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Lentiviral backbone-based hepatitis B virus replicon-mediated transfer favours the establishment of persistent hepatitis B virus infection in mice after hydrodynamic injection

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ABSTRACT

Establishment of a non-transgenic mouse model of persistent hepatitis B virus (HBV) infection is urgently needed. In this study, we constructed novel lentiviral-transfer plasmids containing HBV replicon DNA (pCS-HBV1.3, containing a 1.3-fold-overlength genome of HBV) and employed hydrodynamic injection (HDI) to develop an HBV-persistent mouse model. We explored the impact of host (different mouse strains, BALB/c and C57BL/6), gender, and the plasmid backbone on persistent HBV in mice. Our data showed that HBV antigenaemia (HBsAg, HBeAg) and HBV DNA persisted for >56 days post-injection, while the appearance of anti-HBs antibody in the serum was only found among <30% of female C57BL/6 mice injected with pCS-HBV1.3. Moreover, HBcAg and HBV DNA were also detected in the liver of HDI mice. Compared with previous AAV-backbone based HBV replicon DNA transfer, we found that the HDI transfer with the lentiviral vector-based HBV replicon (pCS-HBV1.3) in this study resulted in a significantly higher level of HBV DNA transfer in the liver and longer persistence of HBV DNA and antigenaemia in the serum. Furthermore, we also showed that immunization of HBV replicon transfer mice with the novel HBSS1-based vaccines was able to overcome tolerance against HBV in mice and induces robust immunity (humoral as well as T-cell responses), followed by the clearance of the HBV viremia. We concluded that lentiviral backbone-based transfer vectors more readily establish persistent HBV infection in mouse models via HDI, providing a new tool useful for the study of HBV infection and immune-based therapies.

1. Introduction

Hepatitis B virus (HBV) is a non-cytopathic prototype for liver-specific pathogens that cause acute and chronic hepatitis. Although an effective vaccine exists, HBV infection is still a serious public health problem; more than 240 million people are chronically infected with HBV worldwide, and about 25% of them die of chronic active hepatitis, cirrhosis, or hepatocellular carcinoma (Lai and Yuen, 2008; Liaw and Chu, 2009). Our understanding of the mechanism responsible for HBV infection and immunity has been hindered by a lack of an appropriate animal model because of the strict host restriction of HBV infection. It is imperative to establish a mouse model that can precisely simulate HBV infection in humans and is convenient for anti-HBV drug and vaccine evaluation.

Although chimpanzees as HBV animal models might offer an opportunity for *in vivo* studies of HBV infection, they are expensive and not readily available (Will et al., 1983). HBV transgenic animals have proven very useful in some immunological studies, but always develop immune tolerance to the dominant HBV antigens (Chisari, 1996; Guidotti and Chisari, 2006; Guidotti et al., 1995). HBV genomes were recently delivered into the livers of mice to generate non-transgenic immunocompetent mouse models for infection of HBV through either hydrodynamic injection (HDI) (Liu et al., 1999; Yang et al., 2002) or viral vector (adenovirus or adeno-associated virus)-based gene transfer (Dong et al., 2010; Sprinzl et al., 2001; von Freyend et al., 2011). Yang et al. (2002) first generated an acute HBV infection murine model by HDI with transposase-based HBV DNA, and Huang et al. (2006) first created an immunocompetent model of persistent HBV infection by HDI into mice with replication-competent HBV DNA based on the plasmid backbone of an AAV vector (pAAV/HBV1.2). The HBV antigenaemia persisted for >6 months in 40% of injected C57BL/6 mice because of an insufficient cellular immune response to the HBV antigen. They then constructed a set of serial HBcAg deletion mutants for HDI

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and found that 90% of the mice that received an HBcAg-mutated HBV genome exhibited high levels of HBV antigenaemia for >6 months (Lin et al., 2010). Chen et al. (2012b) recently demonstrated persistent HBV replication in an FVB/N mouse model by HDI with HBV-replicon plasmids containing 1.3-fold-overlength HBV genomes. An alternative method used viral vectors to transfer the HBV genome into immunocompetent mice (Dion et al., 2013; Sprinzl et al., 2001; von Freyend et al., 2011), and acute or chronic HBV infection was established depending on the dose of adenoviral vector injected. Compared with viral vector-based HBV gene transfer, the plasmid-based HDI approach induces weaker inflammatory responses, and is cheaper and more readily available. Furthermore, it is more convenient to modify the HBV genome, which offers a great opportunity to investigate the viral factors associated with HBV pathogenesis and immunity *in vivo*.

Aside from the sequences and structure of the HBV genome and associated host factors, previous reports on the HDI approach have indicated that persistent HBV replication and the associated immune responses might be influenced by the backbone of the transfer plasmid for the HBV genome (Huang et al., 2006). Lentiviral vectors have emerged as potent and versatile tools for *ex vivo* or *in vivo* gene transfer and allow for stable transgene expression (Miyoshi et al., 1998; Singer and Verma, 2008). To further optimise HDI procedures for establishment of a persistent HBV mouse model, we constructed a novel HBV replicon-transfer plasmid, namely pCS-HBV1.3, based on a self-inactivating lentiviral-transfer vector which contained a 1.3-fold-overlength genome of HBV (Chuai et al. 2012). We then explored and optimised several impact factors via HDI. We herein report an alternative novel protocol for the development of a persistent HBV infection mouse model via HDI and its application for HBV therapeutic vaccination.

2. Materials and methods

2.1. Plasmid constructs

The plasmids pCS-HBV1.3 and pAAV-HBV1.3, both containing a 1.3-fold-overlength genome of HBV, were constructed as described previously (Chuai et al., 2012; Guidotti et al., 1995; Guo et al., 2010); the plasmid maps are shown in Fig. 1. The HBV sequence was of the ayw subtype (GenBank Accession No. AB267090.1). Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Germany) according to the manufacturer's instructions. The purity of the DNA preparations was determined by reading the optical density at 260 and 280 nm.

2.2. Cell culture and transfection

Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum under 5% CO_2 incubation at 37 °C. Huh7 cells were split into six-well dishes and cultured overnight. Upon transfection, the cells were at ~80% confluence. pCS-HBV1.3 and pAAV-HBV1.3 plasmids were transfected into Huh7 cells using FuGENE® HD Transfection Reagent (Promega, USA) according to the manufacturer's instructions. The final amount of plasmid DNA was 2 μg per transfection per well of the six-well plate. The supernatant of all transfected cells was harvested 48 h post-transfection.

2.3. Animal study

Breeder mice (6 weeks old) of the female BALB/c strain and the male or female C57BL/6 strain were purchased from Animal Care Centre, Chinese Academy of Medical Science, Beijing. All experiments were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC). 5 μg of HBV plasmid DNA (pCS-HBV1.3 or pAAV-HBV1.3) was 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 samples were collected at the time points indicated after DNA injection and assayed for HBsAg, HBeAg and HBV DNA; the animals were sacrificed at various time points after injection, and the serum and liver were collected. The serum was stored at −20 °C. The liver tissue was separated into two parts: one was fixed in 40 g/L neutral-buffered formalin, and the other was snap-frozen in liquid nitrogen and stored at −70 °C for subsequent analysis of HBV DNA.

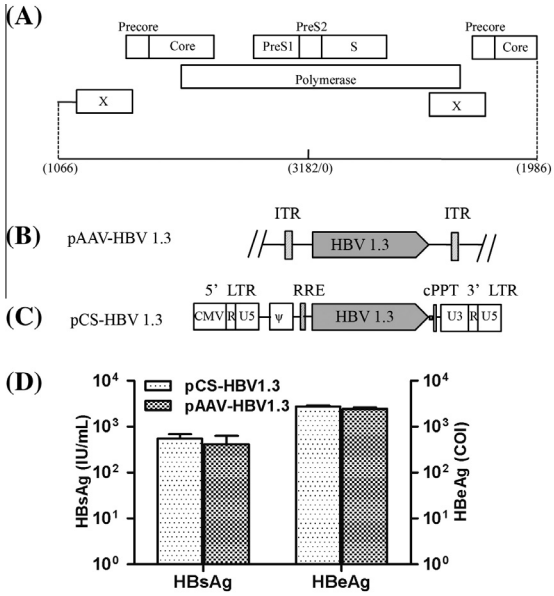

Fig. 1. Construction and characterisation of HBV replicon transfer plasmids (pAAV-HBV1.3 and pCS-HBV1.3). (A) Schematic representation of the replication competent: the HBV 1.3-fold-overlength genome. (B) Schematic diagram of the pAAV-HBV1.3 plasmid. (C) Schematic diagram of the pCS-HBV1.3 plasmid. (D) Detection of HBsAg and HBeAg in the supernatant of pAAV-HBV1.3 and pCS-HBV1.3 transfected Huh7 cells using ELISA. Serum HBsAg levels are expressed as the IU/mL and serum HBeAg levels are expressed as the COI. The results are shown as means ± SD.

HBV replicon HDI mice were applied to evaluate the therapeutic effect of two novel HBV vaccine candidates. The HBSS1 vaccine expressed in CHO cells containing S (1–223 aa) and PreS1 (21–47 aa) was prepared as previous reports (Chuai et al., 2013), and formulated with different adjuvant(s) immediately prior to immunization (Table 1). Poly (I:C) was purchased from Sigma (St. Louis, MO), and dissolved in saline. Briefly, 3 days after the female C57BL/6 mice were hydrodynamically injected with 5 μg of pCS-HBV1.3, HBV transfer *in vivo* were determined by ELISA and real-time PCR assay. Again 2 days later, the HBV transfer mice were intramuscularly injected with adjuvant formulation or two novel HBV vaccine candidates in 100 μL of PBS. Every 2 week after the vaccination, the samples (sera, splenocytes or liver tissue) were collected from the mice for analysis as above or previous report.

Table 1
Immunization groups with HBSS1 vaccine candidates.

Group	Immunogen	Immunization dose (μg)
1	Alum/Poly I:C	100/50
2	HBSS1/Alum	1.25/100
3	HBSS1/Alum/Poly I:C	1.25/100/50

2.4. Detection of HBV antigen and antibody and ALT in serum

Serum levels of HBsAg of the mice were determined using commercially available ELISA kits (Abbott Architect 2000 HBsAg assay). Serum levels of HBeAg, anti-HBc, and anti-HBs were measured using their respective ELISA kit (Kehua, Shanghai, China). The reporting unit for HBsAg was the IU/mL. For HBeAg detection, the obtained S/CO was converted to the cut-off index (COI) detected. According to the manufacturer's instructions, the sample was positive for HBeAg when the COI was $\geqslant 1$. The cut-off value for determining anti-HBc, and anti-HBs was an S/N ratio of $\geqslant 2$. Serum ALT levels were measured using the ALT kit (Cayman Chemical Company, USA). All of these assays were conducted following the manufacturers' instructions.

2.5. Detection of HBV DNA in serum or liver

Serum or live tissue samples were collected at the indicated time points after HDI of pCS-HBV1.3 or pAAV-HBV1.3. The total DNA was extracted from 2 to 10 μL of serum or 10–50 ng of tissue from mice at indicated time and detected for HBV DNA using the HBV DNA PCR Kit (Sansure Biotech, Hunan, China). The reporting unit was IU/mL according to the manufactures instructions.

2.6. Immunohistochemical staining for HBcAg

Liver tissues were collected from mice killed at the indicated time points. Intrahepatic HBcAg was visualised by immunohistochemical staining of tissues embedded in OCT by rabbit anti-HBc antibodies (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and EnVision System-HRP (diaminobenzidine) (Zhongshan, Beijing). Liver sections were also stained with haematoxylin.

2.7. IFN-γ enzyme-linked immunospot (ELISPOT) assay

The IFN-γ ELISPOT assays were performed using the BD mouse IFN-γ ELISPOT set (BD Biosciences) according to the manufacturer's instructions, and as described previously. Splenocytes were prepared from mice receiving HDI at 14 dpi. 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 mg/mL peptides separately, as described previously (Chen et al., 2011); the frequencies of IFN-γ-secreting cells were determined and measured as the number of spot-forming cells (SFC) per million cells.

2.8. Statistical analysis

Results are expressed as mean ± SEM. Unpaired two-tailed Student's *t*-test or Kaplan–Meier analysis was used to compare means between different groups. Statistical analyses for multiple comparisons were conducted using the one-way ANOVA analysis function in the SPSS software package SSPS, ver. 17.0. Values of $^{*}P < 0.05$ or $^{**}P < 0.001$ were considered to indicate statistical significance.

3. Results

3.1. pCS-HBV1.3 and pAAV-HBV1.3 resulted in similar levels of HBsAg and HBeAg expression in transfected Huh7 cells

A previous report showed that plasmid backbones used for HDI affected HBV persistence in the mouse liver. Here, we developed a lentiviral-based HBV vector, pCS-HBV1.3. The backbone of the plasmid was from a self-inactivating lentiviral vector system, pCS-CG, and contained 1.3 copies of the HBV genome. A schematic diagram of the 1.3-fold HBV genome (Fig. 1A) and two HBV replicon-transfer plasmids (pAAV-HBV1.3 and pCS-HBV1.3) for HDI in this study (Fig. 1B and C) are shown in the indicated figures. We first analysed the expression level after *in vitro* transfection of HBV antigen with the HBV-transfer plasmids pCS-HBV1.3 and pAAV-HBV1.3. Both HBsAg and HBeAg could be detected by ELISA in the supernatant of Huh7 cells 48 h post-transfection (Fig. 1D). No significant differences in HBV protein expression were observed after transfection with both HBV replicon-transfer plasmids *in vitro*.

3.2. HBV persistence in vivo after HDI with pCS-HBV1.3 plasmid was influenced by mouse strain and gender

To evaluate the influence of the mouse strain on HBV persistence in mice, 5 μg of pCS-HBV1.3 were hydrodynamically injected into the tail veins of female BALB/c or C57BL/6 mice. Sampling regularly after injection, the expression levels of HBsAg, HBeAg, and IgG against hepatitis B core antigen (anti-HBc) or hepatitis B surface antigen (anti-HBs) in the sera of mice were monitored; in addition, the HBV DNA level was assayed in the serum or liver tissue of mice (Fig. S1). In female BALB/c mice, the level of HBsAg and HBeAg peaked at 5 dpi, and then quickly decreased until 12 d dpi, when it disappeared. All of the BALB/c mice developed detectable anti-HBc within 7 dpi and anti-HBs within 14 days. HBV DNA in serum was positive only from 1 to 14 dpi, but HBV DNA in the liver remained positive beyond 15 dpi. The drop in antigenaemia was accompanied by the appearance of anti-HBc IgG antibody on day 7, and disappearance of serum HBV DNA coincided with the induction of anti-HBs IgG antibody at day 14 (Fig. S1). In contrast, the HBsAg, HBeAg, and HBV viraemia persisted for a longer period (until to 49 dpi) among more than 70% of female C57BL/6 mice injected with 5 μg of pCS-HBV1.3 (Fig. 2), which was much longer than that in the serum of BALB/c mice. We also noted that no anti-HBs IgG was detected until 56 dpi. Thus, the mouse genetic background influences HBV persistence and immune status in HDI mice.

To explore the impact of gender on the HBV persistence in mice that underwent HDI with pCS-HBV1.3, we also evaluated the HBV DNA, antibody, and antigen expression status in male C57BL/6 mice hydrodynamically injected with 5 μg of pCS-HBV1.3. HBsAg was detectable from 1 dpi and persisted for only 28 d, accompanied by the appearance of anti-HBs IgG. HBeAg was also detected at 1 dpi, and disappeared among more than 70% of mice at 28 dpi. Serum HBV DNA was detectable from 1 to 49 dpi (Fig. S2). Overall, antigenaemia in female C57BL/6 mice persisted at a higher proportion and for a longer period of time (70% vs. 30% positive for HBeAg at 49 dpi; 80% positive for HBsAg at 49 dpi vs. all negative at 28 dpi) compared with male C57BL/6 mice (Fig. S2 and Fig. 2).

3.3. pCS-HBV1.3 is superior to pAAV-HBV1.3 for establishment of persistent HBV infection in vivo

To explore the impact of the backbone of the transfer plasmid on HBV persistence *in vivo*, 6-week-old female C57Bl/6 mice underwent HDI with 5 μg of pCS-HBV1.3 or pAAV-HBV1.3. Various HBV replication and infection parameters (including HBV DNA in the serum or liver, HBV antigenaemia, and antibody levels) were evaluated at the indicated times until 56 dpi (Fig. 2). In the sera of mice injected with pCS-HBV1.3, HBsAg and HBeAg became detectable from 1 dpi and >70% persisted for 6 weeks. None of the C57BL/6 mice developed anti-HBs at 56 dpi, although all developed anti-HBc at 7 dpi that remained detectable until 56 dpi. In contrast, mice receiving pAAV-HBV1.3 injection expressed higher

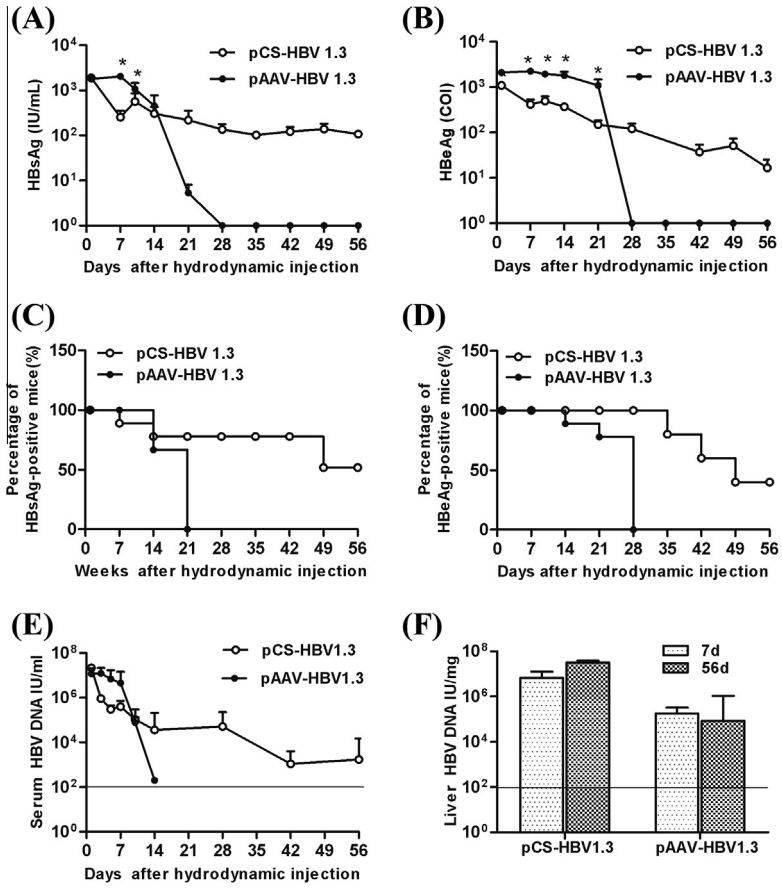

Fig. 2. Dynamics and positive rate analysis of HBV markers (HBsAg, HBeAg, and HBV DNA) in the serum and liver of transfer mice. Five micrograms of HBV replicon DNA (pAAV-HBV1.3 and pCS-HBV1.3) were injected into indicated mice. Six mice per group were analysed sequentially from day 1 to day 56 postinjection as indicated and HBsAg and HBeAg levels and HBV DNA were determined in mouse sera; and at day 7 and day 56 postinjection 4 mice were killed. Detection of HBsAg, HBeAg, in the serum by ELISA and HBV DNA in both the serum and liver by real-time PCR. (A) Titer of serum HBsAg in mice after pAAV-HBV1.3 or pCS-HBV1.3 injection, the levels are expressed as the IU/mL. (B) Titer of serum HBeAg in mice after pAAV-HBV1.3 or pCS-HBV1.3 injection, and the levels are expressed as the COI. (C and D) The positive rate of HBsAg (C) or HBeAg (D) in mice receiving pAAV-HBV1.3 or pCS-HBV1.3 injection at different time points; the data were analyzed by Kaplan–Meier analysis. The date was based on 6 mice each group. (E) Serum HBV DNA in mice after pAAV-HBV1.3 or pCS-HBV1.3 injection and expressed as the IU/mL. (F) Liver HBV DNA were quantified by real-time PCR at day 7 and day 56 postinjection and the data was based 4 mice each group. The results are shown as means ± SD.

levels of HBsAg, HBeAg, and HBV DNA in their sera during the first week (Figs. 2and 3A), but all remained positive for no more than 28 dpi, which coincided with the induction of anti-HBs IgG (Fig. 2). The serum and liver samples from HDI C57BL/6 mice were also assayed for the presence of encapsulated HBV DNA by real-time PCR (Fig. 2). For mice injected with either pCS-HBV1.3 or pAAV-HBV1.3, HBV DNA in the liver was detectable from 1 to 56 dpi. However, the intrahepatic HBV DNA levels in mice injected with pCS-HBV1.3 were significantly higher than those in mice injected with pAAV-HBV1.3 detected at 56 dpi (Fig. 2F). HBV DNA in the serum of mice injected with pCS-HBV1.3 was also detectable from 1 dpi and peaked during the first week pi, then decreased slowly until 56 dpi. However, HBV DNA in the serum of mice injected with pAAV-HBV1.3 was detectable only during the first 10 dpi, and decreased to less than 10^2 IU/mL at 14 dpi. These data suggest that the lentiviral-based HBV-transfer plasmid pCS-HBV1.3 is superior to pAAV-HBV1.3 for establishment of persistent HBV infection via HDI *in vivo*.

All mice transfected using HDI developed anti-HBc at 7 dpi, and HBV DNA remained positive in liver tissue even after disappearance of serum markers (Fig. 2). HBcAg expression in the liver of female C57BL/6 mice injected with pCS-HBV1.3 and pAAV-HBV1.3 plasmid were detected by immunohistochemical staining at 5 dpi. Representative data are shown in Fig. 3. Multiple positive HBcAg-staining foci were observed among a portion of hepatocytes of mice with pCS-HBV1.3 and pAAV-HBV1.3 injection. The liver of C57BL/6 mice with HDI showed normal architecture and obvious inflammatory responses, or evidence of hepatic damage were not detected. In addition, when IFN-γ-producing T cells among the splenocytes in mice at 14 dpi were analysed by ELISPOT assay after S- and C-specific peptide stimulation, the median number of spot-forming cells per million splenocytes in HDI mice was <20 after subtraction of the background (data not shown). All the ALT level in sera of HDI mice was less than 50 U/mL at 7 dpi (data not shown). These data indicate that hydrodynamics-based delivery of the pCS-HBV1.3 plasmid resulted in expression of HBV antigen in the liver and did not cause obvious T cell response and liver damage.

3.4. Robust immunity by novel HBSS1 vaccine formulations resulted HBV viremia clearance in HBV infection mice model

To assess the application of HBV replicon transfer mouse model, two novel HBSS1 vaccine formulations were tested for their ability to induce HBV-specific immunity as well as therapeutic efficacy after establishment of the above HBV persistence infection mice via HDI with pCS-HBV1.3 plasmid (Table 1, Fig. 4). Significant higher level of humoral response(anti-S and anti-PreS1 IgG) as well as specific T-cell response to HBsAg were elicited after two immunizations with HBSS1 vaccine formulations in mice,

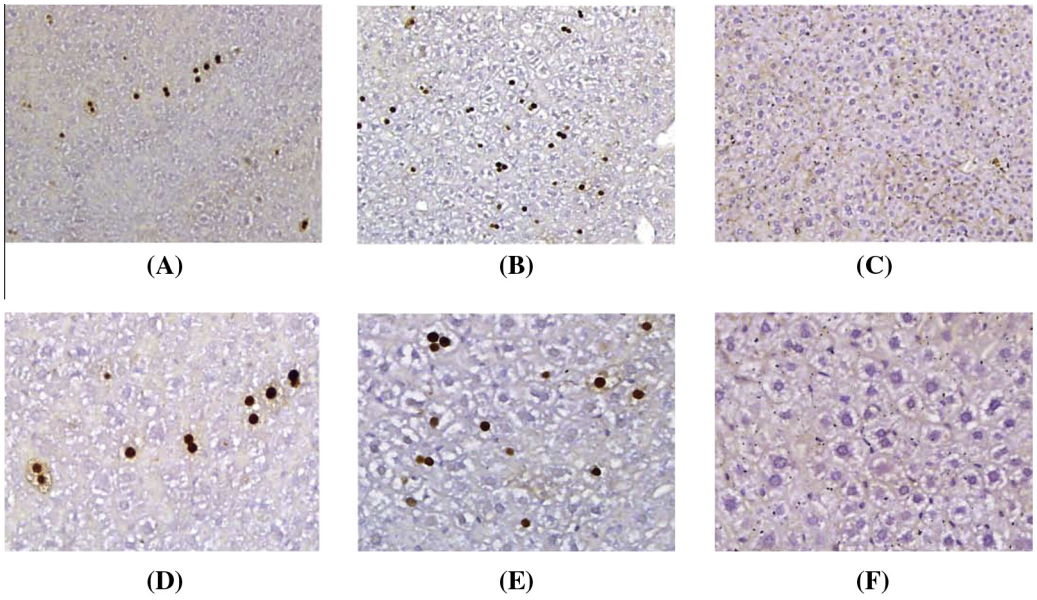

Fig. 3. Expression of HBcAg in the liver of mice after HDI. Immunohistochemical staining for HBcAg of liver sections were taken from C57BL/6 mice injected with 5 μg of pCS-HBV1.3 (A and D), pAAV-HBV1.3 (B and E), and 100 μL of PBS (C and F) at 5 dpi. Samples were prepared and staining with rabbit anti-HBc polyclonal antibody. Results of one representative mouse are shown. (A–C: 100-fold magnification; D–F: 200-fold magnification)

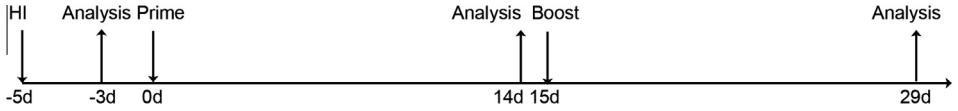

Fig. 4. Therapeutic evaluation schedule for novel HBV vaccines among HBV infection mice model. 5 days before immunisation, HBV infection in C57BL/6 mice were established via HDI. Each group (10 mice/group) was primed twice with mock (adjuvant combinations only) or HBSS1 particles vaccine together with various adjuvant combinations at days 0 and 15. Samples from mice were collected and evaluated at the indicated times.

compared to that with adjuvant formulation (Alum + Poly I:C) only (Fig. 5).

We further investigate HBV infection markers (DNA and relative antigens level) in the serum or liver of mice after HBSS1 vaccination (Fig. 6). Compared to the group with adjuvant formulation only, the clearance of HBV viremia and significant decline of serum HBsAg level was found among the mice of twice immunization with the novel HBSS1 vaccine formulations (Fig. 6B). However, the significant decline of HBeAg in serum was only transiently observed among immunized mice after priming with HBSS1 and combined adjuvant (Alum + Poly I:C) as showed in Fig. 6C. Furthermore, no difference of HBV DNA level in the liver was showed among three groups of mice (Fig. 6A), which indicated that immunisation with the HBSS1 formulation did not influence the existence of HBV DNA in the liver of HBV infection mice.

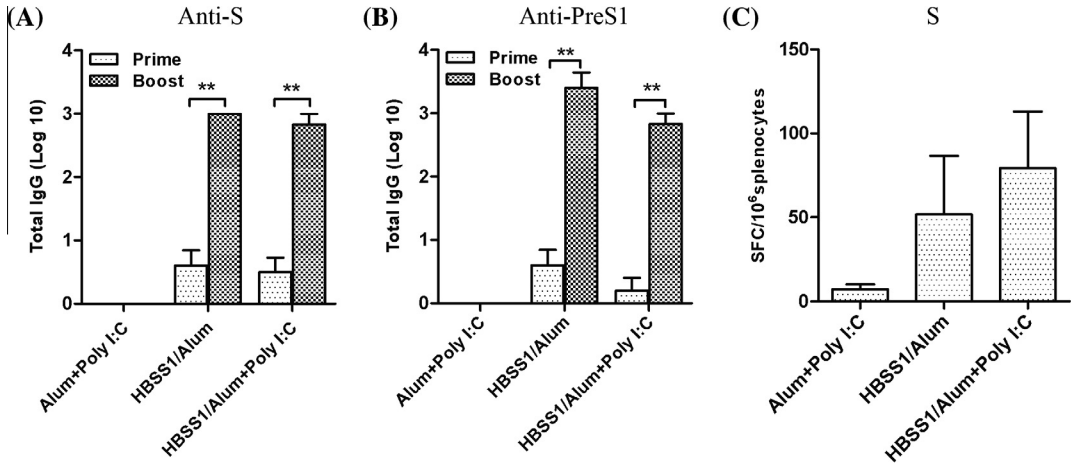

Fig. 5. Characterization of HBV antigens specific immunity induced by novel HBSS1-based vaccines immunization in HBV infection mice model. Titers of anti-HBV antigens IgG in the serum of mice induced by vaccination were determined in ELISA. Specific T-cell responses to HBV antigens were analyzed by ELISPOT assay 14 days after the boost immunization and their numbers were shown as spot-forming cells (SFC) per million splenocytes. The statistic significance of the results was analyzed and indicated: $^{**}P < 0.01$. (A) Titers of anti-S IgG in the serum of mice after prime or boost. (B) Titers of anti-PreS1 IgG in the serum of mice after prime or boost. (C) T-cell response to HBsAg.

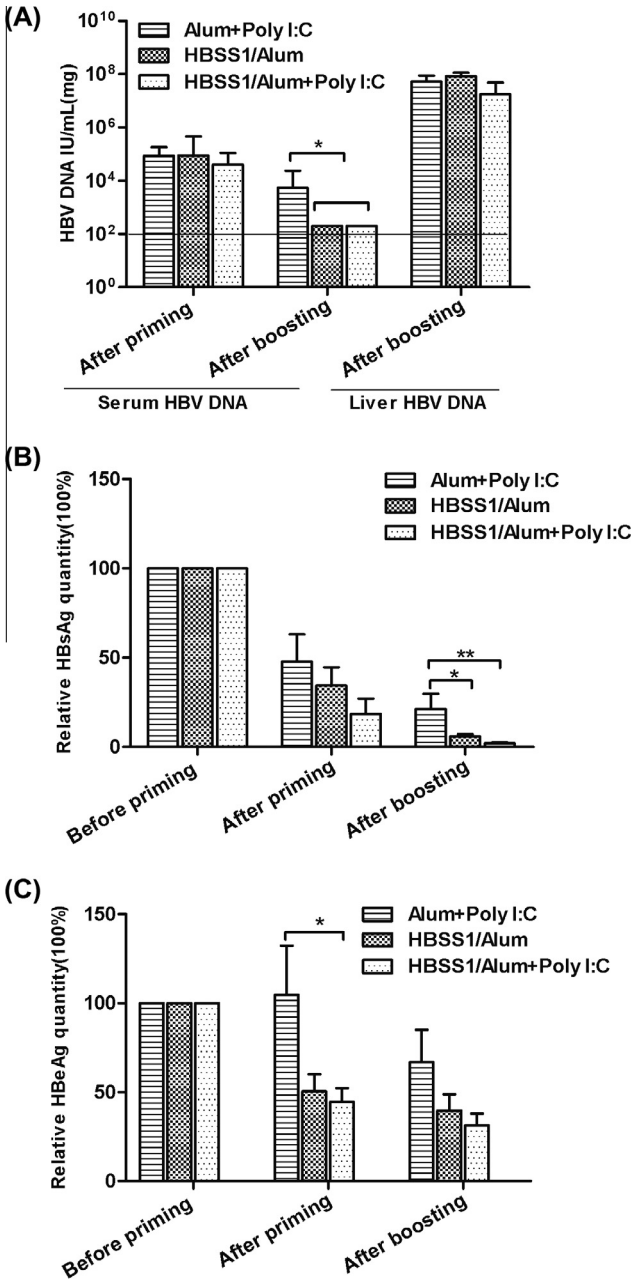

Fig. 6. Detection HBV DNA and HBV antigens in the group of persistence HBV infection mice after immunization with novel HBSS1-based vaccine formulations. Persistence HBV infection in the mice were developed by HDI with 5 μg of pCS-HBV1.3 at 5 days before immunisation (−5 d). The samples were collected from mice at days −3 d (before priming), 14 d (after priming) and 29 d (after boosting), respectively. HBV DNA concentration in the sera or liver of mice were determined by real-time PCR and expressed as IU/mL. HBsAg and HBeAg were estimated in the sera by ELISA and expressed as IU/mL or COI values, respectively. The relative quantity was calculated by the average of levels of antigen in the sera at indicate time to the average of levels of that at pre-immunization with vaccine (3 days after HDI, which was set as 100%). Data are presented as mean and standard deviation. (A) Detection of HBV DNA in the mice. (B) Analysis of relative HBsAg quantity in the sera of mice. (C) Analysis of relative HBeAg quantity in the sera of mice.

4. Discussion

We herein describe an alternative HDI method for the study of HBV persistence in a murine model, based on lentiviral backbone-based HBV replicon transfer. We also investigated several HBV persistence factors in this mouse model via HDI procedures. In mice receiving 5 μg of pCS-HBV1.3 via HDI, serum HBsAg, HBeAg and HBV DNA persisted for more than 56 d. To our knowledge, this is the first HBV-persistent infection mouse model using the lentiviral-based HBV-transfer vector. Furthermore, we first report that immunization of HBV replicon transfer mice with the novel HBSS1-based vaccines is able to overcome tolerance against HBV antigens in mice and induces robust immunity (humoral as well as T-cell responses), followed by the clearance of the HBV viremia as well as reduction of circulating HBsAg antigen levels. However, the induced immunity by these novel HBSS1 vaccines did not suffice to clear HBeAg in serum and HBV DNA in liver of HBV-persistent infection mouse model.

Previous studies have shown that the mouse genetic background influences the outcome after HDI. Huang et al. (2006) found that after HDI with 10 μg of pAAV-HBV1.3 in BALB/c mice, the HBsAg level promptly increased within 1 week but then decreased rapidly, and anti-HBs developed within 14 days. In C57BL/6 mice, the HBsAg level declined more slowly and remained positive at 35 dpi, and none of the mice developed anti-HBs within 28 days. In the present study, we also hydrodynamically injected 5 μg of pCS-HBV1.3 into BALB/c and C57BL/6 mice and obtained results similar to those above. This confirms that host genetic background does indeed influence HBV clearance in mice.

Gender has also been found to affect HBV replication and pathogenesis (Tian et al., 2012; Wang et al., 2009). We also evaluated the gender disparity in HBV replication in pCS-HBV1.3 HDI mice. Serum markers of HBV infection (viraemia and antigenaemia) in male C57BL/6 mice persisted for a shorter period of time than those in female mice; however, the kinetics of anti-HBc IgG and HBV DNA in the liver of male C57BL/6 mice were similar to those of female C57BL/6 mice. In male mice, although serum HBsAg, HBeAg, and HBV DNA could be detected only up to 28 dpi, the levels of these serum markers were higher than those in female mice up to 7 dpi. This is accordance to earlier report (Tian et al., 2012), which also demonstrated that HBV replicated approximately twice as efficiently in male mice than in female mice by using transgenic mice that carried the entire HBV genome and productively replicated HBV in the liver. However, we demonstrated gender disparity in our model, but the underlying mechanism responsible for this phenomenon needs further investigation.

HBV viral sequences and the construct backbone are believed to have contributed to the HBV persistence in this mouse model. The AAV backbone-based transfer plasmid has been widely used for HDI to establish chronic HBV infection mouse models. However, the construct based on AAV backbone HBV-replicon transfer resulted in acute HBV infection among C57BL/6 mice in this study. Our data showed that serum HBsAg, HBeAg, and HBV DNA was undetectable at 28 days in mice receiving 5 μg of pAAV-HBV1.3 via HDI, while anti-HBs was detectable at 28 d after injection. In contrast, the novel lentiviral backbone-based construct (pCS-HBV1.3) that we produced resulted in HBV persistence in immunocompetent female C57BL/6 mice. We found that HBsAg, HBeAg, and HBV DNA persisted for at least 7 weeks in the sera of >70% of HDI female C57BL/6 mice. In the meantime, these mice failed to develop neutralising antibodies (anti-HBs) even at 56 d after injection. However, HBcAg and HBV DNA in liver tissue were detected in both groups. No significant inflammation was observed in the liver. We supposed that the increased persistence of the HBV infection was associated with delayed anti-HBs antibody and presence of lower cellular-mediated immunity induced by pCS-HBV1.3 transfer in the mice. It is also assumed that the required *cis*-acting sequences (3′ and 5′ LTR, packaging signal (ψ), rev responsive element (RRE) and cPPT) in the SIN-lentiviral transfer vector may have contributed to the higher level of HBV replicon in the liver of transfer mice, resulting longer HBV persistence (Kafri et al., 1997; Sinn et al., 2005).

Previous clinical and experimental evidence suggests that the restoration of host immune response to HBV-related antigens may have therapeutic implications in chronic HBV infection (Buchmann et al., 2013; Chen et al., 2012a; Chuai et al., 2013; Michel et al., 2011). HBsAg specific immune responses normally are associated with effective clearance of HBV in chronic HBV patients (Zhou et al., 2010). We also showed that immunization of HBV replicon transfer mice with the novel HBSS1-based vaccine formulations was able to overcome tolerance against HBV in mice and induces robust immunity (humoral as well as T-cell responses), followed by the clearance of the HBV viremia. In addition, no obvious liver damage was detected (data not shown). There seems have a link between the HBsAg specific immunity and the clearance of HBV viremia and strong decline of serum HBsAg level. This is in line with the previous report and may support the concept that HBV specific immunity eliminates the virus also by noncytopathic mechanisms (Chen et al., 2011, 2012a; Chuai et al., 2013; Na et al., 2011). This study also further supports this HBV mice by HDI with pCS-HBV1.3 are good model of chronic HBV infection.

In summary, we established an HBV mouse model by HDI of the lentiviral-based HBV-transfer vector pCS-HBV1.3 into immunocompetent mice. We further optimised factors such as the mouse genetic background and gender. Overall, female C57BL/6 mice that underwent HDI with 5 μg of pCS-HBV1.3 were more suitable for the establishment of a persistent HBV infection model. With the ease of HDI and the convenience of lentiviral transfer plasmid construction, the approach reported herein provides a simple, convenient, and useful tool for functional studies of HBV replication and the development of new treatments and immune-based therapies or therapeutic vaccines for chronic HBV infection. Our study may also provide further insight into developing an immunotherapeutic approach for chronic HBV infection, although the mechanisms of this vaccine-based therapeutic effect on chronic HBV infection is need to be further explored.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.10.019.

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