Antiviral Research 168 (2019) 156–167

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Heterologous prime-boost immunization with vesiculovirus-based vectors expressing HBV Core antigen induces CD8$^+$ T cell responses in naïve and persistently infected mice and protects from challenge

Carolina Chiale[a], Safiehkhatoon Moshkani[a], John K. Rose[b], Michael D. Robek[a,*]

[a] Department of Immunology and Microbial Disease, Albany Medical College, Albany, NY, USA
[b] Department of Pathology, Yale School of Medicine, New Haven, CT, USA

ARTICLE INFO

Keywords:
Virus-based vectors
Prime-boost
HBV
Core antigen
VSV
AAV

ABSTRACT

Chronic hepatitis B virus (HBV) infections cause more than 800,000 deaths per year and currently approved treatments do not cure the disease. Because a hallmark of acute infection resolution is the presence of functional CD8$^+$ T cells to the virus, activation of the immune system with therapeutic vaccines represents a potential approach for treating chronic hepatitis B. In this study, we evaluated the immunogenicity and efficacy of two attenuated vesiculovirus-based platforms expressing HBV Core antigen, the highly attenuated vesicular stomatitis virus (VSV) N4CT1 and a unique vaccine platform [virus-like vesicles (VLV)] that is based on a Semliki Forest virus replicon expressing the VSV glycoprotein. We found that heterologous prime-boost immunization with VLV and N4CT1 induced Core-specific CD8$^+$ T cell responses in naïve mice. When immunized mice were later challenged with AAV-HBV, functional Core-specific CD8$^+$ T cells were present in the liver, and mice were protected from establishment of persistent infection. In contrast, when mice with pre-established persistent HBV replication received prime-boost immunization, functional Core-specific CD8$^+$ T cells were found in the spleen but not in the liver. These results highlight the importance of investigating the therapeutic value of different HBV antigens alone and in combination using preclinical animal models, and understanding the correlation between anti-HBV efficacy in these models with human infection.

1. Introduction

Hepatitis B virus (HBV) infection can be prevented with the approved HBV vaccine that efficiently generates antibodies that block the virus from infecting hepatocytes (Michel and Tiollais, 2010). However, the failure of the vaccine to generate HBV-specific CD8$^+$ T cells makes it ineffective for treating established chronic HBV infections (Guidotti et al., 2015; Pol et al., 2001). Current therapies for chronic HBV inhibit viral replication but do not eliminate the cccDNA transcriptional template (Dolman et al., 2018; Nassal, 2015). Dysfunctional T cells are a hallmark of chronic HBV infection (Das et al., 2008), but the immune system can eliminate the virus if sufficiently activated (Rehermann et al., 1996). Consequently, therapies that successfully restore a functional immune response to HBV represent a potential option for treatment of chronic infection (Kosinska et al., 2017).

Therapeutic vaccines are an attractive approach to treat chronic HBV, but to date, several clinical trials with non-replicating vaccines such as DNA and protein have failed to demonstrate efficacy in humans [reviewed in (Kosinska et al., 2017; Maini and Pallett, 2018)]. Virus-based vaccines constitute a promising opportunity since they stimulate strong antibody and CD8$^+$ T cell responses (Ewer et al., 2016). Nevertheless, the potential pathogenicity of viral vectors is a safety concern that can hinder their use. Vesicular stomatitis virus (VSV) induces strong CD8$^+$ T cell responses (Cobleigh et al., 2012; Barefoot et al., 2009; Rose et al., 2001), and unlike some other viral vaccine platforms, VSV is available in multiple serotypes and does not have a high prevalence in the human population, reducing the possibility of a preexisting immune response to the vector (Lichty et al., 2004). A highly attenuated form of VSV, N4CT1, contains the nucleocapsid (N) gene translocated from the first genome position to the fourth (N4) and a glycoprotein (G) cytoplasmic tail truncation to a single amino acid (CT1) (Clarke et al., 2007; Cooper et al., 2008). This vector provides the same immunogenicity as wild type (WT) VSV but without the potential pathogenicity of a WT virus, and was found to be safe and immunogenic in non-human primates and humans (Matassov et al., 2015; Li et al., 2017; Elizaga et al., 2018). Virus-like vesicles (VLV) are Semliki Forest

* Corresponding author. Department of Immunology and Microbial Disease, Albany Medical College, 47 New Scotland Ave, MC-151, Albany, NY, 12208, USA.
E-mail address: robekm@amc.edu (M.D. Robek).

https://doi.org/10.1016/j.antiviral.2019.05.014
Received 23 March 2019; Received in revised form 24 May 2019; Accepted 29 May 2019
Available online 30 May 2019

virus (SFV)-based replicons that propagate in the cytoplasm and produce infectious spherules containing VSV-G glycoprotein on their surface, which promotes vesicle budding and spread from infected cells (Rolls et al., 1994). These SFV-VSV hybrid vectors have been engineered to express foreign antigens that can act as vaccine platforms (Rose et al., 2014; Reynolds et al., 2015), and like attenuated N4CT1 VSV, VLV are immunogenic but not pathogenic (Rolls et al., 1994, 1996; Rose et al., 2008, 2014; Schell et al., 2011).

The activation of $CD8^+$ T cell responses to HBV antigens correlates with control of the virus in humans (Rehermann et al., 1996). Although this concept is broadly accepted, there is no consensus as to which antigen represents the best target for a therapeutic vaccine. Patients who clear HBV elicit responses to multiple antigens, and no specific antigen is known to be superior for virus control (Rehermann et al., 1995; Chisari and Ferrari, 1995; Bertoletti et al., 1991). In mice, transduction with adeno-associated virus (AAV) encoding the HBV genome (AAV-HBV) efficiently delivers the viral genome to the liver and establishes persistent HBV replication (Yang et al., 2014; Dion et al., 2013). This model has been widely used to demonstrate efficacy of therapeutic immunization approaches, but these studies have all employed as the vaccine antigen either some variant of HBsAg or a combination of multiple HBV proteins without identifying whether immune responses to one particular antigen were required for virus control (Yang et al., 2014; Dion et al., 2013; Bian et al., 2017; Martin et al., 2015; Zhao et al., 2017; Zhu et al., 2016). Similarly, in previous studies, we found that VSV, N4CT1, and VLV vectors expressing the HBV middle surface glycoprotein (MHBs) stimulate specific $CD8^+$ T cell responses that protect from HBV challenge and/or eliminate established HBV replication in mouse models (Reynolds et al., 2015; Cobleigh et al., 2010, 2013; Moshkani et al., 2019).

Compared to other HBV proteins such as HBsAg, immunization with the HBV Core structural protein represents a logical alternative. Core is not highly secreted like HBsAg (Grimm et al., 2011; Glebe and Bremer, 2013), so T cell tolerance to Core in humans might be easier to overcome. Core is also known to promote Th1 responses, and can act as an adjuvant aiding in the activation of immune responses to other HBV antigens (Aguilar et al., 2004). In the woodchuck hepatitis virus (WHV) model, T cell responses to WHV Core are protective (Zhang et al., 2015; Menne et al., 1997; Kosinska et al., 2012). In contrast, we previously found that a single dose of VLV did not induce detectable and functional $CD8^+$ T cell responses to Core in mice, and that a single dose of recombinant WT VSV expressing Core from the fifth genome position failed to protect mice from HBV hydrodynamic challenge (Reynolds et al., 2015).

In this study, we evaluated the ability of VLV and N4CT1 vectors to generate Core-specific $CD8^+$ T cell responses in mice using single dose and prime-boost immunizations. Our results show that heterologous prime-boost immunization with VLV and N4CT1 elicits Core-specific $CD8^+$ T cell responses in both naïve and chronically infected mice. In naïve mice, animals that received prime-boost immunization and were subsequently challenged with AAV-HBV were protected, and this protection was accompanied by increased Core-specific $CD8^+$ T cells in the liver. However, in mice with pre-existing persistent HBV infection, Core-specific $CD8^+$ T cell responses were systemically stimulated by VLV-N4CT1 immunization, but were not functional in the liver.

2. Materials and methods

2.1. Peptide epitopes

T cell stimulation was performed using previously described $CD8^+$ T cell epitopes for HBV Core, HBsAg, and VSV nucleocapsid (N) protein. Core 93 is an immunodominant H2-K^b-restricted $CD8^+$ T cell epitope from Core antigen consisting of amino acids (a.a.) 93 to 100 (MGLKFRQL) (Kuhober et al., 1996). S 353 is an immunodominant H2-K^b-restricted $CD8^+$ T cell epitope from HBsAg consisting of a.a. 353–360 (VWLSVIWM) (Schirmbeck et al., 2003). VSV N was utilized as a control for immune responses to VSV, which comprises a.a. 52–59 (RGYVYQGL) of the N protein, and is also specific for H2-K^b (Van Bleek and Nathenson, 1990). Overlapping pools of peptides for Core and HBsAg were also utilized (Genscript). The Core pool consisted of 11-mer peptides that overlap by 4 a.a. and cover the entire sequence of Core. Similarly, the S pool is a pool of 11-mer peptides overlapping by 4 a.a. that encodes the entire sequence of HBsAg.

2.2. Mice

Six to eight-week-old C57BL/6 mice were purchased from The Jackson Laboratory or Taconic Biosciences. Mice were housed in the Animal Resource Facility at Albany Medical College (AMC), and all experiments were done following protocols approved by the AMC Institutional Animal Care and Use Committee.

2.3. Plasmids, cell lines, and viruses

For DNA immunizations, the plasmid pcDNA(3.1+)-Core was used. VLV-Core was generated as previously described (Reynolds et al., 2015). N4CT1-Core, VSV[5]-Core, and VSV[1]-Core (Fig. 1A) were generated using methods previously described (Clarke et al., 2007; Cooper et al., 2008; Cobleigh et al., 2010; Moshkani et al., 2019; Ramsburg et al., 2005). Protein expression was confirmed by Western blot to Core antigen (Invitrogen and Austral Bioscience) or with polyclonal antibody to VSV. Viral titers were determined by infection of BHK cells with serial dilutions of the virus stocks in a plaque forming assay using standard protocols.

2.4. Immunizations

All immunizations were done intramuscularly in 50 µL PBS. For immunizations with N4CT1 or VSV, mice received 1×10^6 PFU/mouse unless indicated; for VLV, 1×10^7 PFU/mouse were injected; mice that received plasmid were immunized with 20 µg of pcDNA(3.1+)-Core. PBS alone was utilized as a control. Immunizations were done every 3–4 weeks and blood was collected one week after each immunization to assess the presence of Core-specific $CD8^+$ T cells or the levels of HBV antigen depending on each experimental design (Fig. 1D and E). Immunization of naïve mice included a prime with either DNA or PBS, followed by an immunization with VLV or PBS four weeks after the prime, and a second boost with either VSV or N4CT1 four weeks after the first boost (Fig. 1D). For therapeutic vaccination, mice were screened for HBeAg levels 8 weeks after AAV-HBV transduction, grouped, and started with a prime immunization with either DNA or PBS, followed by a boost 3 weeks later with VLV or PBS, and a final boost with VSV or N4CT1 3 weeks after the first boost (Fig. 1E).

2.5. HBV transduction with AAV

Serotype 8 of AAV encoding a 1.3- or 1.2-mer HBV genome was prepared by Vector Biolabs and SignaGen. HBV replication was established in male C57BL/6 mice with transduction via intravenous injection of 1×10^{11} genome copies of AAV-HBV. For therapeutic experiments, persistent HBV replication was defined by stable levels of viral Ags in the serum at weeks 6–8 post AAV-HBV transduction.

2.6. Isolation of intrahepatic leukocytes (IHL)

Mice were euthanized, the portal vein was cut, and livers were perfused with sterile PBS then mechanically dissociated, homogenized with a syringe, and passed through a 100 µm mesh strainer. Cells were pelleted by centrifugation, resuspended with 40% Percoll in serum-free media, and centrifuged for 20 min at $600 \times g$ with no brake.

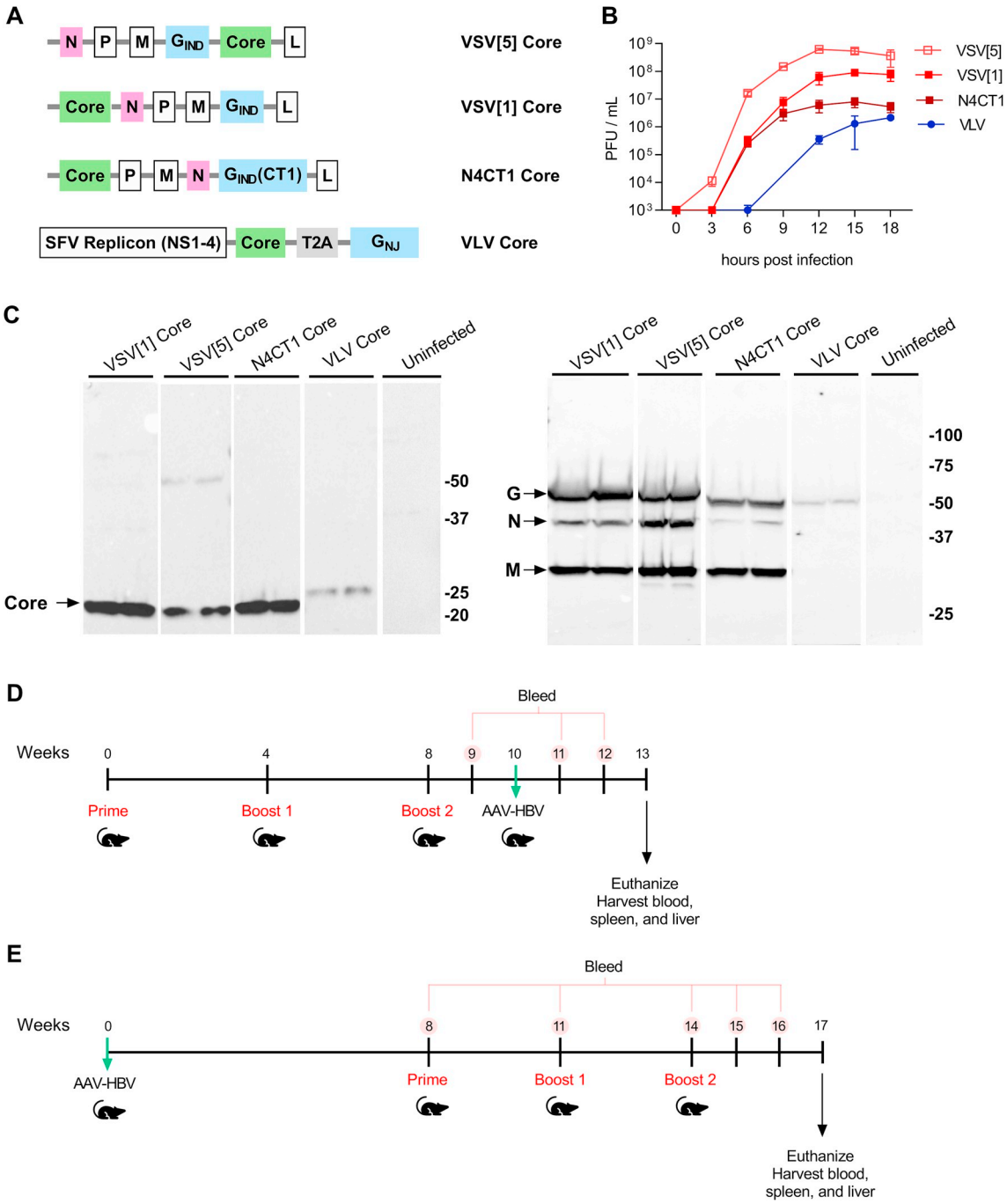

Fig. 1. Antigen expression from VSV/VLV vectors and immunization strategy. (A) VSV and VLV vectors expressing HBV Core antigen utilized in this study. All VSV vectors were VSV-G serotype Indiana (IND), while VLV-Core was serotype New Jersey (NJ). (B) BHK cells were infected with the different VSV or VLV vectors and the media were titered at multiple time points post infection. (C) Expression of Core protein and VSV proteins (G, N, and M) determined by Western blot from lysates of BHK cells infected with either VSV, VLV, or N4CT1 vectors expressing Core (MOI = 0.3). The higher molecular weight of Core expressed from VLV is due to the additional C-terminal amino acids from the picornavirus T2A sequence. (D) Schematic representation of prime-boost immunization in naïve mice. The prime consisted of DNA or PBS, and for each boost, combinations with either VLV, VSV, N4CT1, or PBS were used as described for each experiment. (E) Schematic representation of prime-boost immunization in mice that were transduced with AAV-HBV.

2.7. Intracellular cytokine staining and flow cytometry

Core-specific IFN-γ-producing $CD8^+$ T cells were detected by flow cytometry following stimulation with Core peptides. Spleen cells or IHL were harvested, red blood cells were removed by lysis with ACK buffer, and cells were stimulated with peptide for 5 h in the presence of brefeldin and monensin. Cells were stained for surface markers CD8 and CD3 and intracellular cytokine staining was performed using a Fixation/Permeabilization Kit (BD Cytofix/Cytoperm™). Samples were analyzed using an LSR II cytometer and FlowJo software.

2.8. ELISPOT assay

IFN-γ-producing $CD8^+$ T cells were measured by IFN-γ ELISPOT assay as previously described (Reynolds et al., 2017). Spot formation was quantified with an automated spot counter (Immunospot, Cellular Technology Ltd).

2.9. Alanine aminotransferase (ALT) measurement

The presence of ALT enzyme in the serum was detected using Infinity ALT Liquid Stable Reagent (Thermo Scientific) with Enzyme ER Verifier Kit (Verichem Laboratories) standards, and analyzed using a SpectraMax iD3 microplate reader (Molecular Devices).

2.10. ELISA detection of viral antigens and HBV-specific antibodies

Serum HBeAg, HBsAg, and anti-Core antibody were measured by ELISA (International Immunodiagnostics) following the manufacturer's protocol. Recombinant HBeAg and HBsAg standards were purchased from Fitzgerald Industries.

2.11. Viral RNA detection by qPCR

Liver tissues were collected, snap-frozen in liquid nitrogen, and stored at −80 °C until processed. Following homogenization, RNA was purified using an RNeasy kit (Qiagen), and cDNA was prepared from equal amounts of RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative PCR was performed using Taqman Fast Advanced Master Mix (Applied Biosystems). Reactions were done on a StepOnePlus real time PCR system (Applied Biosystems) using StepOne software v2.3. For HBV detection the following sequences were used: HBV probe 5′-CCT CTT CAT CCT GCT GCT ATG CCT CAT C-3′, antisense 5′-GAC AAA CGG GCA ACA TAC CTT, sense 5′-GTG TCT GCG GCG TTT TAT CA-3' (Garson et al., 2005). GAPDH expression was measured as an endogenous control. Taqman Assay Mix containing probe and specific primers for CD8 or GAPDH (Thermofisher) were utilized. Gene expression was quantified by the comparative $\Delta\Delta C_T$ method.

2.12. Capsid-associated viral DNA preparation and detection

HBV capsid-associated DNA was prepared as previously described (Moshkani et al., 2019; Keasler et al., 2007). HBV genomes were detected by quantitative PCR using the primers and probe described in 2.11 for viral RNA detection.

2.13. Serum viral DNA purification and detection

HBV serum DNA was purified using a High Pure Viral Nucleic Acid Kit (Roche) following the manufacturer's instructions. HBV genomes were detected by quantitative PCR using the primers and probe described in 2.11. Plasmid encoding the HBV genome was used as a standard to calculate DNA copy number.

2.14. $CD8^+$ T cell depletion

Mice were injected with 250 µg i.p. of anti-CD8 antibody (BioXcell, clone 2.43) or isotype control (BioXcell, clone LTF2) twice a week for a period of 3 weeks. Depletion was started three days prior to AAV-HBV challenge, and elimination of $CD8^+$ cells from the blood was confirmed by flow cytometry.

2.15. Statistical analysis

Data were analyzed with GraphPad Prism 7.0 using an unpaired or multiple *t*-test for normally distributed data, or a Mann-Whitney *U* test for results which were not normally distributed, comparing to PBS or non-specific peptide controls. P values are indicated for each experiment.

3. Results

3.1. Attenuated VSV N4CT1 induces $CD8^+$ T cell responses to Core antigen

We previously found that in contrast to other HBV antigens such as MHBs, Core-specific $CD8^+$ T cell responses were not detected in mice after a single immunization with VLV or VSV[5] (Fig. 1A) (Reynolds et al., 2015). The lack of Core immunogenicity could be related to the different replication characteristics of the vectors (Fig. 1B) or to suboptimal antigen expression (Fig. 1C). To investigate if antigen expression and replication could impact immunogenicity, we evaluated multiple VSV constructs with differences in levels of Core antigen expression and vector attenuation. During VSV mRNA transcription, proteins that are encoded in the first position of the VSV genome are more highly expressed due to the generation of a gradient of mRNAs that result from detachment of the viral RNA polymerase (Clarke et al., 2006). Mice were immunized with either N4CT1-Core, a highly attenuated form of VSV that has higher expression levels of Core, non-attenuated VSV that expresses Core from the fifth genome position (VSV[5]-Core), or VSV that expresses Core from the first genome position (VSV[1]-Core) and is mildly attenuated due to the addition of the first position open reading frame (Fig. 1A–C). Subsequently, the presence of Core-specific T cells in the spleen was evaluated 7 days post-immunization. Even though no responses to the immunodominant Core 93 epitope were detected by flow cytometry, the more sensitive ELISPOT assay revealed the presence of IFN-γ-producing $CD8^+$ T cells only in mice immunized with N4CT1-Core (Fig. 2A and B). In contrast, Core-specific $CD4^+$ T cell responses were not detected (data not shown). This result indicates that expression of Core from N4CT1 induces stronger Core-specific $CD8^+$ T cell responses compared to Core expression from non-attenuated VSV.

Since the N4CT1 vector generated a low magnitude of Core-specific $CD8^+$ T cell responses, we reasoned that prime-boost immunizations could increase the extent of the immune response. In addition to heterologous immunization with the viral vectors, we also included a prime with DNA, because DNA immunizations can be safely used in humans, and several studies evaluated prime-boost combinations of DNA and viral vectors with promising results (Li et al., 2017; Kosinska et al., 2012; Fontaine et al., 2015; Chapman et al., 2017). Mice were immunized with a Core expression plasmid, followed by a boost with VLV-Core four weeks later and a second boost with N4CT1-Core, and immune responses were measured in the spleen one week after the final boost. Different serotypes of VSV-G were utilized in the VSV and VLV vectors to avoid neutralization by VSV-G-specific antibody. Although the sample size was small and the responses were low in magnitude, prime-boost immunization induced Core-specific $CD8^+$ T cells that could be readily detected by flow cytometry (Fig. 2C) and ELISPOT (Fig. 2D and E) in the spleen of immunized mice. We further evaluated a prime-boost with VSV[5] instead of N4CT1, and even at a 10 times higher dose of VSV[5] than N4CT1, prime-boost with N4CT1 was more efficient at promoting Core-specific responses than VSV[5] (Fig. 2D and E). Together, these data indicate that Core-specific $CD8^+$ T cell responses are enhanced by heterologous prime-boost immunizations with VLV and N4CT1 vectors.

3.2. Heterologous prime-boost immunization stimulates Core-specific $CD8^+$ T cell responses that expand following HBV challenge

Since Core-specific T cell responses could be induced by heterologous prime-boost, we next measured the ability of this immunization regimen to protect from establishment of persistent HBV infection. Mice were intramuscularly immunized with combinations of DNA, VLV, and N4CT1 vectors expressing Core (Fig. 1D). In addition to the Core 93 epitope used for analysis of immunized naive mice, an overlapping pool of peptides that cover the entire sequence of Core was also utilized to detect the potential presence of Core-specific $CD8^+$ T cells with other

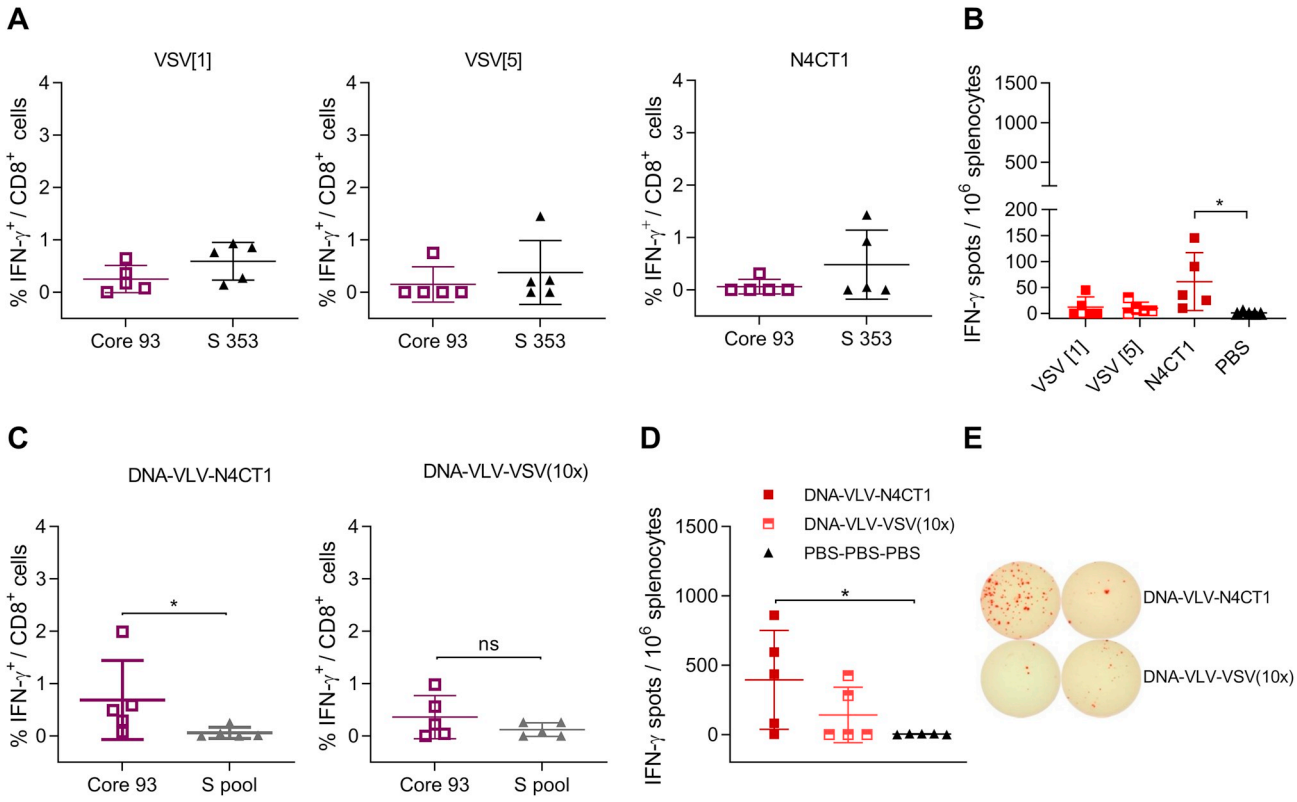

Fig. 2. Attenuated VSV N4CT1 induces stronger CD8$^+$ T cell responses to Core antigen compared to VLV and other VSV platforms. (A–B) Mice received 1×10^6 PFU of either VSV[1]-Core, VSV[5]-Core, or N4CT1-Core, and Core-specific CD8$^+$ T cell responses were measured one week post-immunization by stimulation with Core 93 peptide. (A) Frequency of IFN-γ$^+$CD8$^+$ cells specific for HBV epitopes in splenocytes of mice immunized with either VSV[1]-, VSV[5]-, or N4CT1-Core. (B) ELISPOT analysis of splenocytes from different immunization groups after stimulation with Core 93 peptide. (C–D) Mice received DNA-VLV-N4CT1 or DNA-VLV-VSV[5] at a 10-fold higher dose of VSV[5], and one week after the final immunization, spleen cells from immunized mice were analyzed by flow cytometry and ELISPOT. (C) Frequency of IFN-γ$^+$CD8$^+$ cells specific for HBV epitopes in spleen cells of mice immunized as indicated. (D) IFN-γ-producing cells in the spleen of immunized mice after stimulation with Core 93, and (E) two representative wells from the ELISPOT assay of mice that were immunized with DNA-VLV-N4CT1 or DNA-VLV-VSV[5](10x). *p $<$ 0.05 compared to PBS control or S pool peptide stimulation.

specificities. As we found previously (Fig. 2C), Core-specific CD8$^+$ T cell responses were detected only in mice that received the DNA-VLV-N4CT1 immunizations (Fig. 3A–D). Several variations in the order of VLV and N4CT1 prime-boost immunizations were also evaluated (data not shown), but DNA-VLV-N4CT1 was the most immunogenic, consistent with the notion that priming with less inflammatory vaccines and boosting with more immunogenic platforms can generate immune responses of greater magnitude (Nolz and Harty, 2011).

To establish the efficacy of prime-boost vaccination to induce functionally active CD8$^+$ T cell responses, the immunized mice were challenged with AAV-HBV, and the persistence of Core-specific responses after HBV challenge was determined. Mice that received DNA-VLV-N4CT1 had detectable CD8$^+$ T cell responses to Core after HBV challenge (Fig. 3E and H). In contrast, mice that were not immunized failed to elicit Core-specific T cell responses (Fig. 3G and H), consistent with the tolerogenic establishment of HBV replication previously reported with the AAV-HBV system (Yang et al., 2014; Dion et al., 2013). In mice that received DNA-PBS-N4CT1 immunization, Core-specific T cell responses were not significantly different from background prior to AAV-HBV challenge (Fig. 3B). In contrast, Core-specific responses were detected after challenge (Fig. 3F and H). These results indicate that heterologous prime-boost immunization stimulates Core-specific CD8$^+$ T cell responses that persist and expand after HBV challenge.

3.3. Heterologous prime-boost immunization protects mice from HBV challenge

Prime-boost immunization successfully induced CD8$^+$ T cell responses in naïve mice, and these responses were expanded after challenge with AAV-HBV. To evaluate the functionality of the CD8$^+$ T cell responses, we assessed whether prime-boost immunization could protect the mice from the establishment of HBV replication. HBV replication was monitored by the measurement of serum HBs and HBe antigens and liver HBV RNA/DNA. Mice that received DNA-VLV-N4CT1 had reduced levels of HBsAg and HBeAg in serum compared to the untreated PBS group (Fig. 4A and B). Mice that received DNA-PBS-N4CT1 also showed reduced levels of HBV antigens (Fig. 4A and B), even though Core-specific CD8$^+$ T cell responses could not be detected before challenge (Fig. 3B). Consistent with the lower levels of antigen, mice that received DNA-VLV-N4CT1 or DNA-PBS-N4CT1 also showed reduced levels of HBV RNA in the liver compared to the PBS group (Fig. 4C). In those groups, the levels of CD8 RNA in the liver were increased, suggesting the presence of CD8$^+$ T cells (Fig. 4D). Liver viral DNA was also reduced in mice that received DNA-VLV-N4CT1 compared to the PBS group, but not in those that received DNA-PBS-N4CT1 (Fig. 4E), which could be due to potential differences in the effectiveness of the CD8$^+$ T cell responses.

Because liver damage during hepatitis is caused by CD8$^+$ T cells eliminating virus-infected hepatocytes, serum ALT was measured as a marker of liver inflammation. Mice that received DNA-VLV-N4CT1 had increased ALT levels as early as one week after HBV challenge, which remained elevated at week 2 and returned to normal by week 3 (Fig. 4F). In contrast, mice immunized with DNA-PBS-N4CT1 had normal ALT levels at week 1 post-HBV challenge, showed a peak ALT at week 2, and by week 3 post-challenge continued to be elevated (Fig. 4F). These results further suggest the presence of Core-specific CD8$^+$ T cells in the liver. Consistent with the lack of CD8$^+$ T cell

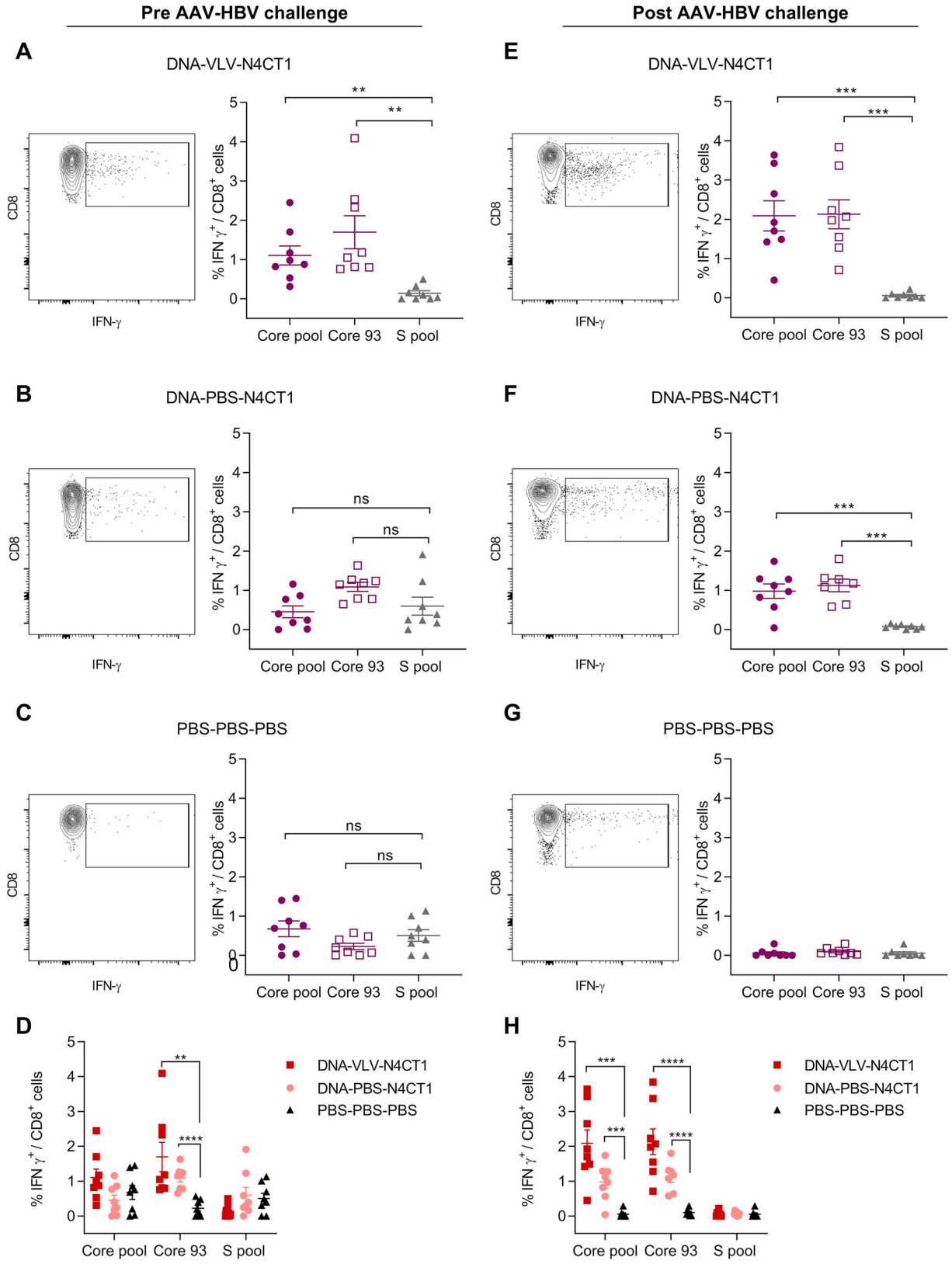

Fig. 3. Prime-boost immunization induces HBV Core-specific CD8$^+$ T cells that are enhanced after AAV-HBV challenge. Mice received prime-boost immunizations with DNA-VLV-N4CT1 (A and E), DNA-PBS-N4CT1 (B and F), or PBS-PBS-PBS (C and G), and were challenged with AAV-HBV two weeks after the final immunization. (A-C, E-G) A representative plot from each group showing responses to the Core 93 epitope is displayed in the left panels, and overall group results are graphed in the right panels. (A–D) PBMC were stimulated with HBV peptides one week following the last immunization. (E–H) HBV Core-specific CD8$^+$ T cells were measured by flow cytometry in spleen cells three weeks following AAV-HBV challenge. **p < 0.01, ***p < 0.001, ****p < 0.0001 relative to the non-specific S pool or PBS.

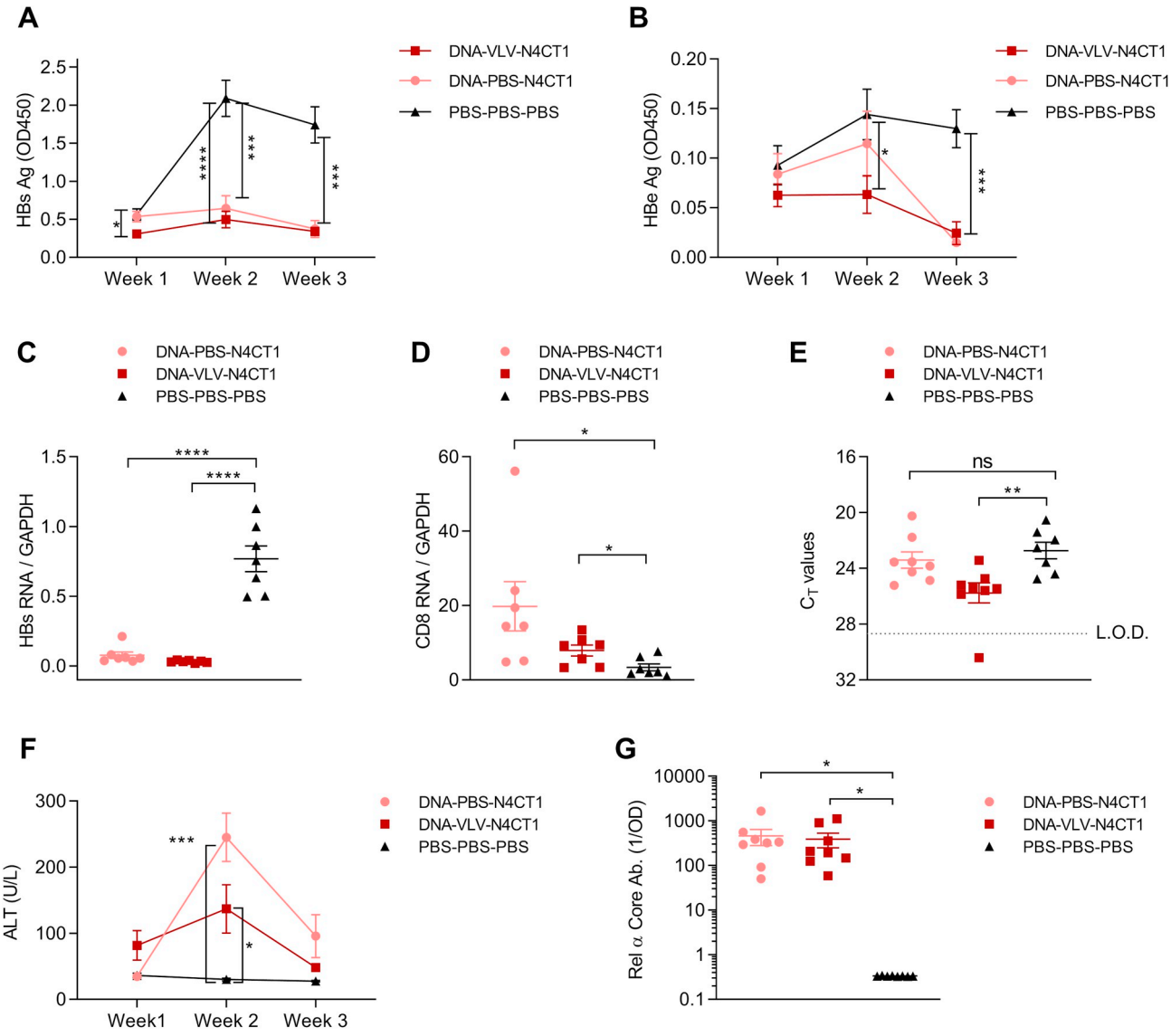

Fig. 4. Immunized mice control HBV replication following AAV-HBV challenge. (A) HBsAg and (B) HBeAg in serum of mice immunized with DNA-VLV-N4CT1, DNA-PBS-N4CT1, or PBS-PBS-PBS. (C) HBV and (D) CD8 RNA levels were measured by RT-qPCR and normalized to GAPDH. (E) HBV DNA in the liver determined by qPCR. (F) ALT levels in the serum of mice that were challenged with AAV-HBV at weeks one, two, and three after challenge. (G) Core-specific antibodies in the serum from immunized mice three weeks after challenge. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 relative to PBS control.

priming in the unimmunized AAV-HBV transduced mice, antibodies to Core antigen were also absent in the PBS group, but were elevated in the immunized groups (Fig. 4G).

Liver $CD8^+$ T cells are important for control of HBV infection (Pallett et al., 2017). To confirm whether liver Core-specific $CD8^+$ T cells are induced by the DNA-VLV-N4CT1 immunization regimen, we performed a CD8 depletion prior to challenging the mice with AAV-HBV, then analyzed for the presence of $CD8^+$ T cells in the liver. Mice that received prime-boost immunization but were depleted of $CD8^+$ cells showed no responses to Core antigen in the spleen as measured by ELISPOT (Fig. 5A). Moreover, recruitment of $CD3^+$ cells to the liver was only observed when mice received DNA-VLV-N4CT1, but not when they received anti-CD8 antibody (Fig. 5C), indicating that the increase in $CD3^+$ T cells observed in the liver with the prime-boost immunization is due to either the recruitment of $CD8^+$ cells to the liver or their local proliferation. Mice that received DNA-VLV-N4CT1 also showed increased levels of $CD8^+$ T cells in the liver (Fig. 5B and D). These cells showed a trend toward higher IFN-γ production when stimulated with the Core peptide pool compared to cells from control mice immunized with PBS (Fig. 5E). Together, these results indicate the efficacy of heterologous prime-boost immunization for activating Core-specific $CD8^+$ T cell responses in the liver and preventing establishment of HBV infection.

3.4. *A single dose of N4CT1-Core fails to induce liver Core-specific $CD8^+$ T cell responses that control virus replication in a model of chronic HBV infection*

Heterologous prime-boost immunization elicited Core-specific $CD8^+$ T cells that protected from HBV challenge in naïve mice. Because we found that Core-specific T cell responses in naïve mice could be expanded after HBV challenge even when responses were not detectable before challenge (Fig. 3B and F), we evaluated the ability of N4CT1-Core to eliminate persistent HBV replication. Mice were transduced with AAV-HBV and immunized with either N4CT1-Core or PBS 8 weeks after transduction. Mice that received a single dose of N4CT1-Core did not control HBV replication, as determined by unchanged HBeAg (Fig. 6A) and HBV DNA (Fig. 6B) levels in the serum. Despite having no effect on HBV persistence, N4CT1-Core immunization

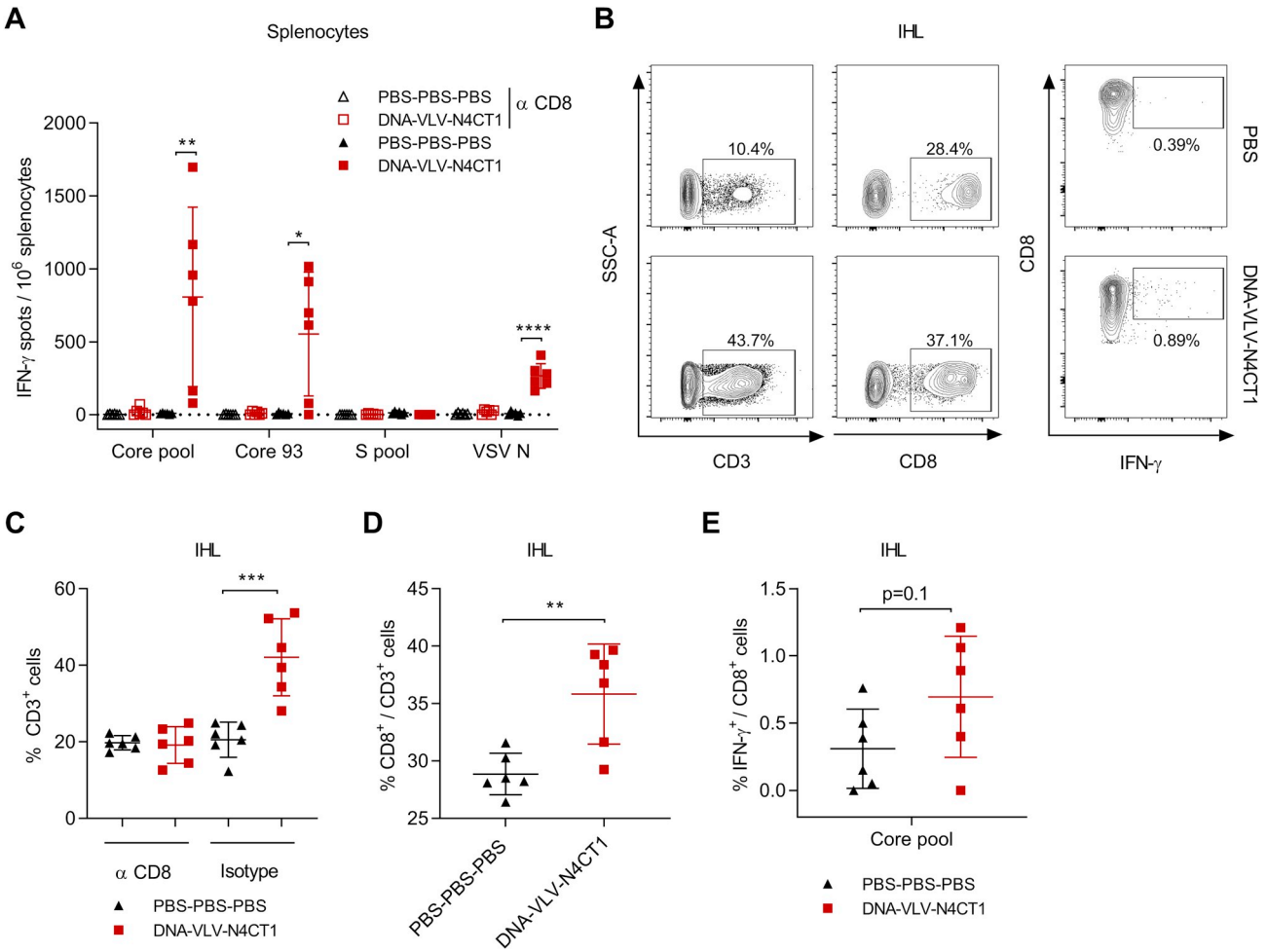

Fig. 5. DNA-VLV-N4CT1 immunization induces CD8$^+$ T cells in the liver. Mice received prime-boost immunization and either anti-CD8 or isotype control antibody. IHL were analyzed three weeks after AAV-HBV challenge. Splenocytes and IHL were stimulated overnight with HBV Core peptides, and subsequently analyzed by flow cytometry. (A) IFN-γ-producing cells in the spleen after peptide stimulation. (B) Gating strategy for analysis of CD8$^+$ T cells in the liver. (C) Percentage of CD3$^+$ T cells in IHL. (D) Percentage of CD8$^+$ T cells in the CD3$^+$ cells of IHL. (E) Percentage of IFN-γ-producing CD8$^+$ T cells after stimulation with the Core peptide pool. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 relative to PBS control.

elicited a weak Core-specific CD8$^+$ T cell response in the spleen (Fig. 6C and D), but no Core-specific responses were detected in the liver of the immunized mice (Fig. 6E and F). These data indicate that a single dose of N4CT1-Core is not capable of controlling HBV replication in the AAV-HBV model of chronic HBV infection.

3.5. Heterologous prime-boost immunization during chronic HBV replication generates systemic Core-specific CD8$^+$ T cell responses that are absent in the liver

Because a single dose of N4CT1-Core did not induce functional Core-specific CD8$^+$ T cell responses in the liver of persistent AAV-HBV mice, and prime-boost immunization in naive mice was more efficient at promoting Core-specific responses, we reasoned that prime-boost immunization with VLV and N4CT1 might stimulate stronger Core-specific CD8$^+$ T cell responses that control HBV replication. Mice were transduced with AAV-HBV and subsequently immunized as described in Fig. 1E. Mice that received DNA-VLV-N4CT1 did not control HBV replication as measured by serum HBeAg (Fig. 7A) and HBV DNA levels (Fig. 7B) despite the presence of Core-specific CD8$^+$ T cell responses in the spleen (Fig. 7C–E). In the liver of mice that received prime-boost immunization, no significant change in the percent of CD8$^+$CD3$^+$ T cells was detected (Fig. 7F), and cells did not produce IFN-γ when stimulated with HBV Core peptides (Fig. 7G), suggesting that prime-boost immunization stimulated Core-specific T cell responses in the periphery that failed to either migrate to the liver or survive in the intrahepatic immunosuppressive environment.

4. Discussion

In previous studies we found that the VLV and VSV[5] vectors stimulated CD8$^+$ T cell responses to the HBV MHBs antigen in mice after a single dose, but failed to elicit Core-specific T cell responses (Reynolds et al., 2015; Cobleigh et al., 2010, 2013; Moshkani et al., 2019). However, other studies have shown that Core can be an immunogenic antigen when expressed by other vaccine platforms (Zhang et al., 2015; Menne et al., 1997; Kosinska et al., 2012), and the unique effects observed with VLV and VSV might reflect Core structural properties combined with expression by a rapidly cytopathic vector. Interestingly, immunization with either VSV[5] or N4CT1 expressing MHBs induces immune responses of similar magnitude (Moshkani et al., 2019), suggesting that specific Core properties are important in the differential immunogenicity that is observed. Core protein assembles into stable empty capsids or virions that expose B cell epitopes that can stimulate antibody responses even in the absence of T cells (Milich and McLachlan, 1986; Whitacre et al., 2009). Although we did not directly measure capsid assembly in the infected cells, the high level of intracellular protein expression that occurs following VSV infection,

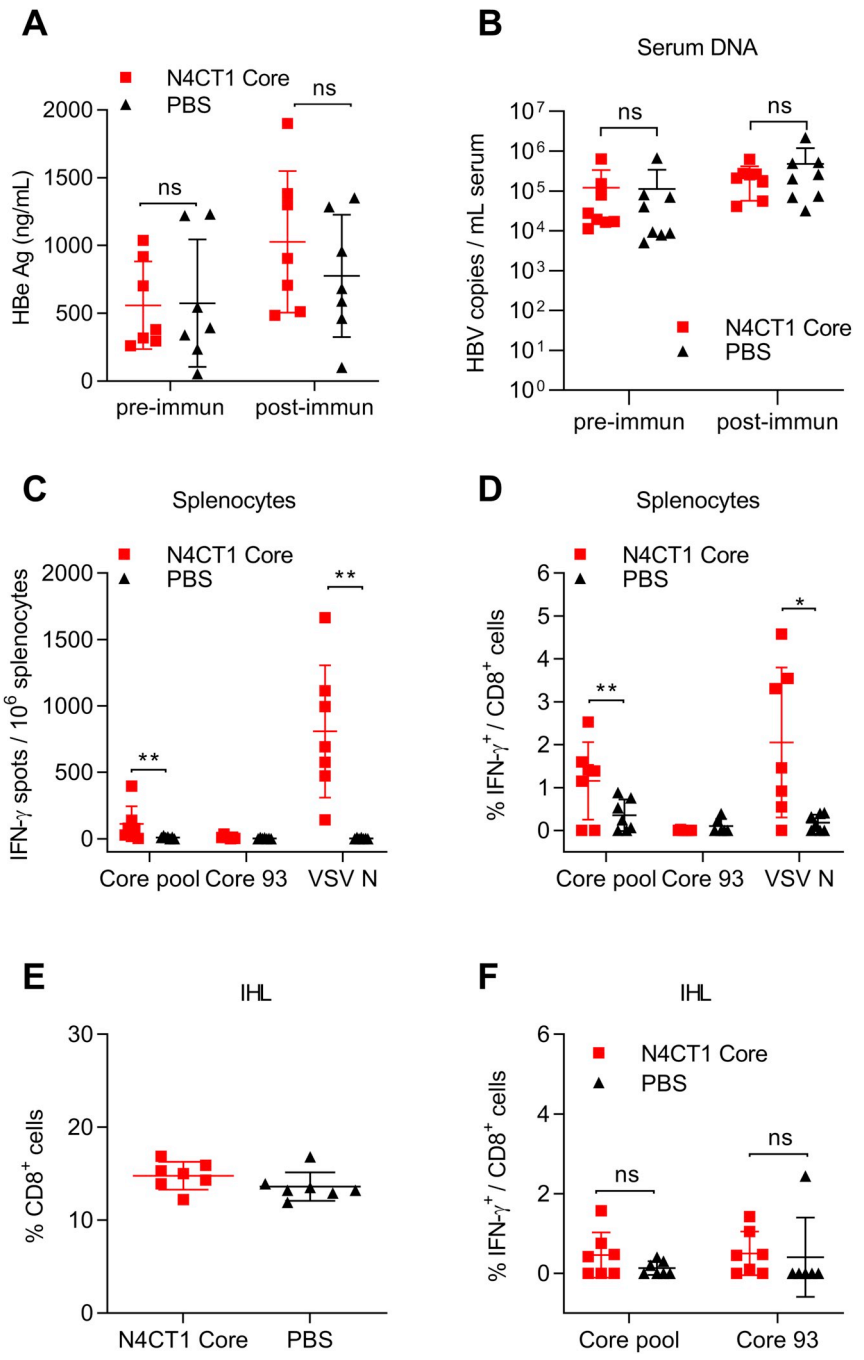

Fig. 6. A single dose of N4CT1 expressing Core antigen fails to induce Core-specific T cell responses in the liver of chronically infected mice. Mice were transduced with AAV-HBV, and eight weeks later received a single dose of N4CT1-Core. Core-specific T cells and antigen levels were analyzed three weeks after the immunization. (A) HBeAg measured in serum pre- and post-N4CT1 immunization. (B) Serum HBV DNA determined by qPCR. (C) IFN-γ-producing cells in the spleen after stimulation with HBV antigens and VSV N measured by ELISPOT. (D) Percentage of IFN-γ-producing CD8$^+$ T cells in the spleen after stimulation with Core pool, Core 93, or VSV N peptide. (E) Percentage of CD8$^+$ cells in the liver of immunized mice. (F) Percentage of IFN-γ-producing CD8$^+$ T cells in IHL after stimulation. *p < 0.05, **p < 0.01 relative to PBS control.

combined with the observation that prime-boost immunization efficiently produced anti-Core antibody responses, is consistent with the formation of particulate antigen.

Core is also the most stable hepadnaviral antigen (Yao and Tavis, 2003; Yao et al., 2003), and this stability combined with expression from rapidly cytopathic vectors might result in inefficient antigen presentation that limits stimulation of T cell responses. VSV[1] displays increased Core expression compared to VSV[5], but compared to N4CT1 shows only a modest attenuation in viral replication (Fig. 1B); in contrast, the N4CT1 vector expresses higher levels of Core similar to VSV[1], but is also highly attenuated. The decreased pathogenicity and slower replication rate of N4CT1 compared to WT VSV is consistent with the hypothesis that the low immunogenicity of Core when expressed by WT virus could be due to reduced antigen processing and presentation as a consequence of more rapid cell death. We found that Core-specific T cell responses were only detectable after immunization with a single dose of N4CT1-Core (Fig. 2B), supporting the notion that for VSV-based vectors, attenuation of the vector and not solely antigen expression level improves immunogenicity of the platform, at least in context of the HBV Core protein. Future studies to address the effect of Core protein stability and vector attenuation would aid in improving vaccine design not only for HBV therapeutics, but also for the use of the vaccine platforms with other antigens.

One potential obstacle of utilizing Core as a therapeutic antigen is that although Core is not abundantly secreted during chronic HBV infection, HBeAg is highly expressed and this antigen shares at least 154 amino acids with Core (Schodel et al., 1993; Ito et al., 2009; Messageot et al., 2003). The T cell responses to HBeAg can cross-react with Core, and previous studies have demonstrated tolerogenic effects of HBeAg on T cell responses to Core (Tian et al., 2016; Chen et al., 2004). In our

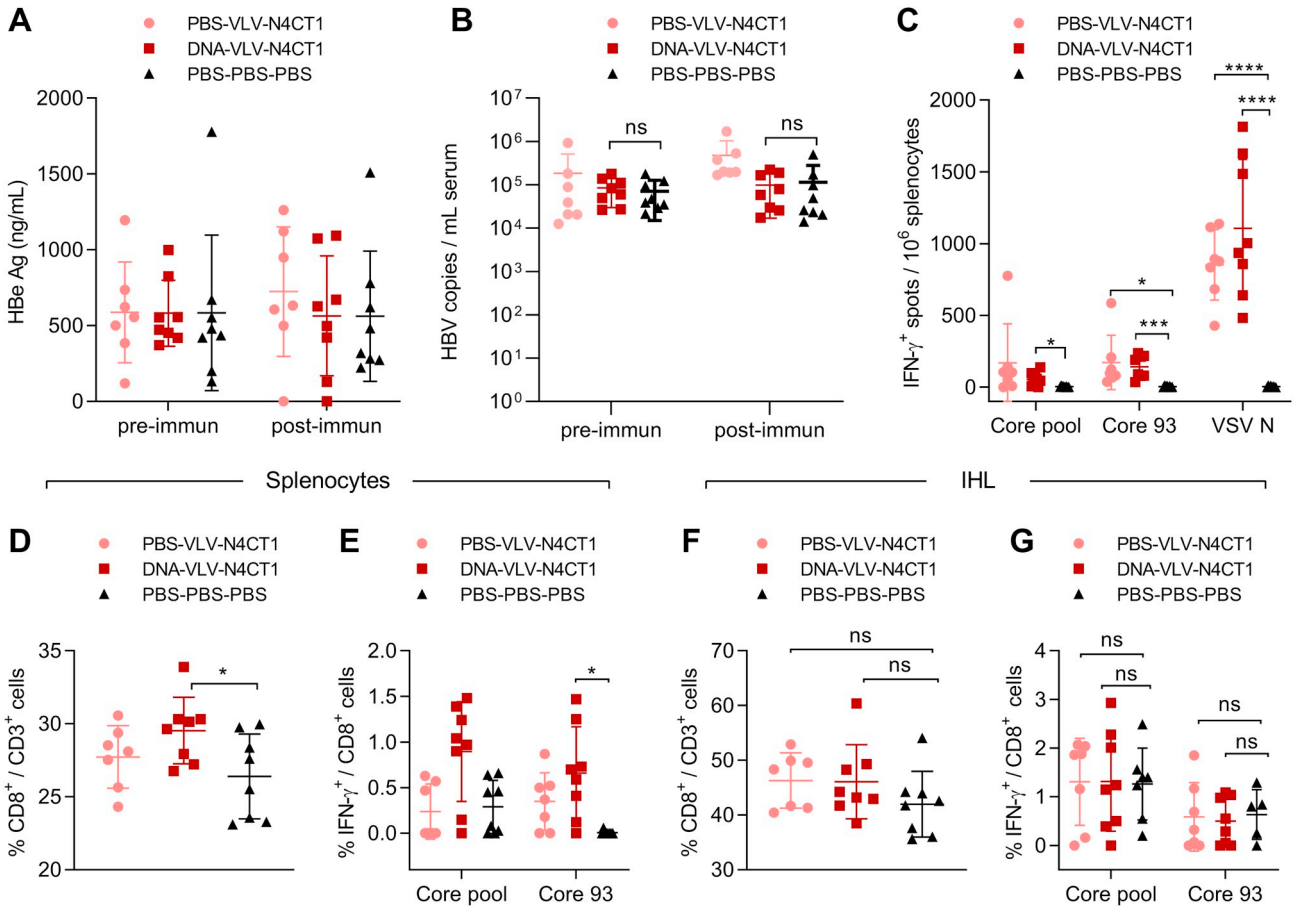

Fig. 7. DNA-VLV-N4CT1 induces systemic Core-specific CD8$^+$ T cell responses that are absent from the liver of mice with chronic HBV replication. Mice were transduced with AAV-HBV and 8 weeks after transduction received heterologous prime-boost immunizations. Core-specific T cells and antigen levels were analyzed three weeks after the final immunization. (A) HBeAg measured in serum pre- and post-prime-boost immunization. (B) Serum HBV DNA determined by qPCR. (C) ELISPOT analysis of spleen cells from mice three weeks after the final boost. (D) Percentage of CD8$^+$ T cells in the CD3$^+$ cells in the spleen. (E) Percentage of IFN-γ-producing CD8$^+$ T cells in the spleen after stimulation with Core peptides. (F) Percentage of CD8$^+$ T cells in the CD3$^+$ cells in IHL from AAV-HBV transduced and immunized mice. (G) Percentage of IFN-γ-producing CD8$^+$ T cells in the IHL from immunized mice after stimulation. *p $<$ 0.05, ***p $<$ 0.001, ****p $<$ 0.0001 relative to PBS control.

therapeutic studies, mice displayed high HBeAg levels that remain constant throughout the duration of the experiments (Figs. 6A and 7A). Despite prime-boost immunization successfully activating Core-specific CD8$^+$ T cells systemically, these cells were not present in the liver. This lack of T cell functionality could potentially be explained by a tolerogenic effect of HBeAg on the elicited T cells. For example, studies by Tian et al. showed that maternal HBeAg caused hepatic macrophages to support HBV persistence in mice, and depletion of these cells leads to activation of CD8$^+$ T cells (Tian et al., 2016).

From our results it cannot be ruled out that the magnitude of the vaccine-induced CD8$^+$ T cell response is simply not strong enough to control HBV replication. However, the magnitude of the antigen-specific T cell responses to Core in the spleen is similar to what we previously found when immunizing mice with N4CT1-MHBs (Moshkani et al., 2019), which in contrast to N4CT1-Core, effectively controlled HBV replication. This would argue against the magnitude of the immune response being important, and favor a hypothesis were the liver suppressive environment and/or tolerogenic effect of HBeAg affects the migration or function of Core-specific T cells in the liver. In the preventative experiments, mice immunized with DNA-PBS-N4CT1 showed protection from HBV challenge (Fig. 4) even though no responses were detected pre-challenge (Fig. 3B). This could be explained by the fact that the pre-challenge T cell responses were measured by flow cytometry in the blood of the mice, and the sensitivity of flow cytometry might not be sufficient to detect the low magnitude of the response that was elicited. This shows that even when low numbers of Core-specific CD8$^+$ T cells are primed in naïve mice, they can recognize HBV antigen and expand, providing protection from challenge. In fact, the kinetics of the immune response were altered in the different immunization groups (Fig. 4). Mice that received DNA-PBS-N4CT1 showed higher levels of CD8 RNA in the liver by week 3 post-challenge, together with higher ALT levels in serum. Compared to those mice that received DNA-VLV-N4CT1, the magnitude of CD8$^+$ T cell responses was lower in the DNA-PBS-N4CT1-immunized mice, and control of HBV DNA was not achieved by week 3 post-challenge, supporting the idea that VLV boost enhanced the magnitude of the immune responses, favoring more rapid control of HBV.

The AAV-HBV model has been increasingly utilized for chronic HBV studies, but the similarities and differences between chronic infection in humans and this model are not completely understood. Our data are consistent with a mechanism in which HBV delivered to hepatocytes via AAV transduction is associated with an immunosuppressive environment in the liver that tolerizes or exhausts the immune responses generated to Core antigen. This may be similar to chronic HBV infection in humans, where T cell responses become dysfunctional in the liver immunosuppressive environment and fail to control viral replication. Future studies are needed to determine whether AAV-HBV transduction induces immune responses that are tolerant to the virus, causes T cell exhaustion in the liver, or avoids initial priming of immune responses, as well as to define differences in the immunotolerance to distinct HBV antigens.

Despite Core-specific T cell responses not being sufficient to eradicate HBV in mice with persistent infection, this study has three important findings. First, our results demonstrated successful activation of Core-specific $CD8^+$ T cell responses with VSV- and VLV-based vaccine platforms, both in naïve mice and in a chronic model of HBV. In contrast to intrahepatic MHBs-specific T cell responses activated by N4CT1 immunization (Moshkani et al., 2019), the Core-specific responses were not able to control established HBV replication, stressing the importance of homing of functional cells to the liver to control infection. Nevertheless, activation of Core-specific T cells by heterologous prime-boost immunization might be combined with other strategies to decrease the immunosuppressive liver environment and/or favor migration of functional T cells to the liver (Kosinska et al., 2013; Liu et al., 2014; Dembek et al., 2018). Second, the inability of Core-specific T cell responses in the AAV-HBV model to eradicate persistent HBV replication raises new unanswered questions with regard to which HBV antigen(s) represent the best therapeutic option for use in humans, whether tolerance to Core antigen might be more difficult to overcome compared to HBsAg in the AAV-HBV model, and whether tolerance to specific HBV antigens in the AAV-HBV model accurately reflects natural chronic infection in humans. Thus, these results highlight the importance of evaluating the efficacy of different therapeutic antigens in preclinical animal models, and understanding the correlation with therapeutic value in human chronic HBV infection. Finally, as the high degree of T cell dysfunction to Core in the AAV-HBV model appears to mimic the profound tolerance to HBV that is observed in human chronic infection, further characterizing the mechanisms of viral persistence in this model may reveal additional pathways to exploit in the development of new therapeutic options.

Competing interests statement

M.D.R and J.K.R. have financial relationships with a company, CaroGen Corporation, that is seeking to commercialize the VLV vaccine platform.

Acknowledgments

We thank Rebecca Knipp, Linda Buonocore, Anasuya Chattopadhyay, Qi Yang, and the Albany Medical College IMD Flow Cytometry Facility for helpful discussions and technical assistance. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number R01AI124006. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Aguilar, J.C., Lobaina, Y., Muzio, V., Garcia, D., Penton, E., Iglesias, E., Pichardo, D., Urquiza, D., Rodriguez, D., Silva, D., Petrovsky, N., Guillen, G., 2004. Development of a nasal vaccine for chronic hepatitis B infection that uses the ability of hepatitis B core antigen to stimulate a strong Th1 response against hepatitis B surface antigen. Immunol. Cell Biol. 82, 539–546.

Barefoot, B.E., Sample, C.J., Ramsburg, E.A., 2009. Recombinant vesicular stomatitis virus expressing influenza nucleoprotein induces CD8 T-cell responses that enhance antibody-mediated protection after lethal challenge with influenza virus. Clin. Vaccine Immunol. 16, 488–498.

Bertoletti, A., Ferrari, C., Fiaccadori, F., Penna, A., Margolskee, R., Schlicht, H.J., Fowler, P., Guilhot, S., Chisari, F.V., 1991. HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. Proc. Natl. Acad. Sci. U. S. A. 88, 10445–10449.

Bian, Y., Zhang, Z., Sun, Z., Zhao, J., Zhu, D., Wang, Y., Fu, S., Guo, J., Liu, L., Su, L., Wang, F.S., Fu, Y.X., Peng, H., 2017. Vaccines targeting preS1 domain overcome immune tolerance in hepatitis B virus carrier mice. Hepatology 66, 1067–1082.

Chapman, R., Jongwe, T.I., Douglass, N., Chege, G., Williamson, A.L., 2017. Heterologous prime-boost vaccination with DNA and MVA vaccines, expressing HIV-1 subtype C mosaic Gag virus-like particles, is highly immunogenic in mice. PLoS One 12, e0173352.

Chen, M.T., Billaud, J.N., Sallberg, M., Guidotti, L.G., Chisari, F.V., Jones, J., Hughes, J., Milich, D.R., 2004. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. Proc. Natl. Acad. Sci. U. S. A. 101, 14913–14918.

Chisari, F.V., Ferrari, C., 1995. Hepatitis B virus immunopathogenesis. Annu. Rev. Immunol. 13, 29–60.

Clarke, D.K., Cooper, D., Egan, M.A., Hendry, R.M., Parks, C.L., Udem, S.A., 2006. Recombinant vesicular stomatitis virus as an HIV-1 vaccine vector. Springer Semin. Immunopathol. 28, 239–253.

Clarke, D.K., Nasar, F., Lee, M., Johnson, J.E., Wright, K., Calderon, P., Guo, M., Natuk, R., Cooper, D., Hendry, R.M., Udem, S.A., 2007. Synergistic attenuation of vesicular stomatitis virus by combination of specific G gene truncations and N gene translocations. J. Virol. 81, 2056–2064.

Cobleigh, M.A., Buonocore, L., Uprichard, S.L., Rose, J.K., Robek, M.D., 2010. A vesicular stomatitis virus-based hepatitis B virus vaccine vector provides protection against challenge in a single dose. J. Virol. 84, 7513–7522.

Cobleigh, M.A., Bradfield, C., Liu, Y., Mehta, A., Robek, M.D., 2012. The immune response to a vesicular stomatitis virus vaccine vector is independent of particulate antigen secretion and protein turnover rate. J. Virol. 86, 4253–4261.

Cobleigh, M.A., Wei, X., Robek, M.D., 2013. A vesicular stomatitis virus-based therapeutic vaccine generates a functional CD8 T cell response to hepatitis B virus in transgenic mice. J. Virol. 87, 2969–2973.

Cooper, D., Wright, K.J., Calderon, P.C., Guo, M., Nasar, F., Johnson, J.E., Coleman, J.W., Lee, M., Kotash, C., Yurgelonis, I., Natuk, R.J., Hendry, R.M., Udem, S.A., Clarke, D.K., 2008. Attenuation of recombinant vesicular stomatitis virus-human immunodeficiency virus type 1 vaccine vectors by gene translocations and g gene truncation reduces neurovirulence and enhances immunogenicity in mice. J. Virol. 82, 207–219.

Das, A., Hoare, M., Davies, N., Lopes, A.R., Dunn, C., Kennedy, P.T., Alexander, G., Finney, H., Lawson, A., Plunkett, F.J., Bertoletti, A., Akbar, A.N., Maini, M.K., 2008. Functional skewing of the global CD8 T cell population in chronic hepatitis B virus infection. J. Exp. Med. 205, 2111–2124.

Dembek, C., Protzer, U., Roggendorf, M., 2018. Overcoming immune tolerance in chronic hepatitis B by therapeutic vaccination. Curr Opin Virol 30, 58–67.

Dion, S., Bourgine, M., Godon, O., Levillayer, F., Michel, M.L., 2013. Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules. J. Virol. 87, 5554–5563.

Dolman, G.E., Koffas, A., Mason, W.S., Kennedy, P.T., 2018. Why, who and when to start treatment for chronic hepatitis B infection. Curr Opin Virol 30, 39–47.

Elizaga, M.L., Li, S.S., Kochar, N.K., Wilson, G.J., Allen, M.A., Tieu, H.V.N., Frank, I., Sobieszczyk, M.E., Cohen, K.W., Sanchez, B., Latham, T.E., Clarke, D.K., Egan, M.A., Eldridge, J.H., Hannaman, D., Xu, R., Ota-Setlik, A., McElrath, M.J., Hay, C.M., Team NHVTNS, 2018. Safety and tolerability of HIV-1 multiantigen pDNA vaccine given with IL-12 plasmid DNA via electroporation, boosted with a recombinant vesicular stomatitis virus HIV Gag vaccine in healthy volunteers in a randomized, controlled clinical trial. PLoS One 13, e0202753.

Ewer, K.J., Lambe, T., Rollier, C.S., Spencer, A.J., Hill, A.V., Dorrell, L., 2016. Viral vectors as vaccine platforms: from immunogenicity to impact. Curr. Opin. Immunol. 41, 47–54.

Fontaine, H., Kahi, S., Chazallon, C., Bourgine, M., Varaut, A., Buffet, C., Godon, O., Meritet, J.F., Saidi, Y., Michel, M.L., Scott-Algara, D., Aboulker, J.P., Pol, S., group AHs, 2015. Anti-HBV DNA vaccination does not prevent relapse after discontinuation of analogues in the treatment of chronic hepatitis B: a randomised trial–ANRS HB02 VAC-ADN. Gut 64, 139–147.

Garson, J.A., Grant, P.R., Ayliffe, U., Ferns, R.B., Tedder, R.S., 2005. Real-time PCR quantitation of hepatitis B virus DNA using automated sample preparation and murine cytomegalovirus internal control. J. Virol Methods 126, 207–213.

Glebe, D., Bremer, C.M., 2013. The molecular virology of hepatitis B virus. Semin. Liver Dis. 33, 103–112.

Grimm, D., Thimme, R., Blum, H.E., 2011. HBV life cycle and novel drug targets. Hepatol Int 5, 644–653.

Guidotti, L.G., Isogawa, M., Chisari, F.V., 2015. Host-virus interactions in hepatitis B virus infection. Curr. Opin. Immunol. 36, 61–66.

Ito, K., Kim, K.H., Lok, A.S., Tong, S., 2009. Characterization of genotype-specific carboxyl-terminal cleavage sites of hepatitis B virus e antigen precursor and identification of furin as the candidate enzyme. J. Virol. 83, 3507–3517.

Keasler, V.V., Hodgson, A.J., Madden, C.R., Slagle, B.L., 2007. Enhancement of hepatitis B virus replication by the regulatory X protein in vitro and in vivo. J. Virol. 81, 2656–2662.

Kosinska, A.D., Johrden, L., Zhang, E., Fiedler, M., Mayer, A., Wildner, O., Lu, M., Roggendorf, M., 2012. DNA prime-adenovirus boost immunization induces a vigorous and multifunctional T-cell response against hepadnaviral proteins in the mouse and woodchuck model. J. Virol. 86, 9297–9310.

Kosinska, A.D., Zhang, E., Johrden, L., Liu, J., Seiz, P.L., Zhang, X., Ma, Z., Kemper, T., Fiedler, M., Glebe, D., Wildner, O., Dittmer, U., Lu, M., Roggendorf, M., 2013. Combination of DNA prime–adenovirus boost immunization with entecavir elicits sustained control of chronic hepatitis B in the woodchuck model. PLoS Pathog. 9, e1003391.

Kosinska, A.D., Bauer, T., Protzer, U., 2017. Therapeutic vaccination for chronic hepatitis B. Curr Opin Virol 23, 75–81.

Kuhober, A., Pudollek, H.P., Reifenberg, K., Chisari, F.V., Schlicht, H.J., Reimann, J., Schirmbeck, R., 1996. DNA immunization induces antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2b mice. J. Immunol. 156, 3687–3695.

Li, S.S., Kochar, N.K., Elizaga, M., Hay, C.M., Wilson, G.J., Cohen, K.W., De Rosa, S.C., Xu, R., Ota-Setlik, A., Morris, D., Finak, G., Allen, M., Tieu, H.V., Frank, I., Sobieszczyk, M.E., Hannaman, D., Gottardo, R., Gilbert, P.B., Tomaras, G.D., Corey, L., Clarke, D.K., Egan, M.A., Eldridge, J.H., McElrath, M.J., Frahm, N., Network, N.H.V.T., 2017. DNA priming increases frequency of T-cell responses to a vesicular stomatitis virus

HIV vaccine with specific enhancement of CD8(+) T-cell responses by interleukin-12 plasmid DNA. Clin. Vaccine Immunol. 24. https://doi.org/10.1128/CVI.00263-17.
Lichty, B.D., Power, A.T., Stojdl, D.F., Bell, J.C., 2004. Vesicular stomatitis virus: re-inventing the bullet. Trends Mol. Med. 10, 210–216.
Liu, J., Zhang, E., Ma, Z., Wu, W., Kosinska, A., Zhang, X., Moller, I., Seiz, P., Glebe, D., Wang, B., Yang, D., Lu, M., Roggendorf, M., 2014. Enhancing virus-specific immunity in vivo by combining therapeutic vaccination and PD-L1 blockade in chronic hepadnaviral infection. PLoS Pathog. 10, e1003856.
Maini, M.K., Pallett, L.J., 2018. Defective T-cell immunity in hepatitis B virus infection: why therapeutic vaccination needs a helping hand. Lancet Gastroenterol Hepatol 3, 192–202.
Martin, P., Dubois, C., Jacquier, E., Dion, S., Mancini-Bourgine, M., Godon, O., Kratzer, R., Lelu-Santolaria, K., Evlachev, A., Meritet, J.F., Schlesinger, Y., Villeval, D., Strub, J.M., Van Dorsselaer, A., Marchand, J.B., Geist, M., Brandely, R., Findeli, A., Boukhebza, H., Menguy, T., Silvestre, N., Michel, M.L., Inchauspe, G., 2015. TG1050, an immunotherapeutic to treat chronic hepatitis B, induces robust T cells and exerts an antiviral effect in HBV-persistent mice. Gut 64, 1961–1971.
Matassov, D., Marzi, A., Latham, T., Xu, R., Ota-Setlik, A., Feldmann, F., Geisbert, J.B., Mire, C.E., Hamm, S., Nowak, B., Egan, M.A., Geisbert, T.W., Eldridge, J.H., Feldmann, H., Clarke, D.K., 2015. Vaccination with a highly attenuated recombinant vesicular stomatitis virus vector protects against challenge with a lethal dose of ebola virus. J. Infect. Dis. 212 (Suppl. 2), S443–S451.
Menne, S., Maschke, J., Tolle, T.K., Lu, M., Roggendorf, M., 1997. Characterization of T-cell response to woodchuck hepatitis virus core protein and protection of woodchucks from infection by immunization with peptides containing a T-cell epitope. J. Virol. 71, 65–74.
Messageot, F., Salhi, S., Eon, P., Rossignol, J.M., 2003. Proteolytic processing of the hepatitis B virus e antigen precursor. Cleavage at two furin consensus sequences. J. Biol. Chem. 278, 891–895.
Michel, M.L., Tiollais, P., 2010. Hepatitis B vaccines: protective efficacy and therapeutic potential. Pathol. Biol. 58, 288–295.
Milich, D.R., McLachlan, A., 1986. The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. Science 234, 1398–1401.
Moshkani, S., Chiale, C., Lang, S.M., Rose, J.K., Robek, M.D., 2019. A highly attenuated vesicular stomatitis virus-based vaccine platform controls hepatitis B virus replication in mouse models of hepatitis B. J. Virol. 93 e01586-18.
Nassal, M., 2015. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 64, 1972–1984.
Nolz, J.C., Harty, J.T., 2011. Strategies and implications for prime-boost vaccination to generate memory CD8 T cells. Adv. Exp. Med. Biol. 780, 69–83.
Pallett, L.J., Davies, J., Colbeck, E.J., Robertson, F., Hansi, N., Easom, N.J.W., Burton, A.R., Stegmann, K.A., Schurich, A., Swadling, L., Gill, U.S., Male, V., Luong, T., Gander, A., Davidson, B.R., Kennedy, P.T.F., Maini, M.K., 2017. IL-2(high) tissue-resident T cells in the human liver: sentinels for hepatotropic infection. J. Exp. Med. 214, 1567–1580.
Pol, S., Nalpas, B., Driss, F., Michel, M.L., Tiollais, P., Denis, J., Brecho, C., Multicenter study, g, 2001. Efficacy and limitations of a specific immunotherapy in chronic hepatitis B. J. Hepatol. 34, 917–921.
Ramsburg, E., Publicover, J., Buonocore, L., Poholek, A., Robek, M., Palin, A., Rose, J.K., 2005. A vesicular stomatitis virus recombinant expressing granulocyte-macrophage colony-stimulating factor induces enhanced T-cell responses and is highly attenuated for replication in animals. J. Virol. 79, 15043–15053.
Rehermann, B., Fowler, P., Sidney, J., Person, J., Redeker, A., Brown, M., Moss, B., Sette, A., Chisari, F.V., 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J. Exp. Med. 181, 1047–1058.
Rehermann, B., Lau, D., Hoofnagle, J.H., Chisari, F.V., 1996. Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. J. Clin. Investig. 97, 1655–1665.
Reynolds, T.D., Buonocore, L., Rose, N.F., Rose, J.K., Robek, M.D., 2015. Virus-like vesicle-based therapeutic vaccine vectors for chronic hepatitis B virus infection. J. Virol. 89, 10407–10415.
Reynolds, T.D., Moshkani, S., Robek, M.D., 2017. An ELISPOT-based assay to measure HBV-specific CD8(+) T cell responses in immunocompetent mice. Methods Mol. Biol. 1540, 237–247.
Rolls, M.M., Webster, P., Balba, N.H., Rose, J.K., 1994. Novel infectious particles generated by expression of the vesicular stomatitis virus glycoprotein from a self-replicating RNA. Cell 79, 497–506.
Rolls, M.M., Haglund, K., Rose, J.K., 1996. Expression of additional genes in a vector derived from a minimal RNA virus. Virology 218, 406–411.
Rose, N.F., Marx, P.A., Luckay, A., Nixon, D.F., Moretto, W.J., Donahoe, S.M., Montefiori, D., Roberts, A., Buonocore, L., Rose, J.K., 2001. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell 106, 539–549.
Rose, N.F., Publicover, J., Chattopadhyay, A., Rose, J.K., 2008. Hybrid alphavirus-rhabdovirus propagating replicon particles are versatile and potent vaccine vectors. Proc. Natl. Acad. Sci. U. S. A. 105, 5839–5843.
Rose, N.F., Buonocore, L., Schell, J.B., Chattopadhyay, A., Bahl, K., Liu, X., Rose, J.K., 2014. In vitro evolution of high-titer, virus-like vesicles containing a single structural protein. Proc. Natl. Acad. Sci. U. S. A. 111, 16866–16871.
Schell, J.B., Rose, N.F., Bahl, K., Diller, K., Buonocore, L., Hunter, M., Marx, P.A., Gambhira, R., Tang, H., Montefiori, D.C., Johnson, W.E., Rose, J.K., 2011. Significant protection against high-dose simian immunodeficiency virus challenge conferred by a new prime-boost vaccine regimen. J. Virol. 85, 5764–5772.
Schirmbeck, R., Bohm, W., Fissolo, N., Melber, K., Reimann, J., 2003. Different immunogenicity of H-2 Kb-restricted epitopes in natural variants of the hepatitis B surface antigen. Eur. J. Immunol. 33, 2429–2438.
Schodel, F., Peterson, D., Zheng, J., Jones, J.E., Hughes, J.L., Milich, D.R., 1993. Structure of hepatitis B virus core and e-antigen. A single precore amino acid prevents nucleocapsid assembly. J. Biol. Chem. 268, 1332–1337.
Tian, Y., Kuo, C.F., Akbari, O., Ou, J.H., 2016. Maternal-Derived Hepatitis B Virus e Antigen Alters Macrophage Function in Offspring to Drive Viral Persistence after Vertical Transmission. Immunity 44, 1204–1214.
Van Bleek, G.M., Nathenson, S.G., 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. Nature 348, 213–216.
Whitacre, D.C., Lee, B.O., Milich, D.R., 2009. Use of hepadnavirus core proteins as vaccine platforms. Expert Rev. Vaccines 8, 1565–1573.
Yang, D., Liu, L., Zhu, D., Peng, H., Su, L., Fu, Y.X., Zhang, L., 2014. A mouse model for HBV immunotolerance and immunotherapy. Cell. Mol. Immunol. 11, 71–78.
Yao, E., Tavis, J.E., 2003. Kinetics of synthesis and turnover of the duck hepatitis B virus reverse transcriptase. J. Biol. Chem. 278, 1201–1205.
Yao, E., Schaller, H., Tavis, J.E., 2003. The duck hepatitis B virus polymerase and core proteins accumulate in different patterns from their common mRNA. Virology 311, 81–88.
Zhang, E., Kosinska, A.D., Ma, Z., Dietze, K.K., Xu, Y., Meng, Z., Zhang, X., Wang, J., Wang, B., Dittmer, U., Roggendorf, M., Yang, D., Lu, M., 2015. Woodchuck hepatitis virus core antigen-based DNA and protein vaccines induce qualitatively different immune responses that affect T cell recall responses and antiviral effects. Virology 475, 56–65.
Zhao, W., Zhou, X., Zhao, G., Lin, Q., Wang, X., Yu, X., Wang, B., 2017. Enrichment of Ly6C(hi) monocytes by multiple GM-CSF injections with HBV vaccine contributes to viral clearance in a HBV mouse model. Hum. Vaccines Immunother. 13, 2872–2882.
Zhu, D., Liu, L., Yang, D., Fu, S., Bian, Y., Sun, Z., He, J., Su, L., Zhang, L., Peng, H., Fu, Y.X., 2016. Clearing persistent extracellular antigen of hepatitis B virus: an immunomodulatory strategy to reverse tolerance for an effective therapeutic vaccination. J. Immunol. 196, 3079–3087.