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HBV antigen and DNA loss from mouse serum is associated with novel vaccine-induced HBV surface antigen-specific cell-mediated immunity and cytokine production

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ABSTRACT

Therapeutic vaccination is a promising strategy for controlling chronic hepatitis B virus (HBV). Here, we tested whether several novel vaccination strategies could be used to induce HBV-specific adaptive immune responses and control/eradicate HBV in a mouse model. Robust HBV antigen-specific antibody responses were elicited by several vaccination strategies using a novel particle vaccine (HBSS1), which expresses a fusion of the S (amino acids [aa] 1–223) and preS1 (aa 21–47) antigens, and/or a recombinant adenovirus rAdSS1 vaccine. However, antigen-specific cell-mediated immunity and high levels of production of multiple cytokines were elicited only by heterologous prime-boost immunization; *i.e.*, priming with the HBSS1 vaccine followed by a rAdSS1 boost. Furthermore, the most rapid loss of serum HBsAg, HBeAg and DNA was achieved by the novel vaccination regimen (priming with HBSS1 formulated with adjuvants [alum plus PolyI:C]), which was strongly associated with more potent and functional HBsAg-specific $CD4^+$ and $CD8^+$ T-cell responses and increased production of interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor-α, IL-12, and IFN-γ-induced protein (IP)-10. Thus, our novel heterogeneous prime-boost vaccine regimen shows promise as a therapeutic strategy against HBV.

1. Background

Despite the availability of an effective prophylactic vaccine since 1981, hepatitis B virus (HBV) infection remains a challenging global public health issue. It is estimated that, worldwide, 257 million people are living with chronic HBV infection and in 2015 an estimated 887,000 people died from complications of hepatitis B (Jackson et al., 2017). The current antiviral drug therapies for chronic hepatitis B (CHB) depend on interferon (IFN)-α or nucleos(t)ide analogs, but these have several limitations. IFN-α therapy only cures approximately 15% of patients treated and can have severe side effects; treatment with nucleos(t)ide analogs suppresses HBV replication but cannot eradicate the HBV genome from the liver. Moreover, long-term treatment with these antivirals may increase the risk of occurrence of drug resistance (Pawlotsky et al., 2008; Zoulim and Locarnini, 2009). Therefore, alternative strategies to treat CHB are urgently needed.

One potential treatment strategy for CHB infection is therapeutic vaccination. Host immune responses, including robust $CD8^+$ T-cell and neutralizing antibody responses, are crucial for the clearance of HBV (Dandri and Locarnini, 2012; Guidotti et al., 2015). In contrast, patients with CHB often show immune-tolerance to HBV proteins, with no production of anti-HBs antibodies and very low or undetectable HBV-specific cytotoxic T lymphocyte responses (Maini and Schurich, 2010). Thus, the use of therapeutic vaccines to stimulate an HBV-specific immune response in chronic HBV carriers is a potential strategy for the control of CHB. However, none of the therapeutic vaccines evaluated to

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date have been able to fully control HBV (Lobaina and Michel, 2017; Mancini-Bourgine et al., 2004; Xu et al., 2013).

Prime-boost vaccination has been used to increase therapeutic hepatitis B vaccine efficacy. Previous study has shown that Protein-prime/Modified Vaccinia virus Ankara (MVA) expressing hepatitis B virus (HBV) antigens-boost vaccination was able to overcome HBV-specific tolerance in HBVtg mice with low and medium but not with high antigenemia. HBV-specific antibody titers, $CD8^+$ T-cell frequencies and polyfunctionality inversely correlated with HBV antigen levels (Backes et al., 2016). We previously constructed a novel protein vaccine (HBSS1) and a recombinant adenoviral vector vaccine (rAdSS1), both of which express a fusion of the S (amino acids [aa] 1–223) and preS1 (aa 21–47) antigens. And our previous study focused on preventive efficacy in immunized mice (naive C57BL/6) with various vaccine regimens, including protein-vaccine HBSS1 with mixed poly(I:C)/alum adjuvant priming, followed by a rAdSS1 vaccine boost (Chuai et al., 2013). Furthermore, the immunized mice were challenged by hydrodynamic injection (HI) of pCS-HBV1.3. High titers of antigen-specific antibodies and neutralizing activity, as well as robust multi-antigen (PreS1 and S)-specific cell-mediated immunity (CMI) were detected in mice primed with HBSS1 and boosted with rAdSS1. Moreover, functional T cell responses with high levels of cytokines and antigen-specific cytotoxic T cell responses ($CD107a^+CD8^+$) were also detected. When challenged by HI of pCS-HBV1.3, the heterogeneous prime-boost regimen showed significant protective efficacy and HBV loss in the serum of mice, as indicated by markedly decreased serum HBeAg and HBV DNA levels (Chuai et al., 2017). However, it is unknown whether this vaccination strategy can be used as a therapeutic vaccine to break immune tolerance and clear HBV in HBV-carrier models. Moreover, the immunological parameters critical for HBV loss and how to achieve a robust therapeutic effect against chronic HBV infection need further study.

HBV-carrier mouse models were established by hydrodynamic injection (HI) (Huang et al., 2006; Yang et al., 2002), which have been used to assess the effectiveness of vaccines and examine the relationship between the immune response and HBV clearance in serum (Yin et al., 2011; Zeng et al., 2013). We previously established an HBV-carrier mouse model by HI of pCS-HBV1.3 into C57BL/6 mice and found that immunization of HBV replicon transfer mice with the novel HBSS1-based vaccines was able to induce robust immunity in mice (humoral as well as T-cell responses), followed by the decrease of the HBV viremia. (Chuai et al., 2014). In this study, we aim to optimize the prime-boost strategies in an HBV carrier mouse model based on the therapeutic efficacy and its correlation with immune response. So, we first established an HBV carrier mouse model that mimics human CHB infection by HI of 5-μg pCS-HBV1.3; Then the HI mice were immunized with the several prime-boost vaccination strategies to optimized which vaccination strategy is the best way to induce HBV-specific immune responses and clear HBV in mice model. Furthermore, we explored the correlation of multiple immunity parameters (antigen-specific antibody response and CMI, cytokine production) induced by prime-boost vaccination strategies and HBV loss in the serum of mice. Our results suggest that the novel HBSS1 prime-rAdSS1 boost regimen could induce high levels of humoral and cellular immune responses and lead to HBV antigen and DNA loss in the serum of HBV carrier mice. The loss of serum HBV antigen and DNA was strongly associated with potent and functional HBsAg-specific $CD4^+$ and $CD8^+$ T-cell responses and increased production of IL-2, IFN-γ, TNF-α, IL-12, and IP-10.

2. Methods

2.1. Mouse model of HBV infection

Female C57BL/6 mice were purchased from the Animal Care Centre, Chinese Academy of Medical Science, Beijing. All experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee. The pCS-HBV1.3 plasmid, containing the full-length HBV DNA, was constructed in our laboratory and the HBV-infection mouse model was established as described previously (Chuai et al., 2014). Briefly, 5 μg pCS-HBV1.3 plasmid was hydrodynamically injected into the tail veins of mice in a volume of 0.9% NaCl, equivalent to 8% of the mouse body weight. The total volume was delivered within 5–8 s. Serum HBV DNA persisted for > 56 days post-injection, and the appearance of anti-HBs antibody in the serum was not found in the mice injected with pCS-HBV1.3.

2.2. Vaccine candidates and adjuvants

The HBSS1 (B genotype; subtype adr, H) and rAdSS1(R) vaccines containing S (1–223 aa) and PreS1 (21–47 aa) fusion gene fragments were prepared as described previously (Chen et al., 2012). The HBSS1 vaccine was expressed in Chinese hamster ovary (CHO) cells and formulated with different adjuvant(s). The rAdSS1(R) vaccines was constructed using a recombinant replication-defective adenovirus serotype 5 vector expressing the fusion protein SS1. Aluminum hydroxide (alum, a) and PolyI:C (p) (Sigma, St. Louis, MO, USA) were used as adjuvants, and were formulated with the HBSS1 vaccine prior to immunization as described previously (Chuai et al., 2013).

2.3. Immunization of HBV-infected mice

Briefly, at 3 days post-inoculation, HBV infection of female C57BL/6 mice was confirmed by enzyme-linked immunosorbent assay (ELISA) for HBV HBsAg and HBeAg and reverse transcriptase-polymerase chain reaction (RT-PCR) assay of the serum HBV DNA level. Two days later, HBV-infected mice were randomly distributed into five groups (8 mice per group) and injected intramuscularly twice under the vaccination schedules shown in Fig. 1A and B. After vaccination, samples were collected for analysis of the immune responses and HBV marker levels (Fig. 1A).

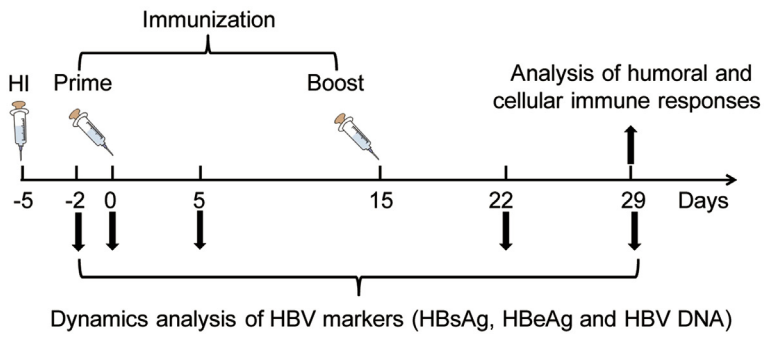
2.4. Analysis of cellular immune responses by enzyme-linked immunospot assay

IFN-γ enzyme-linked immunospot (ELISpot) assays were performed using the BD mouse IFN-γ ELISpot set (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions and as described previously (Chen et al., 2012; Chuai et al., 2013). Briefly, the BD ELISPOT plates were coated with 5 μg/mL of BD NA/LE purified anti-mouse IFN-γ antibody in PBS overnight at 4 °C. Freshly isolated mouse splenocytes (5×10^5 per well) were added to wells in triplicate with 4 μg/mL HBV PreS1 or S peptide pools. The HBV PreS1 relevant peptides (S1–5: DPAFRANTA; S1–6: RANTANPDW; S1–7: NPDWDFNPN; S1–8: NPNKDTWPD; S1–9: GFFPDHQLDPAFRANTANPDWDFNPNKDTWP) and the HBV S antigen relevant peptides (S1: VLQAGFFL; S2: IPQSLDSWWTSL; S3: FLGGTPVCL; S9: FILTRILTI) selected from amino acids 13–49 of S Ag, and S4 LLDYQGMLP, S5 GLSPTVWLS, S6 SILSPFIPLL, S7 VWLSVIWM, and S8 WGPSLYSIL selected from amino acids 97–215 of S Ag) were used in this study(Chen et al., 2012). The plates were incubated overnight at 37 °C in 5% CO2. The frequencies of IFN-γ-secreting cells were determined as the number of spot-forming cells (SFCs) per million cells.

2.5. Intracellular cytokine staining of murine splenic lymphocytes

Approximately $0.5–2 \times 10^6$ splenocytes from each immunization group were collected and cultured for 4 h at 37 °C in RPMI 1640 supplemented with 10% FBS alone (unstimulated), or with 4 mg/mL HBV PreS1 or S peptides pool (as above). And then the intracellular cytokine staining (ICS) was performed using a CytoFix/CytoPerm kit according to the manufacturer's instructions (BD Biosciences) and as described previously (Chen et al., 2012).

A



B

Group of mice for Immunization of HI mice

Groups	Prime(Week 0)		Boost (Week 2)	
	Immunogen	dose	Immunogen	dose
1	pa (Poly I:C+alum)	50μg+100μg	pa	50μg+100μg
2	R (rAdSS1)	1×10^8 vp*	R	1×10^8 vp
3	Hpa (HBSS1+pa)	1.25μg/50μg+100μg	Hpa	1.25μg/50μg+100μg
4	Ha (HBSS1+alum)	1.25μg/100μg	R	1×10^8 vp
5	Hpa (HBSS1+pa)	1.25μg/50μg+100μg	R	1×10^8 vp

* vp: viral particles

Fig. 1. Schedule of recombinant hepatitis B virus (HBV) vaccination and analysis. Female 6–8-week-old C57BL/6 mice were hydrodynamically injected with 5 μg of pCS-HBV1.3 on day −5. On day −2, enzyme-linked immunosorbent assays (ELISAs) and real-time polymerase chain reaction (PCR) were performed to screen for HBV-infected mice. All HBV-infected mice were randomly distributed into five groups (8 mice per group) and primed by intramuscular injection on day 0 (5 days after hydrodynamic injection [HI]). Samples were assessed for HBV-specific immunity and the HBsAg, HBeAg, and HBV DNA positivity rates in the serum and liver at the indicated time points. (A) Vaccination and analysis schedule. (B) Group of mice for immunization of HI mice (n = 8).

2.6. Luminex assay

Mouse splenocytes were stimulated with the same peptide pools used in ELISpot assays. Cell-free culture supernatants collected after 48 h of stimulation were tested for cytokine levels using Luminex multiple bead technology and software according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Luminex assays for IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, transforming growth factor (TGF)-α, IL-12, IL-17, TGF-β, and IFN-γ-induced protein (IP)-10 were conducted as described previously (Wen et al., 2013). The data were acquired and analyzed using a Bio-Plex 200 (Bio-Rad, Hercules, CA, USA).

2.7. Determination of serum HBV DNA levels

Serum samples were collected at the indicated time points and the HBV DNA level was determined using an HBV DNA PCR kit (Sansure Biotech, Hunan, China) as described previously(Chuai et al., 2014, 2017). The results were reported in units of IU/mL according to the manufacturer's instructions.

2.8. Detection of HBV antigens and antibodies

The levels of HBsAg and HBeAg, as well as anti-HBs and -PreS1 antibodies, were determined using commercially available ELISA kits as described previously (Chuai et al., 2014, 2017).

2.9. Statistical analysis

Results are expressed as means ± standard error of the mean. The significance of differences in immune parameters between groups was determined by one-way analysis of variance, and multiple comparisons were performed using the least significant difference *post-hoc* test, when the variance between samples was equal, or Dunnett's T3 test, when the variance was not equal. Group positivity percentages are presented as Kaplan–Meier plots and were subjected to a log-rank (Mantel–Cox) test when necessary. The Spearman rank correlation test was used to assess the relationship between immune responses and HBV loss in the serum of mice. Statistical analyses were carried out using SPSS ver. 21.0 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$ ($^*P < 0.05$, $^{**}P < 0.01$).

3. Results

3.1. HBV antigen-specific immune responses

To investigate the effect of the vaccination regimens on HBV, the serum anti-HBs and -PreS1 IgG levels in HI mice were determined. As shown in Fig. 2A and B, the control HI mice did not produce anti-S/PreS1 antibodies, while all mice that received homologous or heterologous immunization produced high levels of anti-HBs and -PreS1 IgG. No significant difference in IgG titers was observed in HI mice in the homologous (H + H) and heterologous (Ha + R and Hpa + R) immunization groups. These data indicate that the vaccination protocol used in HI mice stimulates significant B-cell responses.

Next, we evaluated the induction of HBV antigen-specific cell-mediated immunity (CMI). The number of IFN-γ-producing cells was determined by ELISpot assay. As shown in Fig. 2C, a high level of HBV S-specific IFN-γ was detected in the Hpa prime-R boost group (average IFN-γ-producing spots [SFU] per million splenocytes, 327), which was significantly higher than in mice that underwent homologous vaccination (R + R; Hpa + Hpa) ($P < 0.05$). However, the IFN-γ responses in mice that received heterologous (Ha + R and Hpa + R) vaccination showed very low frequencies detected for pre-S1 (average SFU per million splenocytes, < 50), this is not, comparable at all to what is detected for S protein. (Fig. 2D); no S- or preS1-specific IFN-γ responses were detected in the control group (pa + pa).

The magnitude and function of the HBsAg-specific $CD4^+$ and $CD8^+$ T-cell responses were evaluated by intracellular cytokine staining (ICS)

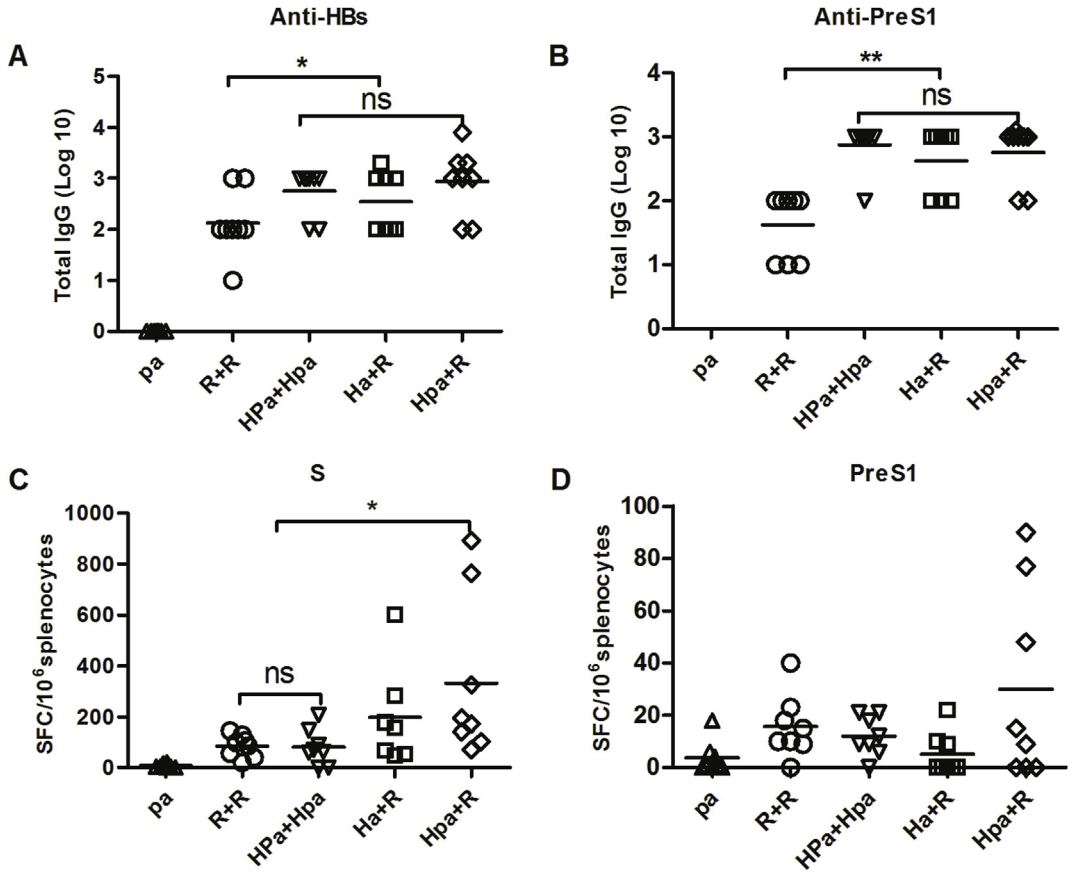


Fig. 2. Vaccination-induced antibody and cellular immune responses in HI mice. Two weeks following the boost immunization, anti-HBV antigen IgG titers in the serum of vaccinated HI mice were determined by ELISA, and T-cell responses to HBV antigens were analyzed by enzyme-linked immunospot assay(n = 8). (A) Anti-HBs IgG2 titers. (B) Anti-PreS1 IgG titers. (C) S-specific interferon (IFN)-γ levels. (D) PreS1-specific IFN-γ levels. *$P < 0.05$ and **$P < 0.01$. ns, not significant. H, HBSS1; R, rAdSS1; p, Poly I:C; and a, alum.

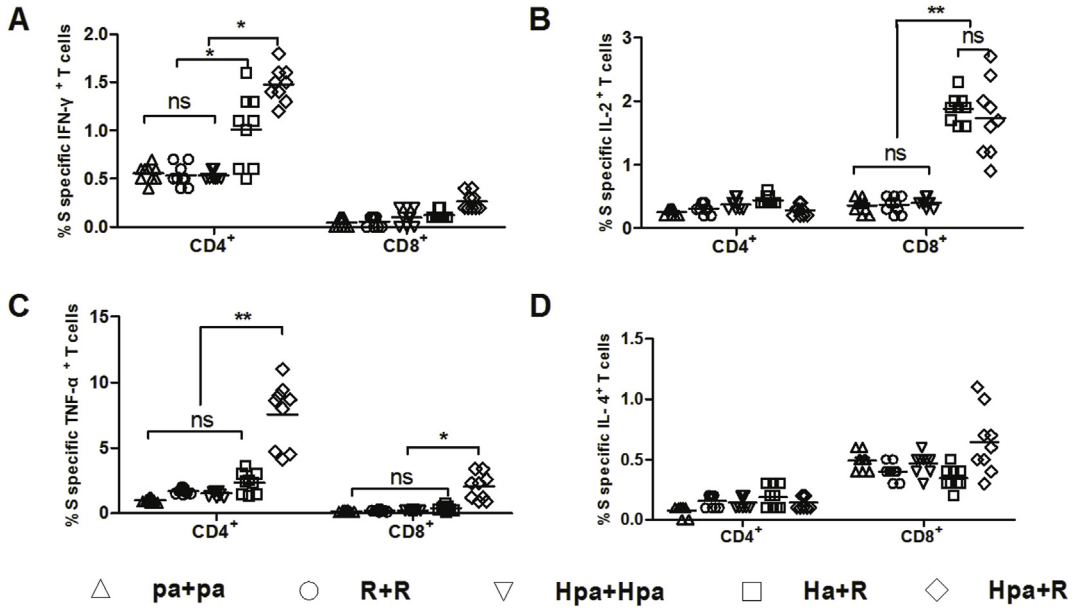


Fig. 3. Intracellular cytokine staining to assess T-cell responses to HBV antigens. Splenocytes were exposed to HBV S peptides, and cytokine production was measured by monoclonal antibody staining and flow cytometry. Mean (± standard error of the mean [SEM]) percentages of IFN-γ-, interleukin (IL)-2-, TNF-α-, and IL-4-producing $CD4^+$ and $CD8^+$ T cells from 3 mice per group following stimulation with S peptides(n = 3). (A) S-specific IFN-γ-producing T cells. (B) S-specific IL-2-producing T cells. (C) S-specific TNF-α-producing T cells. (D) S-specific IL-4-producing T cells. *$P < 0.05$, **$P < 0.01$. ns, not significant. H, HBSS1; R, rAdSS1; p, Poly I:C; and a, alum.

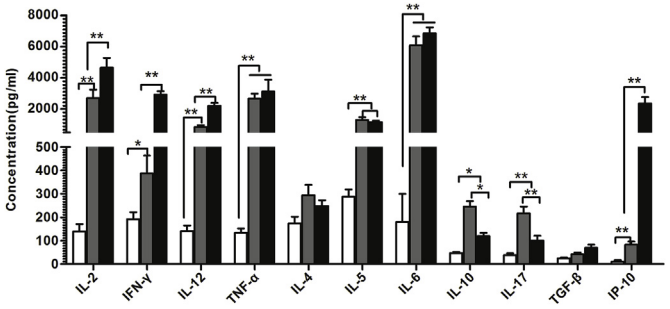


Fig. 4. Cytokine levels in the culture supernatants of splenocytes 2 weeks after the last immunization. Splenocytes (5×10^5) from vaccinated mice were cultured for 2 days with 4 μg/mL peptides, with culture medium as the negative control. Cytokine levels in the supernatants were determined (n = 6). $^{**}P < 0.01$ significant differences in cytokine levels between the Ha + R and Hpa + R groups. H, HBSS1; R, rAdSS1; p, Poly I:C; and a, alum.

of splenocytes 2 weeks after the last immunization. As shown in Fig. 3A and C, compared with those immunized with the Ha prime-R boost regimen, HI mice immunized with the Hpa prime-R boost regimen exhibited greater S-specific production of IFN-γ and TNF-α by $CD4^+$ T cells, and significantly greater production of TNF-α by $CD8^+$ T cells.

3.2. Induction of Th1- and Th2-type cytokine production by the HBSS1 prime-rAdSS1 boost vaccination regimen

Cytokines play an important role in the initiation and regulation of immune responses and so may contribute to the inhibition of virus replication (Grunhage and Nattermann, 2010; Li et al., 2016). Thus, we evaluated cytokine levels in splenocyte culture supernatants at 2 weeks after the second immunization. As shown in Fig. 4, immunization with Ha + R or Hpa + R led to the production of higher levels of IL-2, TNF-α, IL-12, IL-5, IL-6, IL-10, IL-17, TGF-β, and IP-10 than those in mice immunized with pa + pa (control). The levels of Th1 cytokines (IL-2, IFN-γ, IL-12, TNF-α) and IP-10 in the Hpa + R group were significantly higher than those in the Ha + R group ($P < 0.01$), which is consistent with the ELISpot and ICS results. Ha + R immunization induced higher levels of IL-10 and IL-17 than Hpa + R immunization.

3.3. Loss of serum HBV antigens and DNA

The impact of the prime-boost immunizations on HBV loss in the serum of mice was investigated by determining the serum levels of HBsAg, HBeAg, and HBV DNA. As shown in Fig. 5A and B, serum HBsAg levels dramatically decreased in Hpa + R- and Hpa + Hpa-immunized HI mice, and all treated mice became negative at days 7 and 14 after the second immunization; in the Ha + R group, serum HBsAg levels also decreased and 87.5% of the mice became negative 2 weeks after rAdSS1 boosting. In the control group, complete loss of HBsAg occurred in 12.5% of the HI mice after the last immunization. High serum HBeAg levels and HBV DNA titers are markers of active HBV replication in the liver (Zhang et al., 2014). In our model, the heterologous prime-boost immunizations markedly decreased the serum HBeAg level (Fig. 5C and D); furthermore, serum HBV DNA was cleared in the two heterologous prime-boost immunization groups and the Hpa homologous immunization group (Fig. 5E and F), indicating marked inhibition of HBV replication.

3.4. Correlation between immunity and HBV loss in serum of mice

The novel vaccination regimen induced rapid loss of serum HBsAg, HBeAg, and HBV DNA. To explore the immunological mechanism of HBV loss in serum of mice, we next evaluated the correlation of immunity with HBV loss in serum of vaccinated HI mice. The correlations between the immunologic parameters and the HBsAg, HBeAg, and HBV DNA levels after the second immunization (compared with day 0) were analyzed by Spearman rank correlation test. HBsAg, HBeAg, and HBV DNA loss in serum of mice was most strongly correlated with HBsAg-specific cellular immune responses ($CD4^+$ IFN-γ and TNF-α, $CD8^+$ IL-2 and TNF-α); HBV loss in serum of mice was also strongly correlated with the production of Th1-associated cytokines (IL-2, IFN-γ, TNF-α, and IL-12) and IP-10 (Table 1).

4. Discussion

In the present study, we tested the therapeutic efficacy of several prime-boost vaccine regimen in HBV-carrier mice model. Mice vaccinated with novel HBSS1 prime-rAdSS1 boost regimens resulted in high levels of HBV specific anti-HBs and PreS1 antibodies, which were consistent with our previous report of wild mice immunized with the same regimens(Chuai et al., 2013, 2017). However, only the HBV S specific CMI and the production of cytokines were found in the HBSS1 primed-rAdSS1 boosted mice, which led to the rapid loss of HBsAg, HBeAg, and HBV DNA in the serum. The PreS1 specific CMI were very low, which is not, comparable at all to what is detected in our previous study of wild mice immunized with the same regimens (Chuai et al., 2013, 2017).

Reduction of HBsAg may be a key factor for therapeutic success. And HBsAg seroconversion is determined as a status of “functional cure” in clinic, which is associated with a largely deceased risk of developing HCC (Lok et al., 2017). We observed a marked reduction (serum HBsAg, HBeAg and HBV DNA) in HBV replication and HBsAg seroconversion in mice immunized with a novel heterologous prime-boost strategy. However, since transduced HBV genome persists in the extranuclear of hepatocytes as episome form in mice, we did not observe HBV DNA reduction or elimination (PCR for the HBV-DNA) and no histopathology in liver of mice after vaccination (data not shown), which was same as described previously (Chuai et al., 2017; Huang et al., 2006). These results suggest that noncytolytic mechanisms may play a significant role in loss of HBV in serum of mice by vaccination is associated with the appropriate host humoral and cellular immune responses (Chisari et al., 2010). Here, we determined whether strong immunity was induced by our prime-boost regimen and the influence of antigen-specific immunity on HBV loss in serum of HBV-carrier mice. After the second immunization, both the HBSS1 homologous vaccination and HBSS1 prime/rAdSS1 boost vaccination induced comparable levels of HBV antigen-specific IgG (ant-S and ant-preS1) in the sera of HBV-carrier mice, which led to the loss of HBsAg and anti-HBs seroconversion. Anti-HBs and -PreS1 IgG are not only involved in the elimination of viral particles from the circulation but also block the release of viral particles from cells by inducing neutralizing antibodies (Neumann et al., 2010). However, no S- or PreS1-specific antibody was detected in the control group, in which the serum HBsAg levels declined slowly over time.

Neutralizing antibody to HBsAg is considered a hallmark of resolution and protection, but a robust T-cell response is required for clearance of infected cells. Studies in preclinical models of HBV infection such as woodchucks and chimpanzees, as well as in patients, have highlighted the importance of HBV-specific T-cell responses for viral clearance (Chisari et al., 2010; Kosinska et al., 2012; Li et al., 2014). Although the complete loss of HBsAg were discovered in the sera of HBV-carrier mice of HBSS1 homologous vaccination or HBSS1 prime/rAdSS1 boost vaccination group, the various regimens showed different properties in terms of loss of HBV DNA and HBeAg in serum of mice. Hpa + R regimen led to markedly decreased serum HBeAg, and HBV DNA levels, indicating dramatic inhibition of HBV replication. The Hpa + R group exhibited significantly higher levels of S-specific cellular responses than the other groups, which was correlated with HBV antigenemia and viremia decrease. These results were also consistent with our previous report in wild mice immunized with the same regimens.

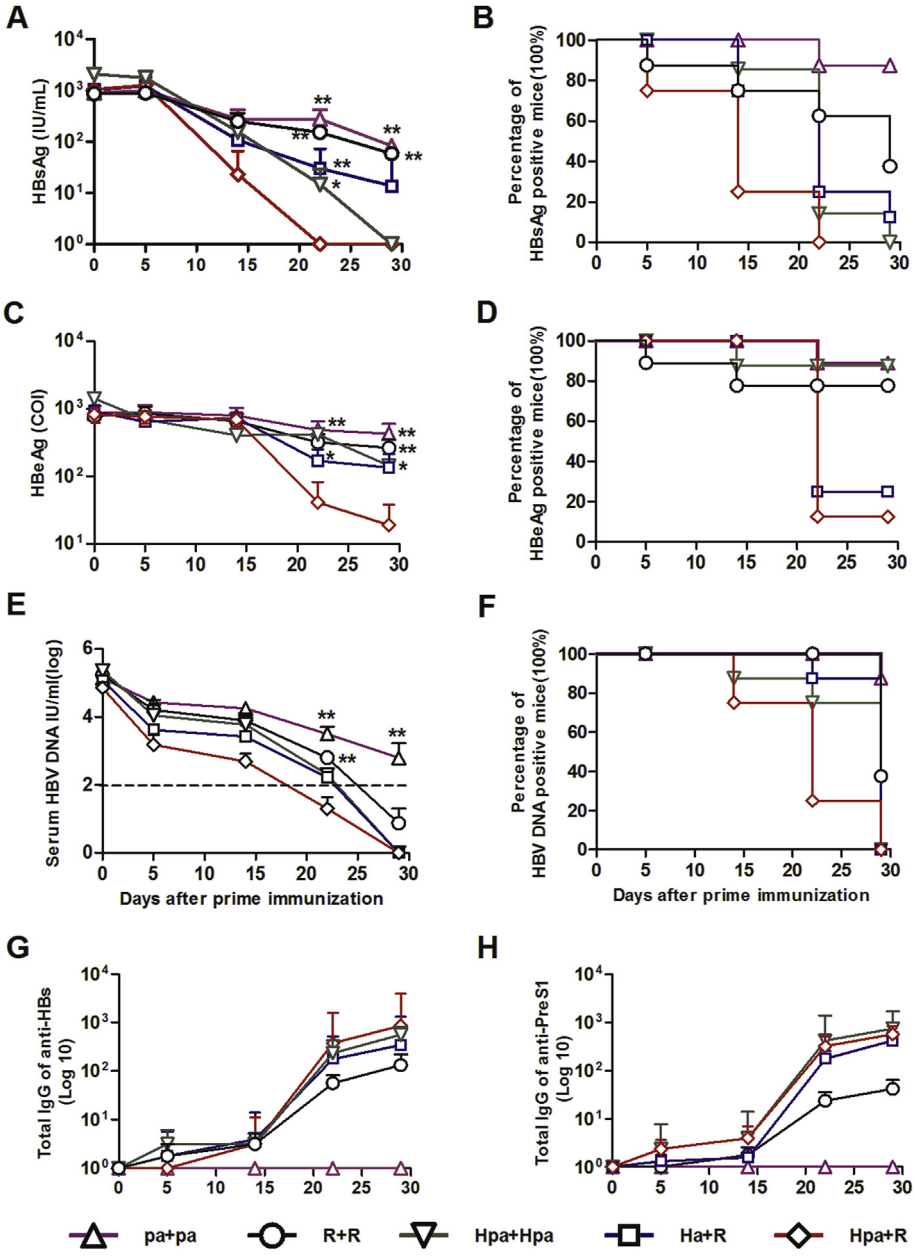


Fig. 5. Kinetics of HBsAg, HBeAg, HBV DNA, anti-HBs and anti-PreS1 in serum. HI mice received the indicated prime-boost regimens or PolyI:C/alum adjuvant as a control. Serum HBsAg (A), HBeAg (C), anti-HBs (G) and anti-PreS1 (H) levels in mice were determined by ELISA. Serum HBV DNA concentrations (E) were determined by real-time PCR and are expressed as IU/mL. The proportions of mice with detectable levels of serum HBsAg (B), HBeAg (D), and HBV DNA (F) were determined after immunization. Data are means ± SEM (n = 8). *$P < 0.05$ and **$P < 0.01$, significant differences compared to the Hpa + R group.

HBV-specific $CD8^+$ T cells facilitate viral clearance by cytotoxic and non-cytotoxic mechanisms. A study in transgenic mice and HBV-infected chimpanzees suggested that the antiviral effect of CMI is mediated mainly by non-cytotoxic mechanisms; *i.e.*, production of IFN-γ, IL-2, and TNF-α, which suppressed HBV gene expression and replication without destroying infected hepatocytes (Phillips et al., 2010). Our data demonstrate that the $CD8^+$ and $CD4^+$ T cells induced by the Hpa + R regimen were highly functional to control and clear serum HBsAg and HBV-DNA. Mice immunized with the Hpa prime-R boost regimen exhibited significantly higher proportions of TNF-α- and IL-2-secreting $CD8^+$ T cells than the other groups. In addition, TNF-α and IFN-γ production by HBV-specific $CD4^+$ T cells was higher in mice that received the Hpa prime-R boost regimen. Furthermore, the levels of several Th1-associated cytokines (IL-2, IFN-γ, TNF-α, and IL-12) were increased in the Hpa prime + R boost group, which correlated with serum HBsAg and HBV DNA loss. Interestingly, IP-10 was also associated with loss of HBsAg, HBeAg, and HBV DNA in serum of mice, in concordance with a previous report that a recombinant HBV vaccine increases the level of IP-10 in patients with CHB and persistent HBsAg, and that higher IP-10 levels are associated with a significant reduction in, or loss of, HBsAg (Shaaban Hanafy, 2017). IP-10, a CXC chemokine, recruits T cells and enhances the production of IFN-γ by activated NK T cells, which facilitate an effective antiviral response (Fabiani, 2015). Therefore, certain cytokines play an important role in the initiation and regulation of the immune responses to HBV.

Vaccination of low titer HBVtg mice did induce HBV-specific adaptive immune responses. However, vaccination of intermediate and high titer mice failed to induce HBV-specific $CD8^+$ T cell responses (Backes et al., 2016). This indicates that there is a mechanism by which HBV inhibits adaptive immune responses. In this study, our data shown

Table 1
Analysis of immune correlates with HBsAg, HBeAg and HBV DNA loss in sera of mice.

	HBsAg		HBeAg		HBV DNA	
	r	*p*	*r*	*p*	*r*	*p*
CMI						
S-ELISpot	0.8891	< 0.0001***	0.6810	< 0.0001***	0.8891	< 0.0001***
$CD4^+$ IFN-γ	0.8787	< 0.0001***	0.6834	0.0003***	0.9241	< 0.0001***
$CD4^+$ TNF-α	0.8017	< 0.0001***	0.5550	0.0060**	0.8460	< 0.0001***
$CD8^+$ IL-2	0.7478	< 0.0001***	0.4577	0.0281*	0.7146	< 0.0001***
$CD8^+$ TNF-α	0.6062	0.0017**	0.3146	0.1437	0.5369	0.0068**
cytokines						
IL-2	0.8982	< 0.0001***	0.6518	0.0008***	0.9173	< 0.0001***
TNF-α	0.9383	< 0.0001***	0.7678	< 0.0001***	0.9365	< 0.0001***
IL-12	0.7365	< 0.0001***	0.4881	0.0181*	0.7652	< 0.0001***
IP-10	0.7296	< 0.0001***	0.6103	0.0020**	0.8845	< 0.0001***
IFN-γ	0.7226	< 0.0001***	0.5583	0.0056**	0.8391	< 0.0001***
IL-4	0.4693	0.0207*	0.5536	0.0061**	0.4754	0.0189*
IL-5	0.5189	0.0094**	0.4246	0.0434*	0.5020	0.0124*
IL-6	0.6322	0.0009***	0.3528	0.0987	0.5861	0.0026**
TGF-β	0.4800	0.0176*	0.2322	0.2863	0.4670	0.0214*
IL-10	0.3852	0.0630	0.3370	0.1159	0.3452	0.0985
IL-17	0.3801	0.0669	0.3118	0.1475	0.3779	0.0686

the titer of HBsAg (> 800 IU/mL) and HBeAg(> 800 COI) were high in serum of HDI mice 5 days before vaccination, which were at same level as high titer HBVtg mice reported. However, both the HBV antigens were declined after the peak titer appeared at day 7, which were different from the HBVtg, for the HBVtg were exposed to HBV antigens for their whole life. So it needs further to test our vaccination strategy also works in a long-standing HBV infection model. Interesting, significant HBV-specific adaptive immune responses and HBsAg seroconversion were still observed in these HBV-carrier mice after vaccination by a novel heterologous prime-boost strategy. The HDI with various HBV genome carrying plasmid has been widely used to establish acute or chronic HBV mice model (Chuai et al., 2014; Huang et al., 2006; Lin et al., 2010). However, some investigators suggest that no detectable antibody or T cell responses in these HDI mice doesn't proof HBV immune tolerance in mice since HBV tolerance needs several weeks to develop(unpublished data). We will further optimize the method to establish a good model with long-standing HBV-replication for HBV immune tolerance to test the efficacy of the immune regimens used in our study. In addition, previous report (Kosinska et al., 2017) indicated that the therapeutic efficacy by vaccination showed significant gender-related differences. So, we may use male C57BL/6 mice in the further study to evaluate the therapeutic vaccination and compared the difference therapeutic efficacy.

In summary, we evaluated a novel therapeutic vaccination strategy in a female HBV-carrier mouse model. The Hpa prime-R boost regimen induced high levels of antibody production and HBV specific T cell responses. The loss of serum HBsAg, HBeAg and HBV DNA was strongly associated with potent and functional HBsAg-specific $CD4^+$ and $CD8^+$ T-cell responses and increased production of IL-2, IFN-γ, TNF-α, IL-12, and IP-10. However, the novel therapeutic vaccination strategy should be further evaluated in nonhuman primate models and chronically HBV-infected patients.

Authors' contribution

X.C, B.X. and W.T. designed research; X.C. Y.D., W.W., Y.Z., Z.G., and W.W. performed research; X.C., B.X., X.Q. and W.T. analyzed data; X.C., X.Q. and W.T. wrote the paper.

Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.11.002.

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