations to expand and to produce cytokines in samples from all chronic HBV patients tested (Boni, C. et al 2007).

Taken together, all above studies show a hierarchical loss of the HBV specific T cell repertoire, which is likely influenced by the levels of HBV replication. In addition, different factors like T cell receptor avidity and the quantity of HBV epitopes generated by antigen processing and presented by different HLA molecules, might affect the immunological pathogenesis of HBV infection.

1.5.4 Dendritic cell function to stimulate anti-viral response

Dendritic cells are professional antigen presenting cells that are central to the induction and regulation of immunity. DC are lineage-negative, MHC-II positive, bone marrow derived mononuclear cells and play a key role in innate and adaptive immune response. Dendritic cells in the periphery capture and process antigens, express lymphocyte co-stimulatory molecules, migrate to lymphoid organs and secrete cytokines to initiate specific immune responses. In human blood, based on staining with CD11c and CD123 markers, DCs are divided into two populations. The first one, $CD11c^{+}$ $CD123^{low}$ cells have a monocytoid appearance and due to their cellular origin they are termed as “Myeloid DCs”, whereas the second one, $CD11c^{-}$ $CD123^{hi}$ cells have morphological features similar to plasma cells and thus termed as “plasmacytoid DCs” (Adams, O'Neill et al. 2005).

DCs are specialized for antigen presentation to and efficient stimulation of B and T lymphocytes. B cells are the precursors of antibody secreting cells and recognize native antigen through their membrane bound B cells receptors. In contrast, T cells recognize antigen only when processed and presented by APC. Intracellular antigens are processed into small peptides by the proteasome in the cytosol of APC and presented on MHC class I molecules which are recognized together with the bound peptide epitope by the TCR of

CD8+ CTLs resulting in their activation. Extracellular antigens, however, are engulfed and processed in endosomes of the APC and in general presented by MHC II molecules to T helper cells (Banchereau and Steinman 1998). In addition to the classical pathway of *intracellular* antigen processing and presentation to MHC class-I, there is an alternative pathway called "Cross-priming" where *exogenous* antigens that are not expected to gain access to the cytoplasm are presented on MHC-I molecules (Albert, Pearce et al. 1998). This Cross-priming mechanism depends on the uptake of apoptotic cells induced by viral infections or tumor growth and leads to presentation of viral or tumor antigens to MHC class I restricted CD8+ T cells. *In vitro* and *in vivo* studies demonstrated that, human dendritic cells are particularly efficient in presenting antigens by this Cross-priming pathway (Albert, Pearce et al. 1998; Albert, Sauter et al. 1998).

In most tissues, DCs are present in an "immature" state which is characterized by low expression of surface molecules such as CD-40, -54, and -86. Because of low level expression of these co-stimulatory molecules, immature DCs are unable to stimulate T cells. However, they are well equipped to capture antigens subsequently leading to their maturation and migration to regional lymphoid organs where they interact with and prime T cells.

Mature DCs (mDCs), in turn, are characterized by strong expression of the surface molecules MHC-II, CD-40, -54, -80, -83, and -86 and are readily capable of priming CTL and Th cells to interact with B cells resulting in antibody production, and with NK cells or macrophages, leading to cytokine production. Mature DCs synthesize high level of IL-12 which enhances both innate (NK cells) and adaptive (B and T cells) immunity. Mature DCs interact with CD8+ T cells and provoke them for epitope specific vigorous proliferation. Moreover, in the presence of mDCs and IL-12, CD4+ T cells turn into IFN-γ producing Th1 cells. This IFN-γ activates macrophages and together with IL-12 it promotes the differentiation of T cells into killer cells. However, these DCs along with IL-4 induce T cells

to differentiate into Th2 cells which secrets IL-5 and IL-4, that are needed for B cell stimulation and specific antibody production (Banchereau and Steinman 1998; Adams, O'Neill et al. 2005). Another important costimulatory molecule present on DC surface is CD 40, which is crucial for B cell growth and differentiation. Moreover, ligation of CD 40 and CD40 ligand (CD40L) expressed on activated T cells is essential during T cell-dependent B cell activation as activation of CD40 up regulates CD80 and 86 expression on DCs increasing their secretion of IL-12 (Caux, Massacrier et al. 1994)

Impaired B cell, Th cell and CTL responses to hepatitis B virus (HBV) encoded antigens are seen in patients with chronic hepatitis B (CHB). The main cause for this reduction in immune response is not clearly determined. One possible explanation might be a functional defect in antigen presentation to T cells by APC leading to incomplete or defective T cell stimulation. *In vitro* and *ex vivo* studies on peripheral blood monocyte derived DCs (MDDC) from chronic HBV patients seemed to indicate that frequencies of DCs and circulating precursor (p)DC1 and pDC2 from HBV-infected patients were significantly decreased compared with healthy donors (Wang, Xing et al. 2001; Beckebaum, Cicinnati et al. 2002; Tavakoli, Schwerin et al. 2004). In addition, some studies reported phenotypical and functional impairment of DC isolated from peripheral blood of chronic hepatitis B patients (van der Molen, Sprengers et al. 2004). However, these findings were challenged by recent studies demonstrating intact alloreactive and antigen-specific T cell stimulation (Tavakoli et al. 2008). Thus, the experimental findings are controversial, and a general DC failure would contrast with the clinical immunocompetence of HBV carriers. In addition, circulating total DC, mDC and pDC were not reduced in chronic HBV carriers. In the same study it was also proven that the expression of co-stimulatory molecules, alloreactive T helper cell stimulation and HBV core specific CTL stimulation was similar as in control DC, whether tested directly *ex vivo* or after *in vitro* maturation. In addition, secretion of cytokines by chronic HBV

derived mDC and pDC in response to CD40 ligation and different TLR agonists were similar to controls (Tavakoli et al. 2008).

Interestingly, HBV DNA and HBV RNA were localized in DCs from chronic HBV patients suggesting that the localization of HBV particles in DCs might cause functional impairment of DCs and thus allows viral persistence (Beckebaum, Cicinnati et al. 2002; Arima, Akbar et al. 2003; Tavakoli, Schwerin et al. 2004). In contrast, a recent study proved that there is no cccDNA was found in these DCs which is the major replicative intermediate of viral replication, suggesting that the Circulating DCs may take up HBV antigens but they do not support viral replication or viral transport in cytoplasm (Untergasser, Zedler et al. 2006). This is in accordance with the lack of HBV mRNA in mDC and pDC (Tavakoli et al. 2008).

Based on these findings and our own data, the role of DCs for the T cell hyporesponsiveness in chronic hepatitis B is unproven and likely neglectible.

1.6 Cell culture and Animal models for HBV infection

The lack of suitable *in vitro* infection systems and convenient animal models has greatly hampered the progress of HBV research. Chimpanzees are the only animals fully permissive and well tested for HBV infection. The generation of HBV transfected human hepatoma cell lines has facilitated the understanding of important aspects of viral replication and gene expression (Sells, Chen et al. 1987). HBV has been successfully grown in primary cultures of human hepatocytes but susceptibility to infection is low and cultured hepatocytes become non-permissive for HBV very fast after plating (Galle, Hagelstein et al. 1994). Recently a highly differentiated hepatoma cell line under specific conditions appears to be susceptible to HBV infection. However more studies are necessary to fully explore this unique cell line (Gripon, Rumin et al. 2002).

HBV related viruses observed in woodchucks (WHV), ducks (DHBV), and ground squirrels (GSHV) offer ample opportunities for *in vivo* studies with naturally occurring hepadnaviruses. Although all these animal models are useful to examine the hepadnaviruses, most of the corresponding animals are difficult to handle in captivity or are not easily available (Chisari and Ferrari 1995). Chimpanzees were the first animals found to be susceptible to HBV infection as demonstrated by injection of serum from humans infected with hepatitis B virus. Though chimpanzees do not develop chronic liver disease, they are the only primates known to develop a cellular immune response similar to that observed in acutely infected humans. Moreover in these animals it was shown that non-cytopathic antiviral mechanisms may contribute to viral clearance during acute HBV infection, indicating that viral clearance is not only due to destruction of infected hepatocytes by CD8+ CTLs (Guidotti, Rochford et al. 1999). However these models involve expensive large animals that are difficult to handle.

The development of transgenic mice that express partial or complete copies of HBV genome has facilitated to study HBV replication, role of viral gene expression in hepatocellular injury and immunopathogenesis of HBV in a small laboratory animal model (Chisari and Ferrari 1995; Guidotti, Matzke et al. 1995). However, mice that overproduce the large envelope polypeptide accumulate nonsecretable filamentous HBsAg particles in the ER of hepatocytes sensitizing them to destruction by physiological concentrations of IFN-γ. These features have made it possible to examine the direct and IFNγ induced pathogenetic consequences of the immune response to HBsAg in this model (Chisari, Filippi et al. 1986). In contrast, HBV transgenic mice are immunologically tolerant to HBV antigens and therefore do not develop chronic liver disease. To break the tolerance to HBV antigens, a replication-competent clone of HBV DNA as a transgene was introduced into a SCID host. Such mice have continued gene expression and virus replication in the serum and liver

throughout their life span. After adoptive transfer of syngeneic splenocytes, these mice clear the virus from liver and serum and develop chronic liver disease (Larkin, Clayton et al. 1999).

To initiate viral replication in mouse livers, hydrodynamic injection of adenovirus vectors containing HBV genome has also been used. In these models it was shown that HBV was rapidly cleared from blood and serum as soon as specific antiviral antibodies and CD8+ CTLs appeared. In contrast, the virus persisted for at least 81 days after transfection of liver cells from NOD/SCID mice background, which lack functional T cells and B cells , showing that the outcome of HBV transfection depended on the host immune response (Yang, Althage et al. 2002)**.** Although infectious virus can be produced in mice, their hepatocytes are not permissive for infection. In HBV transgenic mice all viral RNAs are synthesized from chromosomally integrated copies of the virus, whereas no cccDNA, the major intermediate of viral replication *in vivo* is produced. This limits the use of these models to study antiviral drugs aiming to stop the virus production by eliminating cccDNA (Larkin, Clayton et al. 1999).

In a recent study, *in vivo* HBV infection was achieved by using human hepatocyte transplantation. In this model, partial repopulation of the liver of immunodeficient urokinase-type plasminogen activator (uPA)/recombinant activation gene-2 (RAG-2) mice was achieved with normal hepatocytes isolated from adult human liver. Human hepatocytes were estimated to constitute up to 15% of these uPA/RAG-2 mice livers. When these mice were inoculated with HBV infectious serum, HBV specific markers became detectable in liver and peripheral blood of human hepatocyte recipients indicating establishment of productive HBV infection (Dandri M. et al 2001). This uPA/RAG-2 mouse model was recently used to prove the efficacy of acylated peptides derived from the large HBV envelope (PreS1) protein to block virus entry in vivo. In this study it was shown that HBV infection can be efficiently

restrained through subcutaneous application of a specific HBV preS1 envelope protein in vivo (Petersen, Dandri et al. 2008).

However, while the above animal models did give insight into understanding of different aspects of HBV replication, they are not suitable to understand immunopathogenesis and host anti viral responses.

1.7 Trimera mouse model

Elucidation of the early steps of HBV infection, testing of antivirals and mechanisms of pathogenesis would greatly benefit from an animal system containing HBV permissive human hepatocytes. The main disadvantage of the conventional hu-PBL-SCID mice, where SCID mice were used as recipients for implantation of human PBL, was functional anergy of engrafted human T cells leading to the loss of the ability to generate and/or detect antigen specific human CTL, Th cell or antibody responses (Tary-Lehmann, M. et al 1995). This anergy was caused by the genetic disparity of human PBL donor and recipient mice which lead to poor cross reactivity between human and mouse cytokines, homing receptors and histocompatibility antigens that are crucial for the immune response (Böcher W, Reisner Y. et al 2005).

This problem was overcome by using lethally irradiated normal strains of mice that are radioprotected with SCID mice bone marrow and then transplanted with human PBLs. This resulting human mouse model that comprises three genetically different sources of tissue was therefore termed “Trimera”. In this Trimera model, viremia was induced by transplantation of *ex vivo* HBV infected human liver fragments under the kidney capsule or in the ear pinna. Human HBV viremia and viral replicative intermediates within viable transplanted human hepatocytes were observed for approximately one month, facilitating preclinical *in vivo* studies of new antivirals (Ilan, Burakova et al. 1999). Trimera mice were

further used to investigate vaccination strategies and the therapeutic effects of polyclonal anti-HBs antibodies (Bocher, Galun et al. 2000). Moreover, vaccination with antigen loaded DC, high doses of antigen or DNA plasmid encoding for HBcAg along with PBMC from HBV naïve donors , induced strong HBV specific Th cell and CTL responses in the Trimera mouse system (Bocher, Dekel et al. 2001). Therefore the Trimera system offers the possibility to perform HBV studies in a convenient small animal model of HBV infection.

1.8 Aim and study design

After infection with the hepatitis B virus in adults, more than 90% of the patients spontaneously resolve infection by developing vigorous and multi-specific anti-viral T-cell responses. However in about 5% of them chronic infection evolves, characterized by the lack of such an anti-viral response. Till date, we don't know the exact mechanism for this virus-specific T-Cell defect causing viral persistence and chronic hepatitis (Rehermann, B. and Nascimbeni, M. et al 2005). This T cell failure seems to be virus specific, as chronic HBV patients clinically do not show an obvious immune defect and respond to other vaccines in a similar way as healthy volunteers. It was proposed that Dendritic cells from chronic HBV patients who possess a dysfunction in antigen presentation may be the reason for in sufficient T cell response thus causing viral persistence (van der Molen, R. G. et al 2004). However, these findings were disproved by our own studies (Tavakoli 2004 and Tavakoli 2008), and thus the role of DC function remains controversial (see above). The lack of suitable convenient animal models has greatly hampered understanding of immunopathogenesis and testing of new vaccine candidates.

The HBV Trimera mouse model is based on humanized Balb/c mice that are implanted with PBMC from HBV patients and thus contain a functioning human immune system, including their T cell defect, which may bypass the need of the natural HBV host

(Böcher and Reisner 2005). The main aim of the current study was to assess the potential of therapeutic vaccination with recombinant HBs or HBc particles and antigen loaded autologous dendritic cells (DC) to enhance virus specific immune responses in HBV-Trimera mice implanted with PBMC from patients with different patterns of chronic HBV infection. If successful, such a vaccine might be an interesting candidate to study in clinical trials.

2 Materials

2.1 Antigens and Peptides

EBV BMLF1 (G-L-C-T-L-V-A-M-L)	Proimmune, Oxford, United Kingdom
HBc 18-27 (F-L-P-S-D-F-F-P-S-V)	Neosystem, France
HBs 201-210 (S-L-N-F-L-G-G-T-T-V) = HBs1	Proimmune, Oxford, UK
HBs 251-259 (L-L-C-L-I-F-L-L-F) = HBs2	Proimmune, Oxford, UK
HBs 260-269 (L-L-D-Y-Q-G-M-L-P-V) = HBs3	Proimmune, Oxford, UK
HBs 335-343 (W-L-S-L-L-V-P-F-V) = HBs4	Proimmune, Oxford, UK
HBs 338-347 (L-L-V-P-F-V-Q-W-F-V) =HBs5	Proimmune, Oxford, UK
HBs Antigen (subtype adw)	Aldevron ,S-Fargo, ND,USA
Hepatitis B core Antigen	DiaSorin Biomedica, Italy
Lipopolysaccharides (LPS)	Sigma-Aldrich Chemie, Steinheim, Germany
Phytohemagglutinin (PHA)	Sigma-Aldrich Chemie, Steinheim, Germany
Tetanus Toxoid absorbent for infusion	CHIRON vaccines, Marburg, Germany
Tetanus Toxoid (TT)	CHIRON vaccines, Marburg, Germany
Goat anti Mouse IgG+A+M (H+L)	ZymedLabs, Sanfransisco, USA
HRP-F (ab2)goat anti Mouse IgG (H+L	ZymedLabs, Sanfransisco, USA
Interferon- γ coating Antibody	MABTECH AB, Hamburg, Germany
Interferon- γ Biotin conjugated Antibody	MABTECH AB, Hamburg, Germany
Human anti-HLADR MicroBeads	Miltenyi, Bergisch Gladbach, Germany
Human anti-CD14 MicroBeads	Miltenyi, Bergisch Gladbach, Germany

Human anti-CD 19 MicroBeads	Miltenyi, Bergisch Gladbach, Germany
Human anti-CD4 MicroBeads	Miltenyi, Bergisch Gladbach, Germany
Monoclonal mouse anti HBcAg	CHEMICON International, Inc. Temecula, CA
Peroxidase-conjugated Goat anti mouse IgG	Dianova, Hamburg, Germany

2.2 Antibodies for FACS-analysis

Human anti-BDCA-1 FITC- conjugated	Miltenyi, Bergisch Gladbach, Germany
Human anti-BDCA-4 PE- conjugated	Miltenyi, Bergisch Gladbach, Germany
Human anti-CD11c PE-conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD123 PE- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD14 PE- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD1b/c FITC-conjugated	BioSource, USA
Human anti-CD3 FITC-conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD3 PE-conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD4 FITC- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD40 FITC-conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD40 PE- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD45 FITC- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD8 PE- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD80 FITC- conjugated	BD Pharmingen, Heidelberg, Germany

Human anti-CD83 FITC- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD83 PE- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-CD86 PE- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-HLA-DR FITC- conjugated	BD Pharmingen, Heidelberg, Germany
Human anti-HLA-DR PE- conjugated	BD Pharmingen, Heidelberg, Germany
Mouse IgG1 FITC-conjugated	BD Pharmingen, Heidelberg, Germany
Mouse IgG1 PE-conjugated Antibody	BD Pharmingen, Heidelberg, Germany
HBV $_{\text{pol 573-581}}$ (FLLSLGIHL) PE Pentamer	Proimmune, Oxford, United Kingdom
EBV $_{259\text{-}267}$ (GLCTLVAML) PE Pentamer	Proimmune, Oxford, United Kingdom
HBV $_{\text{env 172-181}}$ (WLSLLVPFV) PE- Pentamer	Proimmune, Oxford, United Kingdom
HBV $_{\text{env 183-191}}$ (FLLTRILTI) PE- Pentamer	Proimmune, Oxford, United Kingdom
HBV $_{\text{core18-27}}$ (FLPSDFFPSV) PE Pentamer	Proimmune, Oxford, United Kingdom

2.3 Chemicals and Reagents

PBS- Instamed	Biochrome, Berlin, Germany
Lymphocyte-Separation medium	PAA Laboratories, Pasching, Austria
Eosin Red	Sigma-Aldrich Chemie, Steinheim,Germany
PBS-EDTA	Cambrex Bio Science, Verviers, Belgium
Para- formaldehyde	Serva Electro GmbH, Heidelberg, Germany
Trypan blue	Sigma-Aldrich Chemie, Steinheim, ermany
Tween 20	Serva Electro GmbH, Heidelberg, Germany
Tetra-Methyl-Benzidine, (TMB)	Sigma-Aldrich Chemie, Steinheim,Germany

Trypsin-EDTA	Gibco- Invitrogen, Karlsruhe, Germany
Türks Lösung	Merck, Darmstadt, Germany
Dymethyl formamide N-N (DMF)	Roth, Karlsruhe, Germany
Dimethylsulfoxid, (DMSO)	Roth, Karlsruhe, Germany
EDTA	Merck, Darmstadt, Germany
FuGENE 6 Transfection Reagent	Roche Applied sciences, Indianapolis, USA
Sulfuric acid H_2SO_4	Merck, Darmstadt, Germany
Hydrogen peroxide H_2O_2	Merck, Darmstadt, Germany
Phosphate-Citrate Buffer Tablets	Sigma-Aldrich Chemie, Steinheim,Germany
PeqGOLD Protein Marker IV	Peqlab Biotechnology, Erlangen, Germany
Roti-Load 1	Roth, Karlsruhe, Germany
Western lightning Reagent plus	PerkinElmer, Zaventem, Belgium
Sodium Acetate	Merck, Darmstadt, Germany
ExtrAvidin-Peroxidase	Sigma Aldrich Chemie,Steinheim,Germany
Isopropyl alcohol (2-Propanol)	Hedinger, Stuttgart, Germany
Ethanol	Roth, Karlsruhe, Germany
3-amino-9-EthylCarbazole	Sigma Aldrich Chemie,Steinheim,Germany
Ciprofloxacin	Bayer AG, Leverkusen, Germany
Protease Inhibitor Cocktail Tablets	Roche, Manheim, Germany
Methanol	VWR International, England
Acrylamid solution	Roth, Karlsruhe, Germany
Glycerin	Roth, Karlsruhe, Germany

Ammonium persulfate (APS)	Sigma Aldrich Chemie,Steinheim,Germany
N,N,N',N'-tetramethylethylenediamine (TMED)	Sigma Aldrich Chemie,Steinheim,Germany
Load buffer	Roth, Karlsruhe, Germany
Roti-Block	Roth, Karlsruhe, Germany

2.4 Lab Ware

MACS Separation columns	Miltenyi, Bergisch Gladbach, Germany
Scalpel	Feather, Japan
Syringes 1mL, 5mL	Braun, Melsungen, Germany
Needles 30mm,12mm	Braun, Melsungen, Germany
Monovette EDTA 9mL	Sarstedt, Nümbrecht, Germany
Monovette Heparin 7,5mL	Sarstedt, Nümbrecht, Germany
Sterile filter system 0,22µm	Millipore, Schwalbach, Germany
Cell culture flasks	Greiner Bio One, Frickenhausen, Germany
Petri-plates	Greiner Bio One, Frickenhausen, Germany
Pipettes 5mL, 10mL, 25mL, 50mL	Corning, NY, USA
Freezing box and Freezing Tubes	Nalgene lab ware, Neerijse, Belgium
Eppendorf Pipette tips and Tubes	Eppendorf AG, Hamburg, Germany
Nylon Filters 100µm	BD Falcon, Heidelberg, Germany
Disposable Tubes 50 mL and 15mL	Greiner Bio One, Frickenhausen, Germany
Tubes for FACS 5mL	BD Falcon, Heidelberg, Germany
MiniCollect Gel serum tubes	Greiner bio-one, Kremsmunster, Austria

ELISpot Plates , 96 wells	Millipore, Schwalbach, Germany
F96 Maxisorb plates for ELISA, 96 wells	NUNC A/S, Roskilde, Denmark
PDV membrane	Millipore, Schwalbach, Germany
Whittman paper	Millipore, Schwalbach, Germany

2.5 Cytokines

GM-CSF Leukine Sargramostim	Immunex, Seattle, WA, USA
Interleukin -2	Chiron B.V., The Netherlands
Interleukin -3	CellGenix, Freiburg, Germany
Interleukin -4	Strathmann Biotech, Hamburg, Germany
Interleukin -7	R&D Systems, Wiesbaden, Germany
Interleukin -15	R&D Systems, Wiesbaden, Germany
Interleukin -6	R&D Systems, Wiesbaden, Germany
Interleukin -1β	R&D Systems, Wiesbaden, Germany
TNFα	R&D Systems, Wiesbaden, Germany
PGE2 (Minprostin E_2)	Pharmacia N.V, Belgium
Interleukin- 12	R&D Systems, Wiesbaden, Germany

2.6 KIT'S

IFN-α ELISA KIT	Bender Med Systems, Austria
ETI-AB-AUK-3 (anti-HBs) Elisa Kit	DiaSorin, Saluggia, Italy

ETI-AB-COREKPLUS–No 137 (anti-HBc)	DiaSorin, Saluggia, Italy
CBA Inflammation Kit	BD, Heidelberg, Germany
CBA TH1/TH2 Kit	BD, Heidelberg, Germany
ANNEXIN V- FITC apoptosis detection Kit	BD Pharmingen, Heidelberg, Germany
Blood Dendritic Cell Isolation Kit II Human	Miltenyi, Bergisch Gladbach, Germany
Plasmid prep Maxi and midi kits	QIAGEN GmbH, Hilden, Germany

2.7 Laboratory equipment

BD CBA Software	BD, Heidelberg, Germany
Cell Quest Software	BD, Heidelberg, Germany
Light microscope	Nikon TMS, Düsseldorf, Germany
Electric Pipette	Eppendorf, Hamburg, Germany
ELISA-Reader	MWG, Ebersberg, Germany
FACScan	Becton Dickinson, Heidelberg, Germany
CO_2 Incubator	Heraeus, Hanau, Germany
MiniMACS separation Set	Miltenyi, Bergisch Gladbach, Germany
Western-blot chambers (mini)	Bio-Rad, Hercules, CA, USA
Multi pipette	Brand GmbH, Wertheim, Germany
Micro Pipette	Gilson International, France
Rotator	Thermocycler Hybaid OmniGene, England
liquid Nitrogen tank	Nunc GmbH, Wiesbaden, Germany

Sterile bench	CleanAirTechniekB.V, The Netherlands
Vortexer	Heidolph Instruments,Schwabach,Germany
Weighing machine	Sartorius, Göttingen, Germany
Cell counting chamber	Laborhandel Labotec, Wiesbaden,Grmany
Centrifuge 1,0R	Heraeus, Hanau, Germany
Glassware	Schott Mainz, Germany
Freezer -20^0C	Bosch GmbH, Germany
Freezer -80^0C	Bosch GmbH, Germany
Frizz 4^0C	Bosch GmbH, Germany
P^H meter (inoLab Level 1)	Semat International Ltd, UK
Stereomicroscope (Stemi 2000)	Carl Zeiss GmbH, , Göttingen, Germany
Individually Ventilated Cage (IVC)	Biozone Ltd, United Kingdom

2.8 Cell lines, Culture Medium and supplements

HepG2
HepG2.2.15
HEK 293
CTL specific for HBc18-27

RPMI 1640	PAA Laboratories, Austria
DMEM	Invitrogen GmbH, Karlsruhe, Germany
X vivo	Cambrex Bio Science, Verviers, Belgium
Fetal Calf serum	PAA Laboratories, Austria
Human Serum (AB)	Transfusion center Mainz, Germany
Penicillin / Streptomycin	PAA Laboratories, Pasching, Austria
L-Glutamine	PAA Laboratories, Pasching, Austria
Trypsin EDTA	Invitrogen, United kingdom

RPMI 5% HUS Medium

RPMI 1640	95 mL
Human serum AB	5 mL
(Heat inactivated at 56^0C for 30 min)	

DMEM 5% FCS Medium

DMEM medium	95 mL
Fetal calf serum	5 mL
(Heat inactivated at 56^0C for 30min)	

2.9 Buffers and solutions

20 X PBS-Buffer

PBS-Instamed	95,5g
Distilled Water	500ml
Autoclaved	
Store at RT	

1 x PBS

20 X PBS	50ml
Distilled water	950ml

PBS 0.05% Tween

Tween 20	500µl
1xPBS	1 Lit

PBS 1% BSA

Bovine serum Albumin	1 g
1xPBS	100ml

MACs Buffer

0.5% BSA	2.5g
2mM EDTA	2 mL (from 500mM stock)
1 molar PBS	4.8g
Distilled water	500 mL

Sterile filtered and stored at 4^0C

1%PFA
Pentamer wash buffer

0.1% BSA
0.1% Na- Azid
In PBS

Pentamer Fix solution

1% FCS
2.5% PFA
In PBS

Ammonium per sulfate (APS) stock 40%

Ammonium per sulfate	400 mg
Distilled water	1 mL

Aliquoted and stored at -20^0C

Avertin stock (100%)

2,2,2 Tribromethanol	10g
t- amyl alcohol	10 mL

Vortex and stored at 4^0C

Avertin working (2.5%)

Avertin stock (100%)	250µL
PBS	10 mL

PBS 1% Na-Citrate

Sterile PBS	500 mL
Na-Citrate	5 g
Sterile filtered	

ELISPOT Coating Buffer

Na-HCO_3	2.93g
Na_2-CO_3	1.59g
Sterile Distilled water	100 mL
Sterile filtered and stored at 4^0C	

ELISPOT developing solution

3-amino-9-ethyl carbozole	1 tablet
Dymethyl formamide (DMF)	2.5 mL
Acetate buffer	47.5 mL

Acetate Buffer

0.2 Mol Na-acetat	11 mL
0.2N- CH3COOH	4.6 mL
Distilled water	46.9 mL

ELISA stop solution

97% H_2SO_4	28 mL
Distilled water	500 mL

3 Methods

3.1 Mice

In this study BALB/cJ, NOD/SCID (NOD.CB17-*Prkdc*^{scid}/J), 1.3 HBV (homozygous), B6 (C57BL/6NCrl), and CB6F1 (CB6F1/Crl) mice strains were used. The BALB/c mice aged between 6 to 12 weeks were obtained from Specific Pathogen Free (**SPF)** breeder stocks of the institutional animal breeding center Uniklink Mainz, Germany.

The NOD/SCID mice were obtained from **SPF** breeder stocks from the institutional animal breeding center Uniklinik Mainz. The breeding of SCID mice was carried out in Individually Ventilated Cages (IVC) units. Female mice were used as bone marrow donars at the age of 4 to 10 weeks. Before using them for bone marrow isolation, NOD/SCID mice were tested for mouse Ig production by ELISA and leaky mice were eliminated from experiments.The B6 and CB6F1 mice were obtained from Charles River Laboratories, Germany. All mice were derived from breeder stocks from Charles River labs and female mice aged between 6 to 12 weeks were used in experiments.

The specific pathogen free (SPF) conditioned mice were maintained behind a barrier in rooms facilitated with High Efficiency Particulate Air (HEPA)-filtered air and were housed in micro-isolator cages or in IVC units. In each cage 5 animals were housed by following guidelines from Institute for Laboratory Animal Research (ILAR) containing sterilized food and water. SPF mice have a defined flora and are free of all known mouse pathogens. Mice were handled according to the regulations from Federation of European Laboratory Animal Science Associations (FELASA) and Gesellschaft für Versuchstierkunde - Society for Laboratory

Animal Science (GV-SOLAS). All animal experiments were approved by the German authorities and were performed in according with German animal protection laws and regulations.

3.2 Blood collection from Mice

Blood was collected from mice by orbital sinus or plexus puncture. Briefly, mice were anesthetized according to their weight. The end of pasture pipette was introduced at the medial canthus of the orbit of mouse, slowly, and with axial rotation, the tip of pipette was gently advanced towards the rear of the socket until blood flows into the pipette. Blood was collected into MiniCollect Gel serum tubes and incubated at 4^0C for 30 minutes. Serum was separated by centrifuging these tubes at 6000rpm for 10 minutes and separated serum was stored at -20^0C until it was used in experiments. Volume of blood collection was varied according to serum requirement of particular experiment.

3.3 Trimera Mouse Model

The Trimera mouse model is based on using lethally irradiated normal strains of mice that are radio protected with SCID mice bone marrow and then transplanted with human PBMC. This resulting humanized mouse model comprises three genetically different sources of tissue (recipient mouse, Bone marrow donor, PBMC) and is therefore termed “Trimera” (Boecher and Reisner 2005).

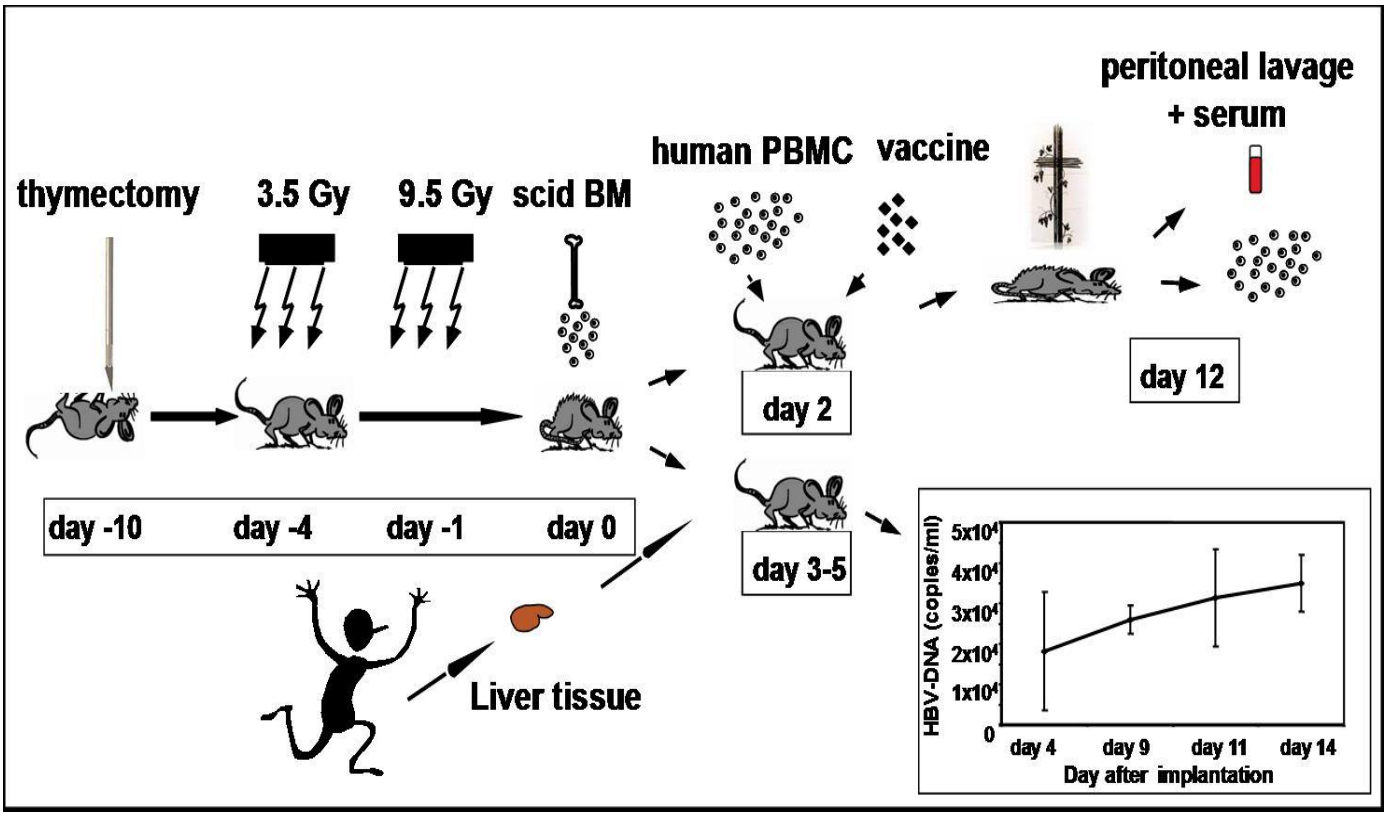


Figure 7: Schematic diagram representing the Trimera mouse system

3.3.1 Mouse IgG ELISA

Mouse Immunoglobulin (Ig) concentration was quantified in serum of NOD/SCID mice to eliminate the occasional leaky mice from experiments. The concentrations of mouse Immunoglobulin were quantified by Sandwich ELISA. Briefly, NUNC maxi sorb plates were coated with Goat anti mouse IgG+A+M (H+L) specific antibody diluted in PBS to (1mg/mL). Plates were incubated with 50µl/well coating antibody at 4^0C for overnight.

Without discarding the coating antibody, plates were blocked with 50µl/well of PBS 1% BSA and incubated for 4 hours at room temperature. After blocking, plates were used immediately or stored at -20^0C for further use (max 3 months). The Serum from NOD/SCID mice was diluted 1**:** 2 with PBS. As a standard, normal mouse IgG was used in different

concentrations. The BALB/c mouse serum and human serum (1**:**2 and 1**:**10,000) were used as positive and negative controls respectively. All samples were diluted in PBS and 50µl/well were pipetted. Plates were incubated at 4^{o}C for overnight and then washed for three times with PBS 0.05% tween with 3 minutes intervals between each washing.

As a secondary antibody, Horse radish Peroxidase {HRP-F(ab')2} goat-anti-mouse IgG (H+L) was used at 1**:**5000 and 50µl/well was pipetted. Plates were incubated at 37^{o}C for 2 hours and were washed three times with PBS 0, 05% Tween. To develop the reaction, Substrate Reagent A and B (BD Pharmingen, diluted 1:1 ratio) was used. Plates were incubated with 50µl/well at room temperature for 5 to 10 minutes and then reaction were stopped by adding 50µl/well ELISA stop solution (BD Pharmingen). The optical density was measured at 450nm without reference value. Serum Ig concentrations were calculated from the standard value. Mice with serum Ig levels >0.05mg/mL were considered as leaky and excluded from experiments.

3.3.2 Thymectomy

All mice used as recipients for the **Trimera mouse model** were thymectomized one week before their use in experiments. Thymus was removed to stop the generation of new mouse T cells during the experiments limiting the human cell engraftment. Briefly, mice were anesthetized based on their weight by intraperitoneal injection of 2.5 % Avertin solution (10g of 2,2,2 tribromethanol in 10ml of t-amyl alcohol). Mice were placed on dissecting board in the dorsal position with their head facing the operator. The arms and legs were restrained by placing them under parallel elastic bands. Mouse's neck was extended by securing the head with a third rubber band placed in its mouth. Neck and upper chest area was swabbed with 70% ethanol.

With scissors, a midline longitudinal skin incision over the supra-sternal notch was done and incision was extended for 2 to 3 cm down the chest. The skin was loosened from the underlying muscle using the bluntend of forceps and skin was reflected to expose the thoracic cage. To see the thymus lobes, the tips of forceps were inserted into the incision and chest was exposed by allowing the forceps to open. Suction cannula was constructed by cutting off the last 5cm of the tip-end from disposable plastic pipette. The tip was placed over the lower pole of one of the two thymus lobes. By using vacuum pump thymus lobes were aspirated into the tip. The chest cavity was checked for thymic- remnants and skin was secured with one or two 9-mm wound clips. Excess blood from the incision was wiped and mouse was placed in a clean cage.

3.3.3 Irradiation of Mice

The Trimera mouse experiments were conducted using BALB/c, CB6F1, and 1.3 HBV and C57B6 mouse strains. Briefly, recipient mice were lethally irradiated by split dose total body irradiation using a Gamma beam source. The irradiation doses are as fallows

Mouse strain	Day -4 (minus 4)	Day -1 (minus 1)
BALB/cJ	3 gray	8.5 gray
CB6F1	4 gray	12.5 gray
1.3 HBV & C57B6	4 gray	11 gray

Table 1 Irradiation doses of Trimera mice

After irradiation, mice were fed with sterile food and sterile water supplemented with ciprofloxacin (20μg/ml). To radio-protect the mice, the day after second irradiation (day 0),

$3x10^6$ bone marrow cells from NOD/SCID mice were transplanted into recipient mice by intravenous injection in a volume of 0.2mL PBS per mouse.

3.3.4 Bone marrow Preparation and Transplantation

Bone marrow from NOD/SCID mice was used to protect the recipient mice from lethal irradiation. The SCID mice aged between 6 to 10 weeks were used for isolation of bone marrow. Briefly, mice were sacrificed by cervical dislocation and disinfected by immersing in 70% alcohol then mice were fixed at hind paw on dissection board with 30mm needles. The limb bones (femur and tibia) were collected by dissecting hind limbs and placed in sterile PBS

Bones were washed three times with PBS to remove excess tissue pieces and then bones were blended in PBS at 5400 rpm/ 90 seconds with the help of homogenizer. To avoid muscle debris in cell suspension, the homogenate was filtered through disposable nylon filters (100mm, 70mm). The filtered cell suspension was washed two times with PBS at 1200rpm/10 minutes. The pellet containing the hematopoietic stem cells was resuspended in PBS. Cells were counted in a hemocytometer by staining with a 0.1% Türks solution. After counting, the cell concentration was adjusted with PBS to get $3x10^6$/ 0.2mL.

With out any air bubbles, 1mL syringes were prepared with cell suspension. The Bone marrow recipient mice were warmed up for 1-2 minutes under red light. Mouse was restrained by placing in mouse restrainer and the lateral tail vein was visualized after swabbing the tail with 70% ethanol .Then with the help of 27-G needle, cells were slowly injected into the lateral vein. Mice were placed in clean sterile cages containing sterile food and sterile water supplemented with ciprofloxacin (20μg/mL). One day after the bone marrow transplantation, these mice were used for either vaccination experiments by transplanting Human PBMC or in viremia

experiments where viremia was obtained by transplantation of the HBV infected liver biopsies under kidney capsule or earpinna.

3.3.5 Transplantation of human PBMC

All vaccination experiments were conducted with HLA-A2 positive healthy donors or patients with different stages of HBV infection. Leukapheresis products were obtained from donors by Blood Transfusion Center of University Hospital Mainz. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. Autologous antigen presenting cells (APC) were separated from PBMC using anti CD14 and CD19 conjugated magnetic micro beads. And these APC were stored frozen and used later in cytokine ELISPOTS**.** All human experiments were approved by regional ethics committee and performed after obtaining written informed consent according to the declaration of Helsinki.

For vaccination, the recipient mice were infused intraperitoneally, with $1x10^8$ of human Peripheral Blood Mononuclear Cells (PBMC), in a volume of 1mL PBS per mouse, along with or without vaccine. On day 9 after vaccination, mice were sacrificed by cervical dislocation, per mouse 5 mL of PBS supplemented with 1% sodium citrate was injected into peritoneum and peritoneal cells were collected. Human cells were separated from peritoneal cells by using Ficoll density gradient centrifugation. These cells were used either in cytokine ELI Spots or for FACS staining.

3.3.6 Induction of viremia by Liver transplantation

Viremia was induced in Trimera mice by transplantation of HBV infected liver biopsies under kidney capsule or earpinna. Trimera mice, from day 4 to 8, were used for these experiments. Liver tissue was obtained from either liver biopsies performed for clinical reasons, or from tumor free margins of surgical liver tumor resections. *Ex vivo* infection of the liver tissue was carried out by incubating 2hrs at 37^0C with 1mL high titer HBV DNA human serum along with 3µg/mL hexadimethrine bromide and 0.5µg/mL of recombinant human interleukin-6 (IL-6). The recipient mice were anesthetized using 2.5% of avertin solution. The position of the kidney was located and a small incision was made on the skin. With the help of forceps, kidney was lifted from surroundings and a 3 to 5mm piece of liver tissue was carefully placed under the kidney capsule. Then incision was closed with auto wound clips. In alternative procedure, a pocket on the dorsum of ear was made by a small incision on the skin at the base of the ear. Then a small curved forceps was placed into the incision and a small space was created between skin and cartilage by dissecting toward the distal edge of the ear. The 5 to 8mm liver implant was eased into the base of the pocket near the distal edge of the ear. Then the surgical incision was gently closed with wound clips. On different time points blood was collected and serum was separated. Then HBV DNA was detected by COBAS Taqman PCR (Roche Diagnostics, Mannheim, Germany)

3.3.7 Collection of peritoneal cells and flow Cytometry

Trimera Mice were sacrificed by cervical dislocation on day 10 after vaccination. Cells were recovered from the peritoneum by lavage with 5mL PBS 1% sodium citrate. Then cells were layered on 6mL of ficoll solution and human cells were separated by density gradient

centrifugation. Carefully the interface ring containing human lymphocytes was collected and washed in PBS at 1200rpm/ 10minutes. These separated human cells were further used in different assays. These cells were stained with anti human-CD4 and CD45 antibodies and engraftment of human cells in Trimera mice was checked by flow cytometry (FACS) analysis.

3.3.8 Pentamer staining for epitope specific CTLs

Pentamer staining was performed to detect epitope specific cytotoxic T cells. Briefly, $1x10^6$ /tube human cells were washed with 2 mL pentamer wash buffer. Then cells were stained with 10μL of each epitope specific pentamer (Proimmune, UK), vortexed and incubated in dark at room temperature for 15 minutes. 2 mL wash buffer was added and cells were washed by spinning them at 1200rpm/10 minutes at room temperature, supernatant was discarded and pellet was resuspended with rest. Cells were counter stained by adding 5μL of CD8-FITC labeled antibody and incubated in dark at 4^0C for 25 minutes. Then cells were washed two times with adding 2mL wash buffer each time. Supernatant was discarded and pellet was resuspended in 200 μL of pentamer fix solution and epitope specific CTLs were measured by FACS.

3.4 Preparation of peripheral blood mononuclear cells (PBMC)

Human peripheral blood mononuclear cells (PBMC) were isolated either from heparinised blood, Buffy coats or from leukopheresis products. Briefly, blood was diluted in 1**:**2 ratio for heparinised blood and Buffy coats, whereas leukapheresis product was diluted in 1**:**6 ratio with PBS. Diluted blood was carefully layered on 12mL of. Ficoll-Paque solution. Blood was centrifuged for 20 minutes at 2000 rpm.without brake. Without aspirating the ficoll solution, the interface ring containing mononuclear cells was carefully collected into new sterile 50mL

tube and after adjusting the volume to 45 mL with PBS, cells were washed at 1200 rpm for 10minutes to get rid of unwanted contaminants. After washing, supernatant was discarded and pellets were collected into one tube and filled to 50mL volume with PBS and washed for 10 minutes at 1200rpm. Then supernatant was discarded and pellet was resuspended in PBS.

Cell counting

10 μL of PBMC suspension was pipetted into a 0.5 mL micro centrifuge tube and 90 μL of 0.4% Trypan Blue stain was added, making a 1**:**10 dilution. Then this cell mixture was carefully loaded into the hemacytometer until the area under the cover slip was filled. Then the cell suspension was allowed to settle in the hemacytometer for at least 10 seconds before counting. Viable cells recognized on their clear color, were counted.

Cryopreservation

Cells were frozen at the concentration of 5–10 x 10^6 viable cells/mL per cryovial. Before starting the freezing procedure, slow-freeze™ containers were kept cold at 4^0C. Cells were centrifuged and resuspended gently in cold freezing medium. 1 mL aliquots of the cell suspension were dispensed into cryovials and placed immediately in a slow-freeze container then placed in a -70°C freezer for max of 14 days. For long term storage, the cryovials were transferred into vapor phase of liquid nitrogen (-135^{o}C).

Thawing Procedure

The cryovials were removed from the freezer or liquid nitrogen container and brought to room temperature. Cells were thawed quickly and suspension from the cryovial was transferred to a 15-mL centrifuge tube containing 10 mL of chilled PBS. Then cells were centrifuged at

1200 rpm/10minutes at 4^0C. The supernatant was discarded without disturbing the cell pellet and the cells were gently resuspended in the appropriate medium for the assay to be performed.

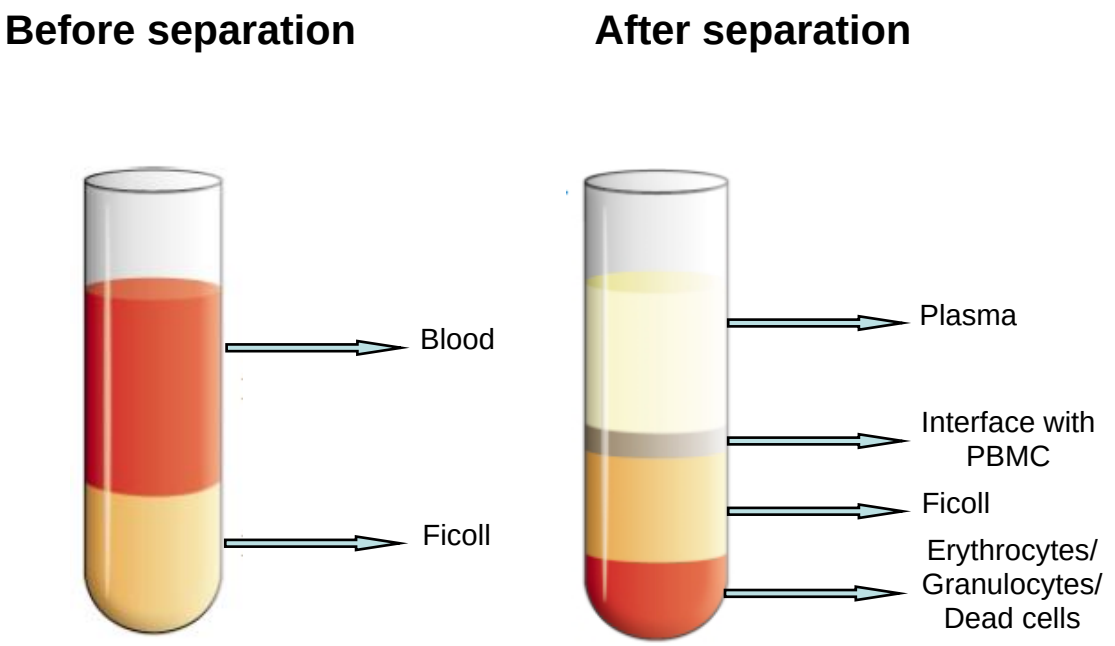


Figure 8: Over view on isolation of PBMC by Ficoll-Paque method.

3.5 Magnetic affinity cell sorting (MACS)

Different human cell populations were separated by Magnetic labeling of human PBMC using receptor specific Micro Beads. Briefly, PBMC were isolated from anti-coagulated peripheral blood or Buffy coats or leukopheresis by Ficoll preparation method. To remove clumps, cells were passed through 70 µm nylon mesh. Cells were resuspended in PBS. Then for 10^7 of total cells 20µL of micro beads and 80µl of MACS buffer was added. Cells were mixed well and incubated for 15 minutes at 4−8 °C. Then 5mL of MACS buffer was added and cells were washed at 1200 rpm/10 minutes at 4^0C. Meanwhile the magnetic column was prepared by

washing with 3 mL of MACS buffer. The supernatant was discarded and pellet was resuspended in 3mL buffer. Cell suspension was applied on top of the column to let the un-labeled cells pass through. The column was rinsed with 2×3 mL of buffer and the effluent was collected as negative fraction. Then column was removed from separator and 5mL of buffer was placed onto the column to firmly flush out positive fraction using the supplied plunger. Depending on the particular experiment, negative or positive fraction of cells was used in further experiments.

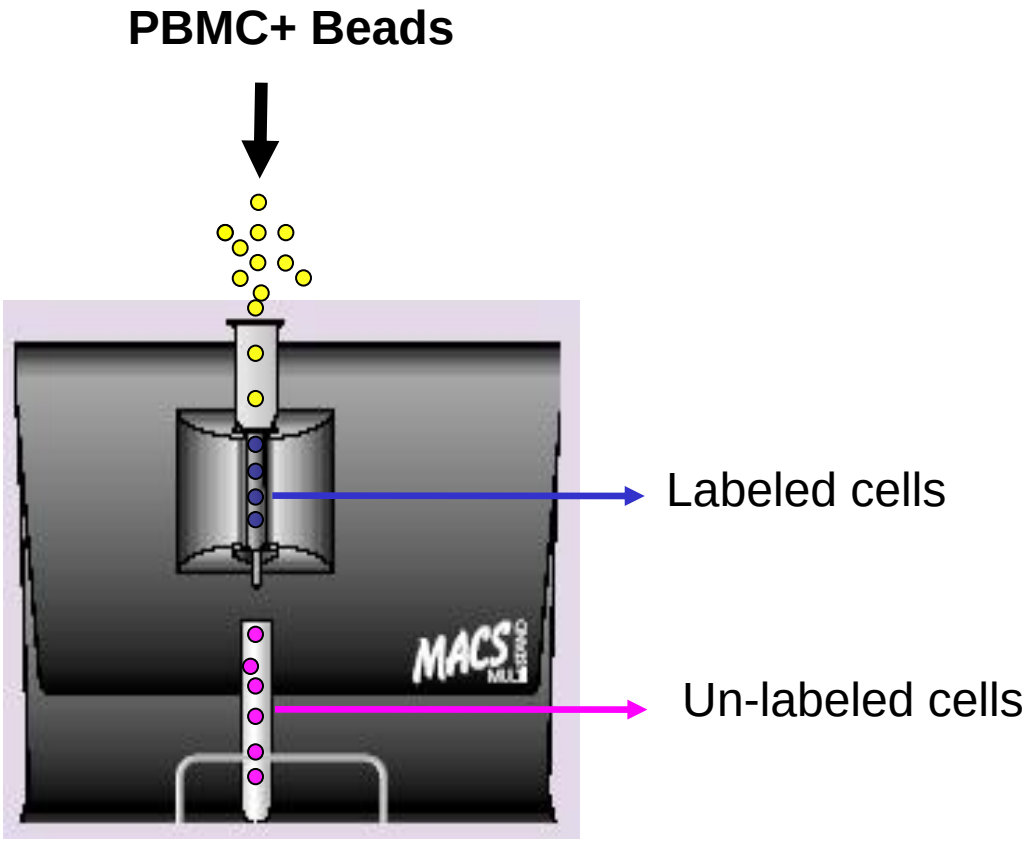


Figure 9: Schematic illustration of Magnetic activated cell separation (MACS) by which cells are separated by surface receptor specificity.

3.6 Dendritic Cell culture

Dendritic Cells were generated by culturing CD14+ve Monocytes. Briefly, Peripheral Blood Mononuclear cells (PBMC) were isolated from heparinised blood using Ficoll density gradient centrifugation. Total PBMC were incubated with anti-human CD14 conjugated magnetic micro beads in a concentration of 20µl beads and 80µl of MACS buffer for 10^7 cells. Cells were incubated for 15minutes at 4^0C and were pelleted at 4^0C by using 1200rpm/10minutes. The labeled CD14+ve monocytes were obtained by positive sorting in presence of magnetic field (MACS). The resulting CD14+ve monocytes were cultured for 6 days in six well plates at concentration of 3 to $4x10^6$ cells per well in 3mL of DC culture medium (X-vivo). The medium was supplemented with 800u/mL GM-CSF, 1000u/mL of human IL-4. Plates were incubated at 37^0C with 5% Co_2. On day three of culture, DC medium was replaced with 1mL of fresh medium supplemented with 800u/mL GM-CSF and 1000u/mL IL-4. Maturation was induced to- Dendritic cells on day 5 of culture by adding DC medium supplemented with 10ng/mL TNF-α, 10ng/mL IL-1β, 1000u/mL IL-6, 1µg/mL PGE_2.

3.7 HBV Transfection

HepG2 or HEK 293 cells were transfected with 1.3 over length HBV plasmid by using Lipofectamine 2000 transfection reagent. Cells were seeded in six well plates with flat bottom. Transfection mixture was prepared by mixing 3µg/well of DNA from 1.3 HBV plasmid to 3µL Lipofectamine reagent and incubated at 37^0C for 15 – 20 minutes. This mixture was prepared in RPMI cell culture medium without any supplements and final volume of transfection mixture was adjusted to get 100µL/well concentration. When the cells were 70% confluent, medium was

replaced with 1mL of fresh medium with out any supplements and 100μL/well of transfection mixture was added. Cells were incubated in incubator at 37^0C with 5% CO_2 for one hour. Then the medium containing the transfection mixture was replaced by 2 mL of fresh medium containing appropriate supplements. On day two after transfection, cells were collected and used for UV apoptosis and Western blot analysis.

3.8 Western blot analysis

Expression of HBV core protein was detected using standard Western blot protocol. Briefly, after HBV transfection, cells were lysed in buffer containing Tris, EDTA, Triton x 100 and protease inhibitors. The protein concentration in the cell lysates was measured and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were run on gels containing 12% polyacrylamide and 0.1%SDS. Proteins were blotted on to a PVDF membrane that was blocked with milk protein to avoid unspecific binding of antibody. To detect core antigen, monoclonal mouse anti HBcAg primary (Chemicon International, Inc) was applied (1:1000) and then peroxidase conjugated anti mouse IgG antibody were added as secondary antibody. Bound antibodies were detected using the western lightning (Perkin Elmer) Western Blot developing solution. The developed protein bands were visualized by transferring to X-ray photo film.

3.9 UV apoptosis

Apoptosis was induced in HepG2.2.15 cells or on day two after HBV transfection for HepG2 cells by UVB irradiation. Therefore, approximately $3x10^6$ cells were harvested in flat

bottomed 6-well plates in 2 mL appropriate medium and when they are confluent, cells were exposed to UVB light for 5 minutes. The distance between cells and UVB lamp was adjusted to get peak intensity of 9000μW/cm^2 and UVB peak emission of 313 nm was used. After UVB exposure, the medium was replaced with fresh medium and Apoptosis was measured in FACS by using Annexin V-FITC apoptosis detection.

3.10 CTL lines

CTL lines specific for HBV core 18-27 were kindly provided by Antonio Bertoletti (UK). These clones were maintained in flat bottomed 48 well plates. These CTL clones were re-stimulated with allogenic irradiated (5000 Rad) PBMC and cultured in RPMI supplemented with 10%HUS, human IL-2 (30 U/mL), IL-7 (10ng/mL) and PHA (1 μg/mL). Cells were maintained in concentrations of 1x10^6 irradiated PBMC per 5x10^5 clones in 500 μL/well of medium. Epitope specificity from these CTL clones was measured using HBc18-27 epitope specific MHC-pentamer in a flow cytometer analysis.

3.11 Cross presentation of DC

In vitro Cross presentation of Ag was tested by IFN-γ ELISPOT. Briefly, Apoptotic HBV transfected HepG2 cells or apoptotic HepG2.2.15 cells were co-incubated with DCs. For immature DC (imDCs), apoptotic cells were co-incubated on day six and cultured for 48 hrs, whereas for matured DC (mDCs), on day 5 of culture maturation was induced by adding cytokine cocktails, after 24h, DC were coincubated with apoptotic cells and cultured for further 48 hrs. DC were separated from contaminating HepG2.2.15 cells by immunomagnetic separation of HLA-DR positive cells and were used in ELISPOT assay. CTL line specific for HBc_{18-27}

epitope was stimulated with either with DC HBV, DC_{18-27}, DC HBc (DC coincubated with HBcAg) or DC HG2 (DC coincubated with mock trasfected HepG2) in ELIPSOT plates coated with anti Human IFN-γ Ab (Mabtech). After 24h of incubation, plates were developed to detect IFN-γ producing cells (see below).

The same cross priming approach was used *in vivo* to stimulate HBV specific Th and CTL responses in HBV Trimera mouse model. In parallel to imDCs and mDCs co-incubated with apoptotic HBV transfected or non-transfected HepG2 cells, the non-coincubated DC were loaded *in vitro* with HBcAg and HBc_{18-27} peptide for 2hrs at 37^0C and used as HBV or control vaccines in vaccination experiments of Trimera mouse system at concentrations of $1x10^6$ DC/ mouse.

3.12 ELISpot analysis of antigen specific T cells

Analysis of antigen-specific T cells was performed by IFN-γ ELISpot analysis. Therefore, 96-well plates with PVDF membrane bottoms (Millipore) were coated overnight with anti-human IFN-γ monoclonal antibody (0.64µg/well). $1x10^5$/well of human PBLs were incubated 48h,in triplicates together with $5x10^4$/well of autologous APC in RPMI 5% HUS. Specific T helper cell response was analysed by stimulation of cells with recombinant HBcAg (1µg/w), HBsAg (1µg/w), purified Tetanus Toxoid (TT, 1µg/w) or Phytohemagglutinin (PHA, 1µg/w). Whereas detection of CTL was performed using synthetic peptides representing the immunodominant HBc epitope (aa 18-27), the EBV protein BMLF1 derived peptide (aa 259-267) or five different HBs peptides. The total volume per well was adjusted to 200µL. After incubation for 48 hrs, cells were discarded and plates were washed and incubated with 100µL of biotinylated anti-human IFNγ mAb (1**:**1000; with PBS 5% FCS). Plates were washed and incubated at room temperature for 30min with 100µL of horseradish peroxidase-Extravidin

(1:500, in PBS 5% FCS). Then, plates were washed and incubated with 100μL of Carbozole/DMF (Dymethyl formamide) in dark at room temperature. Dark red spots were counted as single spot-forming cells (SFC).

4 Results

4.1 Patients and PBMC Donors

PBMC were obtained by leukapheresis from 7 donors with different courses of chronic hepatitis B (HBV-1to -7), one Recovered HBV (RHB-1), and 4 healthy donors (CTR-1 to -4) for details see Table 1) (Fattovich, G. et al 2003). Patients without detectable inflammatory activity in histology or serum and undetectable antiviral T cell responses *ex vivo* have been termed **immunotolerant (IT):** HBV-1**.** Four donors (HBV-2, -4, -5 and -6) were low viremic **inactive HBs carriers** (**ISC**: normal ALT, seronegative for HBeAg, HBV-DNA below 10^4 copies (cps) /ml by COBAS Amplicor HBV (Roche Diagnostics). Donor HBV-3 had histologically confirmed **chronic active hepatitis (CAH)** with minimal inflammation and fibrosis, high ALT, seropositive for HBeAg and $>10^6$ HBV DNA at the time of leukapheresis. One positive control RHB-1 had spontaneously resolved from hepatitis B (normal ALT, anti-HBs and anti-HBc seropositive) Three HBs vaccinated CTR-1, -3 and -4 (anti-HBs seropositive, anti-HBc seronegative) and one non-vaccinated (CTR-2) volunteers served as controls. All donors had been vaccinated with tetanus vaccine years before, were otherwise healthy, and tested negative for anti-HCV and anti-HIV1/2 antibodies. All donors were HLA A2 positive. The study protocol was approved by the institutional ethical committee and all donors gave informed consent to the study in accordance with the Helsinki declaration of ethical guidelines.

	sex	age	Diagnosis*	Histology (inflammation/ fibrosis)	HBeAg	ALT (U/l)	HBV DNA (vge/ml)*	current treatment
HBV-1	M	39	IT	Moderate/numerous septa.	Pos.	25	$>4x10^7$	-
HBV-2	M	34	ISC	Minimal / minimal	neg	36	neg	-
HBV-3	F	58	CAH	moderate / bridging	pos.	153	$7.9x10^7$	-
HBV-4	M	34	ISC	Minimal / minimal	neg.	36	neg	-
HBV-5	M	34	ISC	Minimal / minimal	neg.	36	neg.	-
HBV-6	M	34	ISC	Minimal / minimal	neg.	36	neg.	Lamivudine for 26 months
RHB-1	M	29	RHB	n.t.	neg.	n.t.	nrg	-
CTR-1	F	34	Vaccinated	n.t.	neg.	n.t.	neg.	-
CTR-2	M	26	n.t.	-	-	-	neg	-
CTR-3	M	26	Vaccinated	n.t.	neg.	-	neg.	-
CTR-4	F	26	Vaccinated	n.t.	neg.	n.t	neg.	-

Table 2: Donors clinical data at the time of leukapheresis.

* ISC= inactive HBs carrier; IT= immune tolerant HBV carrier; CAH= chronic active hepatitis; RHB =resolved hepatitis B and CTR= Healthy donor, vaccinated, with recombinant small HBs vaccine.

4.2 Establishing Trimera Mouse Model

4.2.1 Standardisation of Irradiation protocol

In the HBV Trimera mouse system, previously it has been shown that, a split-dose total body irradiation protocol, where an initial low dose is followed by a second lethal dose, most effectively abrogates the recipients bone marrow and optimizes the human PBMC engraftment without relavent increase of the mortality (Ilan, Burakova et al. 1999). After different doses of total body irradiation at days 1 and 4, all mice were radioprotected by transplanting with bone marrow from NOD/SCID mice. Human PBMC were transplanted 1-3 days later (10^8/mouse) i.p. Tetanus Toxoid (TT) was used in all experiments as control vaccine effectively inducing Th type responses and such responses were detected by IFNγ ELispot. Human T cell engraftment was assessed by FACS staining of peritoneal cells for expression of human CD4 and CD45 (Figure 10 and Figure 11).

4.2.2 Thymectomy

The rapid and long lasting engraftment of human PBMC is crucial in the Trimera mouse system. The persistence of residual host T cells, which are generated in the thymus, may interfere with the engraftment of human PBMC. Therefore, preventing the development of such cells by thymectomy may enhance engraftment of the human cells. Thus, thymectomy was performed one or two weeks before irradiation. Low purity of the engrafted human CD4/CD45 in non-thymectomized can be seen in (Figure 12 B). In contrast, thymectomy, before conditioning and transplantation procedure was advantageous and led to improved engraftment of human PBMC.

The positive effect was easily detectable when we analyzed the CD4/ CD45 (97.69%) cell populations (Figure 12 C).

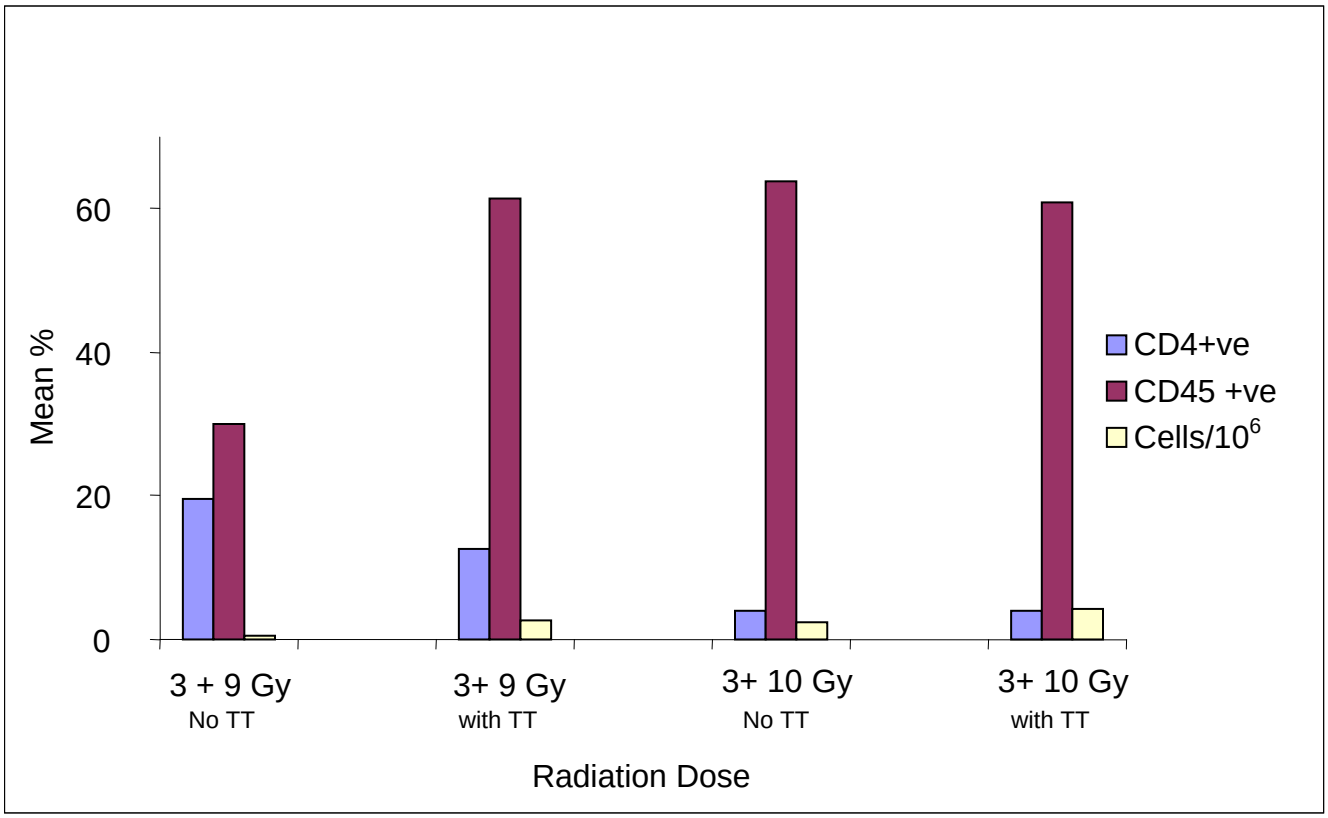


Figure 10: Establishing irradiation protocol for Trimera mice. Balb/c mice were irradiated with different doses of radiation. Then PBMC were obtained by leukapheresis from healthy donor CTR-1 and 108 cells per mouse were transplanted i.p. as described in Material and Methods. Mice were either vaccinated with Tetanus Toxoid (TT) or left unvaccinated. Ten days later, cells were recovered by peritoneal lavage, stained for human CD4/45 and analyzed by FACS. Mean percentages or absolute cell numbers of recovered human cells are shown for the different radiation dose groups.

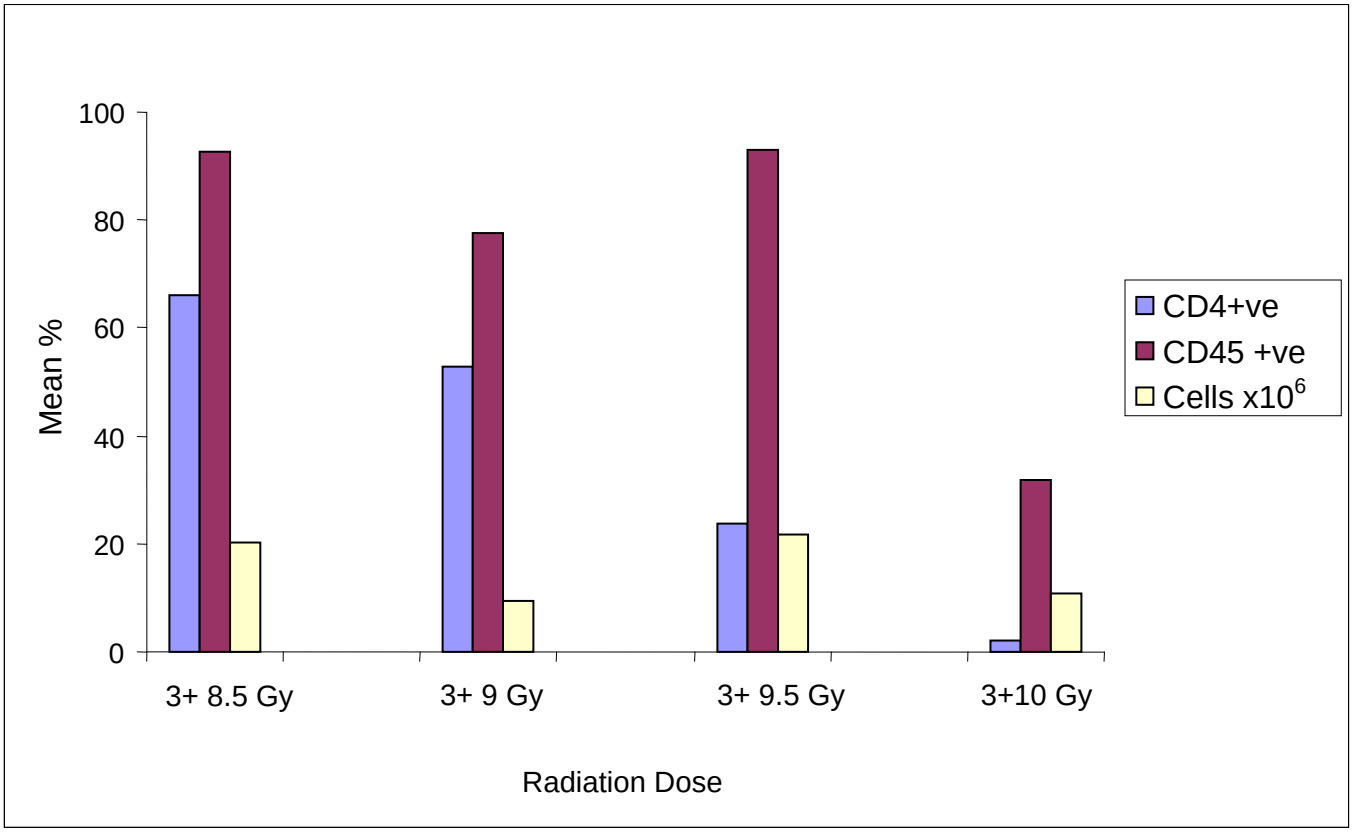


Figure 11: Establishing irradiation protocol for Trimera mice. Balb/c mice were irradiated with different doses of radiation. Then PBMC were obtained by leukapheresis from healthy donor CTR-2. and 108 cells per mouse were transplanted i.p. as described in Material and Methods. Ten days later peritoneal cells were recovered, stained for human CD4/45 and analyzed by FACS. Mean percentages or absolute cell numbers of recovered human cells are shown for the different radiation dose groups (frist dose in all cases: 3 rad).

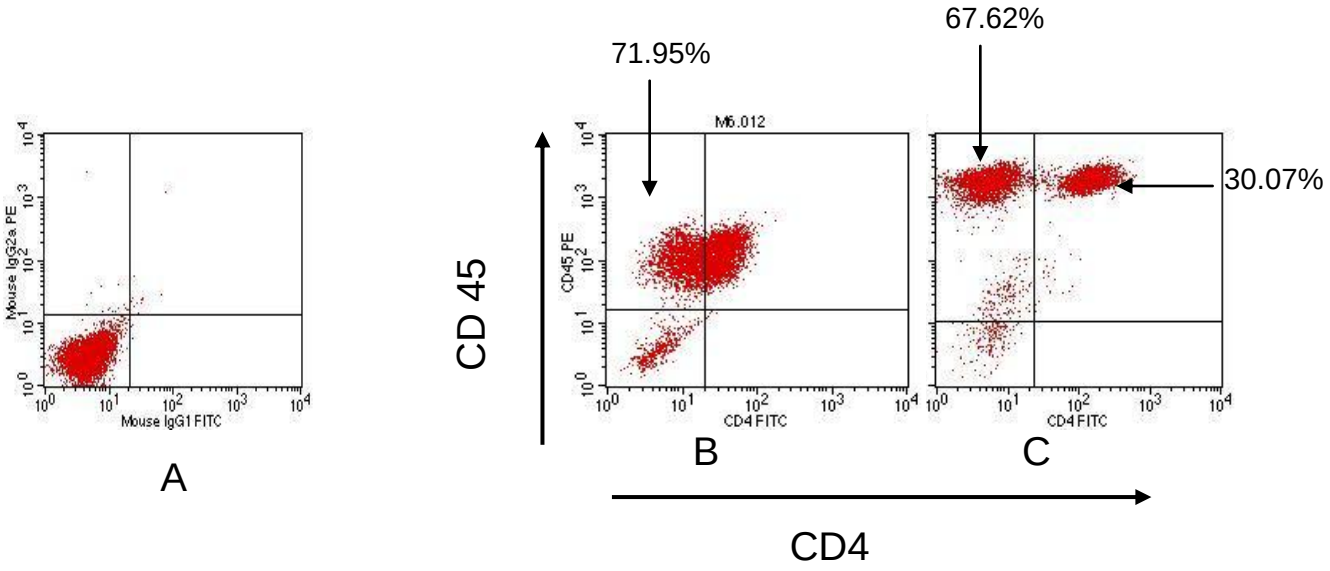


Figure 12 FACS staining of peritoneal cells from Trimera mice. PBMC from healthy donor (CTR-2). obtained by leukapheresis and 108 PBMC were transplanted i.p. per mouse as described in Material and Methods. Ten days later peritoneal cells were obtained and stained with mouse IgG as isotype control (A), and counterstained with human CD4/45 before thymectomy (B) and after thymectomy (C).

4.2.3 Human T cell response after vaccination

In order to assess the potential of the Trimera mouse system to test different therapeutic vaccination regimen for chronic HBV infection, we first analyzed the immunogenicity of various non-HBV vaccines and expansion and function of vaccine specific Th cell and CTL responses. Trimera mice were implanted with PBMC from either healthy HLA A2 positive donors (Figure 13 left panel) or from a RHB donor (RHB-1) (Figure 13 right panel). Mice were vaccinated with Tetanus Toxoid (TT) to assess a MHC class II restricted Th cell response and an immunodominant HLA A2 restricted epitope $EBV_{280-288}$ was used to assess MHC class I - restricted CTL responses. Control mice were left unvaccinated. Ten days after vaccination, all

mice were sacrificed and peritoneal cells were recovered by lavage. Peritoneal cells were re-stimulated in presence of thawn autologus (human) APC in an IFN-γ ELISpot setting. As shown in (Figure 13), groups with TT and EBV vaccination of Trimera mice implanted with PBMC from both healthy and RHB donors revealed strong antigen specific human Th and CTL responses, as compared to the non vaccinated control groups. In addition, after EBV vaccination in Trimera, up to 2.99% of human CD8 +ve cells were specific for the $EBV_{280-288}$ epitope in MHC epitope specific Pentamer staining (Figure 14 C) in contrast, CD8+ve cells from un vaccinated mice lack such epitope specificity (Figure 14 B). These findings demonstrate the potential of the Trimera mouse system to assess both human Th cell and CTL responses after *in vivo* vaccination.

Induction of HBV specific immune responses in the Trimera model

Previous experiments in the Trimera mouse model showed that de-novo induction of virus specific Th cell response can be achieved by vaccination with recombinant HBc particles. However none of these vaccine candidates were unable to stimulate strong virus specific CTL responses. Dendritic Cell has been used in tumor vaccination trials, owing to their capacity to process and cross present exogenous antigens to MHC class I restricted CTLs. In human influenza infection, this unique capacity depends strictly on the phagocytosis of apoptotic infected cells. Moreover in this infection it has been shown that this cross presentation pathway stimulates influenza specific MHC class I restricted CTL responses. (Albert, Sauter et al. 1998). Based on these findings, in our current study we assessed the immunogenicity of autologous antigen loaded DC to stimulate HBV specific CTL in the HBV-Trimera mouse model.

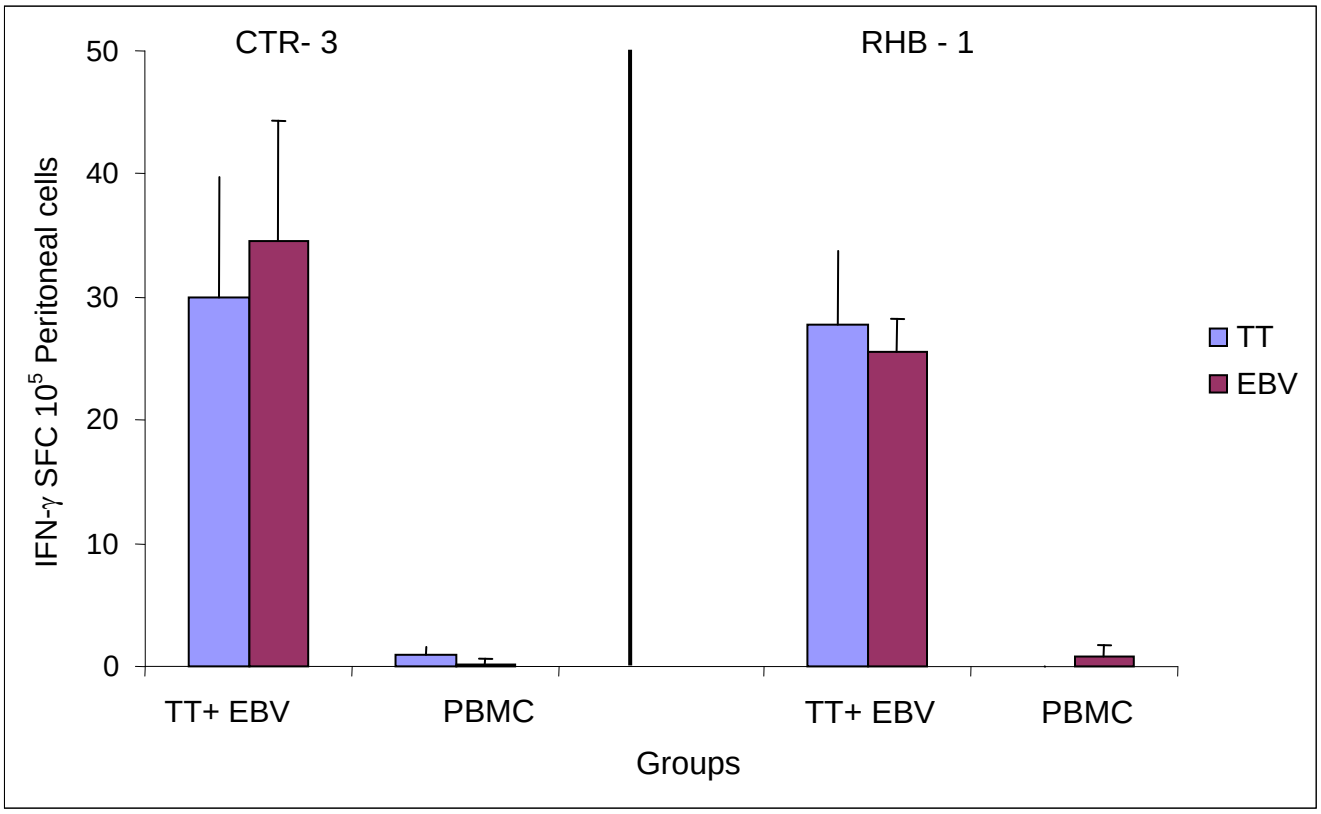


Figure 13: Assessment of Th cell and CTL response in Trimera mice after vaccination. This graph represents two individual experiments. Groups of 6 Trimera mice each were engrafted with 108 PBMC from healthy donor CTR-3 (left panel) or recovered HBV donor RHB-1 (right panel). Mice were vaccinated i.p. with Tetanus Toxoid (TT) and EBV peptide. Ten days later, peritoneal cells were collected and TT specific Th cell and EBV specific CTL responses were analyzed in IFN-γ ELISpots.

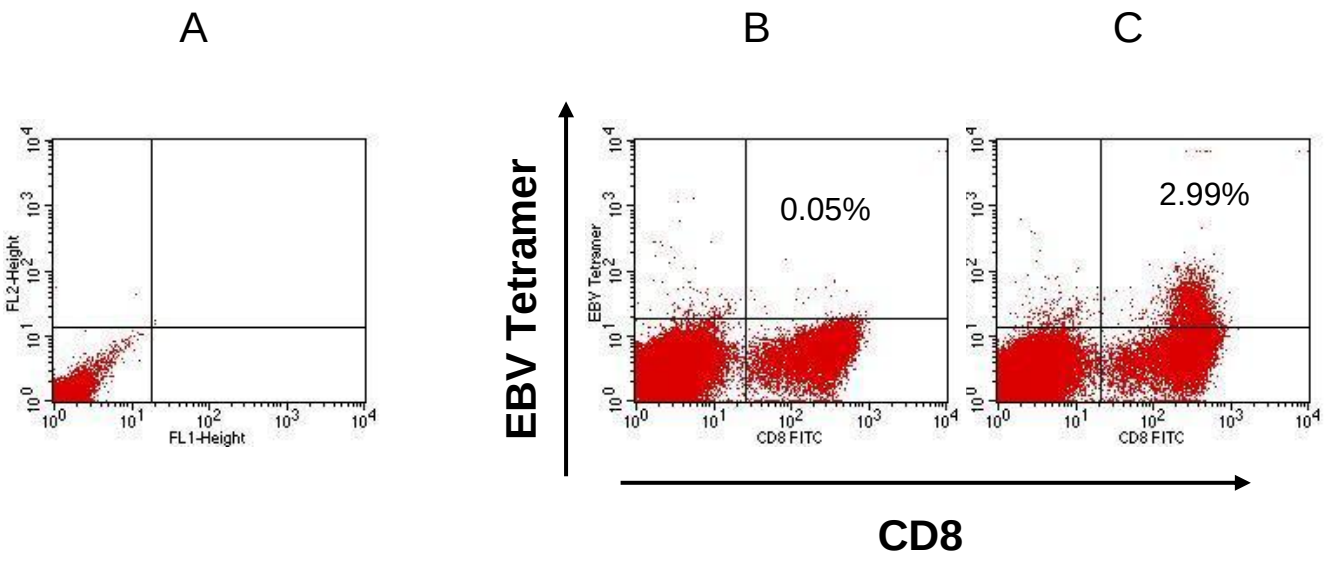


Figure 14: MHC restricted epitope specific Pentamer staining. Trimera mice were transplanted with PBMC from healthy donor CTR#2. PBMC obtained by leukapheresis and 108 cells were transplanted i.p. per mouse along with EBV peptide as described in Material and Methods. Ten days later peritoneal cells were obtained, stained with tetramers specific for the MHC class I restricted epitope EBV280 and anti-human CD8 antibodies.

4.2.4 Transfection kinetics

To achieve HBV expression a cell culture system, a 1.3 HBV plasmid containing linear over length HBV genome was used. This plasmid expresses all viral proteins by replication level comparable to natural infection. Over night cultured human hepatoma cells (HepG2) were transfected with a 1.3 HBV plasmid using Lipofectamine 2000 transfection reagent. After transfection, cells were collected on day 1, 2 & 3 and total protein was prepared by cell lysis. Proteins were separated in SDS-PAGE by loading 30 μg of protein/ well of transfected HepG2 cells, untransfected HepG2 cells or recombinant HBV core protein (as negative and positive controls, respectively). Separated proteins were blotted on PVDF membrane. Expression of HBV

core protein was detected in Western Blot using mouse monoclonal anti-HBV core antibody as primary antibody.

Bound antibodies were visualized using peroxidase conjugated mouse IgG. Western blot analysis revealed that maximum expression of HBV core protein was obtained three days after transfection in HepG2 cells (Figure 15A). Furthermore we compared HBV core protein expression in HepG2 cells after transfection with the non replicative HBV plasmid pCH3142. Western blot analysis revealed that core expression was low in these cells (Figure 15B). Further, we compared HBV core expression in HepG2.2.15 cells (a stable HBV transfected HepG2 cell line) with that in 1.3 HBV or pCH 3142 HBV transfected HepG2 cells. In (Figure 15B), a very strong band corresponding to HBV core protein can be observed in HepG2.2.15 cell lysates when compared to 1.3 HBV transfected or pCH 3142 transfected HepG2 cell lysates.

4.2.5 Apoptosis

As cross-presentation of exogeneous antigens by DC is strictly restricted to apoptosis, apoptosis was induced by UVB irradiation in HepG2.2.15 cells or in HepG2 cells two days after HBV transfection. Cells were exposed to UVB light for 5 minutes by adjusting distance between cells and UVB lamp to get peak intensity of 9000μW/cm^2 and UVB peak emission of 313nm. Apoptosis was measured by FACS using the Annexin V-FITC apoptosis detection kit (BD Pharmingen). Cells positive for both Annexin V and Propedium Iodide (PI) represent necrotic cells whereas Annexin V single positive cells represent apoptotic cells. (Figure 16) shows that 60 % of cells were apoptotic after exposure to UVB light for 5 minutes and only 9% of cells were necrotic. Other exposure times did not further increase apoptosis rate. Based on these findings in our further experiments we used exposure time of 5 minutes.

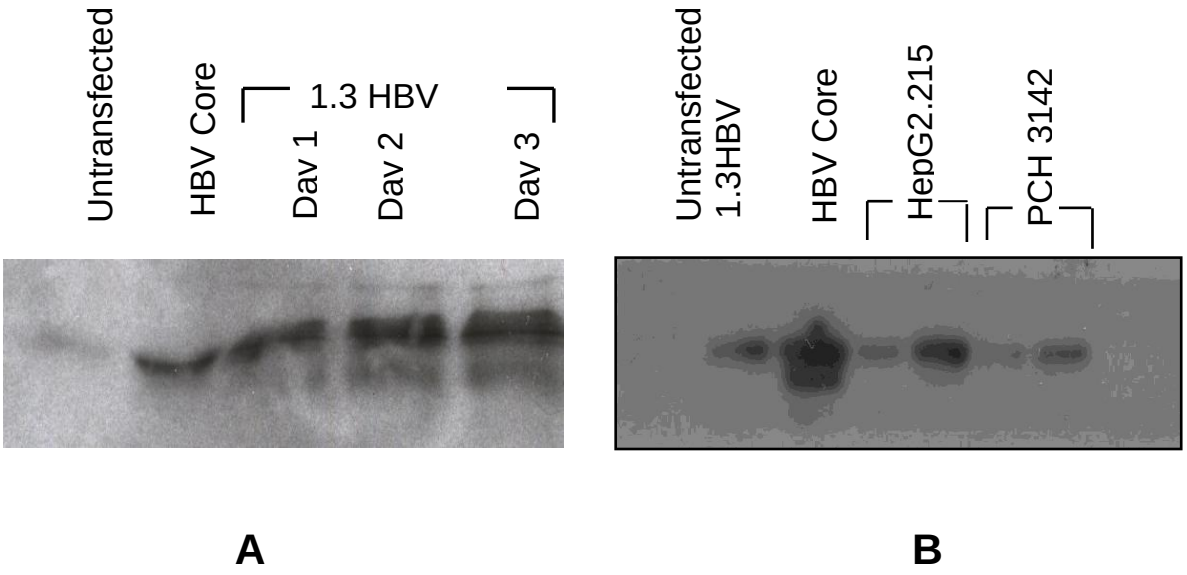


Figure 15: Western blot analysis of HBV transfected HepG2 or stable transfected HepG2.2.15 cells. HBV core protein expression was detected by using mouse anti-HBc antibody.

4.2.6 *In vitro* cross priming

We analyzed the cross-priming capacity of MDDC (Monocyte Derived Dendritic Cells) derived from 3 healthy donors and one chronic HBV carrier for HLA class I restricted cross presentation of HBV antigens to induce HBV specific CTL responses in an *in vitro* co-culture system. Dendritic cells were generated *in vitro* according to standard protocols from CD14 positive monocytes of HLA-A2 +ve donors. On day 7 of culture, these immature DCs were co-cultured with apoptotic HBV or mock transfected HepG2 cells. After 24 hrs of co-culture, DCs were separated by MACS sorting using immune magnetic HLA-DR microbeads. Cross presentation of HBV antigens was assessed by stimulation of HBc_{18-27} specific HLA A2.1 restricted CTL line. The loaded dendritic cells were cocultured with the CTL line in a ratio of 1:3

or 1:4. DC loaded with Mock transfected HepG2 cells or the HBc_{18-27} synthetic peptide served as negative and positive controls, respectively. (Figure 17) shows that apoptotic HBV loaded DC (DC_{HBV}) stimulated the HBc_{18-27} specific CTL, whereas DC loaded with mock transfected cells (DC_{HG2}) were unable to do so. This proves that cross-priming of HBV antigens can be achieved by DC when loaded with apoptotic HBV transfected cells.

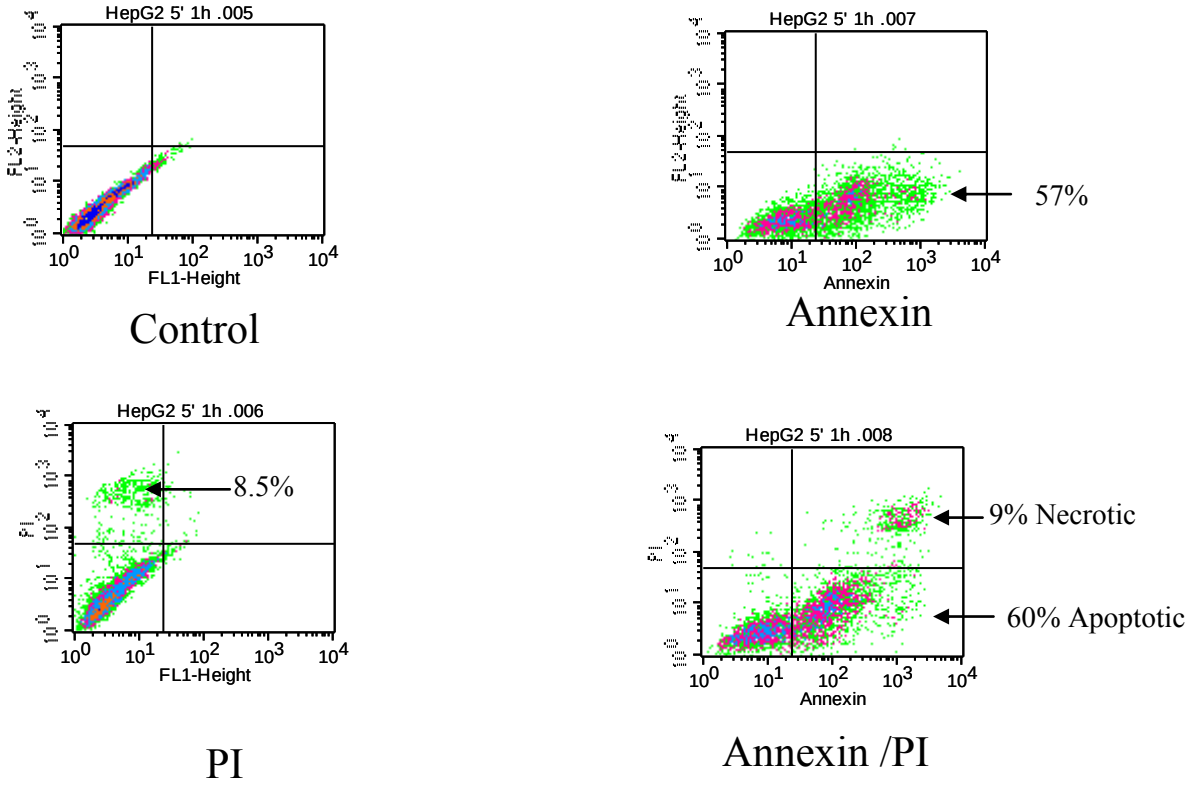


Figure 16: Induction of apoptosis by UVB exposure. HepG2 cells were exposed to UVB light for 5 minutes and apoptotic cells were analyzed by FACS staining for Annexin V and propidium iodide (PI).

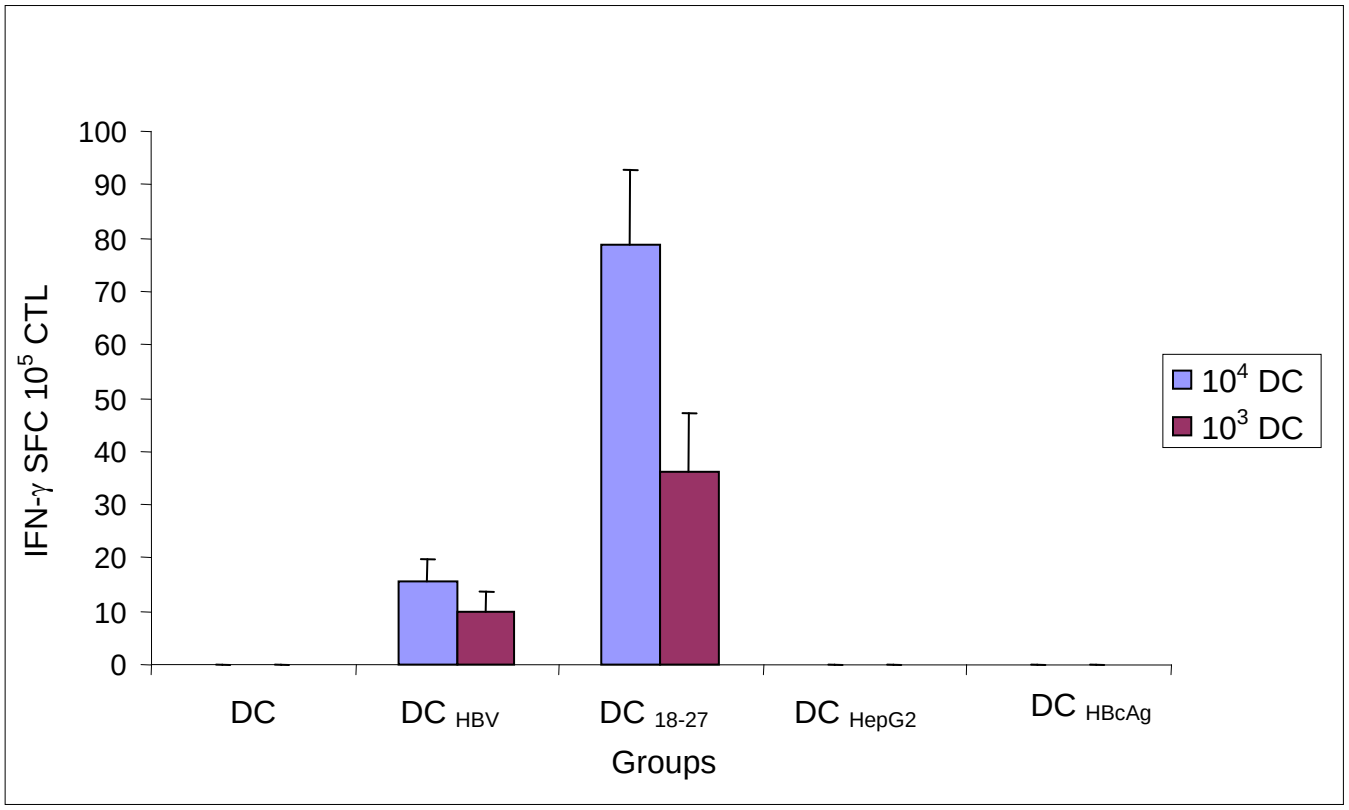


Figure 17: *In vitro* cross presentation by DC. Dendritic cells generated from PBMC of chronic HBV carrier (HBV-2) were loaded with mock- (DC HG2) or HBV-transfected apoptotic HepG2 cells (DC HBV), HBcAg (DC HBc), or HBc18-27 peptide (DC18-27). Then these DC were co cultured with HBc18-27 specific CTL lines and stimulated CTL frequencies were assessed by IFN-γ ELISpot analysis.

4.2.7 Time point for *in vitro* cross priming

After demonstrating the functionality of the cross-priming pathway *in vitro*, we further analyzed different incubation time points to get optimal cross-presentation of HBV antigens by DCs. As mentioned above, DC were prepared from HLA A2 positive donors and loaded with apoptotic HBV transfected HepG2 cells (DC_{HBV}). After 24 hrs and 40 hrs, these apoptotic HBV

loaded DC were cocultured with HBc_{18-27} specific CTL clone and stimulation of these CTL clone was detected by IFNγ ELISpot. As shown in (Figure 18), in 40 hrs coculture, more than two fold increase in IFNγ producing cell frequencies (mean ±133%) was observed when compared to 24 hrs co-culture (mean ± 37%).

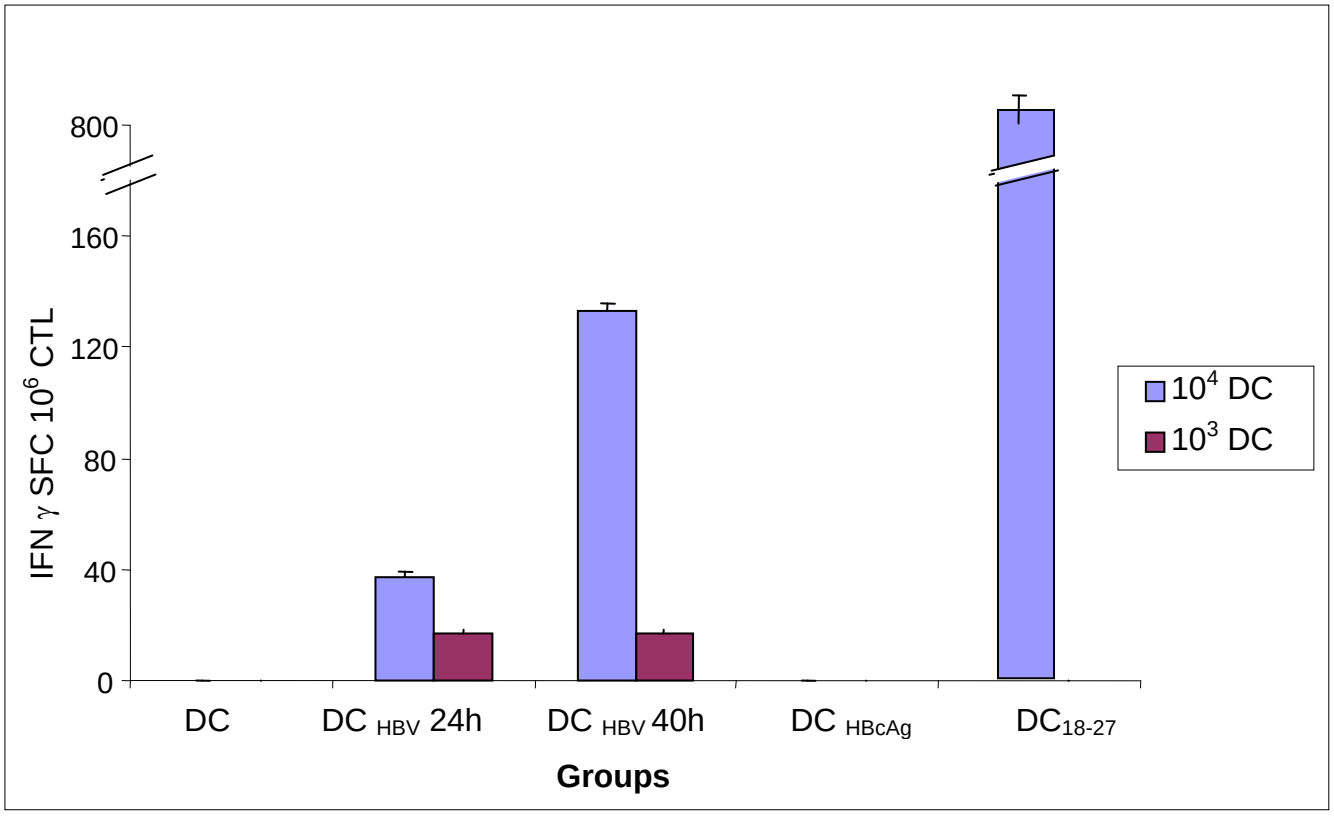


Figure 18: Coincubation time point of *in vitro* cross presentation. As described in methods, Dendritic cells were prepared from healthy donor (CTR-3) PMBC and were loaded with mock (DC HG2) or HBV transfected apoptotic HepG2 cells (DC HBV), HBcAg (DC HBc), or HBc18-27 peptide (DC 18-27) and coincubated either 24 or 40 hrs with a HLA A2-restricted HBc18-27 specific CTL line (DC, CTL ratio, 1:3, and 1:4). Stimulated CTL frequencies were detected by IFN-γ ELISpot analysis.

4.2.8 DC Maturation

Recent literature showed that DC maturation is crucial in antigen uptake, processing, and presentation. Maturation was induced *in vitro*, To study if maturation of DC could increase cross presentation and to determine the role of this DC maturation on DC loading with apoptotic HBV transfected HepG2 cells.

DC maturation was induced by adding a cytokine cocktail containing 10 ng/mL TNF-α, 10 ng/mL IL-1β, 1000 U/mL IL-6, and 1 μg/mL PGE_2, for two days. Phenotypic changes of surface markers on such DC were analyzed by FACS before and after maturation. In **(**Figure 19) it is shown that the expression of HLA-DR was strongly increased in mature (mDC) (80.34%) compared to immature DC (iDC) (57.8%). Similarly, expression of maturation markers (CD86/83) were significantly upregulated in mature DC (CD86= 70.53%, CD83= 12.77%) vs. immature DC (CD86= 44.71%, CD83= 0.17%). These data shows that maturation of DC loaded with apoptotic HBV expressing HepG2 cells can be achieved by culturing DC in presence of pro-inflammatory cytokines.

Further, we checked whether DC maturation influences the cross presentation pathway *in vitro*. We generated DC from a healthy HLA A2 positive donor (CTR-3) and maturation was induced on day 5 of culture then these DC were loaded with apoptotic HBV transfected HepG2 cells. Stimulation of a HBc_{18-27} epitope specific CTL line by immature (iDC) or mature DC (mDC) loaded with HBV apoptotic bodies was analyzed by INFγ ELISpot. DC loaded with HBc_{18-27} synthetic peptide ($DC_{HBc18-27}$) or recombinant HBcAg (DC_{HBc}) served as positive and negative controls respectively (Figure 20).

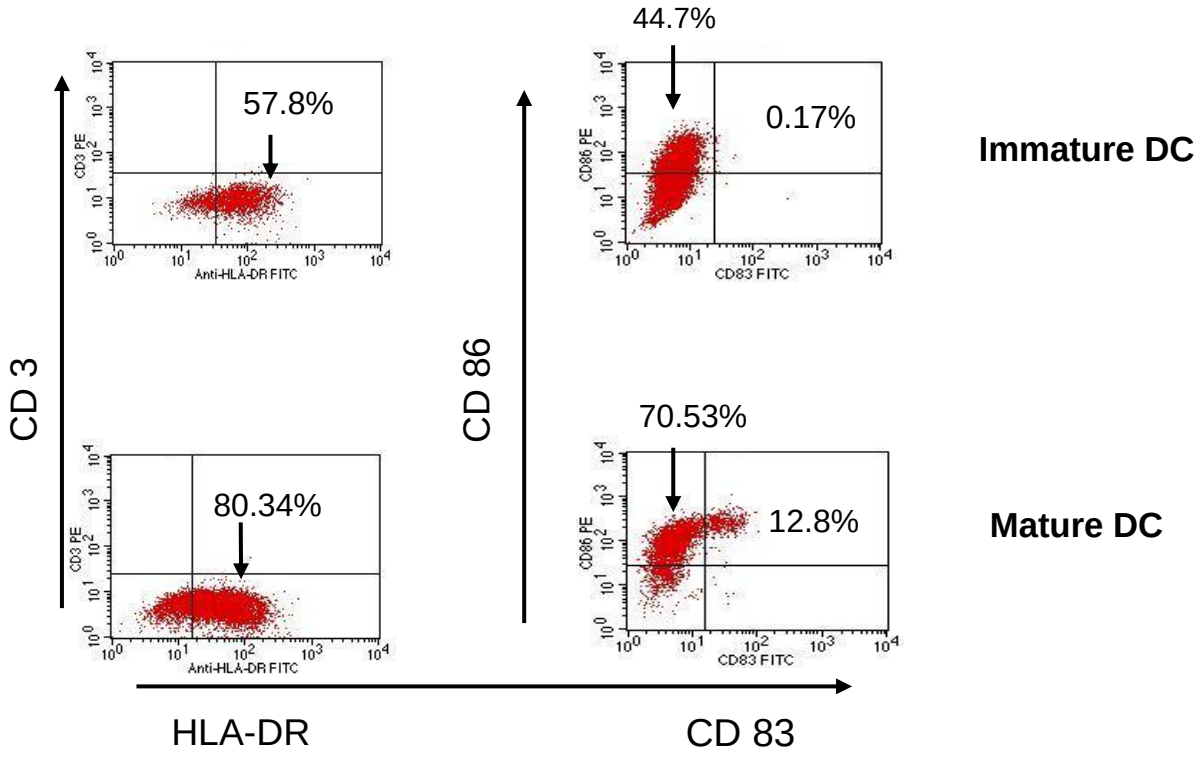


Figure 19: Maturation of DC *in vitro*. Dendritic cells from a healthy donor were cultured *in vitro*; on day 5 of culture, maturation was induced using cytokine cocktail. DCs were stained with human CD 14, 86, 83, and HLADR antibodies and phenotypic changes of surface markers were analyzed in FACS.

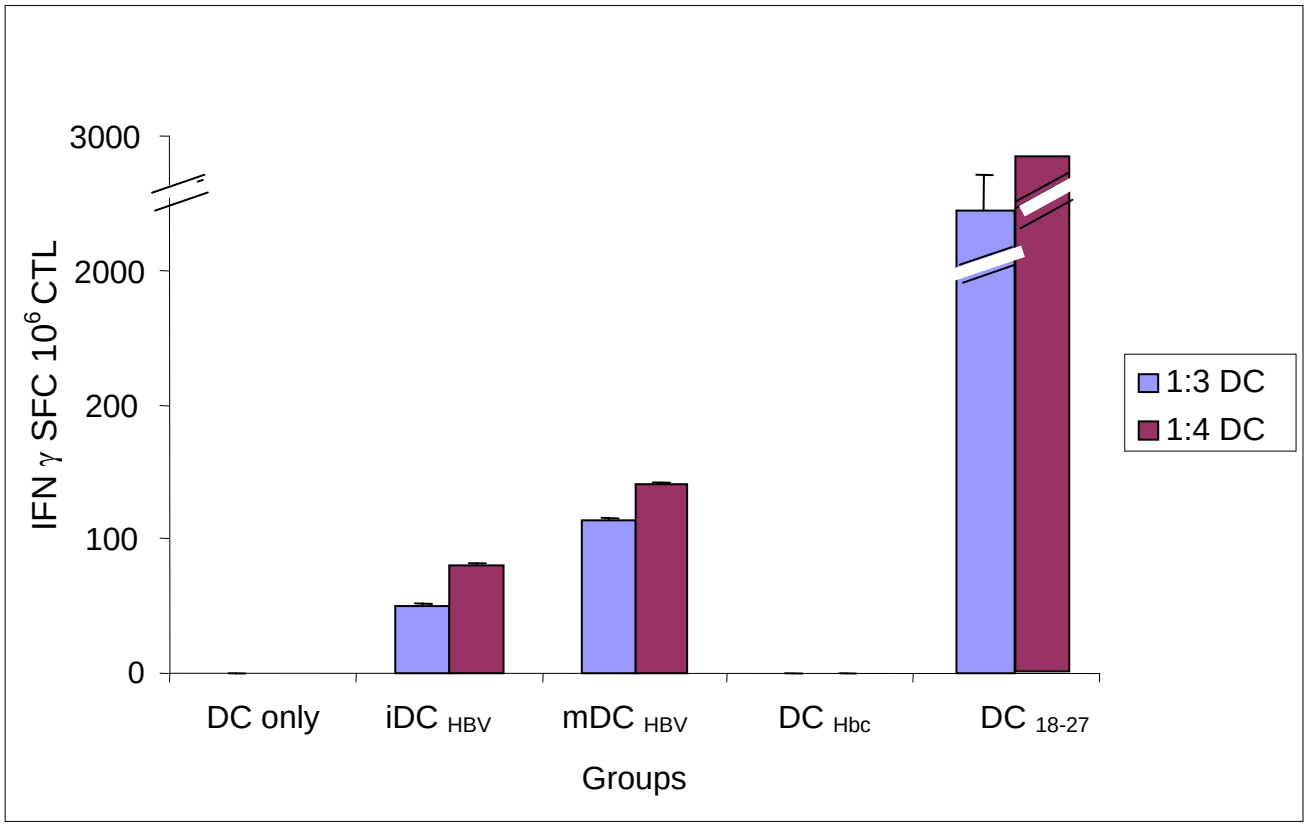


Figure 20**:** *In vitro* cross priming by matured DC. Dendritic cells of healthy donor (CTR-3) were generated as described in methods. Maturation was induced using cytokine cocktail. Both immature and mature DC were loaded with HBV-transfected apoptotic HepG2 cells (iDCHBV and mDCHBV), HBcAg (DCHbc) or HBc18-27 peptide (DC18-27) and coincubated for 40 hrs with a HLA A2-restricted HBc18-27 specific CTL line (DC, CTL ratio, 1:3 and 1:4). Stimulated CTL frequencies were detected by IFN-γ ELISpot analysis

After confirming that the DC maturation increases cross-presentation of expressed HBV antigens, we further assessed the capacity of cross-priming by DC loaded with different apoptotic cell lines transfected with different HBV vectors. As in previous experiments, mature DC were generated from HLA A2 positive healthy donor (CTR-4) and loaded with either apoptotic stabelly HBV-transfected HepG2.2.15 cells or pCH 3142 transfected HepG2 cells.

Loaded DC were cocultured with HBc_{18-27} epitope specific CTL and IFN-γ secretion was analysed by ELISpot. As represented in (Figure 21) the stimulation of HBc_{18-27} CTL line by mature DC loaded with apoptotic HepG2.2.15 cells showed two fold higher SFC frequencies when compared with immature DC. However, the CTL stimulation by mature or immature DC loaded with pCH3142 transfected HepG2 cells were significantly lower in all experiments. These data indicate that maturated DC loaded with apoptotic HepG2.2.15 cells were the strongest CTL stimulators *in vitro* and were therefore further studied *in vivo*.

4.2.9 *In vivo* crosspriming

We have shown in the past that HBV specific Th cells can be stimulated in trimera mice by vaccination with recombinant HBV antigens or DNA plasmids, while CTL responses were weak or absent (Böcher, WO, E. Galun, et al. (2000) and Böcher W, B. Dekel, et al. (2001). However, the major challenge of any therapeutic vaccine is the induction of antiviral CTL responses. Following our *in vitro* findings of cross presentation by DC, we assessed the potential of vaccination with autologous DC loaded with apoptotic HBV expressing HepG2 cells to induce HBV specific Th cell and CTL responses in the Trimera mouse model. Either apoptotic HepG2.2.15 cells stabelly expressing a full 1.3 overlength HBV genome or HepG2 cells transfected with 1.3 HBV plasmid were used to load DC. These experiments were repeatedly performed in trimera mice transplanted with PBMC from patients with different courses of chronic hepatitis B, since the clinical course seems to correlate to the degree of HBV specific T cell failure, with low viremic inactive HBs carriers representing the one end of the spectrum having rather well preserved T cell responses, while immune tolerant patients with high viremia

and normal ALT levels represent the other extreme displaying mutually undetectable T cell reacivties *ex vivo* (Maini, M. K. and Bertoletti, A. et al 2000: Webster, G. J. and Bertoletti, A. et al 2004). Thus, patients with different clinical courses might show differences in the response to therapeutic vaccination.

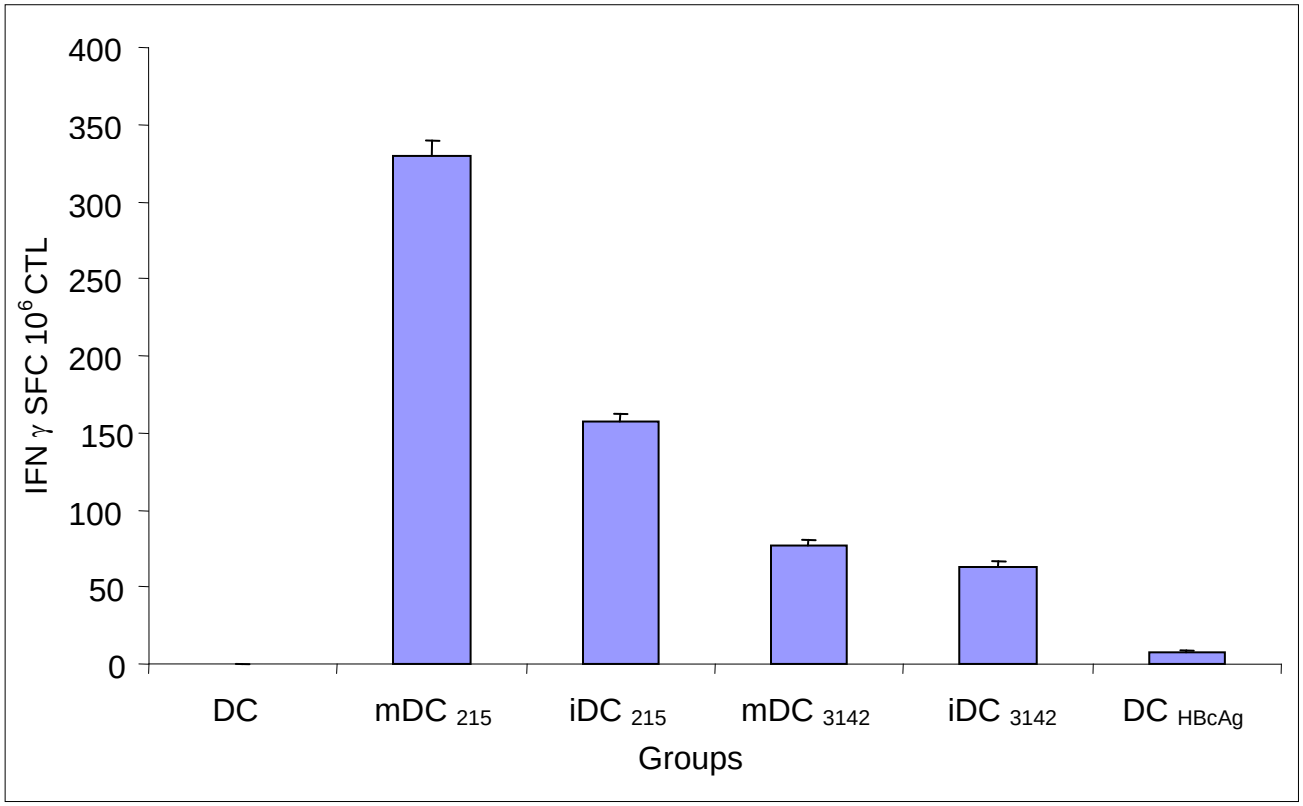


Figure 21: Influence of DC maturation on *in vitro* Cross presentation. Matured Dendritic cells of a healthy donor (CTR-4) were generated as described in methods and loaded with mock- or pCH 3142-transfected apoptotic HepG2 cells, apoptotic HepG2.2.15 cells and HBcAg. Then these DC were coincubated for 40 hrs with a HLA A2-restricted HBc18-27 specific CTL line (DC:CTL ratios of 1:2 and 1:3). Stimulated CTL frequencies were detected by IFN-γ ELISpot analysis.

4.2.9.1 Therapeutic vaccination of Timera mice implanted with PBMC from a HBs carrier (ISC).

Thus, we first studied DC vaccination in Trimera mice engrafted with PBMC from HLA A2 positive HBs carrier (ISC) HBV-2, as these donors should have the best preserved T cell reactivities. Indeed, donor PBMC revealed moderate HBc and low HBs antigen specific Th cell frequencies but only very low or lacking CTL frequencies (Figure 22). One week before.transfer of PBMC into Trimera mice, monocytes were isolated from peripheral blood and cultured in IL-4 and GM-CSF to generate immature DC. For vaccination, DC were pulsed for two days with apoptotic HBV (DC_{HBV}) or mock transfected HepG2 cells (DC_{HG2}) or with HBc antigen or peptide HBc_{18-27} (25 µg/ml each). Before intraperitoneal injection, apoptotic cell loaded DC preparations were purged from contaminating viable HepG2 cells (HLA DR negative) by positive selection of HLA DR expressing DC using immunomagnetic microbeads (MACS). DC loaded with HBV transfected apoptotic HepG2 cells and autologous PBMC were used for vaccination of 5 groups of 5 to 8 mice each. High frequencies of HBc specific Th cells were detected ten days after vaccination with with HBcAg alone. In contrast Th cell frequencies were not increased in mice vaccinated with DC loaded with HBV-transfected HepG2 cells, with HBcAg or with HBc_{18-27} peptide (Figure 23). However, vaccination with DC loaded with HBV-transfected HepG2 cells (DC_{HBV}) did lead to five fold expansion of HBc_{18-27} and HBs specific CTL response compared to DC loaded with mock-transfected HepG2 cells (DC_{HG2}) (Figure 24). DC pulsed with HBc_{18-27} peptide induced the strongest core but no surface specific CTL responses. Similar experiments were performed in trimera mice reconstituted with PBMC from a CAH donor, that revealed similar results (not shown, and HBV-3, Figure 25).

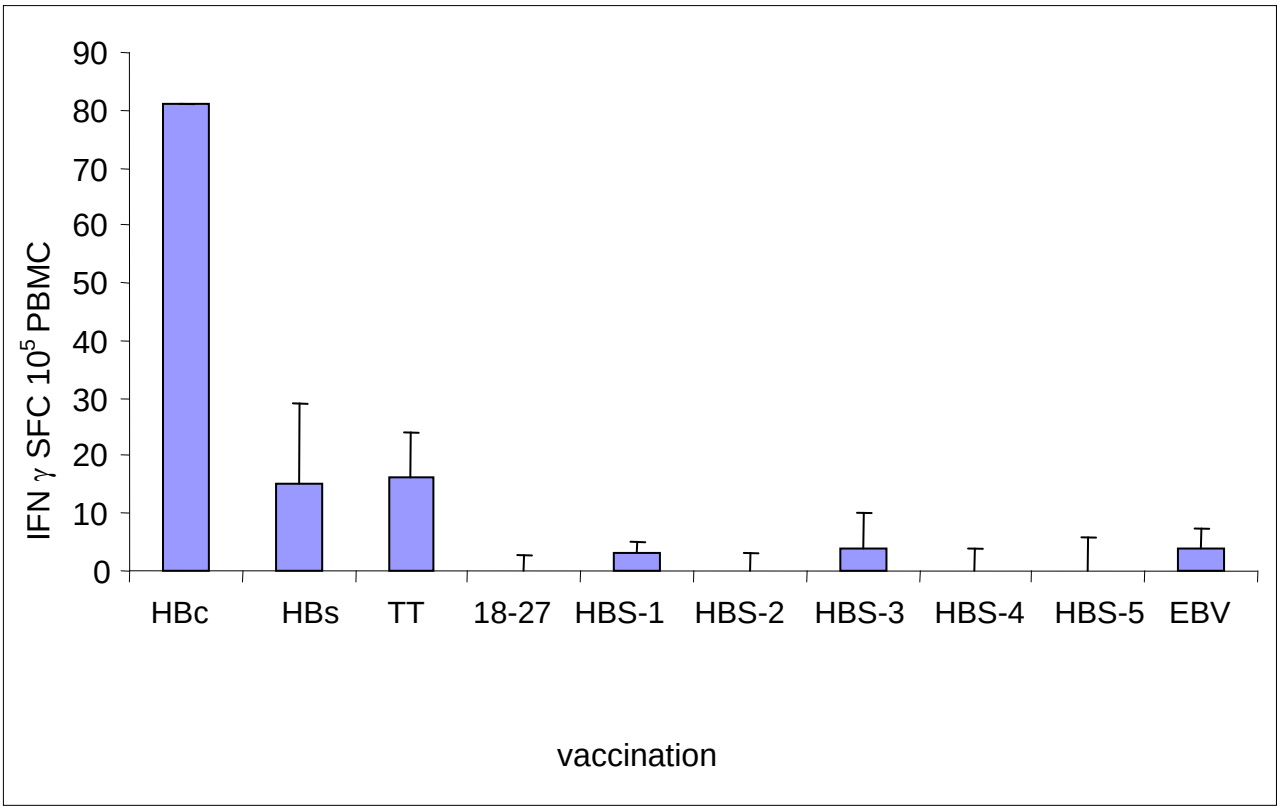


Figure 22: Th cell and CTL response of Donor PBMC in IFN-γ ELISpots. PBMC were stimulated with TT, HBc and HBsAg in context of Th response and for CTL responses, HLA A2 restricted synthetic EBV280-288, HBC18-27 and HBs 201-210 (#1), HBs 251-259(#2), HBs 260-269(#3), HBs 335-343(#4), HBs 338-347(#5) peptides were used.

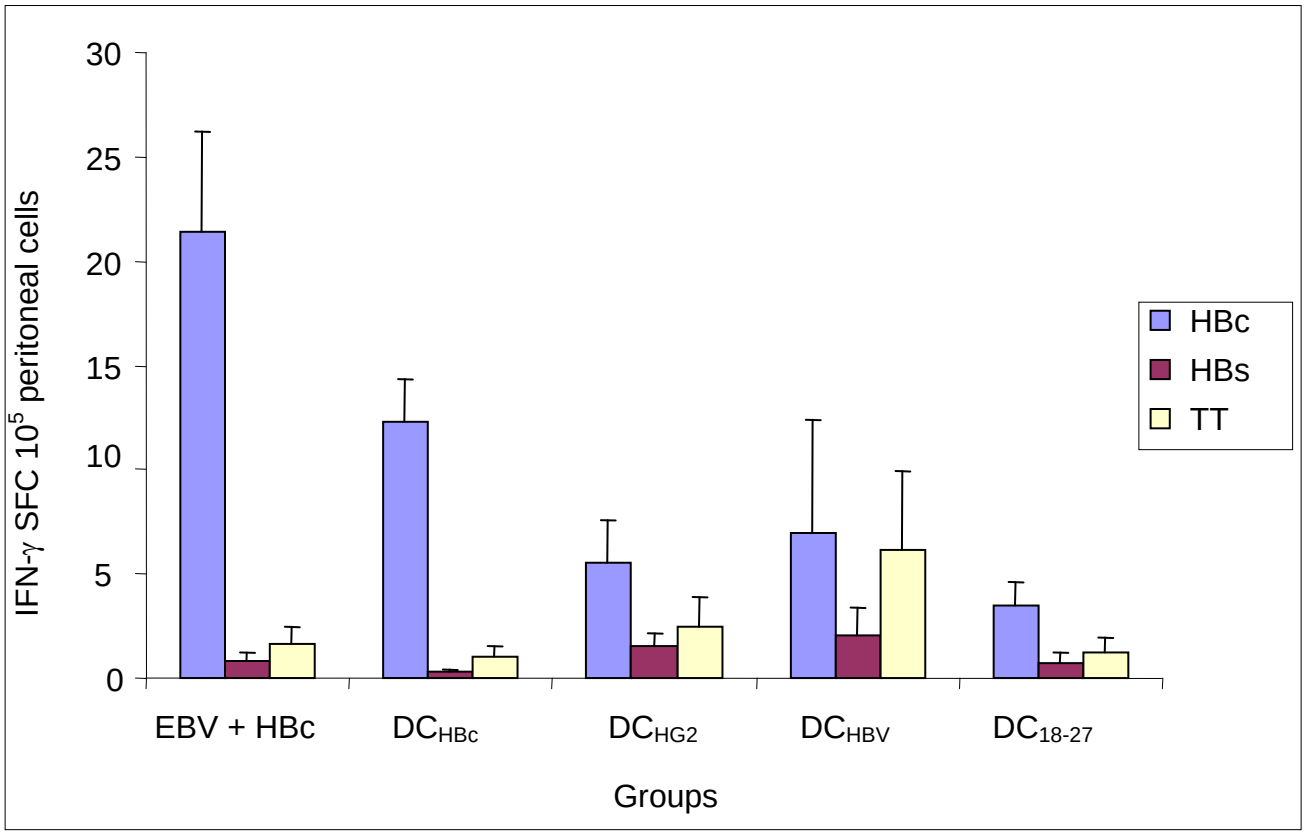


Figure 23: Expansion of HBc, HBs and TT specific Th response after therapeutic vaccination *in vivo*. Each group consists of 5 to 8 mice, engrafted with 108 PBMC from ISC donor (HBV-2). Mice were vaccinated with HBcAg + EBV280-288 peptide (EBV+HBc), autologous DC loaded with HBcAg (DCHBc), DC loaded with apoptotic mock transfected HepG2 cells (DCHG2), DC loaded with apoptotic HBV transfected HepG2 cells (DCHBV) and autologous DC loaded with HBc18-27 peptide (DC18-27). Ten days later peritoneal cells were obtained and HBV specific Th response were analyzed in IFN γ ELIspots.

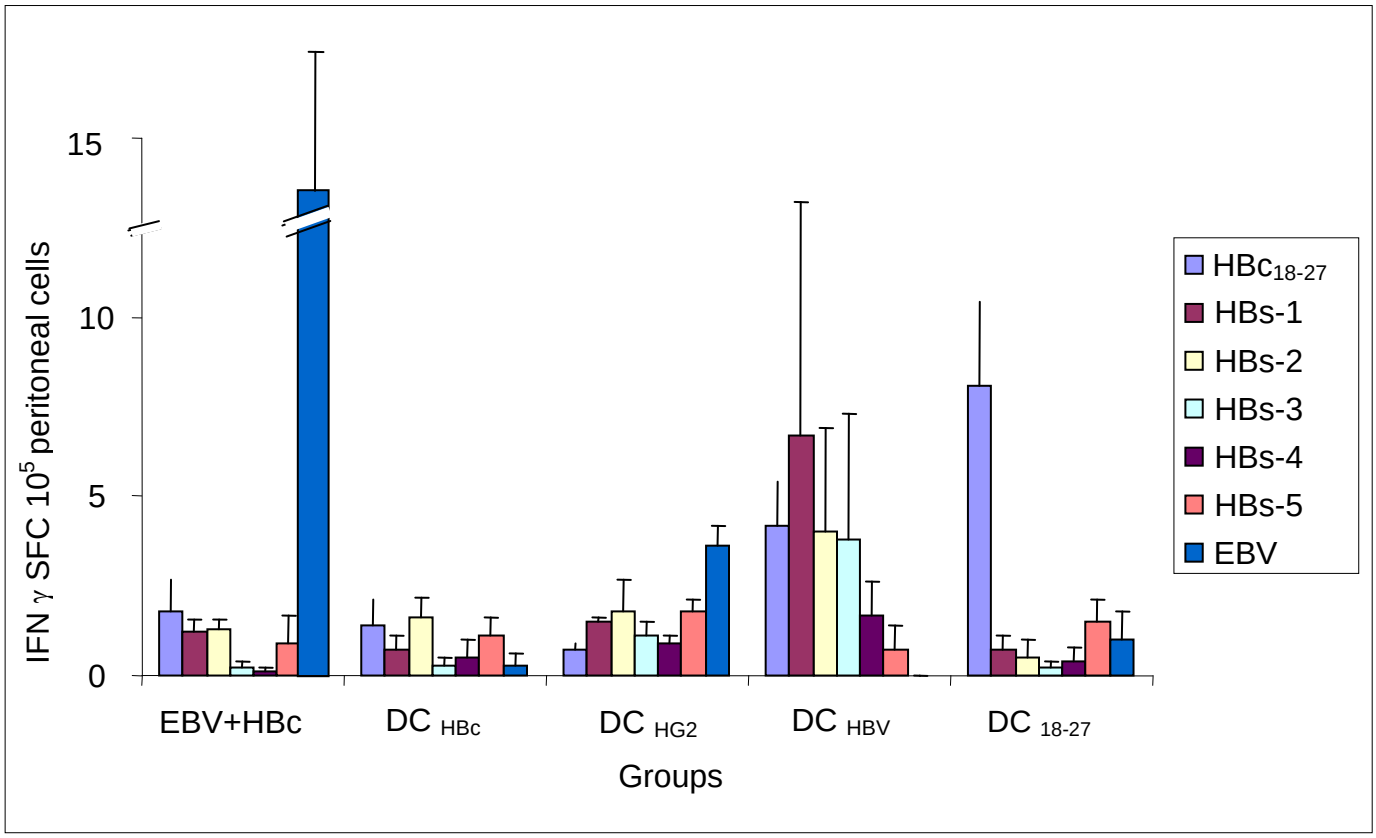


Figure 24: Expansion of HBc, HBs and EBV specific CTL response after therapeutic vaccination *in vivo*. Each group consists of 5 to 8 mice, engrafted with 100 million PBMC from ISC donor (HBV# 2). Mice were vaccinated with HBcAg + EBV280-288 peptide (EBV+HBc)), autologous DC loaded with HBcAg (DC HBc), DC loaded with apoptotic mock transfected HepG2 cells (DC HG2), DC loaded with apoptotic HBV transfected HepG2 cells (DC HBV) and autologous DC loaded with HBc18-27 peptide (DC18-27). Ten days later peritoneal cells were obtained and HBV specific CTL frequencies were analyzed in IFNγ ELIspots.

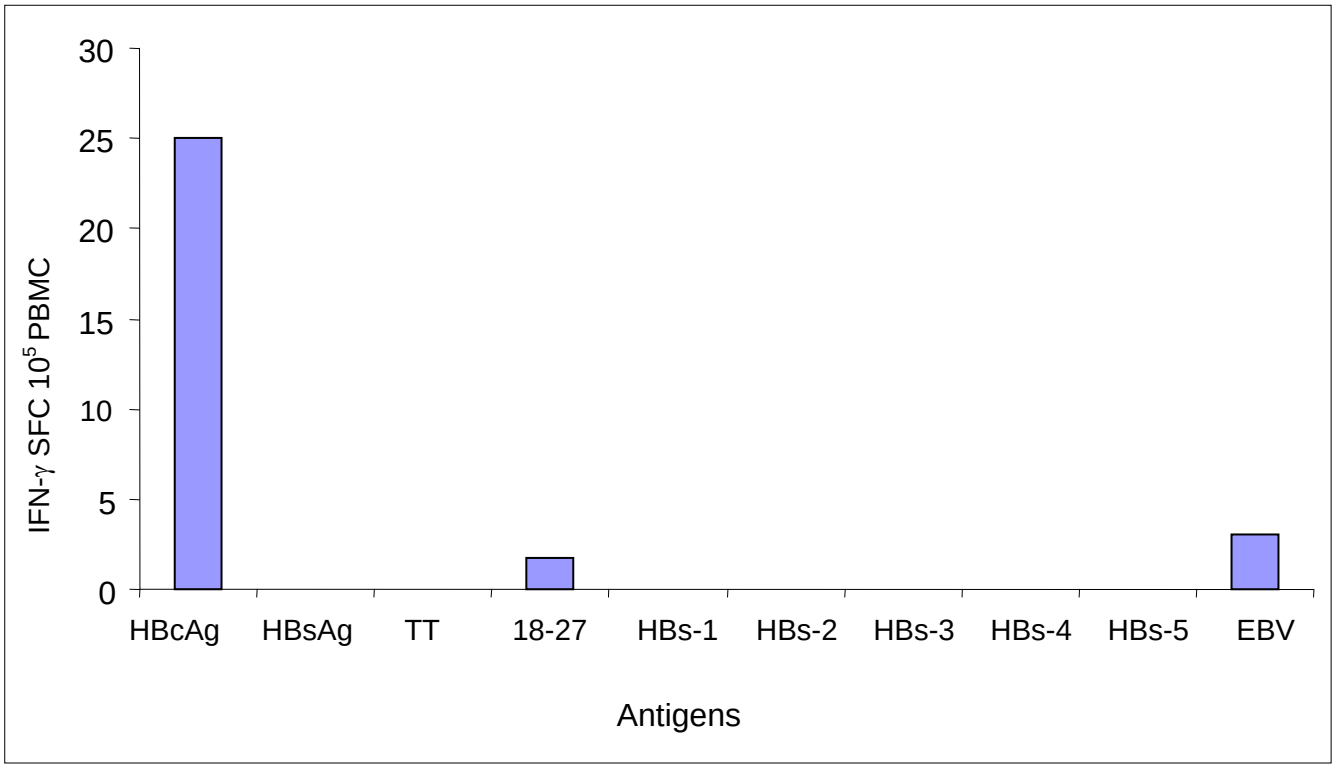


Figure 25: Th cell and CTL response in PBMC of chronic active hepatitis Donor (HBV-3) by IFN-γ ELISpots. PBMC were stimulated with TT, HBc and HBsAg in context of Th response and for CTL responses, HLA A2 restricted synthetic EBV280-288, HBC18-27 and HBs 201-210 (#1), HBs 251-259(#2), HBs 260-269(#3), HBs 335-343(#4), HBs 338-347(#5) peptides were used.

4.2.9.2 Therapeutic vaccination of Timera mice implanted with PBMC from a donor with chronic active hepatitis (CAH)

We further checked the potency of HepG2.2.15 loaded mature DC in trimera mice stimulated with PBMC from CAH donor HBV-3. Donor PBMC showed moderate HBc specific Th cell, low HBc specific CTL and no HBs specific Th or CTL responses (Figure 25). Autologous immature or mature DC_{HBV} were generated as described before and vaccinated at a count of $1x10^6$ DC/mouse. DC loaded with HBc_{18-27} peptide served as positive control whereas vaccination with mDC_{HG2} served as negative control. Strong HBc specific Th responses were found after vaccination with either immature or mature DC_{HBV} without a significant difference (Figure 26). Remarkably, only HepG2.2.15 loaded immature DC (iDC_{HBV}) induced HBs specific Th response. Mice vaccinated with apoptotic HepG2.2.15 cells alone failed to induce such responses. Regarding CTL stimulation, no HBc or HBs specific CTL were detected in the control groups vaccinated with EBV/TT or apoptotic $HG_{2.215}$ cells. In contrast, increasing frequencies of HBc specific CTL were detected after vaccination with HBV loaded immature and mature DC and mDC loaded with 18-27 peptide (Figure 27).

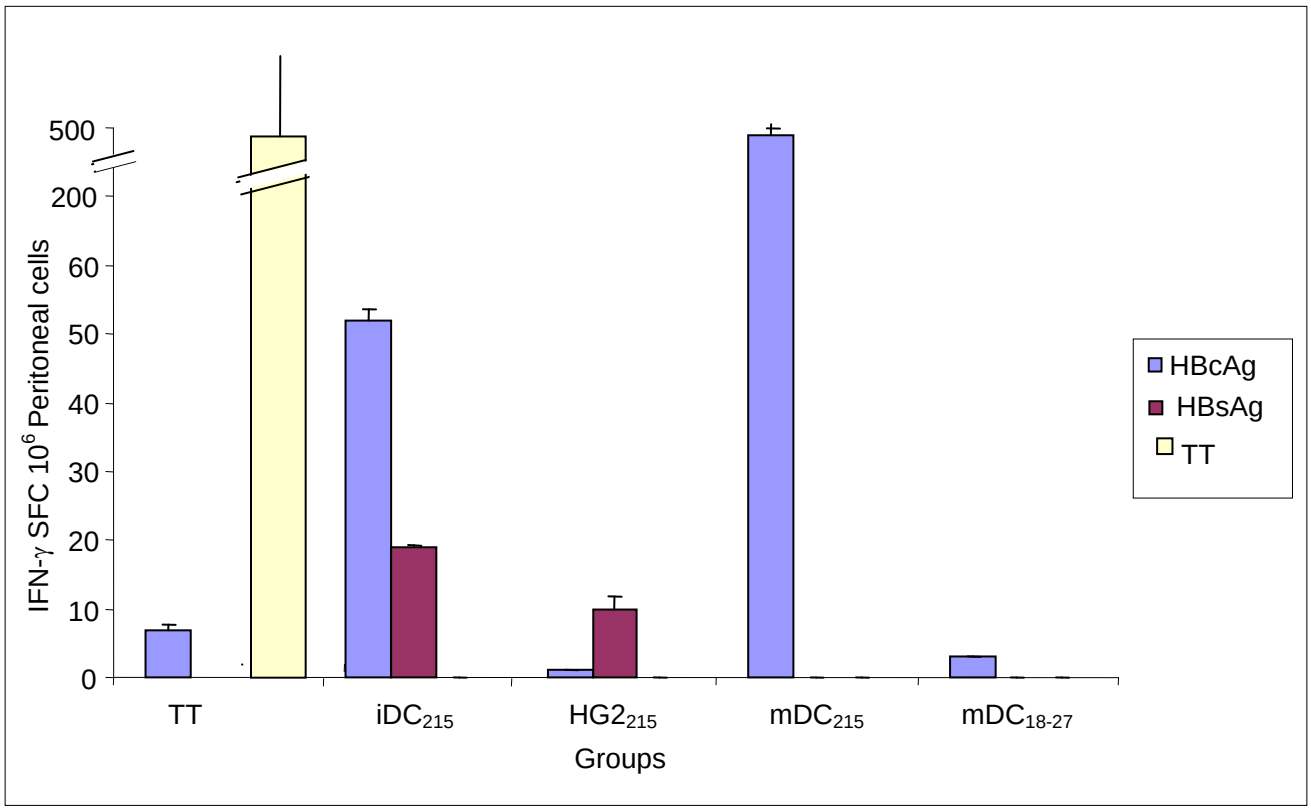


Figure 26: Expansion of HBc and HBs specific Th cells by DC vaccination *in vivo*. Each group consists of 5 to 6 Trimera mice, engrafted with PBMC from donor HBV-3 . Mice were vaccinated i.p. with TT, immature DC loaded with apoptotic HepG2.2.15 cells (iDC2.15), PBMC + HepG2.215 (HG2215) cells , mature DC loaded with apoptotic HepG2.215 cells (mDC2.15) and DC loaded with HBc18-27 peptide (DC18-27). Ten days later peritoneal cells were isolated and HBc, HBs, and TT specific Th responses were analyzed *ex vivo* by IFNγ ELIspots.

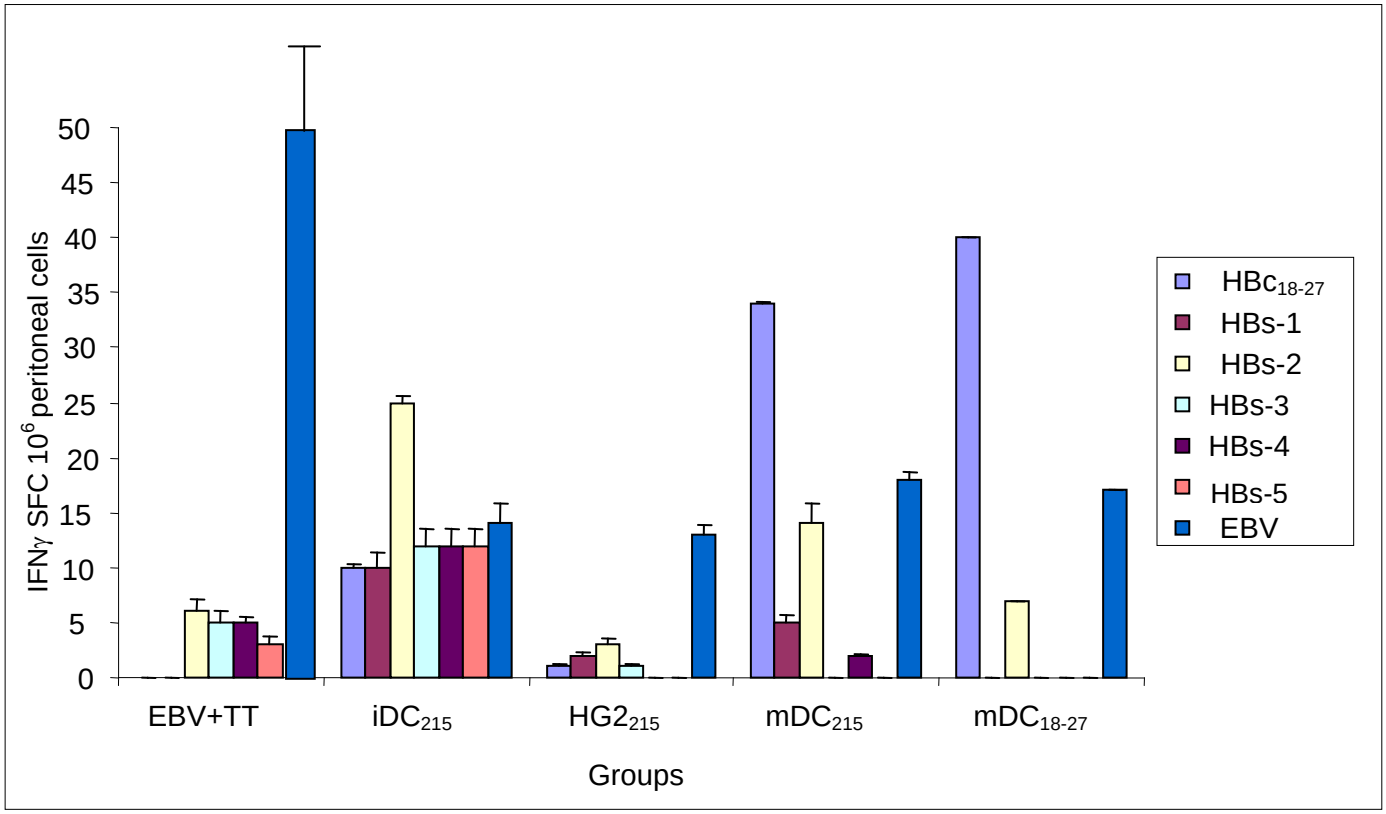


Figure 27: Expansion of HBc and HBs specific CTL by DC vaccination *in vivo*. Each group consists of 5 to 6 Trimera mice, engrafted with PBMC from donor HBV-3 . Mice were vaccinated i.p. with TT, immature DC loaded with apoptotic HepG2.2.15 cells (iDC2.15), PBMC + HepG2.215 (HG2215) cells , mature DC loaded with apoptotic HepG2.215 cells (mDC2.15) and DC loaded with HBc18-27 peptide (DC18-27). Ten days later peritoneal cells were isolated and HBc, HBs, and TT specific Th responses were analyzed *ex vivo* by IFN γ ELIspots

4.2.9.3 Therapeutic vaccination of Timera mice implanted with PBMC from a Immune tolerant Donor (IT).

The above experiment was repeated in trimera reconstituted with PBMC from immune tolerant donor HBV-1. In such donors, it should be most difficult to induce relevant T cell responses. This donor displayed no HBc specific Th cells or CTL and very low HBs specific CTL frequencies in peripheral blood as detected by ELISpot (Figure 28). One week before collection of PBMC for transfer into Trimera mice, monocytes were isolated from peripheral blood and cultured in IL-4 and GM-CSF to generate immature DC. For vaccination, DC were pulsed for two days with apoptotic HBV or mock transfected HepG2 cells (ratio 3:1) or with HBc antigen or peptide HBc_{18-27} (25 µg/ml each). Before intraperitoneal injection, apoptotic cell loaded DC preparations were purged from contaminating viable HepG2 cells (HLA DR negative) by positive selection of HLA DR expressing DC using immunomagnetic microbeads (MACS). Five groups of 5-7 mice each were implanted with PBMC and vaccinated with 10^6/mouse of autologous pulsed DC. Ten days later, HBc, HBs specific Th and CTL responses were assessed by IFN-γ ELISpots. In (Figure 29) strong HBc specific Th cell responses in peritoneum of mice vaccinated with HBcAg, with DC loaded with HBcAg (DC_{HBc}), with HBV transfected HepG2 cells (DC_{HBV}) can be observed. In contrast HBc specific Th cell frequencies were not increased in mice vaccinated with DC loaded with peptide HBc_{18-27} ($DC_{HBc18-27}$). However, (Figure 30) shows significant expansion of HBc_{18-27} and HBs specific CTL responses in mice vaccinated with DC_{HBV}, or with the peptide ($DC_{HBc18-27}$) while no such increase was found after vaccination with HBc or mock-transfected HepG2 loaded DC (DC_{HG2} and DC_{HBc}).

Thus, vaccination with autologous DC loaded with apoptotic HBV transfected HepG2 cells strongly increased core and surface specific CTL frequencies in HBV-Trimera mice over those of control mice or donor PBMC.

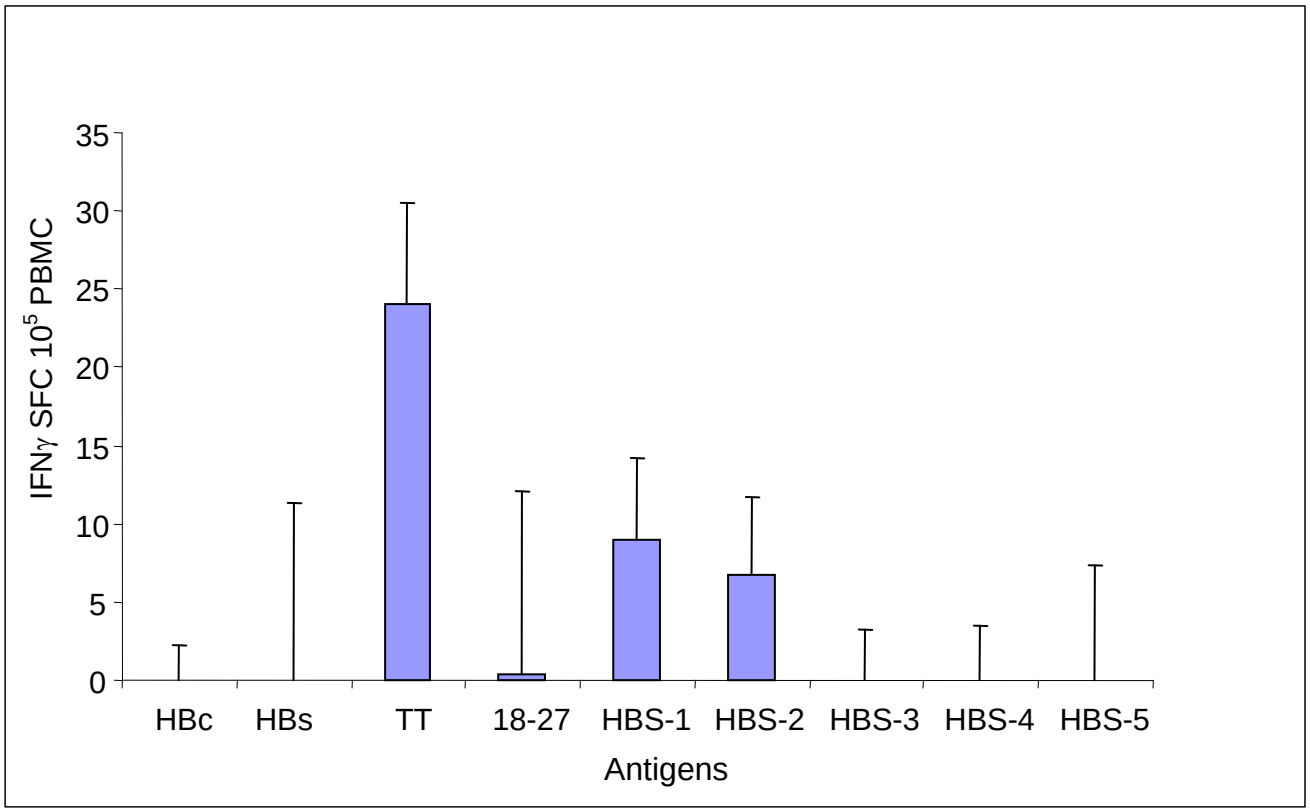


Figure 28: Th cell and CTL response in PBMC of IT Donor (HBV-1) by IFNγ ELISpots. PBMC were stimulated with TT, HBc and HBsAg in context of Th response and for CTL responses, HLA A2 restricted synthetic HBC18-27 and HBs 201-210 (#1), HBs 251-259(#2), HBs 260-269(#3), HBs 335-343(#4), HBs 338-347(#5) peptides were used..

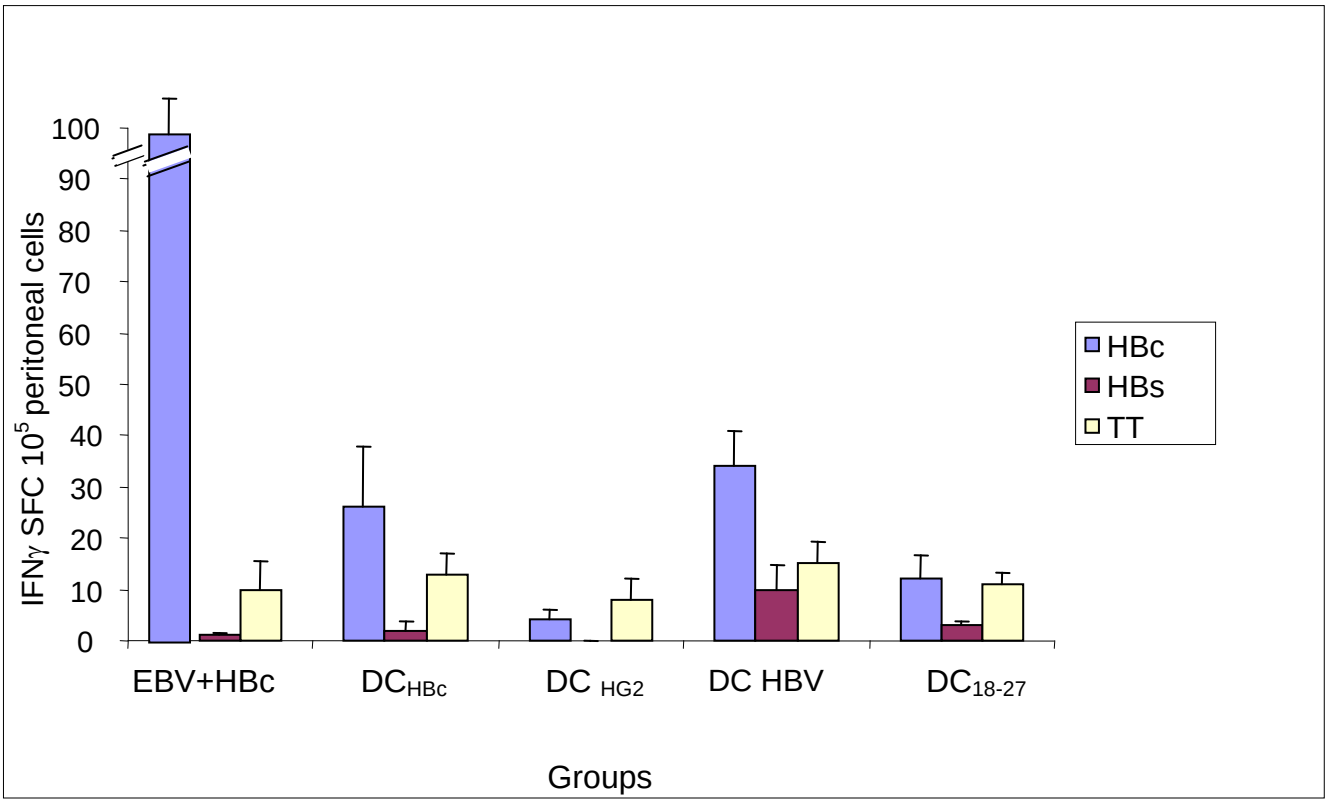


Figure 29: Expansion of HBc and HBs specific Th cell response *in vivo*. Each group consists of 5 to 7 Trimera mice, engrafted with PBMC from IT donor (HBV#1). Mice were vaccinated i.p. with HBcAg + EBV280-288 peptide (EBV+HBc), DC loaded with HBcAg (DCHBc), DC loaded with apoptotic mock transfected HepG2 cells (DCHG2), DC loaded with apoptotic HBV transfected HepG2 cells (DCHBV), DC loaded with synthetic HBc18-27 peptide (DC18-27). Ten days later peritoneal cells were isolated and HBc, HBs, specific Th responses were analyzed *ex vivo* in IFNγ ELISpots

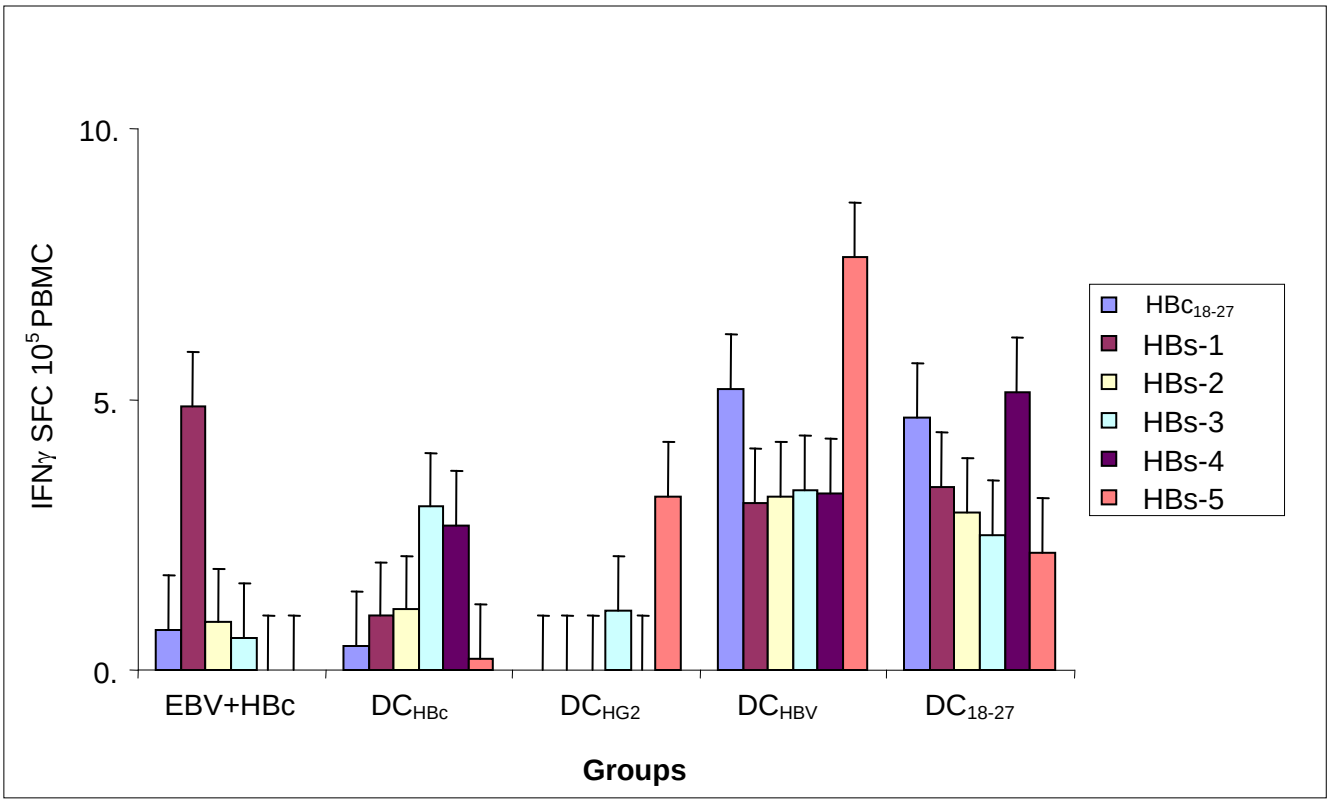


Figure 30: Expansion of HBc and HBs specific CTL cell response *in vivo*. Each group consists of 5 to 7 Trimera mice, engrafted with PBMC from IT donor (HBV#1). Mice were vaccinated i.p. with HBcAg + EBV280-288 peptide (EBV+HBc), DC loaded with HBcAg (DCHBc), DC loaded with apoptotic mock transfected HepG2 cells (DCHG2), DC loaded with apoptotic HBV transfected HepG2 cells (DCHBV), DC loaded with synthetic HBc18-27 peptide (DC18-27). Ten days later, HBc, HBs, specific CTL responses were analyzed *ex vivo* from peritoneal cells restimulated *in vitro* with HBc18-27 peptide and mixture of five HBs peptide epitopes in IFNγ ELISpots..

4.2.9.4 Crosspriming with matured DC

In vitro experiments showed that the efficacy of cross presentation and CTL stimulation can be increased by using mature DC. To check the potential of the vaccination with mature vs. immature DC loaded with apoptotic HBV expressing cell lines, Trimera mice were implanted with PBMC from an inactive HBs carrier HBV-4. DC were generated as described in methods. On day five of DC culture, maturation was induced by adding the inflammatory cytokine cocktail to the culture. Both immature and mature DC loaded with apoptotic HepG2.2.15 or pCH 3142 transfected HepG2 cells, stimulated strong HBc specific and, HBs specific Th responses after vaccination in trimera mice (Figure 31). However, strong HBc and HBs specific CTL responses were induced only by vaccination with apoptotic HepG2.2.15 cells. In contrast, DC loaded with pCH 3142, transfected HepG2 cells failed to stimulate such CTL responses (Figure 32).

This experiment seems to confirm that DC maturation may enhance the cross presentation of HBV antigens *in vivo*.

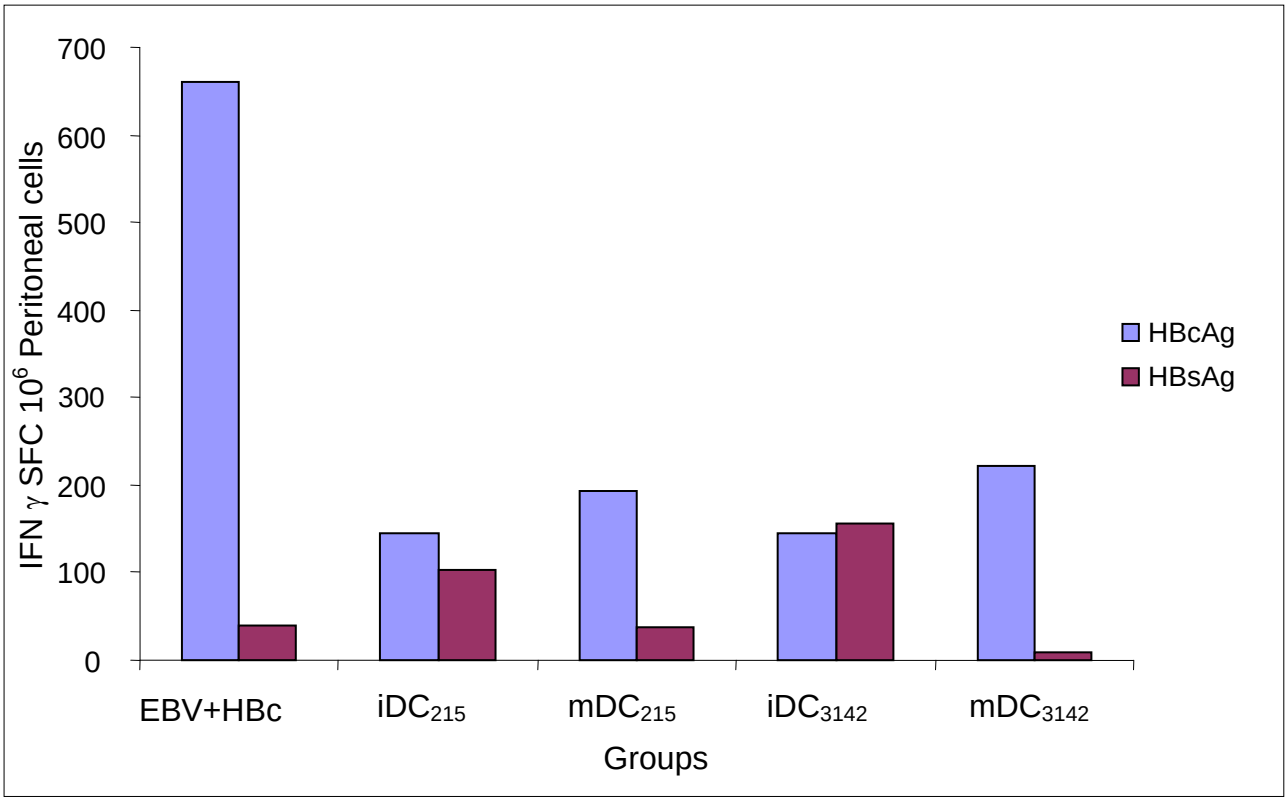


Figure 31**:** Expansion of HBc and HBs specific Th cell response by DC vaccination *in vivo*. Each group consists of 5 to 8 Trimera mice, engrafted with PBMC from ISC donor (HBV-4). Mice were vaccinated i.p. with HBcAg + EBV280-288 peptide (HBc+EBV), mature DC loaded with apoptotic HepG2.215 (mDC215), immature DC loaded with apoptotic HepG2.215 cells (iDC215), mature DC loaded with apoptotic pCH 3142 transfected HepG2 cells (mDC3142), immature DC loaded with apoptotic pCH 3142 transfected HepG2 cells (iDC3142). Ten days later, HBc and HBs specific Th cell responses were analyzed *ex vivo* from peritoneal cells restimulated with recombinant HBcAg and HBsAg in IFNγ ELISpots.

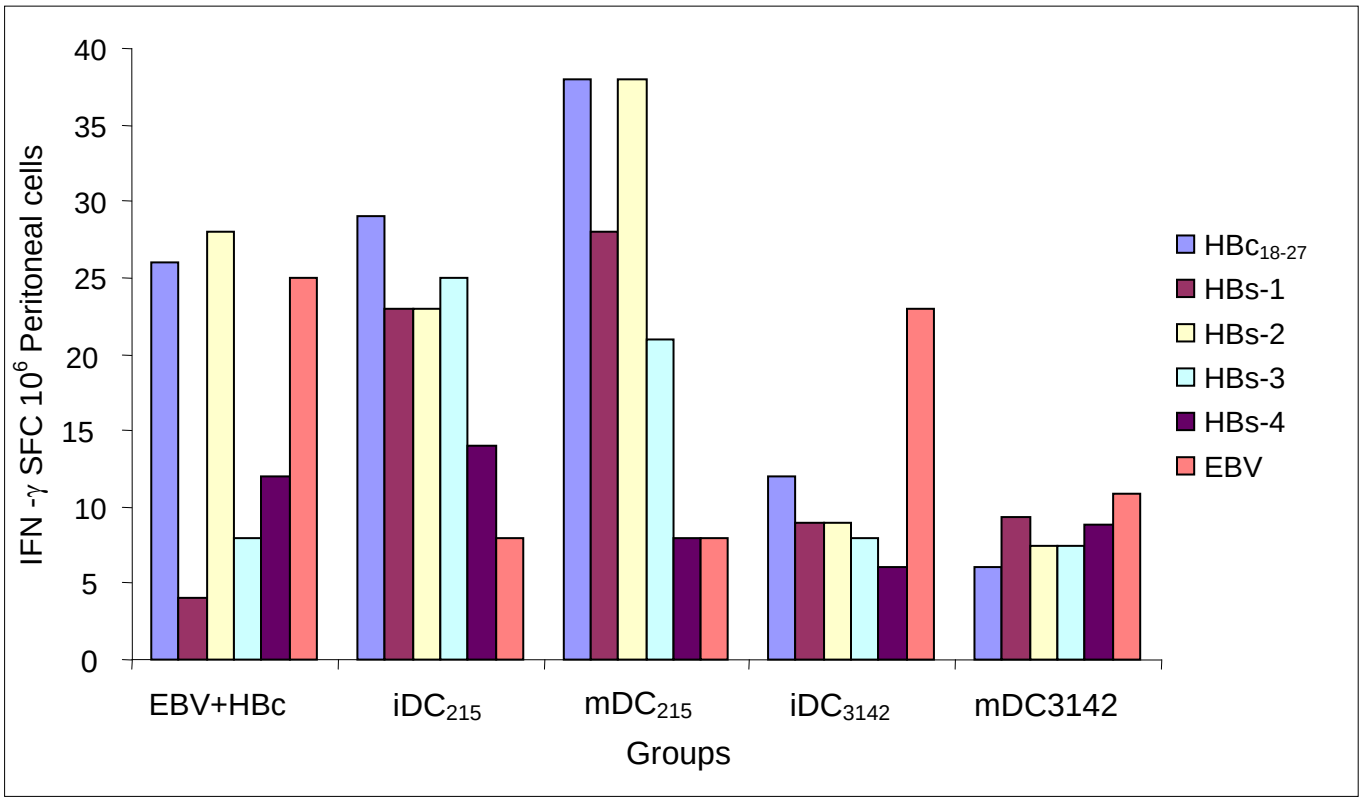


Figure 32 Expansion of HBc, HBs and EBV specific CTL response by DC vaccination *in vivo*. Each group consists of 5 to 8 Trimera mice, engrafted with PBMC from ISC donor (HBV-4). Mice were vaccinated i.p. with HBcAg + EBV280-288 peptide (HBc+EBV), mature DC loaded with apoptotic HepG2.215 (mDC215), immature DC loaded with apoptotic HepG2.215 cells (iDC215), mature DC loaded with apoptotic pCH 3142 transfected HepG2 cells (mDC3142), immature DC loaded with apoptotic pCH 3142 transfected HepG2 cells (iDC3142). Ten days later, HBc, HBs, and EBV specific CTL responses were analyzed *ex vivo* from peritoneal cells restimulated *in vitro* with HBc18-27 , EBV280-288 and mixture of four HBs peptide epitopes in IFNγ ELISpots.

4.3 Mechanisms of antiviral T cell stimulation in trimera mice.

As demonstrated in previous experiments, vaccination with recombinant HBc antigen or autologous DC loaded with apoptotic HBV transfected cells strongly increased both anti viral Th cell response as well as core and surface specific CTL response in the humanized Trimera mouse model. This is in contrast to clinical studies of different protein, peptide or DNA vaccines, that were immunogenic in healthy individuals but not in chronic HBV carriers (Mancini-Bourgine, M., H. Fontaine, et al. (2004); Pol, S., M. L. Michel, et al. (2000); Heathcote, J., J. McHutchison, et al. (1999). Thus, the identification of the mechanisms involved in T cell stimulation in our system might pave the path to new treatment options for patients. The high efficacy of vaccination in our system might be due to:

1. Efficient antigen processing and presentation of HBV antigens by mouse derived APC.
2. Xenoreactively driven cytokines in the peritoneum, possibly reversing antiviral T cell tolerance or leading to improved DC maturation in situ, or
3. Due to lack of HBV viremia or antigenemia in our system that might induce T cell tolerance in patients.

These three hypotheses were further studied in the Trimera mouse system.

4.3.1 Human APC are crucial for T cell stimulation

Although recipient mice are lethally irradiated, mouse derived antigen presenting cells (APC) might persist together with transferred human B cells, monocytes and dendritic cells. Thus, murine APC might cross-present vaccine derived epitopes across the species barrier to human T cells.

Peritoneal, spleen and bone marrow cells were obtained from Trimera mice on day 2, day 4, and day 9 after human PBMC transfer and stained with monoclonal antibodies specific for different murine APC populations and analyzed by flow cytometry. (Figure 33: shows one representative FACS staining for mouse derived B cells and macrophages in Trimera mice. The co-expression of CD86/B220 represents murine B cells and co-expression of CD86/ F480 represents murine macrophages. In the upper panel of (Figure 33: it can be seen that a slight increase in murine B cells from day 2 to day 9 after PBMC transplantation. Moreover, the lower panel of the same figure demonstrates a small increase in percentage of the murine macrophages in Trimera mouse peritonium. Thus, low but increasing numbers of mouse APC persist in peritoneum, spleen, and bone marrow (not shown) of Trimera mice in parallel to implanted human PBMC. Due to the lack of mouse strain-specific antibodies, it could not be distinguished, whether these cells are of NOD/SCID (i.e. bone marrow donor) or Balb/c (i.e. recipient) origin.

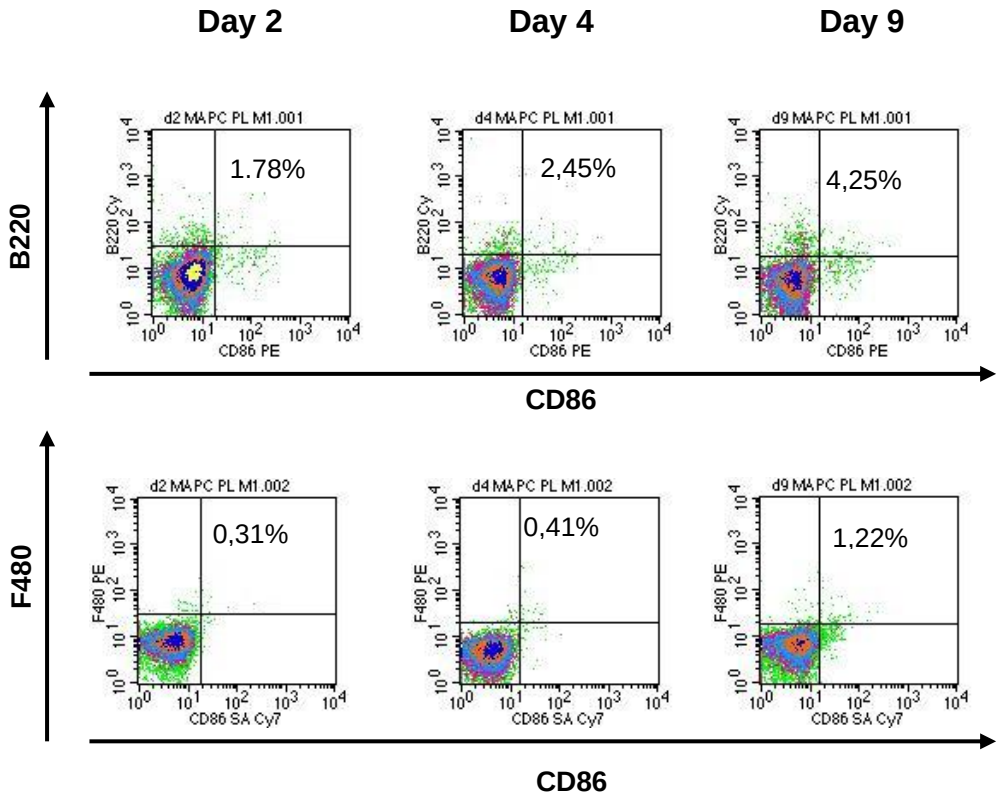


Figure 33: FACS staining of Murine antigen presenting cells (APC) in peritoneum of Trimera mice. Peritoneal cells were collected from Trimera mice 2, 4 and 9 days after transfer of PBMC from healthy donor (CTR-4). Peritoneal cells were stained for murine B cells (B220) and murine macrophages (F4/80/ CD 86) and analyzed by Flow cytometry.

Since complete *in vivo* depletion of mouse APC from Trimera mice was impossible due to lack of specific lytic antibodies, the need of human APC was assessed in trimera mice in APC depletion experiments. Therefore, PBMC obtained from HLA A2.1 recovered HBV donor (RHB-1) were transferred into Trimera mice either unmanipulated or depleted from all HLA DR^+ APC, or selectively from $CD14^+$ monocytes, $CD19^+$ B cells, or BDCA-1^+/-4^+ DC. Depletions were carried out by immune magnetic cell sorting using mAb against the respective surface markers. Vaccination of such Trimera was performed i.p. with Tetanus Toxoid

(100µL/mouse) (to study Th cell responses) and the synthetic $EBV_{280-288}$ peptide (30µg/mouse) (to study CTL responses). Antigen specific human Th cell and CTL frequencies were analyzed 10 days after vaccination in peritoneal cells by IFN-γ ELISpot. As demonstrated in (Figure 34) very strong Tetanus specific Th type and EBV specific CTL responses were induced in vaccinated Trimera mice implanted with total PBMC. Depletion of total HLA-DR^+ APC resulted in a wide reduction in both Th and CTL responses (DR-ve), proving the pivotal role of human APC for antigen specific T cell stimulation. Selective depletion of CD 19 positive B cells resulted in a similar decrease in TT specific Th responses but not of the EBV specific CTL response (CD 19-ve). In contrast, selective depletion of CD 14 positive monocytes resulted in a significant reduction of EBV specific CTL but not TT specific Th cell response (CD 14-ve). Depletion of BDCA-1^+/-4^+ DC led to abrogation of both Th cell and CTL responses (DC -ve) demonstrating the need of DC for T cell stimulation in Trimera mice. In contrast, mice transplanted with PBMC but left with out vaccination showed no responses.

Whereas the contradictory effect of B cell and monocyte depletion on Th cell and CTL response was not reproducible, otherwise similar results were obtained in Trimera injected with PBMC from different healthy donors.

In summary, these experiments demonstrate that human HLA-DR positive APC are needed for T cell stimulation. The residual Th cell and CTL frequencies detected despite near complete depletion of all human APC (< 5% HLA DR^+ cells in PBMC inoculum) might be due to *in vivo* maturation of APC from HLA-DR negative cells transferred with the donor PBMC. However, a minor effect of cross species acting mouse APC cannot definitely be ruled out. DC seem to be the most important APC in our system, whereas the effect of B cells and monocyte

depletion can widely be counterbalanced by the respective other APC types. Thus, human APC, in particular DC, are crucial for human T cell stimulation in our Trimera system

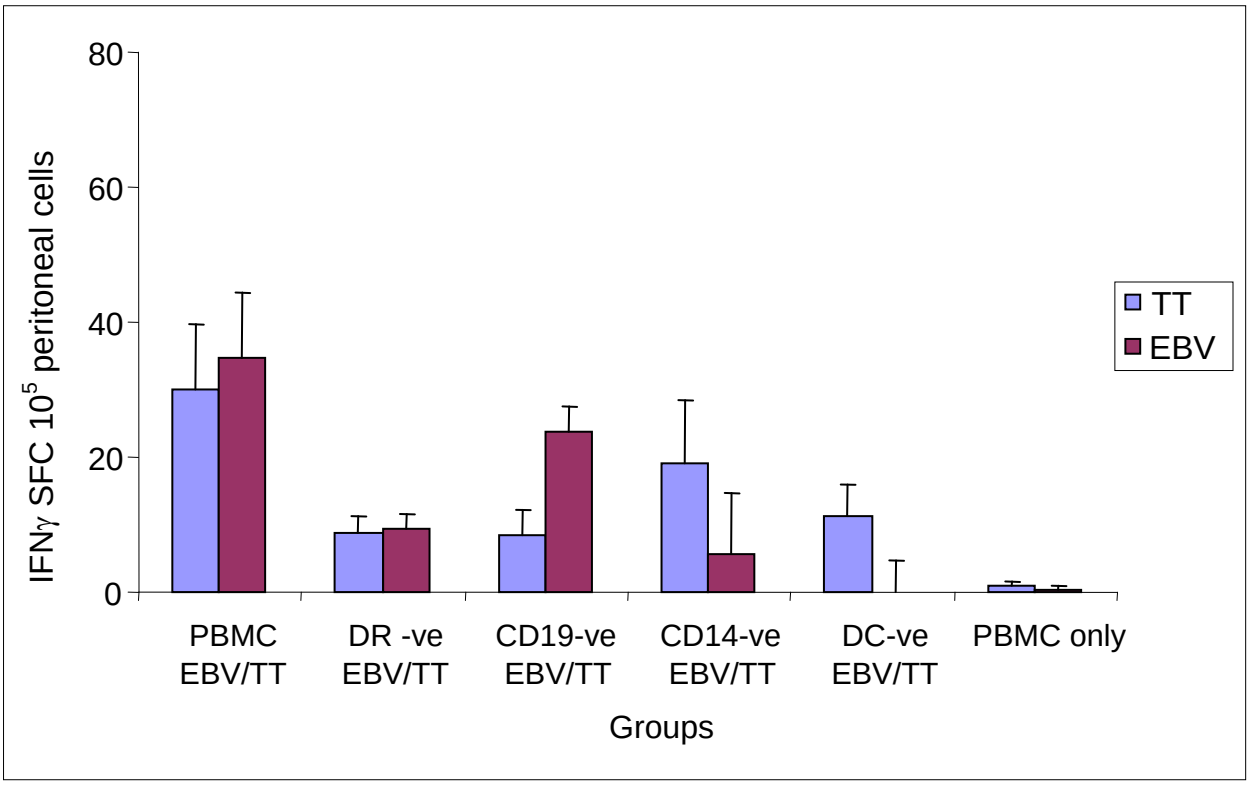
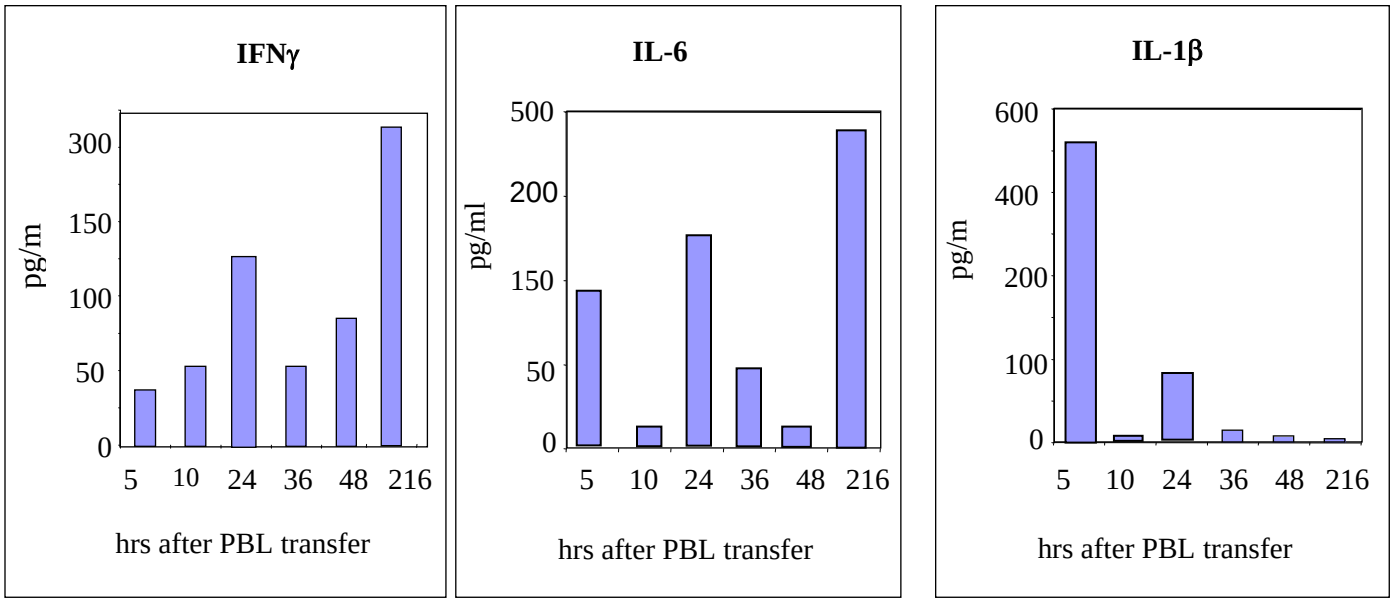


Figure 34: Role of Human APC in to stimulating TT specific Th cell and EBV specific CTL responses *in vivo*. Each group consists of 5 to 7 Trimera mice, engrafted with PBMC from recovered HBV donor (RHB-1). Mice were vaccinated i.p. with TT + EBV280-288 peptide (EBV+TT), control mice left unvaccinated (PBMC only). Vaccination was performed in mice engrafted with total PBMC, PBMC depleted for all HLA DR positive APC (DR-ve), depleted for CD19-positive B cells (CD19-ve), depleted for CD14 positive monocytes (CD14 -ve), or with PBMC depleted for myeloid and plasmacytoid DC (DC -ve). Ten days later, TT and EBV specific T cell responses were analyzed *ex vivo* from peritoneal cells restimulated *in vitro* with Tetanus Toxoid and EBV280-288 peptide by IFN-γ ELISpot.

4.3.2 Human cytokines in peritoneum of Trimera mice

Alternatively, the capacity of patient derived HBV specific Th cell and CTL to expand and secrete cytokines after therapeutic vaccination in our model, that was not found in clinical trials of chronic HBV carriers vaccinated with peptides or recombinant antigens, might be due to xenoreactively driven inflammatory cytokines, which might reverse T cell anergy of HBV patients. Thus, Trimera mice were engrafted with PBMC from healthy donors and peritoneal lavage was performed at several time points after PBMC transfer. Peritoneal washes were analysed for human inflammatory cytokines using a highly sensitive FACS-based cytokine bead array kit.

By these analyses, only the secretion of human IFN-γ, IL-6 and IL-1β was detected, while IL-2 , TNF-α, IL-12, IL-4, IL-5, IL-10 were undetectable (Figure 35). The kinetics of IFN-γ and IL-6 levels showed two peaks, one early after transfer on day 1, and a second late peak around day 9, thus closely correlating to the kinetics of human T cell engraftment in the trimera with an early decrease of transferred T cells until day 2 followed by progressive engraftment and population of the mice to day 9 -12 (Marcus, H. and Gan, J.et al 1995; Bocher, W. O., H. Marcus, et al. 1999). In contrast, IL-1β was detected only very early and disappeared by days 2-3. This cytokine might be derived from early xenoreactively activated monocytes and other APC, that usually disappear from peritoenum within few days after transfer **(**Lubin, I and Y Reisner. et al. 1994**).** However, IL-6 and IL-1β are pro-inflammatory cytokines known to reverse T cell tolerance and improve or recover Th cell function under certain conditions. Thus, their secretion into peritoneum might reverse T cell tolerance of patient derived transformed T cells, and might therefore play an important role for the success of therapeutic vaccination in our model.



IL-2, TNF-α, IL-12, IL-4, IL-5, IL-10: < 20 pg/ml

Figure 35: Human cytokines in peritoneum of Trimera mice. After engraftment of PBMC from healthy human donors into Trimera mice, peritoneal cells were obtained at different time points. Cells were stained for human cytokines using the FACS based CBA kit and analyzed by Flow cytometry.

In situ maturation of transformed DC

Furthermore, under the influence of the above detected cytokines, human DC precurcers, transferred together with the PBMC inoculum, might mature in situ and thereby lead to efficient T cell stimulation. FACS analyses of peritoneal cells recovered from peritoneum of trimera mice implanted with PBMC from healthy donors indeed revealed a progressive increase of HLA-DR expression on the surface of lineage negative HLA-DR positive DC after transplantation .

Moreover, frequencies and numbers of HLA $DR^+/CD83^+$ mature DC increased continuously after PBMC transfer (Figure 36).

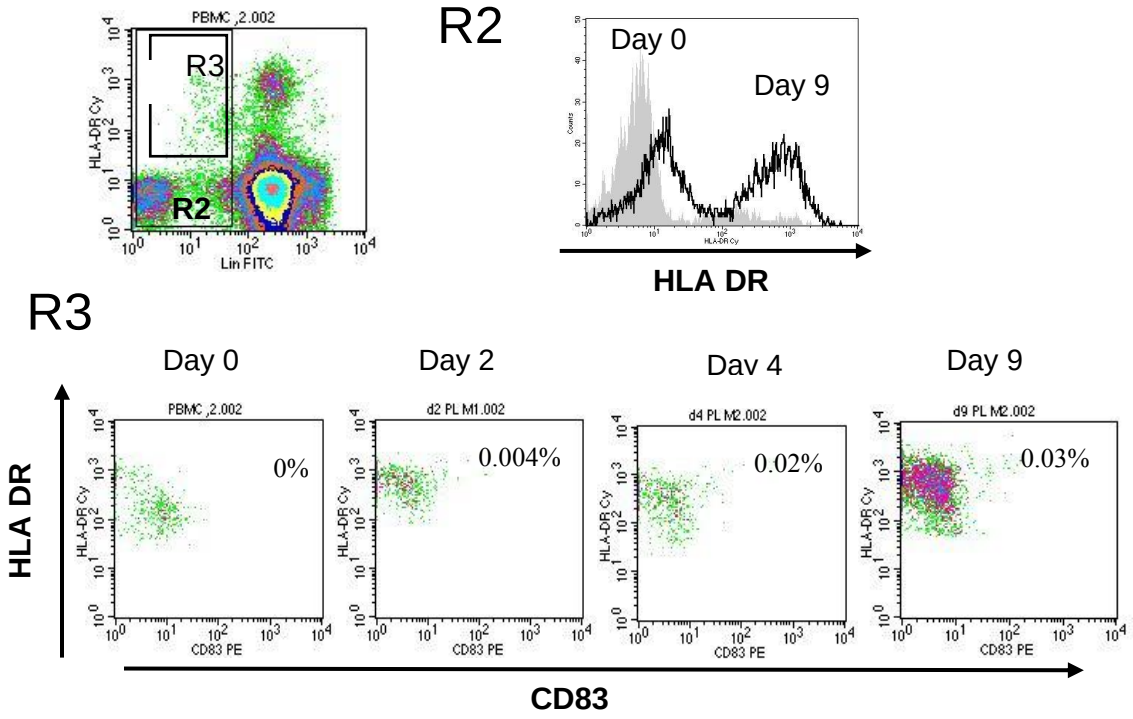


Figure 36: Maturation of Human Dendritic cells in peritoneum of Trimera mice. PBMC from a healthy human donor were engrafted to peritoneum of Trimera mice and peritoneal cells were obtained on day 0, day 2, day 4, and day 9. Then cells were stained with human DC antibodies and analyzed in FACS.

4.3.3 Role of HBV viremia/ antigenemia

The main immunological feature that characterizes chronically infected HBV patients is a state of hyporesponsiveness of HBV specific T cells that might be caused by deletion of specific T cells (exhaustion) or by functional tolerance (anergy). As our trimera mice under the above conditions are negative for HBV DNA and HBV antigens (Böcher, W.O, and Reisner Y. et al 2000), HBV viremia or antigenemia might be responsible for this T cell dysfunction in patients with persistent infection. To assess the influence of the virus and its antigens on such immune responses, we established viremia and/or HBs antigenemia in Trimera model by use of HBV and HBs transgenic mice as recipients for healthy or patient derived PBMC to study the human immune response under conditions of HBV viremia and antigenemia.

Thus, PBMC were obtained from a HLA A2 positive healthy donor (CTR-1). Donor PBMC were transferred into lethally irradiated and SCID bone marrow engrafted 1.3HBV transgenic or wildtype C57B6 mice. These trimera mice were vaccinated i.p. with TT (100µL/mouse) and HBcAg (100µg/mouse) to induce recall or primary Th cell responses or synthetic $EBV_{280-288}$ peptide (30µg/mouse) to induce recall CTL responses. Antigen specific human Th cell and CTL responses were analyzed 10 days after vaccination in peritoneal cells by IFN-γ ELISpot. As demonstrated in (Figure 37), TT specific Th cell and EBV specific CTL responses in 1.3 HBV trimera mice were equivalent to those obtained in wild type Trimera mice. In contrast, after vaccination with HBcAg, in 1.3 HBV transgenic mice only very low Th cell responses to HBc were detectable, while this response was very strong in wild type mice. Thus, strong unspecific T cell responses can be induced in HBV viremic trimera mice, while HBV specific T cell responses are prevented under HBV viremic conditions.

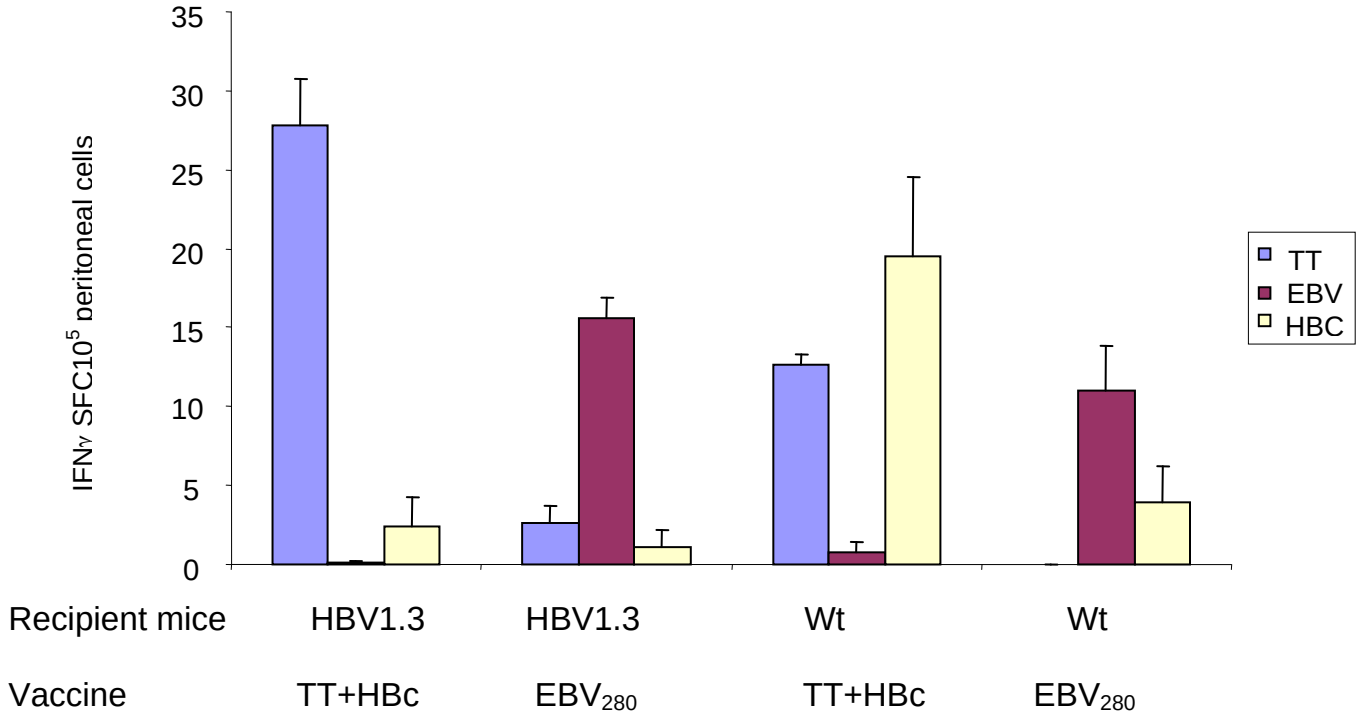


Figure 37: Impact of HBV viremia on development of HBV and control antigen specific T cell responses *in vivo*. Each group consists of 4 to 5 Trimera mice, engrafted with PBMC from healthy donor (CTR-1). HBV 1.3 transgenic or wildtype mice were vaccinated i.p. with TT + recombinant HBcAg (Tg TT+HBc) or with EBV280-288 peptide (Tg EBV). Ten days later, HBc, TT, and EBV specific T cell responses were analyzed *ex vivo* in peritoneal cells restimulated with HBcAg, Tetanus Toxoid, and EBV280-288 peptide by IFN-γ ELISpot.

In order to discriminate the roles between HBV virus including HBV antigens, versus only HBs antigen only (w/o viremia), this experiment was repeated using 1.3HBV tg, HBs tg or wt C57B6 mice as recipients. Such trimera mice were transplanted with PBMC from a healthy donor (CTR-4) and vaccinated i.p. with TT (100μL/mouse), HBcAg (100μg/mouse) and synthetic $EBV_{280-288}$ peptide (30μg/mouse), or with 10^6/mouse autologous DC loaded with HBc_{18-27} peptide. Antigen specific human Th cell and CTL frequencies were analyzed 10 days after vaccination in peritoneal cells by IFN-γ ELISpot. As demonstrated in (Figure 38:), similar TT and EBV specific Th cell and CTL responses were induced in 1.3 HBV transgenic (1.3 HBV Tg) , HBs transgenic (HBs Tg) , and wild type mice trimera (wt). However, vaccination with HBcAg lead to widely reduced HBV specific Th cell responses in both, 1.3HBV tg or HBs tg trimera mice when compared to wild type control mice , indicating that HBs antigen might play the key role in the HBV associated T cell failure of chronic HBV carriers.

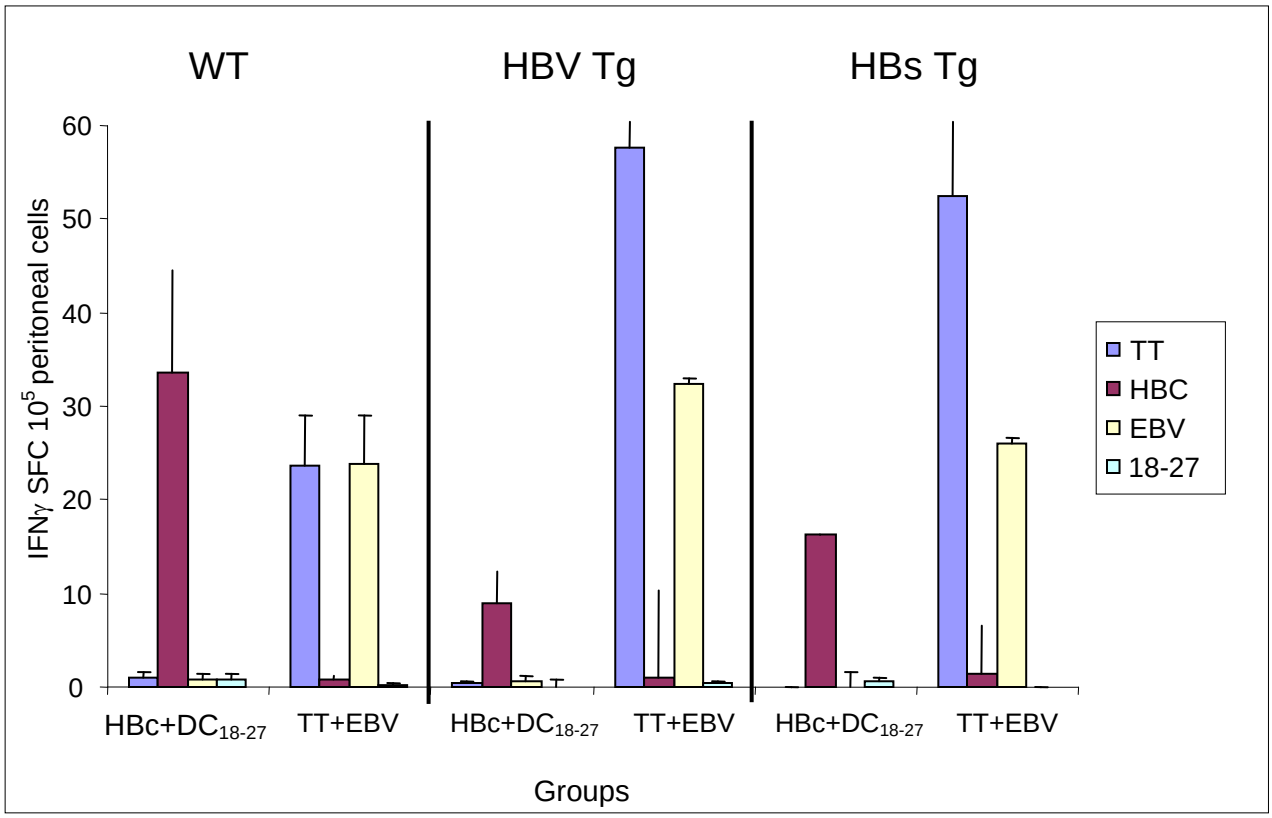


Figure 38: Influence of HBV viremia on HBc, TT specific Th cell, and HBc, EBV specific CTL responses *in vivo*. Each group consists of 3 to 4 Trimera mice, engrafted with PBMC from CTR donor #4. All three phenotypic mice (1.3 HBV, Hbs and control mice) were vaccinated i.p. with autologous DC18-27+ recombinant HBcAg (HBc+ DC18-27) and with TT/EBV280-288 peptide (TT+EBV). Ten days later, HBc, TT, and HBc, EBV specific T cell responses were analyzed *ex vivo* from peritoneal cells restimulated *in vitro* with HBcAg, Tetanus Toxoid, and HBc 18-27, EBV280-288 peptide in IFN-γ ELISpots.

5 Discussion

The close association of strong and multispecific antiviral CTL and Th cell responses with control of viral replication in acute and chronic HBV infection suggests that any means to stimulate efficient immune responses might represent an effective and specific new treatment strategy. This hypothesis has been confirmed in adoptive transfer experiments of anti-HBV immunity by transplantation of bone marrow from naturally immunized donors into chronically HBV infected leukaemia patients, that lead to clearance of HBV infection in a timely association with immune reconstitution (Lau, Suri et al. 2002). However, clinical trials to recover anti-HBV immunity in chronic hepatitis B by therapeutic vaccination with lipopeptide, recombinant HBs protein or DNA vaccines have failed to induce sustained responses so far, although the vaccine candidates had successfully been tested in healthy volunteers (Heathcote, McHutchison et al. 1999; Mancini-Bourgine, Fontaine et al. 2004).

These different vaccine responses of healthy individuals and chronic HBV patients are probably caused by the antiviral T cell failure of the latter patients. To test immunogenicity of vaccine candidates under conditions of a patient's immune system failing to eliminate HBV infection, we employed the Trimera mouse model for preclinical vaccination studies, that allows to transfer PBMC of chronic HBV patients together with their undefined T cell failure into recipient mice (Böcher and Reisner 2005). A strong immune response to an experimental vaccine in such mice should therefore better predict the response in patients than that of healthy individuals.

In contrast to previous clinical trials of HBs-antigen or -DNA vaccination, we focused in our studies on HBV core vaccines for the following reasons: (i) HBs antigen is a weak Th cell

antigen in acute and chronic hepatitis due to its low content of Th cell epitopes (Ferrari, Penna et al. 1990; Chisari and Ferrari 1995); (ii) HBs antigen was inefficient in inducing specific Th cell responses in patients (Mancini-Bourgine, Fontaine et al. 2004) and HBV trimera mice (Böcher, Galun et al. 2000); (iii) while HBs specific CTL are detectable in patients with any course of chronic HBV infection, HBc specific Th cells and CTL are associated with control of viral replication in acute (Ferrari, Penna et al. 1990; Jung, Diepolder et al. 1995; Maini, Boni et al. 1999) and chronic hepatitis B (Webster, Reignat et al. 2004) as well as after adoptive transfer (Lau, Suri et al. 2002).

In our previous experiments, vaccination with recombinant core particles lead to a strong expansion of HBc specific Th1 cells in Trimera mice implanted with PBMC not only from healthy donors but also from a patient with chronic active hepatitis B, while the DNA vector pCI/C was a weak Th cell stimulator (Böcher, Dekel et al. 2001). However, Trimera mice implanted with PBMC from both types of donors developed strong Th1 cell responses after protein vaccination, while the DNA vector again lead only to weak Th cell stimulation. In these experiments the unmethylated type A oligodesoxynucleotide CpG_{2216} was added to some of the vaccinated mice, due to its capacity to induce DC maturation, IFNγ secretion and CTL induction *in vitro* (Krug, Rothenfusser et al. 2003; Rothenfusser, Hornung et al. 2004), but could not further enhance the HBc stimulated Th1 cell response in our system. Moreover, to check the capacity of these two vaccines to induce core specific CTL responses, experiments were performed using HLA A2 positive PBMC donors, since for this haplotype the highly immunodominant CTL epitope $HBc_{18\text{-}27}$ has been described (Bertoletti, Chisari et al. 1993). However, HBc vaccination with neither of these two vaccines did lead to strong expansion of CTL in Trimera mice implanted with PBMC from an inactive HBs carrier, although strong Th

cell responses were induced in the same mice. This was not due to a selective CTL engraftment failure in our model, since EBV peptide vaccination lead to very strong expansion of corresponding CTL in the same mice. Thus, an irreversible HBV specific CTL failure could be suggested, leading to a lack of CTL recovery despite strong Th cell expansion.

However, most exogeneous protein antigens are processed by antigen presenting cells in the HLA class II pathway only. Moreover, the DNA vector, which was very effective in CTL induction in mice (Geissler, Tokushige et al. 1997), was only a very weak Th cell inducer in our humanized mouse model (Böcher, Dekel et al. 2001), as are DNA vaccines in clinical trials (Moorthy, Pinder et al. 2003; Hejdeman, Bostrom et al. 2004; Mancini-Bourgine, Fontaine et al. 2004). For these reasons, the tested antigens might simply be unsuitable to stimulate class I restricted CTL. This hypothesis was tested in Trimera mice implanted with PBMC from a naturally HBV-immunized donor with detectable memory CTL in peripheral blood. The lacking efficacy to restimulate HBc specific CTL even in such mice despite effective restimulation of EBV specific CTL responses argues strongly for the lacking capacity of the employed vaccines to induce HLA class I restricted CTL. An effective way to include exogenous antigens and vaccines into the HLA class I compartment might be by dendritic cell mediated crosspresentation. DC are the only cells capable to take up exogeneous antigens expressed by apoptotic virally infected or tumor cells and to present these phagocytosed antigens to HLA class I restricted CTL (Albert, Sauter et al. 1998). While apoptosis of the phagocytosed cells is required for antigen uptake and cross presentation, strongest CTL induction is provided after maturation of DC induced by simultaneously present necrotic cells (Sauter, Albert et al. 2000)

A possible explanation for the HBV specific T cell failure of chronic HBV carriers might be a defect in antigen presentation to T cells by DC leading to insufficient HBV specific T cell

stimulation. *In vitro* and *ex vivo* studies on monocytes derived DCs (MDDC) from chronic HBV patients yielded conflicting results concerning frequencies, phenotype and function of such *in vitro* generated DC. However our own extensive studies, despite showing some phenotypic alterations, excluded a relevant functional defect of MDDC (Tavakoli, Schwerin et al. 2004). These results were confirmed in more recent studies using myeloid and plasmacytoid DC from chronic HBV carriers.

Thus, due to their capacity of cros-presentation and functional integrity, autologus DC from HBV carriers might represent an effective vaccination approach. Therefore, we aimed to increase therapeutic CTL responses by vaccination with autologous DC loaded with apoptotic HBV transfected HepG2 or HepG2.2.15 cells in HBV Trimera mice implanted with PBMC from donors with different chronic HBV disease patterns, since their different extend of immune control might determine different vaccine responses. While inactive HBs carriers (low DNA, low ALT) have a rather strong antiviral CTL response in blood and liver partly controlling viral replication, immunotolerant patients with very high viral load and low ALT have hardly any detectable T cell response *ex vivo* leading to unrestricted viral replication (Maini, Boni et al. 2000; Bertoletti and Ferrari 2003; Webster, Reignat et al. 2004).

To get optimum *in vivo* results, first we optimized the Trimera mouse conditioning. Previous studies in Trimera mice have shown that, a split-dose protocol with an initial low dose irradiation followed two days later by a lethal dose, most effectively abrogates the bone marrow in recipient mice, without excessive mortality (Ilan, Burakova et al. 1999). We therefore first established the optimum doses of irradiation to obtain the maximum of human PBMC engraftment with optimal survival rate. Among different doses tested, a dose of initial 3 Gy followed by 8.5 Gy at day 2 showed optimum human cell engraftment with lowest mortality. The

purity of engrafted human cells was significantly increased after thymectomy of recipient mice. Based on these data, we thymectomized all mice one week before irradiation with 3+8.5 Gy for Balb/c strains and 4+ 11 Gy for C57B6 strains.

Efficient expression of HBV encoded antigens is crucial for sufficient T cell stimulation by DC. Thus we first tested transfection efficacy of a full length 1.3 HBV plasmid expressing all HBV proteins. In HepG2 cells by western blot analysis of HBV core protein or by simultaneous transfection of HepG2 cells with a control GFP vector, we observed the highest expression of HBV core protein on day three after transfection (> 65% of cells). Apoptosis of the transfected cell is an important factor in crosspriming since it was shown that DC can only cross-present antigens derived from apoptotic bodies but not from necrotic cells. Thus we induced apoptosis to HepG2 cells by exposing them to UVB light. Cells exposed to UVB for 5 minutes and analysed 2 hours later for apoptosis, revealed the highest apoptotic and least necrosis rate. In repeated experiments with this exposure time we obtained an average of 60% apoptotic cells. Thus, this protocol was chosen for induction of apoptosis in further experiments.

Initially, we tested our crosspriming approach *in vitro*, by feeding immature DC derived from HLA A2 positive healthy donors with apoptotic HBV- or mock-transfected HepG2 cells. The employed HBV1.3 over length vector guaranteed the expression of all viral antigens, including HBc and HBs antigens. These loaded DC indeed were highly effective in stimulating HLA A2 restricted HBc_{18-27} specific CTL. Further we tested this phenomenon in DC from chronic HBV carriers and obtained similar results as in controls suggesting that this crosspresentation pathway is active even in chronic HBV patients and supporting our previous report, that DC from chronic HBV patients are functionally competent (Tavakoli, Schwerin et al. 2004). However the transfection efficacy may vary between individual experiments possibly

leading to variations in crosspriming efficacy by DC. We overcame this problem by using a stable HBV-transfected human hepatoma cell line (HepG2.2.15) (Sells, Chen et al. 1987).

Recent literature suggest that DC maturation is essential in antigen presentation to prime naïve T cell subsets (Banchereau and Steinman 1998). Thus, we induced maturation of DC preparations loaded with HBV transfected HepG2 cells in order to enhance their T cell stimulatory potency. After *in vitro* DC maturation, the *in vitro* stimulation of the HBc_{18-27} specific CTL clone was indeed enhanced two-fold when compared to immature DC.

After confirming the functionality of the cross presentation pathway for HBV antigens *in vitro*, we tested the capacity of DC to induce CTL *in vivo* in the Trimera model. First, we generated DC from an immune tolerant donor (IT) who displayed no HBc specific Th cells or CTL and very low HBs specific CTL frequencies in peripheral blood. When DC were loaded with apoptotic HBV transfected HepG2 cells and transferred into Trimera mice engrafted with autologous PBMC, strong expansion of HBc, HBs specific Th cell and CTL responses could be detected in such mice by IFNγ ELISpot. Then we tested this *in vivo* cross priming experiment with an inactive HBs carrier (ISC) and a chronic active hepatitis donor (CAH) who had very low HBc specific Th and undetectable CTL and no HBs specific T cell response. When Trimera mice implanted with PBMC from such donors were vaccinated with DC loaded with apoptotic HBV expressing HepG2.2.15 cells, we could induce strong HBc specific Th and CTL responses along with strong and multiple HBs epitope specific CTL responses, indicating that the virus specific T cell frequencies which are barely detectable *ex vivo* in these patients, can rapidly be recovered under experimental conditions in Trimera mice arguing against deletion of such cells in patient PBMC. Thus, in PBMC from patients with different courses of chronic HBV infection, that is characterized by different strength of their antiviral T cell responses, sufficient expression of

HBc, or HBs specific Th cell and CTL responses can be achieved by therapeutic DC vaccination *in vivo*.

Based on our *in vitro* findings, from inactive HBs carrier the effect of DC maturation was also tested *in vivo*. In vaccination experiments with Trimera mice implanted with PBMC, we compared the efficacy of immature DC vs. mature DC as potential vaccine candidates. Maturation was either induced by means of poly (I;C) or a pro-inflammatory cytokine cocktail. We observed that DC maturation by means of cytokines or poly (I:C) in our system had no consistent and reproducible influence on the strength of the virus specific immune responses. This data indicates that *in vitro* DC maturation is not necessary in cross-presenting the antigens *in vivo*. This immune stimulatory effect in all *in vivo* experiments was indeed due to DC, since magnetic separation of HLA DR expressing DC before injection into recipient mice had eliminated virtually any contaminating HLA DR negative HepG2 cells. Moreover, trimera mice vaccinated with DC loaded with mock transfected HepG2 cells or HBc particles did not, while DC loaded with the peptide HBc18-27 did induce very strong HBc (but not HBs) specific CTL. This is in contrast to our previous observation of lacking CTL responses in HBV-trimera mice when the peptide is administered directly i.p., and to recent clinical findings of patients being vaccinated with a HBc18-27 lipopeptide vaccine (Heathcote, McHutchison et al. 1999). Thus, DC loaded with synthetic peptides might represent an alternative approach of therapeutic vaccination. However, the major disadvantage of the peptide approach is the restriction to well characterized epitopes and patients with the HLA A2 haplotype, while the approach using HBV transfected cells provides all HBV antigens and might be active in patients with any HLA type. The weak capacity of the DC vaccines to induce Th cells can be counteracted by co-vaccinating with recombinant HBc particles, possibly further strengthening the CTL response.

The potential application of our findings in clinical trial strongly depends on the mechanistic understanding of the immunogenic efficacy of DC vaccination in our system. One possible advantage of trimera mice over human might be an HBV antigen presentation more active mouse APC, as, monocytes and dendritic cells. By FACS staining, indeed we observed a slight increase of murine monocytes and B cells in trimera mice with time after PBMC transfer. Although the percentage of murine APC was very minute, it is necessary to determine the way of antigen presentation in Trimera mice. Thus we engrafted the Trimera mice with PBMC from HLA A2 positive healthy donors. We transferred the PBMC with either un-manipulated PBMC or PBMC depleted of different human APC populations by immunomagnetic beads (MACS) against CD14, CD19, HLA DR or BDCA-1,-3,-4 (DC). We vaccinated the Trimera mice i.p. with recombinant HBc antigen, Tetanus Toxoid (TT), or EBV peptide280-288. Depletion of total human APC (HLA DR) from transferred PBMC, virtually abolished the vaccine-induced human Th cell and CTL responses. Depletion of DC (BDCA-1/-3/-4), B cells (CD19) or monocytes (CD14) widely reduced the T cell responses indicating that the antigen presentation in Trimera mice is mediated by human antigen presenting cells but not murine APC. Moreover, these data indicate that all three human APC populations contribute to effective stimulation of HBV specific human T cell responses in Trimera mice. Thus, mouse derived APC persist in Trimera mice but do not seem to play a major functional role in the generation of human T cell responses, as these depend on the presence of human APC in the PBMC transplant.

Finally, the HBV specific T cell failure of chronic HBV carriers is believed to account for viral persistence. There was proposed to be the generation of T cell tolerance induced by the presence of HBV virions or antigens which are not present in the trimera system under the described conditions. (Chen, M. and Sallberg, M. et al 2005; Milich, D. R. et al 1997).

To assess the possible influence of virus or viral antigenson T cell function, we performed vaccination experiments in transgenic mice expressing HBV viremia and antigens or only the HBs antigen. *In vivo* data from such mice showed that the Th and CTL responses to TT and EBV control vaccines were not at all affected by HBV expression in recipient mice, whereas the responses to HBV antigens were virtually absent in HBV transgenic, while only reduced in HBs transgenic recipient mice. These findings strongly favor the hypothesis that the virus or viral antigens are directly involved in the HBV specific T cell tolerance. Further experiments are running to discover the involved mechanisms in this first *in-vivo* model for induction of specific T cell tolerance by a human pathogenic virus.

6 Summary

Hepatitis B is a liver disease caused by Hepatitis B virus (HBV). It ranges in severity from a mild illness, lasting a few weeks (acute), to a serious long-term (chronic) illness that can lead either to liver disease or liver cancer. Acute infection is self limiting in most adults, resulting in clearance of virus from blood and liver and the development of lasting immunity. However 5% of acutely infected patients do not resolve primary HBV infection, leading to chronic infection with persistent viral replication in the liver. The strength of the initial antiviral immune response elicited to Hepatitis B determines the subsequent clinical outcome. A strong and broad T cell response leads to spontaneous resolution. Conversely, a weak T cell response favours viral persistence and establishment of chronic disease. While treatments using interferon-alpha or nucleos(t)ide analogues can reduce disease progression, they rarely lead to complete recovery. The lack of a suitable small animal model hampered efforts to understand the mechanisms responsible for immune failure in these chronic patients.

In current study we used Trimera mice to study the efficacy of potential vaccine candidates using HBV loaded dendritic cells in HBV chronic infection *in vivo*. The Trimera mouse model is based on Balb/c mice implanted with SCID mouse bone marrow and human peripheral blood mononuclear cells (PBMC) from HBV patients, and thus contains the immune system of the donor including their HBV associated T cell defect.

In our present study, strong HBV specific CD4+ and CD8+ T cell responses were enhanced by therapeutic vaccination in chronic HBV patients. These T cell responses occurred independently of either the course of the disease or the strength of their underlying HBV specific T cell failure. These findings indicate that the Trimera mouse model represents a novel experimental tool for evaluating potential anti-HBV immunotherapeutic agents. This *in vivo* data indicated that both the HBV specific CD4+ cell and CD8+ responses were elicited in the periphery. These HBV specific T cells proliferated and secreted cytokines upon restimulation in Trimera mice. The observation that these HBV specific T cells are not detectable directly *ex vivo* indicates that they must be immune tolerant or present at a very low frequency *in situ*. HBV specific T cell responses were suppressed in Trimera mice under viremic conditions, suggesting that viral factors might be directly involved in tolerizing or silencing antiviral T cell responses. Thus, combination of an effective vaccine with antiviral treatment to reduce viremia might be a more effective therapeutic strategy for the future. Such approaches should be tested in Trimera mice generated in HBV or HBs expressing transgenic mice before conducting clinical trials.

7 Abbreviations

Ab	Antibody
Avg	Average
Ag	Antigen
AHB	Acute Hepatitis B
ALT	Alanin-Aminotransferase
Anti-HBc	Antibody against HBcAg
Anti-HBe	Antibody against HBeAg
Anti-HBs	Antibody against HBsAg
APC	Antigen presenting cell
BDCA	Blood Dendritic cell antigen
BSA	Bovines Serum albumin
CAH	Chronic active Hepatitis
CBA	Cytometric bead assay
cccDNA	covalently closed circular DNA
CD	Cluster of differentiation
CD40L	CD40 Ligand
CTL	Cytotoxic T-Lymphocyte
DC	Dendritic Cell
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid

Abbreviations

EBV	Ebstein-Barr-Virus
EDTA	Ethylen-diamin-tetra-acetat
ELISA	Enzyme-Linked-ImmunoSorbent Assay
FACS	Fluorescence-activated cell-sorting
FCS	Fetal Calf Serum
FELASA	Federation of European Laboratory Anir Associations
FITC	Fluorescein-Isothiocyanat
GPT	Glutamyl pyruvic transaminase
GV- SOLAS	Gesellschaft für Versuchstierkunde- Society for Laboratory Animal Science
HBcAg	Hepatitis B core Antigen
HBeAg	Hepatitis B envelope Antigen
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B Virus
HBxAg	Hepatitis B x Antigen
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HEPA	High Efficiency Particulate Air
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HUS	Human Serum

Abbreviations

IFN	Interferon
Ig	Immunoglobulin
imDC	Immature Dendritic cell
IL	Interleukin
ILAR	Institute for Laboratory Animal Research
ISC	Inactive HBs carriers
IVC	Individually ventilated cages
MACS	Magnetic cell sorting
MDC	Myeloid Dendritic Cell
mDC	Mature Dendritic cell
MDDC	Monocyte derived Dendritic cell
MHC	Major Histo compatibility complex
MLR	Mixed lymphocyte reaction
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid Dendritic cells
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PFA	Paraformaldehyd
PHA	Phytohemagglutinin

RHB	Resolved hepatitis B
RNA	Ribonucleic acid
TCR	T-cell-Receptor
T_H	T-Helper cell
Tg	Transgenic
TLR	Toll-like receptor
TMB	Tetra-Methyl-Benzidine
TNF	Tumor necrosis factor
WHO	world health organisation
SPF	Specific pathogen free
SFC	Spot forming Cells
SCID	Severe Combined Immunodeficiency Disease
MU	Million units
PEG	Polyethylene glycol

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9 Publications:

Boris Alabyev, **Raja Vuyyuru** and Tim Manser (2008). Influence of Fas on the regulation of the response of an anti-nuclear antigen B cell clonotype to foreign antigen. Int Immunol. Oct; 20(10) - 1279-87.

Raja Vuyyuru, Fareed Rahman, Wibke Schwerin, Michael Geissler, Denis Strand, Peter R.-Galle and Wulf O. Böcher. Therapeutic Vaccination with Dendritic Cells in the Trimera Mouse Model of Chronic Hepatitis B Virus Infection (submitted).

Raja Vuyyuru, Chandra Mohan, Tim Manser and Ziaur S. M. Rahman. Lupus susceptibility locus *Sle1* alters peripheral B cell tolerance checkpoints operative in the AFC and GC pathways- (submitted).

Poster presentations at conferences:

Raja R. Vuyyuru, N. Blust, S. Herzog-Hauff, S. Tavakoli, P.R. Galle, W.O. Böcher. Human antigen presenting cells stimulate HBV specific CTL and Th cells in the trimera mouse model. American Association for the Study of Liver Diseases (AASLD), Boston, USA, Oct 2006.

Raja R. Vuyyuru, N. Blust, S. Herzog-Hauff, P.R. Galle, W.O. Böcher. Vaccination with immature or mature dendritic cells induces strong HBV specific Th cell and CTL responses in HBV trimera mice. American Association for the Study of Liver Diseases (AASLD), Boston, USA. Oct 2006.

Reddy Vuyyuru, Ingmar Mederacke, Soheila Tavakoli, Dieter glebe, Peter Galle, W.O. Böcher. HBV infection of myeloid and plasmacytoid dendritic cells in patients with chronic hepatitis B virus (HBV) infection. Annual meeting on the Molecular Biology of Hepatitis B Viruses, Heidelberg, Germany. September 2005.

10 Erklärung:

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die in der Arbeit angegebenen Hilfsmittel und Quellen verwendet habe.Die Thymektomie, CBA Analyse, APC Färbung und die humanen APC Depletionsexperimente habe ich zusammen mit Nina Blust durchgeführt.

Mainz, 18.11.08 Vuyyuru Raja Sekhar Reddy