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Heat shock protein gp96 enhances humoral and T cell responses, decreases Treg frequency and potentiates the anti-HBV activity in BALB/c and transgenic mice

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ABSTRACT

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV). Broad repertoire and strong magnitude of HBV-specific T cell responses are thought to play key roles for virus control and clearance. Previous studies together with ours showed that heat shock protein gp96 as adjuvant induces antigen specific T cell responses, yet little is known for its anti-viral properties. Here, we investigated the role of gp96 mediated cellular and humoral immunity in antiviral effects in HBV transgenic mice. Immunization with HBV surface (HBsAg) and core (HBcAg) antigens combined formulation along with gp96 induced robust antiviral T-cell and antibody immunity against HBsAg and HBcAg. Compared with non-immunized control, immunization with gp96 adjuvant vaccine led to decrease of serum HBs level and HBc expression in hepatocyte by 45% and 90% at maximum, respectively, and decreased serum HBV-DNA level to below or close to the detection limit 4 weeks after the last immunization, suggesting the therapeutic effect. A significant enhancement in cellular responses towards HBcAg and increased infiltration of CD8⁺ T cells in liver of transgenic were observed under treatment with gp96 compared with no treatment ($P < 0.05$ or 0.01). Treatment with gp96 was capable of reducing Tregs by overall 30–40%. The superior immune responses induced with the aid of gp96 correlated with improved antiviral effect by vaccination with HBsAg and HBcAg. We conclude that gp96 may contribute to enhanced antiviral immunity in transgenic mice at least partly by Treg down-regulation. HBcAg may act as potent adjuvant for Th1 response. Our study reveals the novel property of gp96 in immune modulation and its potential use for breaking immunotolerance in immunotherapy of chronic HBV infection.

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1. Introduction

While efficient preventive vaccines are now available against hepatitis B virus (HBV) infection, there are still more than 350 million chronic carriers of HBV worldwide [1]. In China alone about 93 million people are infected with HBV [2]. Chronic HBV infection continue to be a major public health problem as around 15–40% of infected patients will develop life-threatening complications such as cirrhosis, liver failure and hepatocellular carcinoma (HCC). The present antiviral therapies for chronically infected patients, which include interferon (IFN)- α and nucleoside or nucleotide analog (e.g. lamivudine, adefovir, and entecavir), are costly and yet only partially efficient, and drug-resistant HBV mutants frequently arise [3,4]. This underlines the need for development of therapeutic vaccines to complement or even replace current antiviral treatments.

Since clearance of HBV is mediated by robust viral specific CTL and helper T cell responses during resolution of acute and self-limited HBV infections, while chronic infection is characterized by weak T-cell responses, T cells are believed to play key roles for virus control and clearance [5–7]. Recombinant protein- [8–12], epitope [8], DNA or viral vector-based vaccines [13–16] with a particular focus on priming and activation of HBV-specific T cell immunoresponse, have been engineered and tested both in animal models and clinical trials.

Hepatitis core (HBcAg) and surface (HBsAg) proteins are the main structure antigens of HBV. It is well documented that during resolved HBV infection, strong and broadly specific CD8⁺ T cells targeting epitopes from both antigens are readily detected in comparison of weak and monospecific T cell response in chronic infection [6,7,17], indicating that HBcAg- and HBsAg-specific T-cell responses are linked with their potential to control HBV infection. Moreover, during HBV infection, HBcAg-specific CD8⁺ cells have been found to be associated viral control [6].

Previous research showed that heat shock protein gp96, when complexed with virus- or tumor-derived antigens, induced MHC

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class-I-restricted cytotoxic T-lymphocyte (CTL) responses by cross-presentation of antigenic peptides to MHC class I molecules, making gp96 a powerful adjuvant for the generation of CD8⁺ responses [18–20]. Consistent with these studies, our recent work also demonstrated that gp96 or its N-terminal fragment as adjuvant has the ability to augment HBV specific CTL responses [21]. We explored the mechanisms of gp96 mediated balance between CTL and regulatory T cells, which may potentially facilitate development of an effective gp96-based therapeutic vaccine.

There is growing literature showing chronic HBV infection is an immune-mediated disease. The presence of high level viral replication leads to a progressive functional decline of virus-specific CD4⁺ T-cell response, and ultimately to CD8⁺ T cell anergy and exhaustion [2,7,9,17,22]. Several factors could contribute the state of HBV-specific T-cell tolerance as seen in chronic HBV patients, including regulatory T cells (Tregs), dendritic cells and expression of programmed death-1 (PD-1) receptor [22–24]. Based on these observations, in our current study a rational therapeutic approach was designed for sequential administration using heterologous prime–boost immunization format to maximize induced CTL response. Two antigens with strong immunogenicity HBsAg and HBeAg were chosen along with adjuvant gp96 as the therapeutic vaccine to evaluate HBV-specific T-cell immunity first in BALB/c mice. Then we investigated if the gp96-based therapeutic vaccine could induce HBV-specific T-cell responses in HBV transgenic mice by comparison of the CTL responses between these two mouse models. We monitored the activity of CTL and Treg to determine capacity of gp96 to break HBV tolerance and its antiviral effects in HBV transgenic mice.

2. Materials and methods

2.1. Plasmid DNA constructs

Plasmid pTHBV containing the whole HBV genome (GenBank: EU562217, genotype C) was kindly provided by Prof. Dongping Xu [The 302 Hospital, Beijing]. pTHBV was taken as the template to amplify the HBs (encoding surface protein) and HBe (encoding core protein) sequences. The human gp96 cDNA (a kind gift of Dr P.K. Srivastava, University of Connecticut School of Medicine, USA), HBs and HBe gene were inserted into a mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), and they were respectively designated as pcDNA-gp96, and pcDNA-HBs, pcDNA-HBe. To construct plasmid pEGFP-gp96, pEGFP-HBs and pEGFP-HBe, the gp96, HBs and HBe gene were cloned into the pEGFP-N2 vector (Invitrogen) using a set of primers (Table 1). All the constructs were confirmed by enzyme digestion and DNA sequencing. Plasmids were expanded in DH-5 α (*Escherichia coli*) and purified using the Endo Free Qiagen kit (Qiagen, CA, USA) to remove the bacterial endotoxins.

2.2. Preparation of murine gp96 and the recombinant HBeAg and HBsAg

HSP gp96 was obtained from healthy murine livers as described [21]. The HBe gene was cloned into BamHI/XhoI digested digested pGEX-6p-1 vector (GE Healthcare), and the recombinant HBeAg was expressed and purified as GST fusion protein in *E. coli*. Contaminating endotoxin in all proteins was removed accordingly [21]. Briefly, all proteins were treated with detergent Triton X-114 and endotoxin was removed by phase separation. The remaining endotoxin was further eliminated with detoxi-gel endotoxin removing gel (Pierce). All materials were decontaminated prior to use, and pyrogen-free water was used in all experiments. The endotoxin levels were quantified using the Limulus Amebocyte Lysate (LAL) assay

(BioWhittaker). The concentration of endotoxin in both gp96 and HBsAg preparations were ultimately decreased to below 10 EU/mg of protein. Recombinant HBsAg (genotype C) produced by *Saccharomyce cerevisiae* was obtained from Beijing Tiantan Biological Products Co., Ltd. Beijing, China.

2.3. Plasmid transfections and analysis of gene expression

293T cells grown in 10-cm dishes were transfected with various constructs. Two days later, 100 μ l of culture medium was collected from each well and detected for the HBsAg or HBeAg level by ELISA kits (Kewei Diagnostic, Beijing, China), or cells were harvested and lysed for Western Blot analysis of gp96 [21]. For fluorescence assay of gp96, HBs and HBe expression, HepG2 cells were transfected with pEGFP-gp96, pEGFP-HBs, pEGFP-HBe or pEGFP N2 as the control. Plates were incubated at 37 °C for 6 h. Complete RPMI 10% was added to each well and the level of GFP expression of each construct was assessed by fluorescence microscopy at 24 h.

2.4. Mice immunization

Female BALB/c mice (6–8 weeks old) were purchased from Peking University, Beijing. Female HBV transgenic BALB/c mice were purchased from Transgenic Engineering Lab, Infectious Disease Center, Guangzhou, China [25]. Three different formulations were used for immunizations: DNA immunization, protein immunization and DNA prime/protein boost immunization. Six- to eight-week-old mice were immunized in regenerating tibialis anterior muscles with 50 μ g of plasmid pcDNA-HBs, pcDNA-HBe in PBS, or subcutaneously with 10 μ g of HBsAg and HBeAg in PBS with or without gp96 adjuvant (10 μ g of protein or 50 μ g of plasmid). The injection volume was adjusted to 200 μ l. Each group contained at least six BALB/c mice or five transgenic mice. All injections were performed at weeks 1, 2 and 4. Table 2 shows the immunization regimens of each group. As both HBsAg and HBeAg can naturally aggregate into particles, and HBeAg itself can act as a potent adjuvant [10,11], no adjuvant such as mineral oil was included in the immunization protocol.

2.5. Monitoring HBs, HBe humoral immune response by ELISA

Blood was collected at different time points after immunization by retrobulbar puncture. Quantitation of mouse anti-HBs antibody, anti-HBe antibody was performed by ELISA. Briefly, 96-well ELISA plates (Costar, Bethesda, MD, USA) were coated overnight at 4 °C with 10 μ g/ml recombinant HBsAg and HBeAg. After washing with PBST buffer (PBS containing 0.05% Tween 20), the wells were blocked with 5% nonfat dry milk in PBST buffer. Another wash was conducted before sera of serial dilutions in PBST buffer were added to the Ag-coated wells. Sera were incubated for 2 h at 37 °C. After washing with PBST buffer, bound serum Abs were detected using HRP-conjugated rat anti-mouse IgG, IgG1 and IgG2a (SEROTEC, Oxford, UK) at a dilution of 1:2000–5000 dilution. The reaction was stopped with 1 M H₂SO₄, and the extinction was determined at 490 nm.

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

BALB/c mice or HBV transgenic mice were sacrificed 1 or 4 weeks after the third immunization, respectively. Splenocytes were harvested from individual mice. ELISPOT assay was performed according to the protocol supplied by the manufacturer. In brief, 96-well PVDF plates (BD-Pharmingen, San Diego, CA) were precoated with the coating Ab overnight at 4 °C and blocked for 1 h at 37 °C. Purified splenocytes ((2–5) \times 10⁵ cells/well) and HBsAg, HBeAg, or control protein BSA (10 mM) as negative control or 5 μ g/ml of

Table 1

Sequence of primers used to construct expression plasmids.

Plasmid	Primers sequence	Restriction site
pcDNA-gp96	Sense: 5'-CTAGGATCCATGGACGATGAAGTTGATGTG-3' Antisense: 5'-CTAGAATTCCTTACAATTCATCTTTTCAG-3'	BamH I EcoR I
pEGFP-gp96	Sense: 5'-CTAGAATTCACCATGGACGATGAAGTTGATGTG-3' Antisense: 5'-CTAGGATCCATTACAATTCATCTTTTCAG-3'	EcoR I BamH I
pcDNA-HBs	Sense: 5'-CTAGGATCCATGGAGAACATCACATCAGG-3' Antisense: 5'-CTACTCGAGATTAATGTATACCCAAAGACAA-3'	BamH I BamH I XhoI I
pcDNA-HBc	Sense: 5'-CTAGGATCCATGGACATTGACCCGTATA-3' Antisense: 5'-CTACTCGAGACATTGAGATTCGCCGAG-3'	BamH I XhoI I
pEGFP-HBs	Sense: 5'-CTAGAATTCATGGAGAACATCACATCAGG-3' Antisense: 5'-CTAGGATCCATAATGTATACCCAAAGACAA-3'	EcoR I BamH I
pEGFP-HBc	Sense: 5'-CTAGAATTCATGGACATTGACCCGTATA-3' Antisense: 5'-CTAGGATCCAAACATTGAGATTCGCCGAG-3'	EcoR I BamH I

ConA as positive control were added to the well and incubated at 37 °C for 48 h. Each test condition was performed in triplicate. The spots were counted and analyzed with the ELISPOT Reader (Biosys, Germany).

2.7. Lymphoproliferative responses

A single cell suspension of splenocytes was seeded in complete RPMI medium 1640 supplemented with 10% FCS. Cells were incubated in U-bottomed, 96-well microtiter plates (Costar, Cambridge, MA) at a density of 2×10^6 cells/ml in the presence of 10 µg/ml of recombinant proteins (HBsAg, HBcAg), or 5 µg/ml of ConA as positive control and BSA as negative control. After 3 days of incubation, 1 µCi of [³H]-thymidine (Amersham, UK) was added to each well and incubated overnight. Cells were collected on filters with a harvester and [³H]-thymidine incorporation was determined by liquid scintillation counting. All tests were performed in triplicate, and proliferative responses were expressed as their stimulation indices (SI), which represent the ratio of mean proliferation after stimulation to the mean proliferation of medium controls.

2.8. Flow cytometry

The indicated populations of T cells (10^6 cells per sample) were stained with various fluorochrome-conjugated Ab against interested surface markers. FITC-conjugated anti-mouse CD8, PerCP-Cy5.5-conjugated anti-mouse CD3 and PE-conjugated anti-mouse CD25 were purchased from eBioscience. For intracellular staining of Foxp3, cell membranes were permeabilized with permeabilization buffer. APC-conjugated anti-mouse Foxp3 were purchased from eBioscience. Three or four color flow cytometric analyses were performed using FACSCalibur and CellQuest software (BD Biosciences) after staining.

2.9. Immunohistochemistry

For immunohistochemical staining, HBV transgenic mice were sacrificed 5 weeks after the third immunization and the liver tissue was fixed using formalin and embedded by paraffin for immunohistochemistry staining. Aceton-fixed cryosections (5 µm)

were incubated with the rabbit monoclonal anti-mouse CD8 (Santa Cruz, CA, USA) or anti-HBc antibody (Cowin Biotech Co., Ltd., China) for overnight at 4 °C after blocking endogenous peroxidase activity with 0.3% H₂O₂. Biotinylated goat anti-rabbit immunoglobulin (Zhongshang Goldenbridge Biotech, Beijing, China) were then added on the slides overnight at 4 °C. For the enumeration of cells, high-powered fields (hpf, 200×) were used for counting positive cells. Only brightly colored cells were counted. For each liver, means were calculated for the positive cell numbers in 3 microscopic fields distributed over three sections by the two independent observers.

2.10. Virological assessment

HBsAg and HBeAg were determined by commercial enzyme immunoassay kits (Kewei Diagnostic, Beijing, China). HBV Fluorescence Quantitative Polymerase Chain Reaction (PCR) Diagnostic Kit (Shanghai Kehua Bio-engineering Co., Ltd., China) was used to quantify HBV DNA levels in serum of mice.

3. Results

3.1. Preparation and analysis of DNA constructs and the recombinant proteins

To compare the desired immune response and anti-HBV effect, we used both DNA or protein immunization and prime-boost strategies in BALB/c mouse and HBV transgenic mouse model. We made various HBV vaccine constructs for constitutive expression of gp96, HBsAg, and HBcAg. The gp96, HBs and HBc genes were inserted into a mammalian expression vector pcDNA3.1, and they were respectively designated as pcDNA-gp96, pcDNA-HBs, and pcDNA-HBc. To test whether the HBV and gp96 DNA constructs could be expressed properly, 293T cell line was transfected with reagent control (with no plasmid), the empty vector (pcDNA3.1 as mock), and pcDNA-gp96. 48 h after transfection, protein levels (Fig. 1A) was analyzed by Western blot. Compared with control and mock, transfection with pcDNA-gp96 led to increase of gp96. For further confirmation of the gp96 DNA delivery and expression in HepG2 cells, gp96 gene was inserted into pEGFP-N2. Gp96 expression was evaluated by detecting EGFP fluorescence under

Table 2

DNA, protein and prime-boost immunization in BALB/c mice and HBV transgenic mice in the different formulations.

Group	First week	Second week	Fourth week
1	DNA(HBs/HBc)	DNA(HBs/HBc)	DNA(HBs/HBc)
2	DNA(HBs/HBc + gp96)	DNA(HBs/HBc + gp96)	DNA(HBs/HBc + gp96)
3	Protein(HBs/HBc)	Protein(HBs/HBc)	Protein(HBs/HBc)
4	Protein(HBs/HBc + gp96)	Protein(HBs/HBc + gp96)	Protein(HBs/HBc + gp96)
5	DNA(HBs/HBc)	Protein(HBs/HBc)	Protein(HBs/HBc)
6	DNA(HBs/HBc + gp96)	Protein(HBs/HBc + gp96)	Protein(HBs/HBc + gp96)
Control	Empty vector	Empty vector	Empty vector

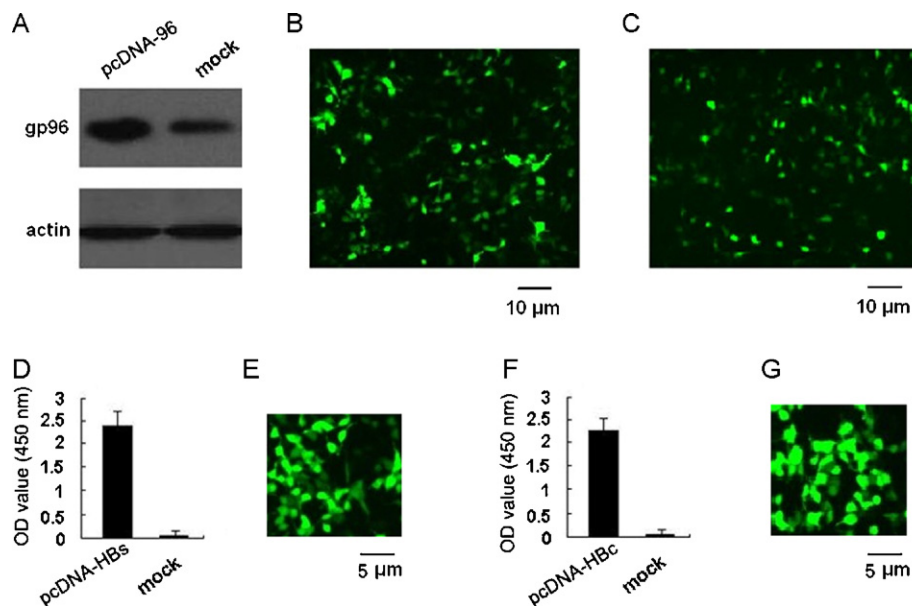


Fig. 1. Production of DNA constructs. (A) Western blot analysis to characterize the expression of vaccine plasmid pcDNA-gp96. 293T cells were transfected with pcDNA3.1 (mock), pcDNA-gp96. 48 h after transfection, cells were harvested, and cell lysates were immunoblotted with an anti-gp96 Ab. Actin was used as loading control. (B) and (C) Epifluorescent micrograph of GFP expression by HepG2 cells. HepG2 cells were transfected with pEGFP-N2 (B) as positive control or pEGFP-gp96 plasmids (C). GFP expression was assessed by fluorescence microscopy 24 h after plasmid delivery. The experiments were performed twice and similar results were obtained. (D) and (F) ELISA analysis to characterize the expression of vaccine plasmid pcDNA-HBs, pcDNA-HBc or pcDNA3.1 as mock. Expressed antigens of HBsAg (D) and HBcAg (F) in the media were detected by ELISA 48 h after transfection. Results are presented as means \pm SD from 3 independent experiments. (E) and (G) Fluorescent micrograph of GFP expression by transfection with EGFP-HBs and EGFP-HBc in HepG2 cells.

fluorescence microscopy at 24 h. Transfection of HepG2 cells with pEGFP-N1 (Fig. 1B) and pEGFP-Gp96 (Fig. 1C) conferred transgene expression in about 70–80% and 60–70% of cells respectively, which indicates that the pcDNA3 construct for gp96 expression is efficient. High level of expression product of HBV surface or core protein was detected by ELISA in HepG2 cells at 48 h after transfection with pcDNA-HBs (Fig. 1D) or pcDNA-HBc (Fig. 1F). Meanwhile, HBs and HBc expression were also evaluated by detecting EGFP fluorescence at 24 h. Transfection of HepG2 cells with pEGFP-HBs (Fig. 1E) and pEGFP-HBc (Fig. 1G) conferred transgene expression in about 80% (Fig. 1E) and 75% (Fig. 1G) of cells respectively.

The preparation of HBcAg expressed in *E. coli* was analyzed by a Coomassie Blue-staining and immunoblotting with an anti-HBc mAb (Fig. 2A). HSP gp96 was obtained from healthy murine livers as described previously (Fig. 2B) [21].

3.2. Gp96 enhanced stimulation of T cell and humoral immune responses in BALB/c mice

We designed 3 immunization strategies: DNA immunization, protein immunization and DNA prime/protein boost immunization, and tested first in BALB/c mice which one was the most potent for inducing CTL response. Female BALB/c mice (6–8 weeks old) were immunized with HBs and HBc DNA constructs, or HBs and HBc protein, or DNA-prime/protein-boost vaccination regimen for 3 times, with or without gp96 adjuvant. CTL expansion in response to HBsAg and HBcAg was quantitated 1 week after the third immunization. Fresh splenocytes from immunized mice were analyzed by ELISPOT assay to quantify antigen specific T cells. ConA served as the positive control. As can be seen in Fig. 3A, compared with treatments without gp96 adjuvant, the numbers of spot-forming cells (SFCs) to HBsAg and HBcAg increased greatly both in DNA (group 2) or protein vaccination (group 4) with gp96 (both $P < 0.05$). Notably, the most remarkable T cell responses were observed in mice with DNA-prime/protein-boost vaccination (group 6). We used a con-

trol protein BSA for evaluation of the background of ELISPOT. T cells from immunized mice could not be stimulated by the negative control protein (Fig. 3A), suggesting that gp96 induced T cells are antigen specific. As T cell proliferation is an important in vitro parameter of in vivo immune, we further measured splenocyte proliferation by [3 H]-thymidine incorporation assay (Fig. 3B). Under gp96 treatment, increased cell proliferation was observed in mice immunized with DNA-prime/protein-boost vaccination (group 6) compared with no treatment (group 5) ($P < 0.05$). Unlike the results in ELISPOT assay, there was only a trend of increase in splenocyte proliferation under gp96 treatment in DNA and protein immunization groups, but the trend did not reach statistical significant.

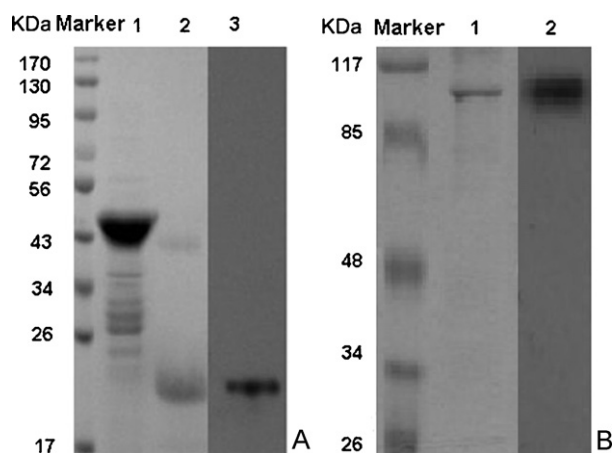


Fig. 2. Expression and purification of recombinant protein. (A) Recombinant GST-HBcAg expressed in *E. coli* (lane 1) and precision protease cleaved HBcAg were subjected to SDS-PAGE and stained with Coomassie Blue (lane 2), or immunoblotted with an anti-HBcAg Ab (lane 3). (B) Purified gp96 from murine livers was analyzed on a SDS-PAGE gel and stained with Coomassie Blue (lane 1) or immunoblotted with an anti-gp96 Ab (lane 2).

This is probably because [^3H]-thymidine incorporation assay measures the proliferation of the whole population of splenocytes while ELISPOT measures the activity of T cells.

Meanwhile, the anti-HBsAg levels were monitored by ELISA assay at 1-week interval after the first immunization. A significant adjuvant effect of gp96 was observed in protein and DNA prime/protein boost immunization groups at as early as week 3 after first immunization (Fig. 4). At week 4, co-immunization with gp96 (group 4) led to increase of IgG (Fig. 4A), IgG1 (Fig. 4B) and IgG2a (Fig. 4C) by 0.5-, 2.7- and 1.2-fold respectively ($P < 0.01$ for IgG and IgG1, and $P < 0.05$ for IgG2a), compared with immunization with HBs and HBc alone (group 3). Notably, under gp96 treatment, DNA-prime/protein-boost vaccination (group 6) also significantly enhanced IgG, IgG1 and IgG2a production compared with no treatment (group 5) (all $P < 0.01$). Similar results were observed until week 5 when the mice were sacrificed. The data above indicate that gp96 enhances both T cell and humoral immune responses against HBsAg and HBcAg in BALB/c mice, and compared with DNA immunization, protein and DNA-prime/protein-boost vaccination with gp96 adjuvant are more efficient for T cell and humoral immune responses.

3.3. HBs and HBc-specific T cell responses induced by gp96 in HBV transgenic mice

Next, we investigated if the gp96-based vaccines could induce HBV-specific CTL responses in HBV transgenic mice which are HBV-immunotolerant. HBV transgenic mice were vaccinated with HBsAg and HBcAg or DNA-prime/protein-boost regimen at weeks 1, 2 and 4 respectively, with or without gp96 adjuvant. Fresh splenocytes from immunized mice were collected and analyzed at week 8. The DNA, protein and prime/boost vaccine formulations effectively elicited T cell responses to HBsAg and HBcAg determined by ELISPOT assay, while only very low virus-specific T-cell immunity could be detected in control mice (Fig. 5A), indicating that the transgenic mice are immunotolerant to HBV. Moreover, significant difference in HBcAg-specific T-cell activation was observed between mice treated with and without gp96 in DNA (group 2 vs group 1, $P < 0.05$), protein (group 4 vs group 3, $P < 0.05$) or prime/boost (group 6 vs group 5, $P < 0.05$) immunization. Similar trend was observed for HBsAg-specific T-cell activation, but the difference did not reach statistical significance, which suggests the ability of gp96 to induce HBcAg-specific T cells is more prominent than HBsAg-specific T cells in HBV transgenic mice. We also found a significantly higher number of total CD8⁺ T cells in spleens of gp96 treated mice compared with no treatment (Fig. 5B), which let us further detect CTLs in liver. Liver tissues from transgenic mice were examined by immunohistochemistry staining of CD8⁺ expression (Fig. 5C). As can be seen in Fig. 5D, a dramatic increased number of liver-infiltrating CD8⁺ T cells were observed between mice treated with and without gp96 in prime/boost immunization groups (group 6 vs group 5, 35 ± 4 vs 15 ± 4 , $P < 0.01$), protein immunization groups (group 4 vs group 3, 25 ± 3 vs 9 ± 2 , $P < 0.01$), and DNA immunization groups (group 2 vs group 1, 13 ± 3 vs 8 ± 2 , $P < 0.05$). To investigate antigen-specific humoral responses, the serum level of specific IgG, IgG1 and IgG2a antibodies to HBsAg and HBcAg was determined by ELISA at week 8. All protein, DNA and prime/boost vaccine elicited humoral responses to HBsAg and HBcAg (Fig. 5E and F). However, unlike enhanced CTL responses by gp96, we did not see similar outcome in antibody responses. No significant difference of serum antibody levels was seen between gp96 treated and untreated mice although there was a trend of increase of IgG, IgG1 and IgG2a against HBsAg when treated with gp96. The results suggest that HBV transgenic mice immunized with HBsAg and HBcAg protein or prime/boost vaccine generated specific T cell

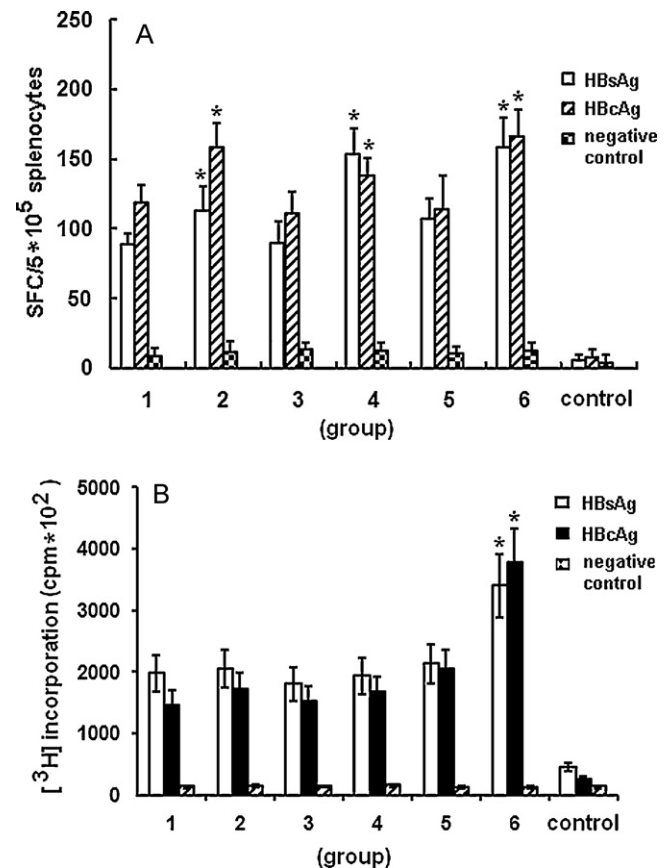


Fig. 3. gp96 promotes HBV-specific T cell response. (A) ELISPOT assay. BALB/c mice were immunized with DNA constructs (groups 1 and 2), protein (groups 3 and 4) or DNA prime/protein boost (groups 5 and 6) with or without gp96 adjuvant, as indicated in Table 2. Immunization with empty vector served as a control. Splenocytes from immunized mice were stimulated with HBsAg and HBcAg, or BSA as negative control for background evaluation. (B) T-cell proliferation assay. Splenocytes were cultured and stimulated with HBsAg, HBcAg or BSA as the negative control, and cell proliferation was determined by [^3H]-thymidine incorporation analysis. Data show means \pm SD of 6 mice. * $P < 0.05$ by *t*-test, compared with no gp96 immunization. Data are representative of two independent experiments.

and humoral responses, and T cell response can be greatly enhanced by gp96 treatment.

3.4. Down-regulation of Treg by gp96 in transgenic mice

CD4⁺CD25⁺FoxP3⁺ Treg suppress the activation, proliferation and effector functions of many cell types including CD4⁺ and CD8⁺ T cells. We therefore detected Treg in the spleen to understand the possible involvement of Treg in the gp96 induced T cell response and break of immune tolerance in HBV transgenic mice (Fig. 6A). Treg are defined as the population of CD4⁺CD25⁺FoxP3⁺ T cells as a percentage of total CD4⁺ T cells. A significant difference in the percentage of Treg was observed between mice immunized with or without gp96 in prime/boost groups (group 5 vs group 6, 9.09 ± 0.67 vs 6.61 ± 0.72 , $P < 0.05$) or protein immunization groups (group 3 vs group 4, 11.39 ± 0.74 vs 6.63 ± 0.58 , $P < 0.01$) (Fig. 6B). Treatment with gp96 led to decrease of Treg populations by 27% and 41% in prime/boost and protein immunization groups, respectively. Compared with control, immunization with HBsAg and HBcAg alone did not cause a significant change in number of Treg in both groups. The result indicates that enhancement of T-cell response by gp96 is associated with dramatic decline of Treg activity. The data also suggests that the ability of gp96 to break immunotolerance in HBV transgenic mice.

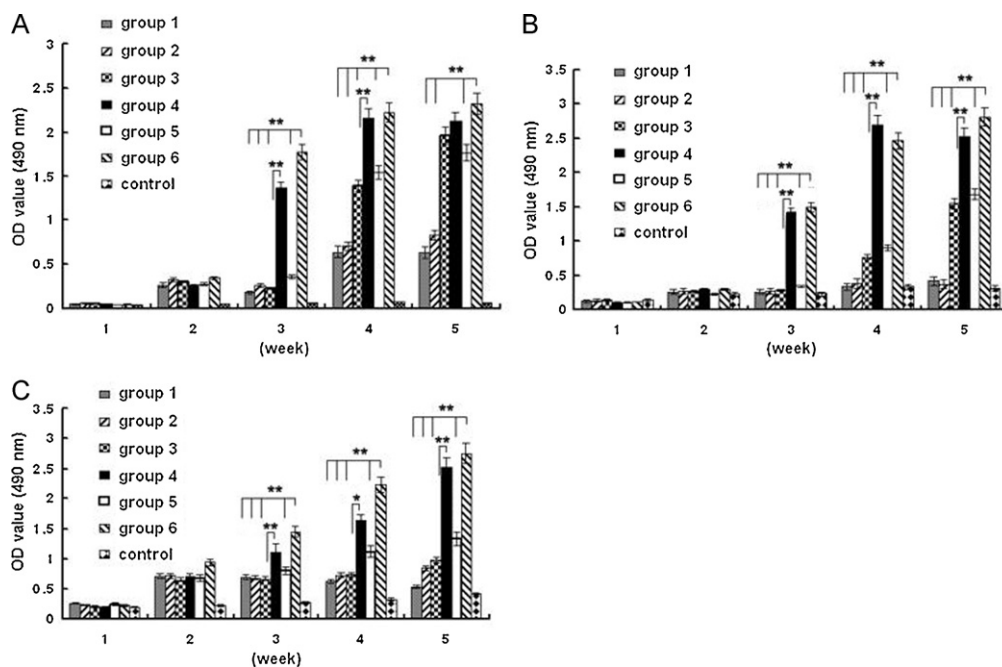


Fig. 4. Humoral immune responses induced by gp96. BALB/c mice were immunized as in Table 2. Serum anti-HBsAg IgG (A), IgG1 (B), and IgG2a (C) antibody titers were detected by ELISA using serial dilution at 1-week interval after the first immunization. IgG, IgG1 and IgG2a titers from 1:2500, 1:2000 and 1:2000 dilution sera respectively. Data show means \pm SD of 6 mice. * $P < 0.05$; ** $P < 0.01$ by *t*-test, compared with no gp96 immunization. Data are representative of two independent experiments.

3.5. Therapeutic effect against HBV by immunization with gp96 adjuvant vaccine

Then we evaluated if the immune responses induced by gp96 adjuvant vaccine could lead to inhibition of HBV. Serum HBs antigen, HBV-DNA and alanine transaminase (ALT) levels were determined at 1-week interval after the first immunization. Compared with control, the level of HBs began to decrease at week 6 in all immunized groups. Notably immunization with HBsAg and HBcAg along with gp96 (group 6) led to 45% of decrease in HBs level compared with control. A significant difference of HBs levels was observed between mice immunized with or without gp96 in prime/boost groups (group 6 vs group 5, 1.3 ± 0.11 vs 1.5 ± 0.07 , $P < 0.05$) or protein immunization groups (group 4 vs group 3, 1.5 ± 0.10 vs 1.75 ± 0.12 , $P < 0.05$) (Fig. 7A). Similar result was obtained in the HBV-DNA assay (Fig. 7B). Compared with control, all immunization groups had a dramatic decrease (>100-fold) of serum HBV-DNA below or close to the detection limit determined by quantitative PCR at week 8. A significant difference of HBV-DNA levels was observed between mice immunized with or without gp96 in prime/boost groups (group 6 vs group 5, $P < 0.05$). The serum ALT levels in all immunization groups began to rise at week 3, peaked at week 5, and returned to normal level at week 8, while stable ALT levels were observed in control mice (Fig. 7C).

The virological and ALT patterns were similar in all immunization groups in which decrease of serum HBs usually followed the decrease or normalization in serum ALT levels of viral replication. Consistent to the results of serum HBs antigen and HBV-DNA assay, all immunized groups had a dramatic decrease of HBcAg expression in hepatocytes of HBV transgenic mice (Fig. 7D). The percentage of HBcAg positive hepatocytes in liver of control mice was about 70%, which then reduced to below 40% in all immunization groups. Of note, immunization with HBsAg and HBcAg along with gp96 (groups 4 and 6) led to about 90% of decrease in HBcAg expression compared with control. A significant difference of HBcAg positive hepatocytes was observed between mice immunized with or without gp96 in prime/boost groups (group 6 vs group 5, 7% vs 12%,

$P < 0.05$) or protein immunization groups (group 4 vs group 3, 8% vs 17%, $P < 0.05$) (Fig. 7E). The results suggest that T-cell responses induced by gp96 could greatly inhibit HBV replication in the liver of transgenic mice.

We also observed that HBsAg alone with gp96 did not induce sufficient immunoresponse against HBV replication in transgenic mice (data not shown), suggesting HBcAg as a high immunogenic antigen and potent adjuvant for Th1 response.

4. Discussion

In this study, the capacity of heat shock protein gp96 to induce humoral and cellular immunoresponses and inhibit HBV replication was evaluated in BALB/c mice and HBV transgenic mice after immunization with combined HBsAg and HBcAg formulation along with gp96. A marked enhancement in antibody and cellular responses towards both HBsAg and HBcAg were detected under coadministration of gp96. The superior immune responses induced with the aid of gp96 correlated with improved antiviral effect by vaccination with HBsAg and HBcAg. Compared with non-immunized transgenic mice, immunization with gp96 adjuvant vaccine reduced serum HBs level and HBc expression in liver tissue by 45% and 90% at maximum, respectively, and decreased serum HBV-DNA level more than 1000-fold to below or close to the detection limit. Treatment with gp96 elicited an overall 30–40% decrease of Tregs which negatively regulate cellular and antibody immunity during HBV infection [23,26,27]. Our study reveals the novel property of gp96 in immune modulation and its potential use for breaking immunotolerance in immunotherapy of chronic HBV infection.

We found that co-administration of two vaccine antigens comprising HBsAg and HBcAg in both prime–boost and protein forms, induced effective immunity against HBcAg and HBsAg in HBV transgenic mice, indicating combination with these two antigens could be an effective approach in the enhancement of antiviral immunogenicity. HBcAg and HBsAg are the main structural pro-

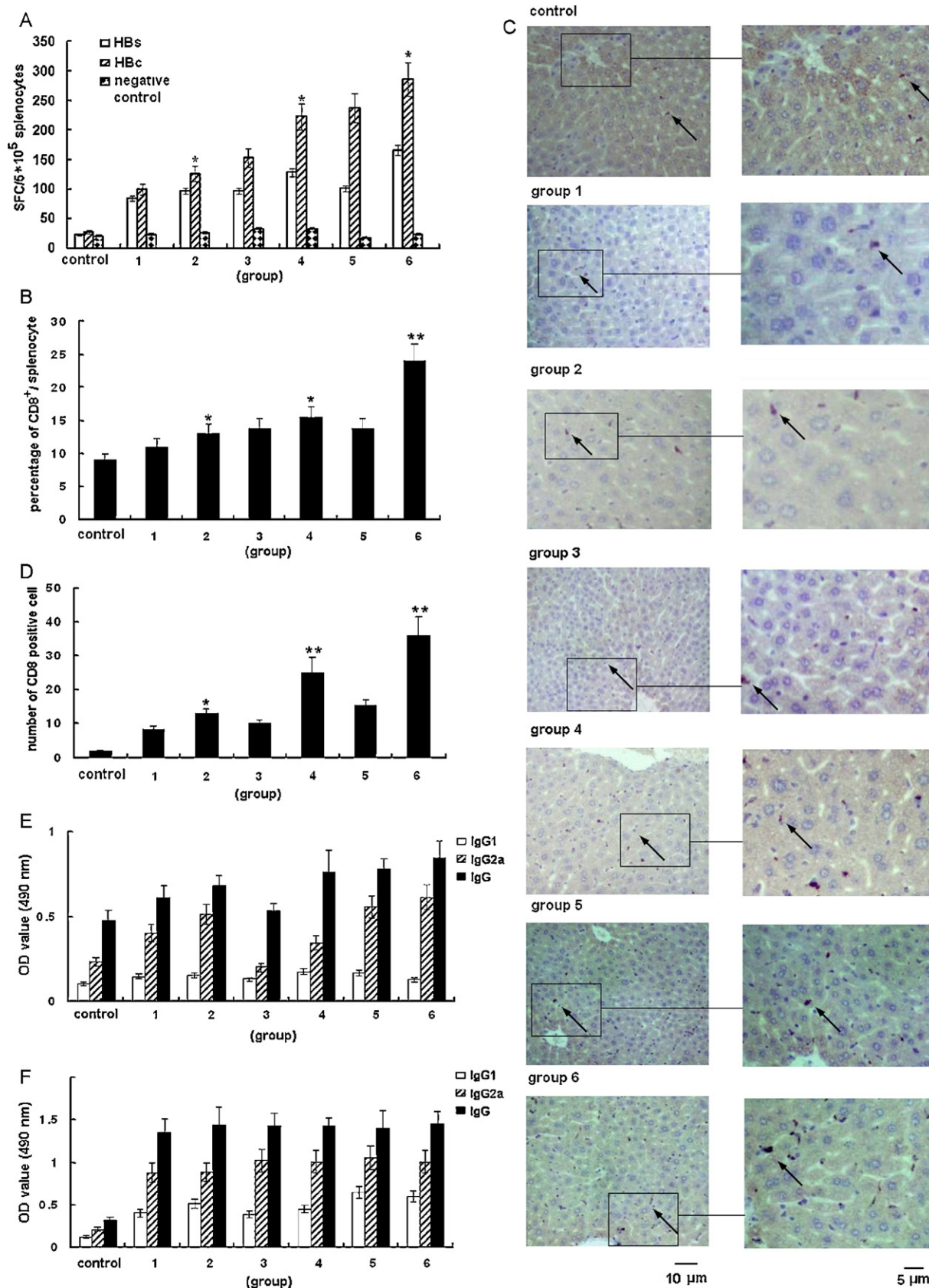


Fig. 5. gp96 induces HBV specific T cell response in HBV transgenic mice. (A) ELISPOT assay. HBV transgenic mice were immunized as indicated in Table 2. Splenocytes from immunized mice were stimulated with HBsAg, HBcAg or BSA as negative control for background evaluation. Flow cytometric (B) and immunohistochemistry (C and D) analysis were performed to quantify CD8+ T cell population from the spleen and T cell infiltration in the liver of transgenic mice. Serum anti-HBsAg (E) and anti-HBcAg (F) IgG, IgG1, and IgG2a antibody titers were detected by ELISA using serial dilution. Anti-HBsAg and anti-HBcAg antibody titers from 1:1500 dilution sera. Data show means \pm SD of 5 mice. * $P < 0.05$; ** $P < 0.01$ by *t*-test, compared with no gp96 immunization. Data are representative of two independent experiments.

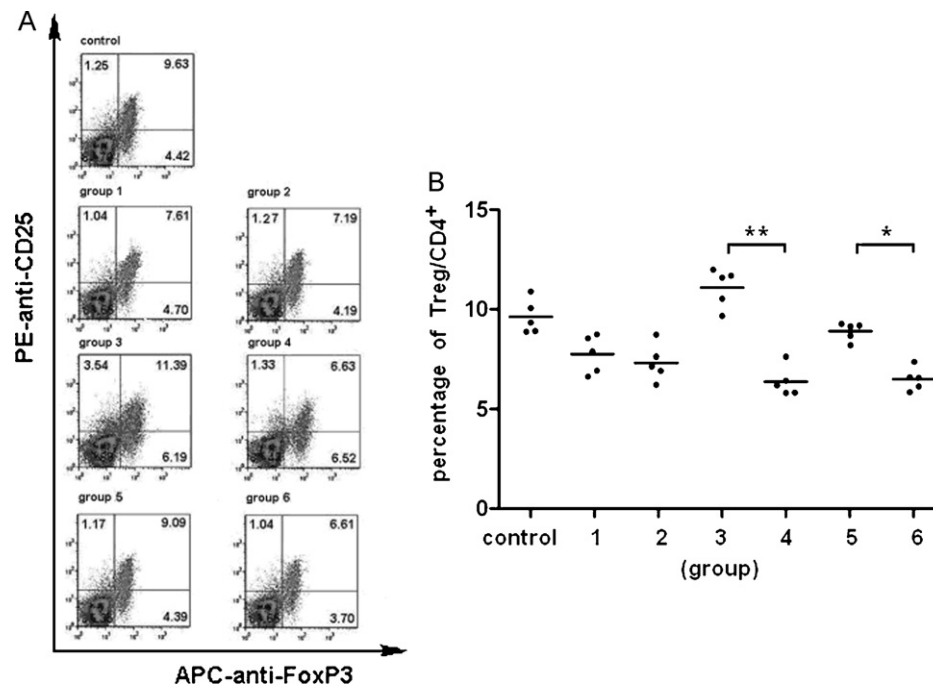


Fig. 6. CD4+CD25+FoxP3+ Treg down-regulation by immunization with gp96. (A) Splenocytes from immunized mice were stained with CD3, CD4, CD25 and intracellular Foxp3. CD25 and Foxp3 expression of CD4+CD3+ cells are shown. (B) Comparisons of the frequencies of CD4+CD25+FoxP3+ Treg among mice immunized with or without gp96. Data show means \pm SD of 5 mice. * $P < 0.05$; ** $P < 0.01$ by *t*-test, compared with no gp96 immunization. Data are representative of two independent experiments.

teins of HBV, which constitute key immunogens for experimental animals as well as in humans infected with HBV [7,8,11,18,28]. A nasal vaccine candidate comprising HBsAg and HBCAg has been developed and tested phase I clinical trial [10,11]. These studies showed that besides serving as an immunogenic target, HBCAg had unique ability to act as a potent Th1 adjuvant to HBsAg due to its unique organizing aggregated structure. The HBCAg expressed in *E. coli* forms particles resembling those in HBV-infected liver cells, which have recently been demonstrated to drastically improve the immunogenicity of HBV X protein (HBx) derived epitopes [29]. In agreement with above studies, we found that the formulation containing a mixture of HBsAg and HBCAg, both in DNA and protein forms, demonstrated potent immunogenicity in BALB/c and HBV transgenic mice, and coadministration of gp96 with HBsAg alone did not elicited effective anti-HBV responses in transgenic mice (data not shown). DNA vaccine did not exhibit superior efficacy over protein vaccine for T cell activation (see Fig. 3), as generally expected, which is probably due to the potent Th1-inducing activity of HBCAg.

Based on our earlier studies [21,30] demonstrating the successful application of gp96 as adjuvant to augment CTL responses, in this study we focused on antiviral T-cell response mediated by gp96 in HBV transgenic mice. Although it well documented that HBCAg as an antigen of choice to increase the immunogenicity of epitopes and the variety of the immune response in mice or clinical trial in healthy adults, few have investigated the antiviral efficacy of therapeutic vaccination using HBCAg in combination with other antigens such as HBsAg in HBV transgenic model or clinical trials in the treatment of chronic hepatitis B. It is possible that due to immunotolerance and impaired immune function, vaccination effective in healthy volunteers with no HBV exposure could only induce comparatively weak T-cell responses in chronically infected patients. Therefore, novel strategies are needed to break immunotolerance and induce vigorous cellular immune responses which are sufficient enough to eradicate HBV infection.

In this study, we observed the potential of gp96 as effective adjuvant for improving antiviral efficacy of the HBsAg and HBCAg

based vaccine. Co-administration with gp96 induced 34% or 17% increase in HBCAg specific cellular immunity in HBV transgenic mice with protein or prime/boost immunization respectively, compared with no gp96 treatment (Fig. 5A). There is considerable evidence that HBCAg-specific T cell response plays key role in the control of HBV infection due to its antigenic conservation and immunogenicity [7,10,31–33]. In addition, we observed an overall 2-fold increase of liver-infiltrating CD8+ T cells in gp96 treated mice with protein or prime/boost immunization. Accordingly, serum HBs level in transgenic mice at week 8 decreased by 14% or 16%, serum DNA copies decreased by 2.5-fold or 100-fold, and HBCAg expression in hepatocytes reduced by 53% or 42% in protein or prime/boost immunization groups respectively, under gp96 treatment compared with no treatment (Fig. 7). These encouraging data are consistent with previous reports regarding the potency of gp96 based therapeutic vaccines against cancer and infectious diseases, including in clinical trials [34,35].

Another intriguing question arises from our observations that the frequency of Tregs declined in the spleen of HBV transgenic mice under low dose (10 μ g) of gp96 treatment. As high amount of HBsAg was detected circulating in the serum (Fig. 7A), while only very low of HBsAg-specific humoral and T-cell immunity could be detected in HBV transgenic mice (Fig. 5), which could contribute to the maintenance of immune tolerance to HBV. These data suggest that the HBV transgenic mice in the study are generally immunotolerant to HBV, which can be employed as a model of chronic HBV infection. Immune tolerance is a primary barrier to the development of effective vaccines to eradicate HBV in host of chronic infection. Tregs are believed to play a major role in host immune tolerance during HBV chronic infection [23]. Conceivably, down-regulation of Treg may be one of the contributing factors for highly effective gp96 vaccines, as Treg may interfere with HBV specific T cell responses which play critical role in viral elimination after infection [24]. Similar result was obtained by Schreiber et al., showing that gp96-Ig immunization induced immune responses against established tumors by decreasing CD11b+Gr-1+ cells and FoxP3+ cells, and thus subjugating tumor-induced suppression of CTL

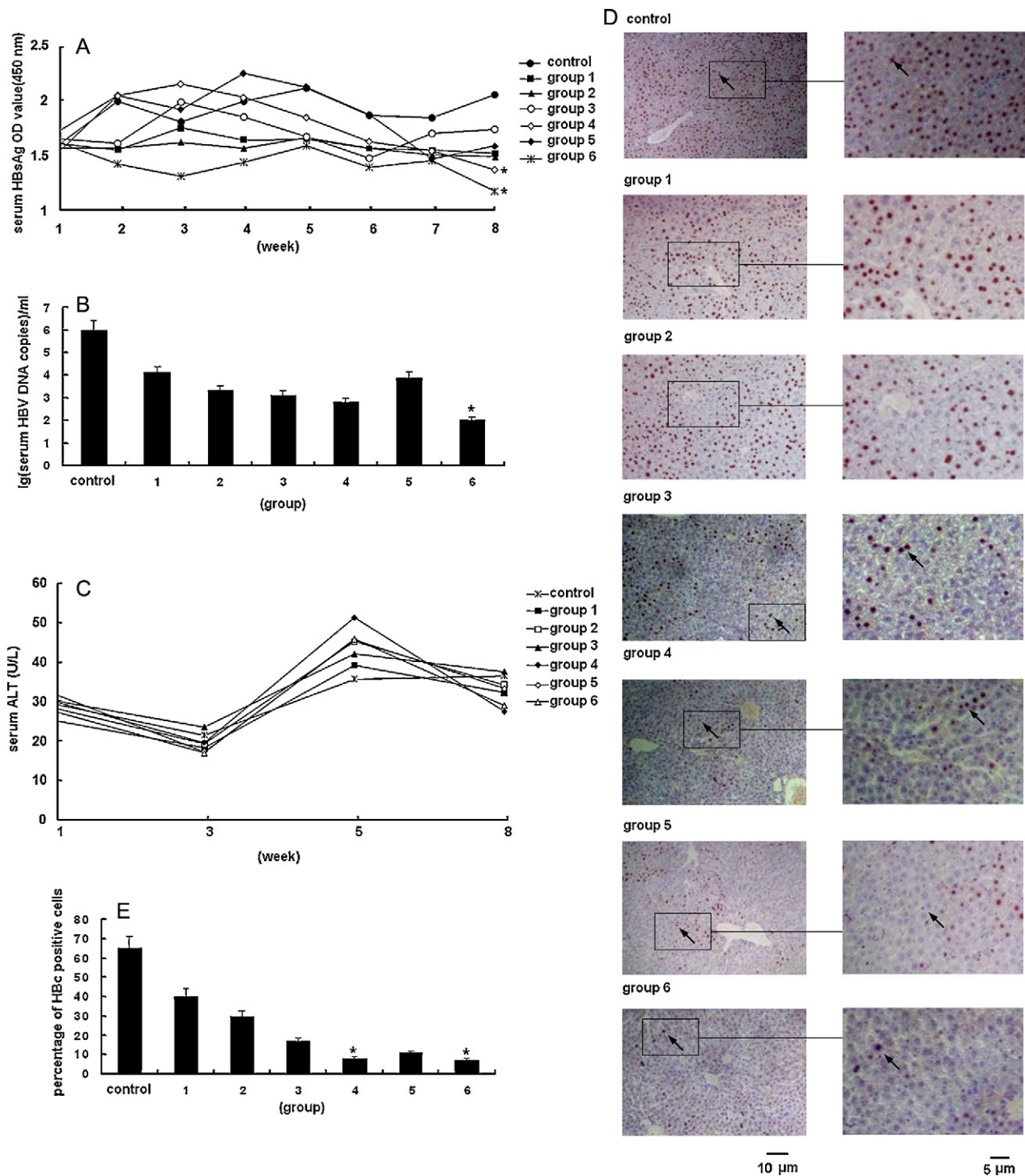


Fig. 7. Inhibition of HBV replication in transgenic mice by immunization with gp96. (A) Serum HBsAg was measured by ELISA. (B) HBV DNA copies from Serum were determined by real-time PCR analysis. (C) sALT was detected at the indicated times post-immunization. (D) Immunohistochemistry analysis of HBCAg expression in the liver of transgenic mice. (E) Comparisons of the percentage of HBC positive hepatocytes among mice immunized with or without gp96. Data show means \pm SD of 5 mice. * $P < 0.05$ by *t*-test, compared with no gp96 immunization. Data are representative of two independent experiments.

expansion [36]. Our previous study showed that high dose (100 μ g) of gp96 immunization could result in detrimental bystander effects on HBV-specific T cells stimulation by activation of Treg in HBV-naïve mice, indicating a balance between Treg and CTLs mediated by gp96. More studies are needed to understand the immunomodulatory role of gp96 in various states of HBV infection, which may help to optimize efficiency of gp96-based vaccines against HBV infection.

In conclusion, this study has significant implications in the application of gp96 based immunotherapy against chronic HBV infection. In the light of strong cellular immunoresponse and significant antiviral effects in BALB/c mice and HBV transgenic mice, the gp96 based HBsAg-HBCAg combined formulations appear to be promising candidates for development of therapeutic hepatitis B vaccine. These results may also provide bases for designing and evaluating effective gp96-based vaccines aimed at

eliciting T-cell responses for prophylactic and therapeutic applications.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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