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Vaccines Targeting PreS1 Domain Overcome Immune Tolerance in Hepatitis B Virus Carrier Mice

Yingjie Bian,[1,2] Zheng Zhang,[3] Zhichen Sun,[1,2] Juanjuan Zhao,[3] Danming Zhu,[4] Yang Wang,[5] Sherry Fu,[5] Jingya Guo,[1] Longchao Liu,[1,2] Lishan Su,[6] Fu-Sheng Wang,[7] Yang-Xin Fu,[1,5] and Hua Peng[1]

Strong tolerance to hepatitis B virus (HBV) surface antigens limits the therapeutic effect of the conventional hepatitis B surface antigen (HBsAg) vaccination in both preclinical animal models and patients with chronic hepatitis B (CHB) infection. In contrast, we observed that clinical CHB patients presented less immune tolerance to the preS1 domain of HBV large surface antigen. To study whether targeting the weak tolerance of the preS1 region could improve therapy gain, we explored vaccination with the long peptide of preS1 domain for HBV virions clearance. Our study showed that this preS1-polypeptide rather than HBsAg vaccination induced robust immune responses in HBV carrier mice. The anti-preS1 rapidly cleared HBV virions *in vivo* and blocked HBV infection to hepatocytes *in vitro*. Intriguingly, vaccination of preS1-polypeptide even reduced the tolerized status of HBsAg, opening a therapeutic window for the host to respond to the HBsAg vaccine. A sequential administration of antigenically distinct preS1-polypeptide and HBsAg vaccines in HBV carrier mice could finally induce HBsAg/hepatitis B surface antibody serological conversion and clear chronic HBV infection in carrier mice. *Conclusion*: These results suggest that preS1 can function as a therapeutic vaccine for the control of CHB. (HEPATOLOGY 2017;66:1067-1082)

Persistent HBV infection still represents a substantial threat to public health, despite the existence of effective prophylactic vaccines. More than 2 billion people are infected with hepatitis B virus (HBV), and 350 million become chronic HBV carriers worldwide. Nearly 1 million people die from hepatitis B–related diseases every year.[1] Thus, there remains an urgent need for effective treatment strategies to limit the enormous burden of viral hepatitis on global health. The HBV genome encodes three overlapping viral surface antigens, named small (S), middle (M), and large (L) proteins, respectively. "S protein," known as hepatitis B surface antigen (HBsAg), is the common C-terminal domain of these three proteins, which is the most abundant surface antigen. M protein is derived from a transcript initiating at the upstream start codon of HBsAg. L protein is composed of the N-terminal preS1 domain and the adjacent M protein.[2] HBsAg is the most active component of conventional HBV vaccines. Although it elicits strong

Abbreviations: AAV, adeno-associated virus; AHB, acute hepatitis B; ALT, alanine aminotransferase; ANOVA, analysis of variance; APC, allophycocyanin; AST, aspartate aminotransferase; cDNA, complimentary DNA; CHB, chronic hepatitis B; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; ELISPOT, Enzyme-Linked ImmunoSpot; gDNA, genomic DNA; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; anti-HBs, antibody to hepatitis B surface antigen; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; L-HBsAg, large HBsAg; rHBsAg, recombinant HBsAg; HBV, hepatitis B virus; HC, healthy controls; HRP, horseradish peroxidase; IFA, incomplete Freund adjuvant; IFNγ, interferon-gamma; IgG, immunoglobulin G; IHC, immunohistochemical; kb, kilobase; LNs, lymph nodes; mAb, monoclonal antibody; MACS, magnetic cell separation system; PBMCs, peripheral blood mononuclear cells; PMM, primary hepatocytes maintenance medium; SUMO, Small Ubiquitin Modifier; WT, wild type.

Received November 23, 2016; accepted April 20, 2017.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29239/suppinfo.

Supported by the National Key Basic Research Program of China (nos. 2012CB910203 and 2012CB519000) and the National Grand Program on Key Infectious Diseases (no. 2012ZX10002006) to Y.-X.F. and H.P.; National Nature and Science Foundation of China (nos. 81471579 and 81641063) to H.P.; and NIH funding (R01AI095097) to L.S. and Y.-X.F.

immunogenicity as a preventive vaccine in healthy people, the current HBsAg vaccines cannot induce antibody to hepatitis B surface antigen (anti-HBs) for viral-clearance in chronic hepatitis B (CHB) patients.[3] High levels of viral antigens in circulation have been shown to induce host immune tolerance with impaired dendritic cell, natural killer, or T-cell and B-cell functions and thus contribute to HBV persistence.[4-7] How to break or bypass immune tolerance and induce anti-HBV immune responses is still a major challenge in the development of HBV therapeutic vaccines.

The correlation between high viral antigen load and dysfunction of the immune system in chronic infections has been documented.[7,8] A recent study further showed that the threshold of antigen expression is the dominant factor in determining the fate of T cells in the liver.[9] However, in certain mouse models, studies also demonstrated that some antigens of HBV could induce effective immune responses because of trace expression quantity or high immunogenicity, like the HBV nonstructure protein polymerase[10] and the "nontolerogen," hepatitis B core antigen (HBcAg).[11-13] These implied the potential alternative HBV vaccine candidates other than the tolerized HBsAg and hepatitis B e antigen (HBeAg) antigens. In HBV infection, the defective viral particles containing HBsAg generally outnumber infectious HBV virions by up to 1,000:1.[2] Unlike HBsAg, the preS1 region exists primarily in mature infectious HBV virions and thus its level is much lower than that of HBsAg.[14] Besides, the preS1 domain mediates the viral interaction with the cellular receptor for hepatocyte entry[15,16] and plays essential roles in the assembly and release of HBV virions,[17] making it a potential target for HBV therapy.

Though the B-cell and T-cell epitopes of the preS1 sequence are well characterized[18-20] and short preS1 peptides have been shown to protect from HBV infection to the chimpanzee,[21] the tolerized state of preS1 and its unique contribution for vaccination in chronic HBV infection has not been well defined. Given that the preS1 domain contains HBV-binding epitopes for cell entry and exists at much lower levels than HBsAg, we sought to determine whether it presented a much weaker tolerized status than that of HBsAg in CHB hosts, and thus may be feasible to efficiently induce immune responses to the preS1 domain for protective anti-HBV immunity. Indeed, we observed both antibody and specific T-cell responses to the preS1 region other than HBsAg in clinical CHB patients. The anti-preS1 antibody correlated well with the reduction of preS1 and HBV DNA. In a further study, a murine CHB model with persistent viremia and immune tolerance to the viral antigens was used.[22,23] We observed that the preS1-polypeptide was a potential immunogen in HBV carrier mice and its vaccination could clear HBV virions. Unexpectedly, we observed that preS1-polypeptide vaccination even reduced the tolerized status of HBsAg, providing a potential therapeutic strategy for treating CHB patients.

Materials and Methods

PATIENTS

In this study, patient clinical information is summarized in Table 1. Sera and peripheral blood

ARTICLE INFORMATION:

From the [1]IBP-UT Group for Immunotherapy, CAS Key Laboratory for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China; [2]University of Chinese Academy of Sciences, Beijing, China; [3]Research Center for Biological Therapy, Beijing 302 Hospital, Beijing, China; [4]Alphamab Co. Ltd., Suzhou, China; [5]Department of Pathology, UT Southwestern Medical Center, Dallas, TX; [6]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC; and [7]Treatment and Research Center for Infectious Diseases, 302 Hospital of Chinese PLA, Beijing, China.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Yang-Xin Fu, M.D., Ph.D.
Department of Pathology, UT Southwestern Medical Center
ND6.200A, UTSW
6000 Harry Hines Boulevard
Dallas, TX 75235-9072
E-mail: Yang-xin.fu@utsouthwestern.edu
Tel: + 1-216-648-6537
or

Hua Peng, Ph.D.
Key Laboratory of Infection and Immunity
Institute of Biophysics Chinese Academy of Sciences
15 Datun Road
Chaoyang District
Beijing 100101, China
Tel: + 86-10-64881152
E-mail: hpeng@moon.ibp.ac.cn

TABLE 1. Clinical Characteristics of Study Cohort

Patients	Age (years ± SD)	Sex (n = Male, %)	Viremia (IU/mL, ± SD)	HBeAg (n = Positive, %)	Anti-HBeAg (n = Positive, %)	ALT (IU/mL ± SD)	AST (IU/mL ± SD)
HC (n = 8)	44.43 ± 13.66	n = 4, 50	—*	n = 0, 0	—*	—*	—*
AHB (n = 10)	34.78 ± 11.44	n = 6, 60	1.46E+06 ± 2.99E+06	n = 2, 20	n = 8, 80	757.00 ± 437.67	337.78 ± 302.92
CHB (n = 25)	25.2 ± 8.89	n = 19, 76	1.55E+08 ± 1.5E+08	n = 25, 100	n = 0, 0	225.28 ± 337.88	110.24 ± 126.34

*Data not tested.

mononuclear cells (PBMCs) isolated from blood samples of healthy controls (HC; n = 8), acute hepatitis B (AHB) patients (n = 10), and CHB patients (n = 25) were collected and stored by the Beijing 302 Hospital (Beijing, China). Serum levels of anti-HBs and anti-preS1 were measured by enzyme-linked immunosorbent assay (ELISA). HBsAg, HBeAg, and preS1 antigen levels were measured by ELISA. HBV DNA in serum was measured by qPCR. Consent for collection of serum and PBMC samples was given by each patient in writing and authorized by the hospital ethics review committee.

MICE AND REAGENTS

C57BL/6 mice were purchased from Vital River Laboratories Animal Technology Co. (Beijing, China). HLA-A2.1 transgenic mice (C57BL/6-Tg(HLA-A2.1)1Enge/JNju) were purchased from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Mice were maintained under specific pathogen-free condition in the BSL-2 + animal facility, and animal experiments were followed with protocol no. DWSWAQ_ (ABSL-2) 2012205 at the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China). Six- to 8-week-old male mice were used in all experiments. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Biomedical Research Ethics Committee of the Institute of Biophysics of the Chinese Academy of Sciences.

Recombinant proteins used in this study include PreS1 standard-polypeptide (PrimeGene Biotechnology Co., Ltd, Shanghai, China), recombinant HBsAg (rHBsAg; Key-Biotechnology Co., Ltd, Beijing, China) for vaccination, and HBsAg (ayw serotype; PrimeGene Biotechnologya) for Enzyme-Linked ImmunoSpot (ELISPOT) testing. The large-HBsAg (L-HBsAg)-containing protein for vaccination was a commercial product that was purified from patient sera (Key-Biotechnology). Both the commercial standard preS1 and the lab-produced preS1 polypeptides are the proteins of 12 Kdalton and contain only the sequence of the preS1 domain of HBV large-HBsAg, which is comprised of preS1, preS2, and S regions. ELISA kits were used for testing HBsAg, HBeAg (Shanghai Kehua Bio-engineering Co., Ltd, Shanghai, China), and preS1 (Shanghai Alpha Biotechnology Co., Ltd, Shanghai, China). For the ELISA to test anti-preS1, plates were coated with the commercial standard preS1 protein at 2 μg/mL in phosphate-buffered saline and samples were added at 1:10, 1;100, and 1:1,000 dilutions. Then, a secondary antibody of goat-anti-mouse immunoglobulin G (IgG; horseradish peroxidase [HRP] conjugated; Cwbiotech, Beijing, China) was added for chromogenic reaction. The purchased anti-preS1 antibody was an IgG2a monoclonal antibody, which was raised against HBV preS1 antigen (Santa Cruz Biotechnology, Santa Cruz, CA). The anti-preS1 XY007 monoclonal antibody (mAb) was screened and produced in our lab by yeast display techniques. The peptides for anti-HBs (ayw) testing 111-140 amino acids (PGSSTTSTGPCRTCMTTAQ_GTSMYPSCCCT) were synthesized by China Peptides Co., Ltd (Shanghai, China).

VIRUS AND ADENO-ASSOCIATED VIRUS/HBV1.3 INFECTION

The HepG2-*hNTCP* cell line and HBV-D (subtype, ayw) virus were kindly provided by Professor Wenhui Li (National Institute of Biological Sciences, Beijing, China). The AAV-HBV1.3 virus was purchased from the Beijing FivePlus Molecular Medicine Institute (Beijing, China). This recombinant virus carries 1.3 copies of the HBV genome (genotype D, serotype ayw) and is packaged in adeno-associated virus (AAV) serotype 8 capsids. Adult C57BL/6 and C57BL/6-Tg (HLA-A2.1) mice were injected with

the experimentally indicated amounts of recombinant virus (diluted to 200 μL with saline) by tail vein injection. After 4 weeks or more, stable HBV carrier mice were used for vaccination.[22] Mice were bled through the ophthalmic vein at the indicated time points in the respective experiments to monitor HBsAg, HBV preS1 antigen (preS1), HBs antibody, preS1 antibody, and HBV genomic DNA (gDNA) in serum.

PreS1-POLYPEPTIDE VACCINE PREPARATION

The coding sequence of preS1-polypeptide was synthesized by PCR from complimentary DNA (cDNA) extracted from livers of AAV-HBV1.3-infected mice. We used the following primers: 5′-CG*GGATCC*at ggggcagaatctttccacca and 3′-CCG*CTCGAG*ctaggcct gaggatgagtgtttct.[24] Then, the sequence was subcloned into plasmid SUMO-pET-28a to yield the expression plasmid. The plasmid was transformed into *Escherichia coli BL21(DE3)* cells. Expression of fusion protein was induced by 1 mmol/L of isopropyl-beta-D-thiogalactopyranoside at 37°C and left overnight. Then, the induced cells were harvested and sonicated. The supernatant of the cell lysates was purified by Ni-NTA (GE Healthcare, Wilkes-Barre, PA) column chromatography. Finally, the 6 × His-SUMO (Small Ubiquitin MOdifier) tag was cut by SUMO-protease, and preS1 was separated by molecular size exclusion and ion-exchange chromatography (Superdex-75, and HiTrap QFF; GE Healthcare).

VACCINATION

Alum adjuvant (Alhydrogel 2%) and MPLA Synthetic VacciGrade were purchased from InvivoGen (Toulouse, France). CpG-1826 (TCCATGACGTT CCTGACGTT) was synthesized by Life Technologies Corporation (Carlsbad, CA, USA). Incomplete Freund's adjuvant (IFA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Supporting Fig. S1). The adjuvants were mixed with 5 μg of rHBsAg or 10 μg of preS1 for use in vaccination. All vaccines were injected subcutaneously.

HBV-DNA AND RNA DETECTION

Serum HBV DNA was extracted from 200 μL of serum and measured following the manufacturer's instructions (careHBV; Qiagen, Hilden, Germany). Liver HBV DNA was extracted from 50 mg of liver tissue using a gDNA kit (Tiagen Biotech, Beijing, China). Total RNA was extracted from livers of AAV-HBV1.3-infected mice with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed (RT-PCR) using the RevertAid First Strand cDNA synthesis Kit (Thermo Scientific, Waltham, MA, USA). Samples were analyzed by using qPCR with the following HBV-specific primers: HBV-HBsAg–real–F:5′-CACATCAGGATTCCTA GGACC-3′; HBV-HBsAg-real-R: 5′-GGTGAGT GATTGGAGGTTG-3′. HBV-3.5kb-real-F:5′-GA GTGTGGATTCGCACTCC- 3′; HBV-3.5kb-real–R:5′-GAGGCGAGGGAGTTCTTCT-3′. HBV-total-real-F:5′-TCACCAGCACCATGCAAC-3′; and HBV-total-real-R:5′-AAGCCACCCAAGGCA CAG-3′. Real- time PCR was performed using the SYBR Premix Ex Taq kit (Takara, Japan) with an ABI Fast 7500 Real-Time PCR System.

HBV INFECTION AND INHIBITION ASSAYS *IN VITRO*

In vitro HBV infection and inhibition assays were performed as reported.[16] Briefly, 1×10^7 copies of genome-equivalent HBV were inoculated into culture medium of 1×10^5 HepG2-*hNTCP* cells in 48-well plates in the presence of the preS1 antibody or anti-preS1 serum and incubated for 24 hours. Cells were then washed with medium three times and maintained in primary hepatocytes maintenance medium (PMM) medium (William's E medium [Gibco, Waltham, MA, USA] with 5 μg/mL of transferrin, 5 ng/mL of sodium selenite, 3 μg/mL of insulin [insulin/transferrin/sodium selenite; Corning, NY, USA], 2 mM of L-glutamine, 10 ng/mL of epidermal growth factor [Sigma-Aldrich, St Louis, MO, USA], 2% dimethyl sulfoxide, 100 U/mL of penicillin, and 100 μg/mL of streptomycin). Medium was changed every 2 days. Viral infection at different time points was analyzed by measuring viral antigen and viral RNAs in culture medium.

ELISPOT ASSAY

Lymph nodes (LNs), spleen, and liver were harvested at the indicated time points after immunization with rHBsAg or preS1-polypeptide vaccines. Intrahepatic lymphocytes were isolated by enzymatic digestion. Briefly, liver tissues were digested by collagenase IV (Roche, Basel, Switzerland) at 37°C for 15 minutes.

The suspension was centrifuged at 30*g* for 1 minute to remove hepatocytes. Lymphocytes were then pelleted by centrifugation at 400*g* for 10 minutes and further purified with 40% and 70% Percoll solutions by centrifugation at 800*g* for 20 minutes at room temperature. Cells were collected from the interface, and red blood cells were removed with ammonium-chloride-potassium buffer to make a single-cell suspension.

CD4-deleted and CD8-deleted lymphocytes were collected through magnetic separation with anti-APC (allophycocyanin) Microbeads (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), following the manufacturer's protocol. Briefly, splenocytes were first stained with the APC-conjugated primary antibody, and cells were then magnetically labeled with anti-APC microbeads. Then, the cell suspension was loaded onto a magnetic cell separation system (MACS) column, which was placed in the magnetic field of a MACS separator. The magnetically labeled $CD4^+$ or $CD8^+$ cells were retained within the column. The unlabeled cells were collected as CD4-deleted or CD8-deleted cells.

PBMCs from HBV patients were stored in liquid nitrogen. And after being thawed at 37°C, lymphocytes were pelleted by centrifugation at 400*g* for 5 minutes in 10 mL of complete medium. Then, cells were put into medium to make a single-cell suspension. For detecting antigen-specific immune response, lymphocytes were incubated for 48 hours at 37°C in complete medium containing full-preS1 protein or HBsAg peptide pools that covers all identified HBsAg T-cell epitopes in an interferon-gamma (IFNγ) ELISPOT plate (Merck Millipore, Billerica, MA). After incubation, the IFN-γ secretion was analyzed using a biotinylated anti-IFN-γ antibody and streptavidin-HRP (BD Biosciences, Franklin Lakes, NJ). Finally, the spots were visualized with 3′-amino-9-ethylcarbazole substrate and quantified with an autoanalyzing system.

STATISTICAL ANALYSIS

Error bars in data represent mean ± SEM. Data were analyzed using an unpaired two-tailed *t* test or two-way analysis of variance (ANOVA) by the GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$). The correlation between HBsAg and preS1, anti-preS1 and preS1, and anti-preS1 and HBV DNA in clinical HBV patients was analyzed using two-tailed correlation test. A value of $P < 0.05$ was considered a statistically significant correlation.

Results

PreS1 DOMAIN PRESENTS MORE IMMUNOGENICITY THAN HBsAg IN CLINICAL CHB PATIENTS

To dissect the tolerized status toward preS1 domain and HBsAg in HBV infection, we compared the levels of antigens and antibodies in clinical HBV patients. We observed that preS1 antigen presented in much less quantity (~10-fold) than HBsAg in serum (Fig. 1A). Anti-preS1, but not anti-HBs, was detectable in both AHB and CHB patients (Fig. 1B), corresponding to the specific T-cell response to preS1 domain, but not HBsAg, in CHB patients (Fig. 1C). All these suggest that CHB hosts have less tolerance toward the preS1 domain than HBsAg.

In the clinic, the appearance of anti-preS1 in patients implies a better recovery from AHB.[25-27] So, we investigated whether there were relationships between anti-preS1 and reduction of HBV infection. We observed that anti-preS1 was negatively correlated with preS1 antigen (Fig. 1D), as well as HBV DNA (Fig. 1E). The results implied that the immune responses to the preS1 domain might be associated with a potential recovery from CHB infection. All these raise the possibility that preS1, presenting in less quantity, together with its essential role in formation of HBV virion, might serve as an immunogenic antigen and thus be an effective therapeutic vaccine to clear HBV virions in CHB infection.

THE LEVEL OF PreS1 ANTIGEN IS SIGNIFICANT LOWER THAN THAT OF HBsAg IN THE MURINE HBV MODEL

We have reported a murine model with intravenous infection of AAV-HBV1.3, which partially mimics immunological characteristics of chronic HBV infection.[22,23] Tolerance to HBsAg was induced by its level in serum and could be reversed by reducing its titer with a neutralizing mAb, leading to immune responses to the conventional HBsAg vaccine.[28] We compared the antigen levels of preS1 and HBsAg in the same model. PreS1 antigen was 10-fold lower than HBsAg in the peripheral (Fig. 2A), regardless of the HBV infection doses (Fig. 2B), similar to that in clinical patients. We

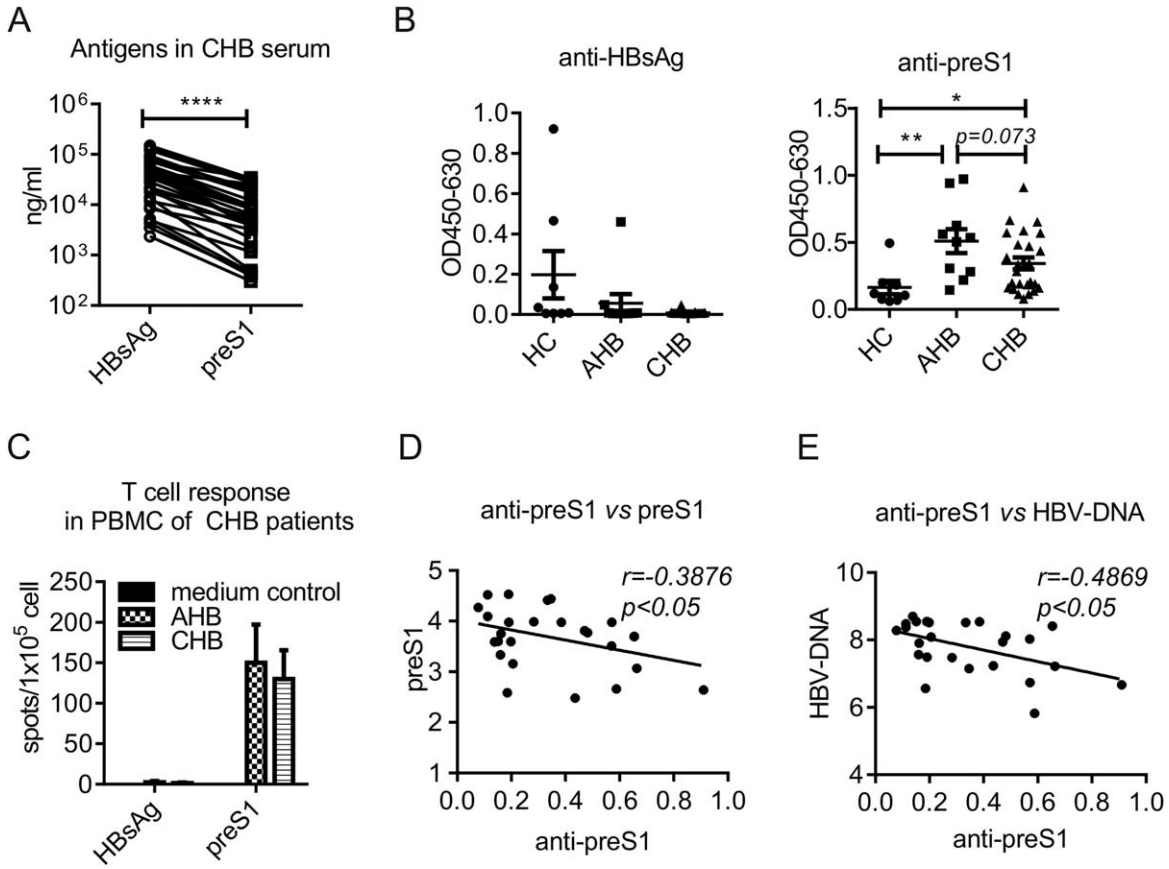

FIG. 1. PreS1 domain is less immune tolerized than HBsAg in CHB patients. (A) HBsAg and preS1 antigen levels in CHB patients (n = 25) were tested by ELISA. The correlation between HBsAg and preS1 was analyzed using a two-tailed correlation test. (B) Anti-HBs and anti-preS1 in HC (n = 8), AHB (n = 10), and CHB (n = 25) were tested by ELISA (dilution 1:10). (C) PBMCs from patients (AHB, n = 2; CHB, n = 14) were collected and then stimulated with 5 μg/mL of HBsAg or preS1. Specific T-cell response to HBsAg and preS1 were tested by IFN-γ secreting ELISPOT. (D,E) In CHB patients, the correlation between anti-preS1 (OD450-630) and preS1 ($\log_{10}$ ng/mL) and the correlation between anti-preS1 (OD450-630) and HBV DNA ($\log_{10}$ IU/mL) were analyzed. Error bars in data represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$ by a two-tailed correlation test (A,D,E) or unpaired *t* test (B). Abbrevistion: OD, optical density.

also observed that preS1 antigen presented in much less quantity than HBsAg in liver (Fig. 2C).

PreS1 domain has been reported to be contained mainly on HBV virions in clinical samples.[14] To confirm this in the murine model, HBV carrier mice were intraperitoneally injected with the mAb, XY007, specific to the preS1 region. We observed that XY007 efficiently cleared both serum preS1 antigen and HBV DNA to an undetectable level, but there was no significant change for level of HBsAg (Fig. 2D). Thus, the preS1 antigen presents in low quantity in the HBV murine model, similar to that in CHB patients. All these raises the possibility that preS1 might function as a potential breakthrough point for breaking HBV immune tolerance.

PreS1 DOMAIN IS NOT TOLERIZED IN HBV CARRIER MICE

We first determined the immunogenicity of the preS1 domain by vaccinating C57BL/6 mice with preS1-polypeptide formulated in the indicated adjuvants (Supporting Fig. S1). Then, the immune responses of preS1-polypeptide vaccine and the conventional HBsAg vaccine were compared in HBV carrier mice. As mentioned, though HBsAg vaccine elicits strong immune response in wild-type (WT) mice, it did not induce HBsAb seroconversion in carrier mice (Fig. 3A). And there was no induction of specific T-cell response to HBsAg in contrast to its

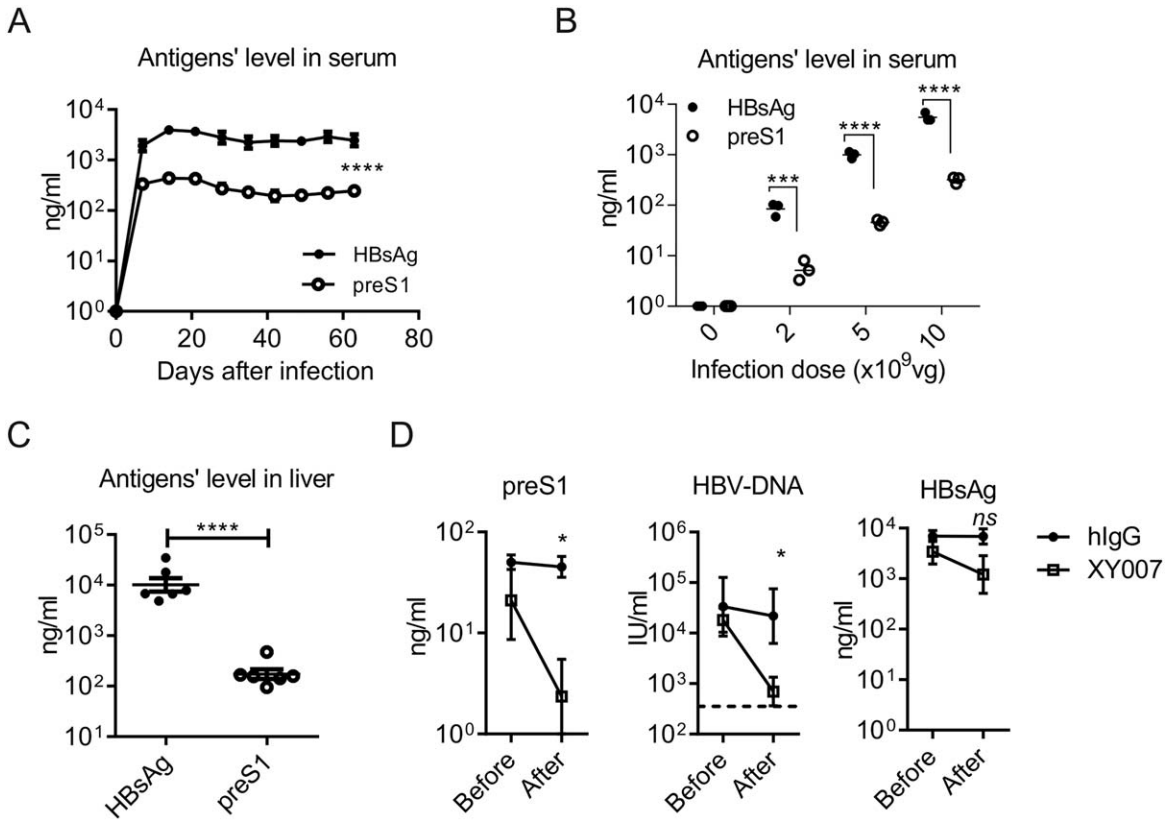

FIG. 2. PreS1 antigen is much lower than HBsAg in HBV carrier mice. (A) C57BL/6 mice (n=4/group, 6-8 weeks old, male) were intravenously infected with 1×10^{10} viral genome equivalents(vg) of AAV-HBV1.3 viruses in 200 μL of saline. Blood samples were collected every week after infection. Antigen levels of HBsAg and preS1 were detected by ELISA. (B) Three doses of virus ranging from 2×10^9 to 1×10^{10}vg were delivered to C57BL/6 mice (n = 3/group, 6-8 weeks old, male). Four weeks later, the antigen level in the serum was measured by ELISA. (C) C57BL/6 mice were infected with 1×10^{10} vg of viruses. Mice were sacrificed to collect liver tissue 8 weeks later. Protein levels of preS1 antigen and HBsAg in grinded tissue were detected by ELISA. (D) Carrier mice were intraperitoneally injected with 200 μg of mAb XY007 specific to the preS1 domain, and 1 day later, preS1 and HBsAg antigen levels in serum were tested by ELISA; HBV DNA in serum was extracted and was tested by qPCR as the manufacturer mentioned. One representative result of three independent experiments for panel A (N = 12/group), or four for panel B (N = 16/group), or three for panel D (N = 15/group) is shown. Error bars in data represent mean ± SEM. "*ns*" means "no significant difference." $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$ by two-way ANOVA (A) or unpaired *t* test (B,C,D). Abbreviation: hIgG, human immunoglobulin G.

robust immune response observed in WT mice (Fig. 3B). Thus, inducing HBsAb is a challenging clinical goal for the conventional HBsAg vaccine to achieve because of the immune tolerance induced by the circulating HBsAg.

However, unlike the tolerized HBsAg antigen, HBV carriers that were vaccinated with preS1-polypeptide generated specific antibodies to the preS1 domain, the level of which is comparable to that in WT mice (Fig. 3C). We also determined the specific T-cell responses to the preS1 region in draining LN, spleen, and liver after preS1-polypeptide vaccination, all of which were similar to the responses developed in WT mice (Fig. 3D). These results suggest that, contrary to HBsAg, the preS1 domain is not a viral tolerogen in the HBV carrier model and could be used as a therapeutic vaccine for CHB infection.

PreS1-POLYPEPTIDE VACCINATION EFFECTIVELY PREVENTS HBV INFECTION

In the clinical setting, the preS1 domain alone had never been used as a preventive vaccine for HBV infection. To determine whether preS1-polypeptide

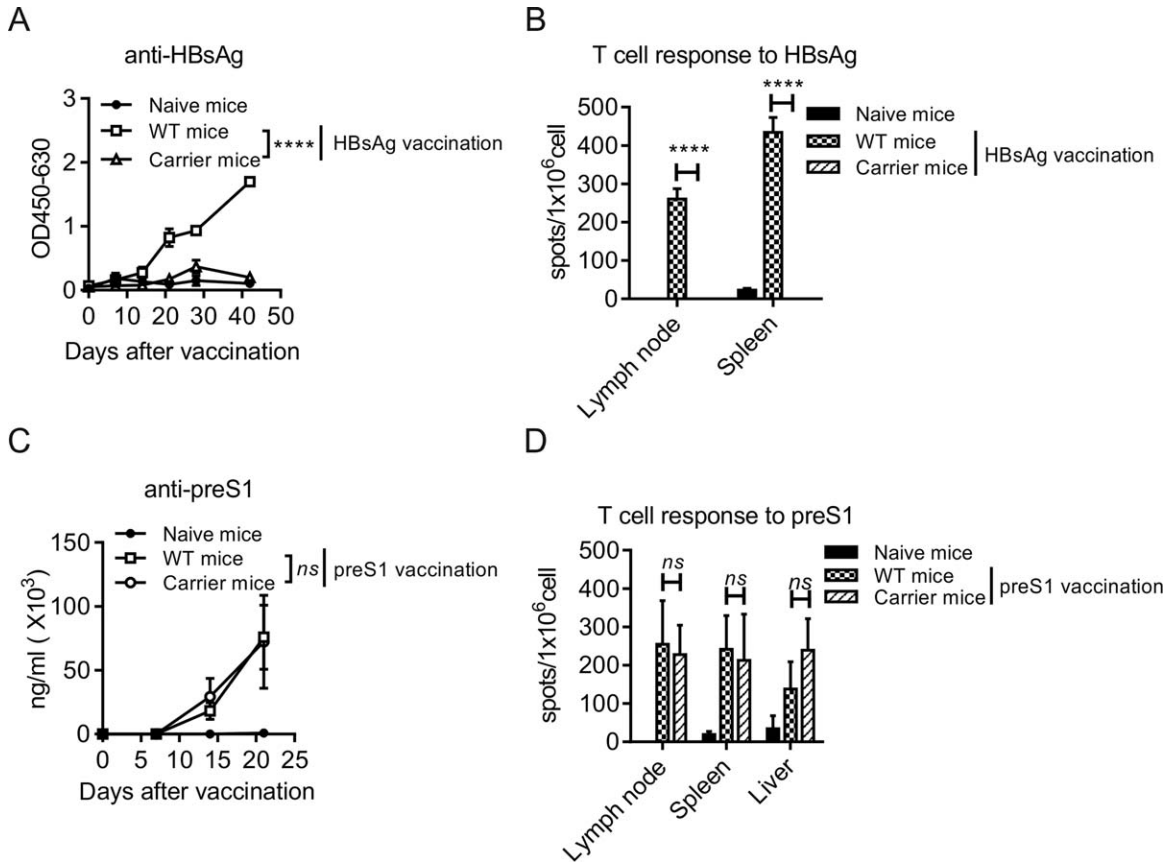

FIG. 3. Unlike HBsAg, the preS1 domain is not tolerized in HBV carrier mice. C57BL/6 (n = 4/group, 6-8 weeks old, male) WT, and stable HBV carrier mice were vaccinated with preS1-polypeptide or HBsAg formulated in IFA adjuvant, respectively. (A) Anti-HBs in serum after HBsAg vaccination in WT and carrier mice were determined at the indicated time points by ELISA. (B) In HBsAg-vaccinated WT and carrier mice, 2×10^5 lymphocytes from LN and spleen were collected 28 days after the second vaccination. Then, cells were stimulated with 5 μg of full-length HBsAg or BSA, and the specific T-cell responses to HBsAg were tested by IFN-γ ELISPOT. (C) Anti-preS1 in serum after preS1-polypeptide vaccination in WT and carrier mice were determined at the indicated time points by ELISA. (D) In preS1-polypeptide-vaccinated WT and carrier mice, 2×10^5 lymphocytes from LN, spleen, and liver were collected 28 days after the second vaccination. Then, cells were stimulated with 5 μg of preS1 polypeptide or BSA. Specific T-cell responses to the preS1 domain were tested by IFN-γ ELISPOT. One representative result of three (WT mice, N = 14) or four (carrier mice, N = 14) independent experiments is shown. Error bars in data represent mean ± SEM. "*ns*" means "no significant difference." $^{****}P < 0.0001$ by two-way ANOVA (A,C) or unpaired *t* test (B,D). Abbreviations: BSA, bovine serum albumin; OD, optical density.

vaccination could be used for HBV prevention, mice were inoculated subcutaneously with preS1-polypeptide as the indicated time schedule in Fig. 4A. This vaccination induced preS1-specific antibody (Fig. 4B) and cleared the preS1 antigens in serum after AAV-HBV infection, similar to the clinical HBsAg vaccination (Fig. 4C). Most important, the HBV DNA in serum was also undetectable at the end of the experiment (Fig. 4D), indicating the viral clearance by endogenous antibody to the preS1 domain. These results demonstrate that preS1-polypeptide vaccination can effectively prevent HBV infection *in vivo*.

As HBV is a hepadnavirus that infects only humans and a few primates,[29] mouse models cannot be used to test the direct HBV infection/reinfection to the host liver *in vivo*. To investigate whether the preS1 antisera could block HBV entry/re-entry to hepatocytes, we infected HepG2-*hNTCP* cells with HBV viruses in the presence of anti-preS1 serum and incubated for 24 hours (Fig. 4E), as reported.[16] The production of both HBsAg and HBeAg in cell culture media was significantly reduced when anti-preS1 serum was added, but not the control antibody (Fig. 4F). It indicated that the HBV infection to hepatocytes was

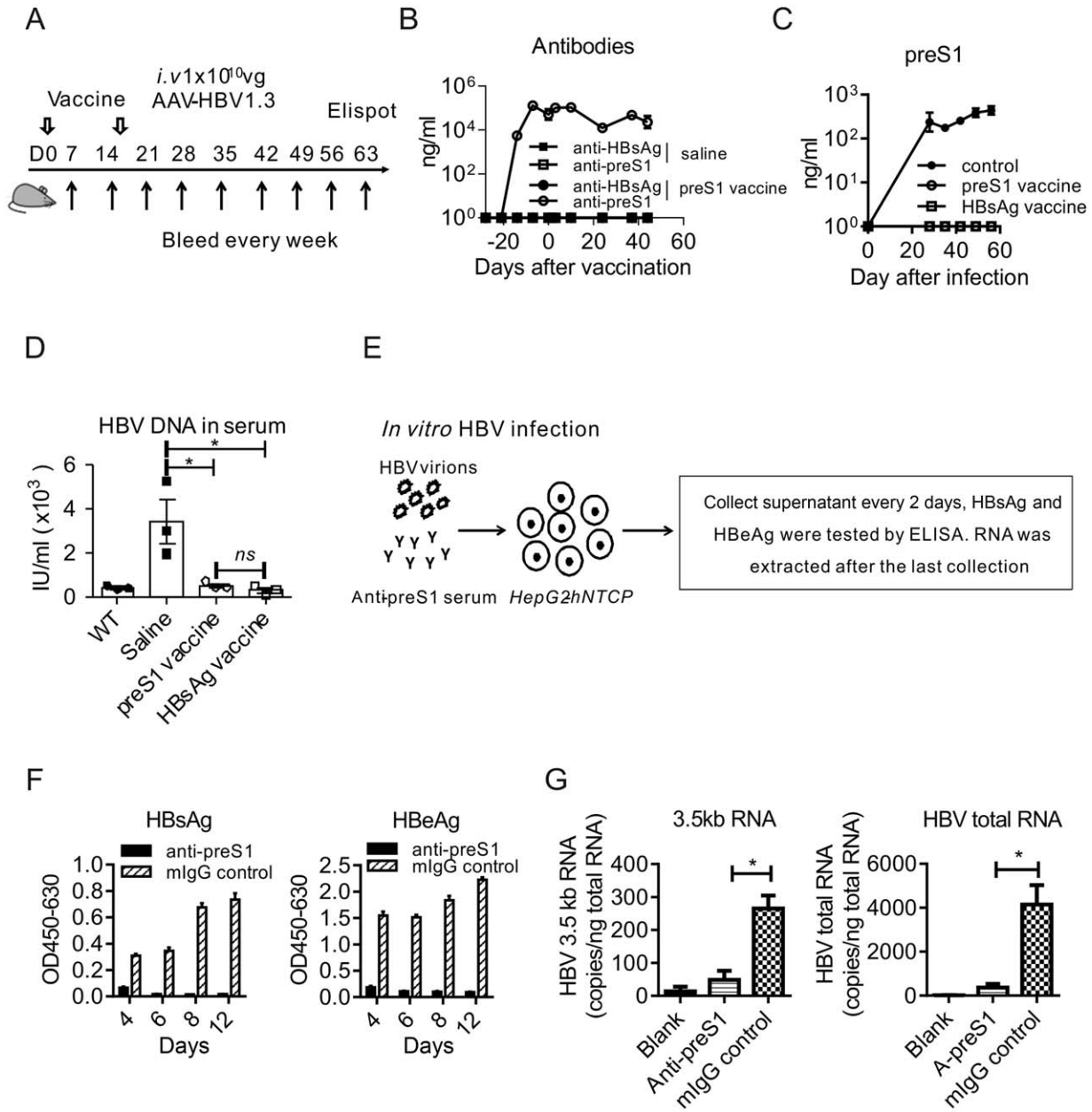

FIG. 4. PreS1-polypeptide functions as an effective preventive vaccine for HBV infection. (A) Time schedule for preS1-polypeptide vaccination to prevent HBV infection. (B) Only anti-preS1, but no anti-HBs, was detected after preS1 vaccination and HBV infection in preS1-polypeptide-vaccinated mice. (C) PreS1 antigen level in serum after HBV infection was tested by ELISA. (D) HBV DNA in serum was extracted per the manufacturer's instructions and tested by qPCR. (E) Schematic diagram of blocking HBV infection to hepatocytes *in vitro*. Briefly, HepG2-*hNTCP* cells were inoculated with 1×10^7 genome equivalents of HBV in the presence of anti-preS1 serum or control mouse IgG and incubated for 24 hours. Then, cells were washed with medium three times and maintained in PMM medium. The supernatant of the culture was collected and medium was changed every 2 days. Levels of HBsAg (F) and HBeAg (G) in the supernatant were measured by ELISA. HBV viral RNAs in infected cells were extracted at the indicated time points; HBV-specific 3.5-kb RNA (H) and HBV total RNA (I) were quantified by qPCR. One representative result of three independent experiments is shown for panels A, B, C, and D (N = 12/group) and for panels E, F, G, H, and I (N=18 wells/group). Error bars in data represent mean ± SEM. "*ns*" means "no significant difference." $^*P < 0.05$ by unpaired *t* test (D,H,I). Abbreviations: IgG, immunoglobulin G; OD, optical density.

blocked by antisera to the preS1 domain. This was further demonstrated by the reduction of HBV viral replicative intermediates, including the 3.5-kilobase (kb) HBV RNA and the total HBV RNA (Fig. 4G). To further confirm that anti-preS1 alone could inhibit the infection and subsequent replication of HBV in hepatocytes, we repeated the experiment by mAb XY007 (Supporting Fig. S2). Thus, using the murine HBV model and HepG2-*hNTCP* infection system, we demonstrated that preS1-polypeptide was potentially an effective vaccine for preventing HBV infection.

PreS1-POLYPEPTIDE SERVES AS A THERAPEUTIC VACCINE IN HBV CARRIER MICE

To determine the therapeutic effects of preS1 polypeptide vaccine, we challenged HBV carrier mice with preS1 vaccine, and boosted with the same dose 14 days later. Antigen and antibody responses were tested at the indicated time points (Fig. 5A). As mentioned, HBsAg vaccine could not induce immune response in HBV carrier mice. Even on 35 days postvaccination with HBsAg, none of the carrier mice showed a significant reduction in preS1 antigen (Fig. 5B) and HBV-DNA in serum (Fig. 5C). On the contrary, preS1-polypeptide vaccination induced anti-preS1 immune response, and then preS1 antigen decreased sharply after induction of anti-preS1. It was cleared completely on day 14 postvaccine boost (Fig. 5B). Moreover, anti-preS1 resulted in HBV-DNA clearance in serum (Fig. 5C). The *in vitro* HBV inhibition assay showed that anti-preS1 in HBV carrier mice resulted in a significant reduction in the secretion of HBsAg and HBeAg in the HepG2-*hNTCP* cell *in vitro* system (Fig. 5D). RNA levels of HBV in HepG2-*hNTCP* also indicated that anti-preS1 induced in carrier mice blocked HBV infection/reinfection to hepatocytes efficiently (Fig. 5E).

We repeated the experiment in the HLA-A2.1/ transgenic HBV carrier mice model.[23] The same as in the C57BL/6 carrier model, vaccination with preS1 polypeptide, rather than HBsAg, induced clearance of the preS1 antigen. ELISPOT results for T-cell response corresponded well to antibody response (Supporting Fig. S3). Together, these results indicated that preS1 antigen is not tolerized in HBV carrier mice. The viral-specific immune response to the preS1 domain can clear HBV viral particles and potentially block HBV infection/reinfection to hepatocytes.

ADMINISTRATION OF PreS1-POLYPEPTIDE PARTIALLY RESTORES HOST IMMUNE RESPONSES TO HBsAg

In the study of applying preS1-polypeptide as preventive vaccination for HBV infection, we unexpectedly observed that the level of HBsAg significantly decreased (Fig. 6A). However, we could not detect significant anti-HBsAg in serum (Fig. 4B). The decrease, but not clearance, of HBsAg may result from the weak anti-HBsAg response induced by preS1-polypeptide vaccination, which could not completely neutralize the large-scale HBsAg circulating in serum. To test this hypothesis, we detected the specific B-cell response to HBsAg by ELISPOT and observed that preS1-polypeptide vaccination indeed induced HBsAg-specific antibody response (Fig. 6B). Given that the preS1 domain is always coexisting with HBsAg in HBV L-HBsAg during infection, the nontolerized preS1-specific T cells could cross-reactivate the tolerized B cells to HBsAg,[20] which thus might partially restore host immune response toward HBsAg. To test the hypothesis, we detected the serum level of HBsAg in carrier mice after preS1 vaccination. Intriguingly, the preS1-polypeptide vaccination definitely reduced HBsAg (Fig. 6C) and induced the specific B-cell response to HBsAg, even in HBV carrier mice (Fig. 6D).

Thus, we proposed that preS1 vaccination may open a therapeutic window for the hosts who are deeply tolerized to the HBsAg vaccine. We designed a sequential vaccine of preS1-polypeptide before HBsAg in HBV carrier mice to test this hypothesis. The vaccination schedule was as shown in Fig. 7A. Compared to HBsAg vaccination alone, the priming with preS1-polypeptide before HBsAg vaccine cleared both preS1 and HBsAg antigens in HBV carrier mice (Fig. 7B). We determined the antibody in serum and observed that such a combination induced specific B-cell response to HBsAg and made anti-HBs seroconversion (Fig. 7C), a marker for clinical cure to CHB infection. And, most important, specific T-cell response to HBsAg could also be detected both in spleen and liver (Fig. 7D). The specific T-cell response induced the reduction both of the HBV RNAs and DNA in hepatocytes (Fig. 7E-G). This response to HBsAg was also accompanied by the diminished immunohistochemical (IHC) staining of HBV core antigen in the liver (Fig. 7H). Thus, the strategy of priming with preS1 before HBsAg vaccination might

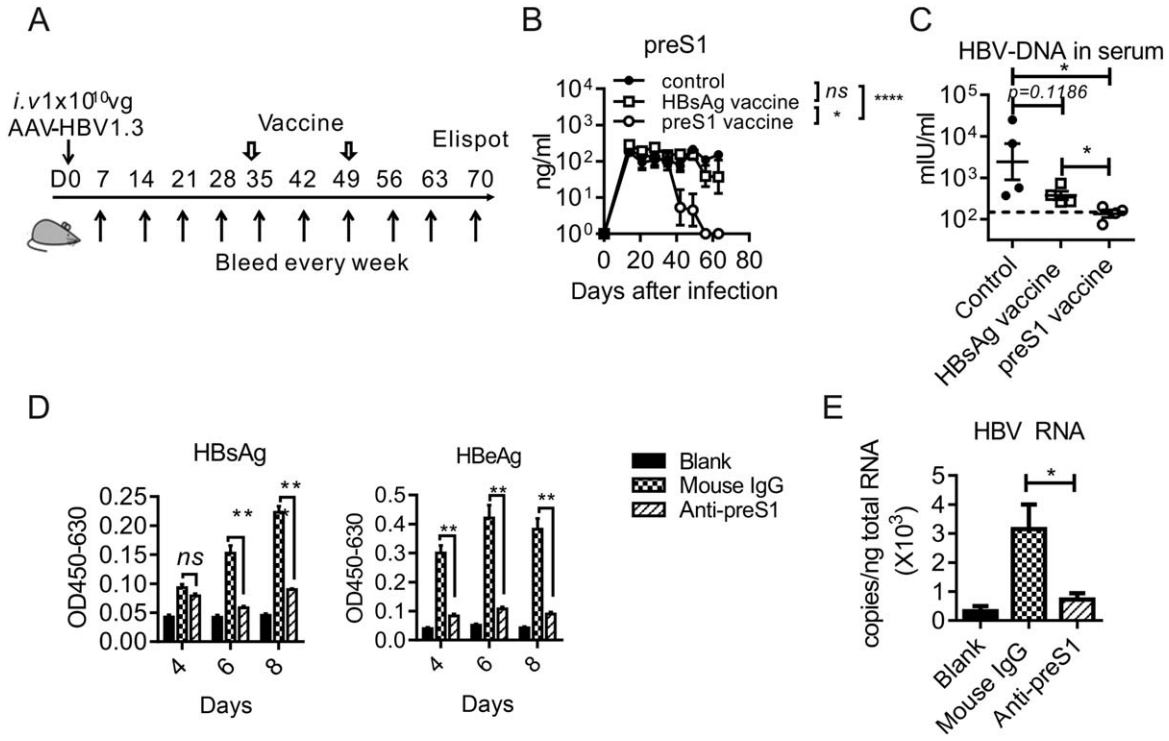

FIG. 5. PreS1-polypeptide functions as therapeutic vaccine in HBV in carrier mice. (A) Time schedule for testing preS1 antigen tolerance in HBV carrier mice. C57BL/6 (n = 4/group, 6-8 weeks old, male) WT, and stable HBV carrier mice were vaccinated with preS1-polypeptide or HBsAg formulated in IFA adjuvant, respectively. (B) After preS1-polypeptide or HBsAg vaccination in carrier mice, preS1 antigen levels in serum were determined by ELISA at the indicated time points. (C) HBV DNA in serum of each group was extracted and determined by qPCR. (D) Anti-sera induced in HBV carrier mice with preS1 vaccination blocked HBV infection to HepG2-*hNTCP in vitro*. Levels of HBsAg and HBeAg in supernatants were measured by ELISA at the indicated time points. (E) Total RNAs of infected cells were extracted on day 10 of HBV infection *in vitro*, and HBV-specific RNAs were measured with qPCR. One representative result of four independent experiments is shown for panels A and B (N = 21/group), three for panel C (N = 12/group), and three for panels D and E (N = 12 wells/group). Error bars in data represent mean ± SEM. "ns" means "no significant difference." $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ by two-way ANOVA (B) or unpaired *t* test (C,D,E). Abbreviations: IgG, immunoglobulin G; OD, optical density.

serve as an effective treatment for clinical CHB virus infection in the future.

Discussion

In this study, we have explored whether preS1-polypeptide vaccination is a potential treatment for CHB infection. We first analyzed the levels of preS1 antigen and HBsAg in clinical HBV patients and compared the immunogenicity of these two viral antigens in HBV carrier mice. The preS1 domain of L-HBsAg presents strong immunogenicity for both B-cell and T-cell responses in contrast to HBsAg, the major toleragen in CHB patients. Actually, the appearance of anti-preS1 indicated a potential recovery from HBV infection. By using the HBV carrier model, we confirmed the immunogenicity of preS1 polypeptide. The anti-preS1 induced by preS1-polypeptide cleared HBV DNA in carrier mice and blocked HBV infection/reinfection to hepatocytes. Furthermore, preS1-polypeptide vaccination even weakened HBsAg tolerized status and the subsequent vaccination with HBsAg could induce anti-HBs seroconversion in HBV carrier mice.

Unlike preS1 polypeptide, HBsAg has been widely used as a prophylactic vaccine against HBV, but it cannot induce immune responses to clear HBsAg in CHB patients and even in some healthy recipients.[4,30-32] To further enhance vaccine immunogenicity and immune protection from HBV infection, the L-HBsAg containing preS1 domain has been included in

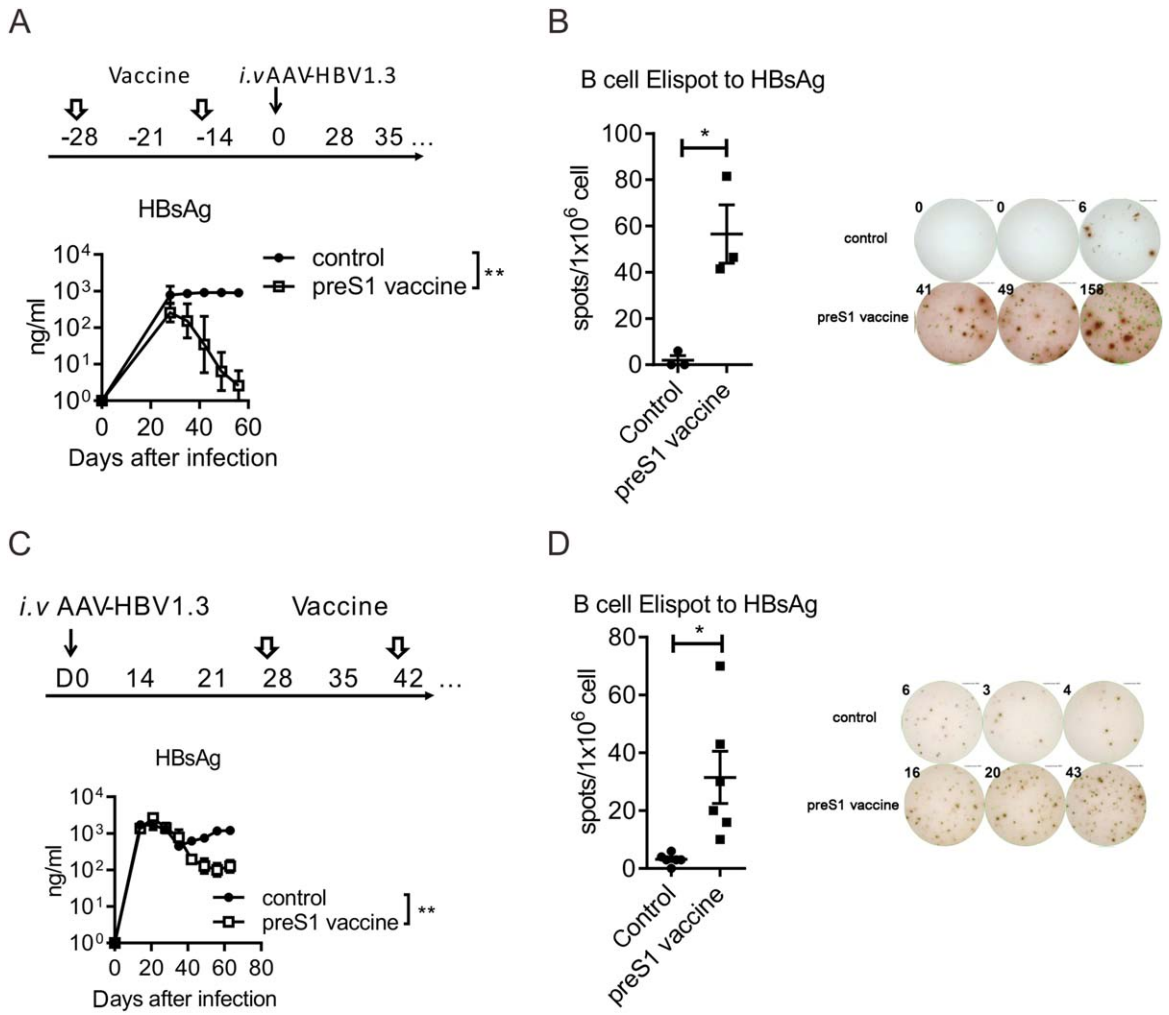

FIG. 6. PreS1-polypeptide vaccination induce the B-cell response to HBsAg in HBV mice. (A) After preS1-polypeptide vaccination, mice were infected with HBV and the level of HBsAg in serum was tested by ELISA, which indicated that preS1 prevaccination diminished HBsAg level in serum. (B) In preS1-prevaccinated mice, 2×10^5 splenocytes were collected 28 days after HBV infection. Specific B-cell response to HBsAg was tested by B-cell ELISPOT assay. (C) In HBV carrier mice, preS1-polypeptide was vaccinated at days 28 and 42 after AAV-HBV inoculation. HBsAg variations in carrier mice were tested by ELISA. (D) In preS1-polypeptide-vaccinated HBV carrier mice, 2×10^5 splenocytes were collected. Specific B-cell response to HBsAg was tested by B-cell ELISPOT assay. One (A,B,C) or two (D) representative results of three (N = 11/group) independent experiments are shown. Error bars in data represent mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$ by two-way ANOVA (A,C) or one-tailed *t* test (B,D). Abbreviation: *i.v*, intravenous.

the third-generation HBV vaccines.[33-35] However, by using the HBV carrier mouse model, we observed that, same as the conventional HBsAg vaccine, the L-HBsAg-containing protein vaccination showed no therapeutic effects in the tolerized model either (Supporting Fig. S4). Failing in induction of immune response to the preS1 domain by the L-HBsAg-containing protein vaccination might be attributed to an overwhelming immune tolerance to HBsAg, a physical link of preS1 domain to HBsAg, or too little amount of preS1 region containing in the vaccine.[36,37] It is also possible that alum, the currently clinically used adjuvant, might not be potent enough to induce detectable responses. Whether the preS1 region itself alone can be applied as a vaccine has not been directly tested. Here, we found that the antigenicity of preS1 was weaker than that of HBsAg; it can only induce robust responses with stronger adjuvants,

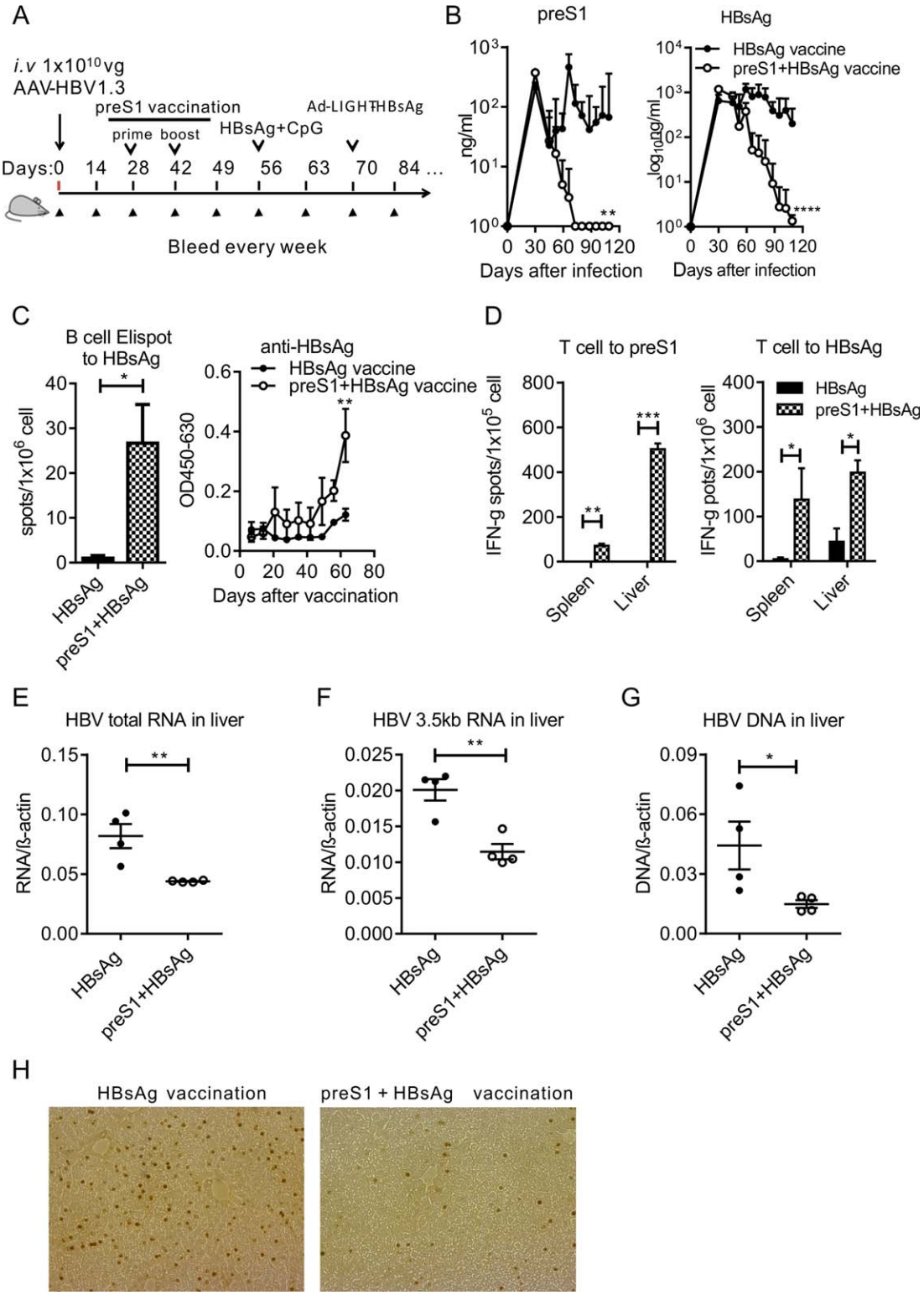

FIG. 7. HBsAg boost immunization amplifies preS1-polypeptide-induced anti-HBV response for viral clearance. (A) Time schedule for preS1-polypeptide combination with HBsAg as vaccine to treat HBV carrier mice. (B) After carrier mice were vaccinated with preS1-polypeptide+HBsAg, the preS1 and HBsAg antigens in serum were tested by ELISA. (C) B-cell response to HBsAg was tested by B-cell ELISPOT, and anti-HBs in serum was tested by ELISA. (D) Mice were sacrificed on the last day, and 2×10^5 lymphocytes from LN, spleen, and liver were collected. Then, cells were stimulated with 5 μg of preS1 polypeptide, HBsAg, or BSA, and specific T-cell response was measured by IFN-γ secretion T-cell ELISPOT assay. (E) HBV total RNAs and (F) intermediate products 3.5-kb RNA in liver tissue were determined with qPCR. (G) HBV DNA in liver was determined by qPCR. (H) IHC staining for HBcAg in hepatocytes (Magnification: 200X). One representative result of three (N = 11/group; B,C,D) or two (N = 7; E,F,G,H) independent experiments are shown. Error bars in data represent mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ by two-way ANOVA (B,C) or unpaired *t* test (D,E,F,G). Abbreviations: Ad, adenovirus; BSA, bovine serum albumin; i.v, intravenous; OD, optical density.

like IFA. However, this adjuvant limits preS1-polypeptide vaccination usage in the clinical setting. We are exploring several other formulation for preS1 polypeptide vaccination, including a nanovaccine that contains preS1 region to enhance its immunogenicity.

In the clinic, anti-preS1 appears early in the course of AHB.[38] Even the occasional appearance of anti-preS1 in a few patients with chronic aggressive hepatitis or treated with antiviral agents indeed correlates well with better health improvement.[39] However, the random clinical analyses of the appearance of anti-preS1 in CHB have not been conclusive for the correlation between changes in immune status and viral persistence. In CHB infection, soluble circulating HBsAg induces lymphocytes anergy that fail to receive the secondary danger signals for sustaining their activation. However, the preS1 domain is contained on HBV virions, which viral DNA can significantly enhance the immunogenicity of preS1. By comparing the immune status between preS1 and HBsAg in clinical patients, we observed that preS1 has much less immune tolerance. And the appearance of antibody and T-cell responses to the preS1 domain in clinical patients correlated with a better prognosis of the significant decrease of serum HBV DNA. Recently, an entry inhibitor peptide derived from the preS1 region, Myrcludex-B, has been investigated as a treatment for HBV infection. This peptide has been shown to inhibit HBV infection *in vivo* and hinder the amplification of the covalently closed circular DNA pool in initially infected hepatocytes.[40] Here, we demonstrated that preS1-polypeptide vaccination can induce a strong antibody response in HBV carrier mice, thus making it a unique candidate vaccine for treating HBV infection. Compared with Myrcludex-B, such vaccination would be much more potent in inducing intrinsic anti-preS1 and would provide long-term protection from HBV infection. Further clinical trials for targeting preS1 region are warranted.

In fact, $CD4^+$, but not $CD8^+$, T-cell responses were induced by preS1-polypeptide vaccination (Supporting Fig. S5A,B), with no liver damage indicated by normal alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum (Supporting Fig. S5C,D). Consistently, the only cytotoxic T lymphocyte (CTL) epitope in the preS1 region identified is for human HLA-A11-restricted.[41,42] Lack of CTLs after preS1-polypeptide vaccination may have liver escape from unexpected injuries, meanwhile the preS1-vaccine-induced anti-preS1 antibodies in the peripheral circulation can clear HBV virions, block HBV reinfection to healthy hepatocytes in patients, and thus dilute and even diminish the HBV-infected hepatocytes gradually. Unexpectedly, we observed that preS1-polypeptide vaccination partially restored immune response to HBsAg during HBV infection and thus opened a therapeutic window for the host to fully respond to the HBsAg vaccine. To amplify the response to HBsAg induced by preS1-polypeptide vaccination and induce stronger and multispecific T-cell responses for complete viral clearance in HBV carrier mice, the sequential combination of preS1-polypeptide before the HBsAg vaccination was administered. We showed that preS1-polypeptide priming before vaccination with HBsAg restored both the B-cell and T-cell response to HBsAg in the periphery and liver, ultimate resulting in the HBsAb seroconversion and gradual clearance of HBV in the liver. IHC staining also showed a dramatic decrease in the number of core-positive hepatocytes in livers of HBV carrier mice. This strategy indeed makes HBsAg, the preventive vaccine, achieve an unexpected therapeutic effect in HBV carrier mice. However, it is not yet clear how preS1 region vaccination helped to restore immune response to HBsAg. In HBV infection, the preS1 domain is always fused with HBsAg within L-HBsAg; thus, T cells to the preS1 region may help in the immune response to HBsAg.[20,43] Moreover, the immune complex of endogenous anti-preS1 with HBV virions may further enhance anti-HBV immune response. The function of the B-cell and T-cell responses to the preS1 domain in the restoration of anti-HBV immunity is under investigation. In summary, preS1-polypeptide vaccine can reverse HBV tolerance in HBV carrier mice and may serve as a potentially effective therapeutic strategy for treating CHB infection.

Acknowledgments: Y.B., Y.-X.F., and H.P. designed the experiments. We thank Dr. Wenhui Li for providing the HepG2-hNTCP cell line and HBV virus (ayw subtype) for HBV infection experiment *in vitro*. We thank Daryl Harmon for editorial assistance.

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Author names in bold designate shared co-first authorship.

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