

Supplemental Materials and Methods

Vaccine preparation

To produce the chimeric particulate antigens displaying the sA epitopes (HBC-S119-125, HBC-S113-127, HBC-S115-125, HBC-S113-135 and HBC-S111-148), the coding sequences of these peptides were cloned into the pC149/mut vector¹ by annealing oligos ordered from Sangon Biotech (Shanghai) Co., Ltd. (Supplemental Table 1). The expression and purification of these proteins were similar to those of pC149/mut¹. The antigens were mixed with alum adjuvant following the previously described protocol².

The CR-T3-SEQ13 gene fragment was synthesized by GENEWIZ, Inc. (Suzhou, China). The gene was cloned into the pTO-T7 expression vector, which was constructed in our lab³; correct plasmids expressing CR-T3-SEQ13 were transformed into *E. coli* strain ER2566¹. The CR-T3-SEQ13 protein and vaccine formulation used in this study were provided by our partner, Xiamen Innovax Biotech Co., Ltd.

Epitope mapping of antibodies induced by CR-T3-SEQ13

Design and synthesis of peptides. The alanine scanning epitope mapping strategy was used to identify key residues for antibody binding. Alanine was introduced for each residue of HBs-aa117-132; the peptides were synthesized by Xiamen Jingju Technology Co., Ltd. (Supplemental Table 2).

Polyclonal antisera and mAbs against CR-T3-SEQ13. The sera were collected from the CR-T3-SEQ13-immunized mice, rabbits and cynomolgus

monkeys for analysis of the characteristics of the polyclonal antibodies induced by CR-T3-SEQ13. The mAbs were obtained from immunized BALB/c mice using standard mouse hybridoma technology⁴ and screened by ELISA using CHO-HBsAg-coated 96-well plates (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.).

ELISA. The peptides were coated onto 96-well plates at a concentration of 100 ng/well. The binding activities of the polyclonal sera and mAbs were detected by indirect ELISA, and serial dilutions were prepared to find a proper concentration of samples that bound to SEQ13-WT with a readout OD value ($OD_{450nm}-OD_{630nm}$) that ranged from 1.0 to 2.0. Then, the fold change in the binding activity caused by each mutation was calculated as follows: $OD_{mut}/OD_{WT}-1$. Finally, a heatmap was created using GraphPad Prism 7.

Detection of HBV markers

The HBV DNA levels in the mouse serum specimens were measured using a real-time qPCR assay from Premix Ex Taq™ (Takara, Dalian, China). The primer sequences were 5'-TTTCACCTCTGCCTAATCAT-3' and 5'-TCAGAAGGCAAAAAGAGAGTAACTC-3'. The probe sequence was 5'-Hex-CCTTGGGTGGCTTTGGGGCATGGA -1-3'.

The HBsAg chemiluminescent quantitation kit and HBeAg quantitation ELISA kit were purchased from Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. The anti-HBs and anti-carrier titers were detected using indirect ELISA kits developed by Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.

For the immunohistochemistry assay, mouse livers were fixed in formalin (3.7% formaldehyde in PBS) for analysis. Intrahepatic HBsAg and HBcAg were detected using immunohistochemical staining with the anti-HBsAg mAb 83H12 in our lab and polyclonal rabbit anti-HBcAg (DAKO, B058601), respectively. The MaxVision™ HRP-Polymer IHC Kit (Maixin, Fuzhou, China) was used.

Detection of Anti-HBs and Anti-SEQ13 antibody by indirect ELISA

The titers of anti-HBs and anti-SEQ13 were measured by indirect ELISA. Wells were coated with the recombinant HBsAg protein (CHO cell-derived, Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China) or synthesized SEQ13 peptide (Jingju, Xiamen, China) at 200 ng per well, and nonspecific binding was blocked with phosphate-buffered saline (PBS) that contained 2% bovine serum albumin (BSA) and 10% sucrose. A series of 5-fold series dilutions of serum that ranged from 50-6250 were prepared. For the test, 100 μ l of specimens were added to the reaction well and incubated for 60 minutes at 37°C, followed by washing and reaction with horseradish peroxidase (HRP)-conjugated anti-mouse pAb (Thermo Scientific, Rockford, USA). After reaction of chromogenic substrate and stop solution, read the absorbance of each well at 450 nm and 630 nm. The maximum dilution fold that yield $(OD_{450\text{ nm}} - OD_{630\text{ nm}}) > 0.1$ will be used to calculate the antibody titer = $(OD_{450\text{ nm}} - OD_{630\text{ nm}}) / 0.1 \times$ dilution fold.

Cryo-EM and three-dimensional (3D) reconstruction of CR-T3-SEQ13

An aliquot of a 3 μ L purified CR-T3-SEQ13 sample (3mg/mL) was deposited onto a glow discharged Quantifoil holey carbon grid (R2/2, 200 mesh, Quantifoil Micro Tools). After 6 s blotting to remove extra sample, the grid was plunge-frozen into liquid ethane using a FEI Vitrobot. A total of 1704 cryo-EM images were recorded on an FEI Falcon II direct detector camera at 93,000 nominal magnification in an FEI TF30 microscope at 300 kV, with defocus settings determined to be between 0.8 and 3.2 μ m using GCTF⁵, and with an electron dose of ~ 25 e/ \AA^2 . Particles were then manually picked with the program Eman2⁶. The origin and orientation parameters for each of these particle images were estimated by means of model-based procedures and an initial model was generated with the random model method⁷. After several rounds of reference-free 2D and 3D classifications using Relion2.0⁸. 5886 good particles were selected for final 3D refinement with Relion2.0. The map resolution was determined based on gold standard criteria of 0.143 FSC cutoff⁹. Figures were prepared with UCSF Chimera¹⁰.

Combinational treatment experiments

The treatment groups included (1) ETV alone, (2) TDF alone, (3) CR-T3-SEQ13 alone, (4) ETV plus CR-T3-SEQ13, and (5) TDF plus CR-T3-SEQ13. The anti-HBV effects of these five regimens were assessed in the HBV-Tg mouse model. HBV-Tg mice were selected at 8 weeks of age, and 4 female and 4 male mice per group were treated as per the corresponding regimen. ETV or TDF powder was administered orally in the drinking water of the mice

(water consumption was calculated as 5 mL per day for each mouse). The average ETV dosage was 3.2 mg/kg/day, and the average TDF dosage was 100 mg/kg/day. Two weeks after oral administration of the analogues, CR-T3-SEQ13 was injected via the lateral muscles of the hind limbs. According to the immunization schedule, CR-T3-SEQ13 was given at weeks 0, 2, 3, 4, 5, and 6. The starting time point of the HBV injection was deemed week 0. The blood collection time points used to monitor the serum HBsAg and HBV DNA levels in the mice were days -14, -9, -6, and -3 and weeks 0, 1, 2, 3, 4, 5, 6, 7, and 8.

***In vivo* potency of anti-CR-T3-SEQ13 sera or purified polyclonal IgG**

To evaluate the broad spectrum anti-HBV efficacy of the polyclonal antibodies induced by CR-T3-SEQ13, sera from BALB/c mice immunized with CR-T3-SEQ13 were collected according to the following procedure. Twenty BALB/c mice were immunized with CR-T3-SEQ13 (12 µg/dose, a total of 6 injections) and sacrificed at week 8. To construct HBV carrier mice carrying HBV genotypes A, B, C and D, HBV replicon plasmids of the four predominant HBV genotypes were transferred into the livers of BALB/c mice by hydrodynamic injection through the tail vein. Then, the sera from the immunized BALB/c mice containing the polyclonal anti-CR-T3-SEQ13 Abs were injected via the tail vein (250 µL per mouse) into the HBV carrier mice for each of the four genotypes tested. Orbital blood was collected pretreatment (0) and at 2 and 12 hours posttreatment, and short-term dynamic changes in the HBsAg levels were monitored.

To further evaluate the *in vivo* anti-HBV efficacy of the antibodies induced by CR-T3-SEQ13 in cynomolgus monkeys, the resultant antibodies were purified from sera from immunized cynomolgus monkeys. Protein G medium was used to purify IgG polyclonal antibodies, and an indirect ELISA was performed to test the anti-HBs binding activity of the polyclonal antibodies. The activity was calibrated to a level equivalent to that of commercial HBIG (Sichuan Yuanda Shuyang Pharmaceutical Co., Ltd.). The binding titer of the monkey serum polyclonal antibodies obtained by purification in this experiment was equivalent to 10 IU/mL of HBIG. Purified polyclonal anti-CR-T3-SEQ13 antibodies or HBIG (1 mL) were injected into the HBV-Tg mice via the tail vein. Blood was collected at 12 h, 24 h, 2 d, 3 d, 4 d and 6 d post injection to monitor changes in the serum HBsAg levels in the mice.

***In vitro* neutralizing assay**

A HepG2-NTCP cell line that supported HBV infection was generated by a sleeping-beauty transposon-based system with co-expression of the fluorescent protein mCherry and puromycin via all-in-one plasmid transfection¹¹. The supernatant was collected from the HepAD38¹² cells and concentrated by ultracentrifugation, and the HBV inoculum was quantified by real-time PCR. For the HBV infection neutralization assay, a two-fold dilution gradient was created from 1/10 to 1/10240 for both the monkey antiserum and HBIG (1 IU/mL); then, the dilutions were mixed with the HBV inoculum and incubated for 1 hour at 37°C. The cells were infected with a 300- μ L mixture supplemented with 4%

polyethylene glycol 8000 and 2% DMSO; approximately 200 VEG/cell of HBV was used. The cultures were incubated overnight, and then the cells were gently washed three times with 1x phosphate-buffered saline (PBS) and cultured for an additional 6 days. To measure HBV infection, HBeAg in the culture supernatants was detected at day 6 using an HBeAg ELISA kit.

Statistical analysis

All experimental data are presented as the mean \pm SEM. A two-sided, unpaired t-test was used. Statistical significance was defined as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Significance is indicated in the figures.

References

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