生 物 工 程 学 报
Chinese Journal of Biotechnology
http://journals.im.ac.cn/cjbcn
December 25, 2013, 29(12): 1765−1775

医学与免疫生物技术

电转联合热休克蛋白佐剂疫苗引发抗 HBV 的免疫应答

徐亚星[1,2], 王彦中[1,2], 赵报[3], 张小俊[1,2], 范红霞[1,2], 李星辉[1,2], 孟颂东[1]

1 中国科学院微生物研究所 中国科学院病原微生物与免疫学重点实验室, 北京 100101
2 中国科学院大学, 北京 100049
3 中国科学技术大学, 安徽 合肥 230026

徐亚星, 王彦中, 赵报, 等. 电转联合热休克蛋白佐剂疫苗引发抗 HBV 的免疫应答. 生物工程学报, 2013, 29(12): 1765–1775.
Xu YX, Wang YZ, Zhao B, et al. Activation of anti-HBV immune activity by DNA vaccine via electroporation using heat shock proteins as adjuvant. Chin J Biotech, 2013, 29(12): 1765–1775.

摘 要: 针对 HBV 感染的治疗性 DNA 疫苗虽然具有很好的应用前景, 但目前抗病毒效果并不高, 表明在病毒长期感染过程中存在免疫抑制机制。以 HBV 的表面蛋白(HBsAg)和核心蛋白(HBcAg)为 DNA 疫苗抗原, 采用 gp96 和 HSP70 作为佐剂联合电转以提高疫苗的活性。将 gp96 为佐剂的 HBsAg/HBcAg DNA 疫苗免疫 HBV 转基因鼠后引发抗原特异性的细胞免疫和体液免疫应答。使用 gp96 和 HSP70 佐剂引起 Treg 下调 20%。与没有免疫的小鼠相比, 以 gp96 和 HSP70 为佐剂的 DNA 疫苗显著降低血清中病毒 S 抗原水平和 DNA 拷贝数, 大幅降低小鼠肝脏中 HBc 的表达。该研究为设计以 gp96 为佐剂的乙肝治疗性 DNA 疫苗提供了依据。

关键词: gp96, HSP70, 乙肝 DNA 疫苗, 免疫治疗

Activation of anti-HBV immune activity by DNA vaccine via electroporation using heat shock proteins as adjuvant

Yaxing Xu[1,2], Yanzhong Wang[1,2], Bao Zhao[3], Xiaojun Zhang[1,2], Hongxia Fan[1,2], Xinghui Li[1,2], and Songdong Meng[1]

1 *CAS Key Laboratory of Pathogenic Microbiology and Immunology*, *Institute of Microbiology*, *Chinese Academy of Sciences*, *Beijing* 100101, *China*
2 *University of Chinese Academy of Sciences*, *Beijing* 100049, *China*
3 *School of Life Sciences*, *University of Science and Technology of China*, *Hefei* 230026, *Anhui*, *China*

Abstract: Although DNA vaccination is now a promising strategy against hepatitis B virus (HBV) infection, this approach has

Received: March 20, 2013; **Accepted:** April 10, 2013
Supported by: National Natural Science Foundation of China (Nos. 31230026, 30970146, 91029724, 81021003, 81102018), Key Projects in the National Science & Technology Program (No. 2013ZX10002001-003-003).
Corresponding author: Songdong Meng. Tel: +86-10-64807350; E-mail: mengsd@im.ac.cn
国家自然科学基金(Nos. 31230026, 30970146, 91029724, 81021003, 81102018), 国家科技支撑计划重点项目(No. 2013ZX10002001-003-003) 资助。

relatively modest antiviral effect, indicating that immunosuppressive mechanisms may occur in the long-term established infection. In this study, we studied the immunogenicity and anti-HBV efficiency of a combination of HBV surface (HBsAg) and core (HBcAg) DNA vaccine, enhanced by heat shock protein (HSP) gp96 or HSP70 and mediated by *in vivo* electroporation. Immunization with gp96 adjuvanted HBsAg/HBcAg DNA formulation induced potent T cell and antibody immunity against HBsAg and HBcAg. Notably, treatment with gp96 or HSP70 as adjuvant resulted in reduction of Treg populations by around 20%. Moreover, compared with nonimmunized control mice, immunization with gp96 or HSP70 adjuvanted DNA vaccine dramatically decreased serum HBsAg and viral DNA levels, and HBcAg expression in liver. These results may therefore provide an effective strategy for designing gp96-based DNA vaccine for immunotherapy of chronic HBV infection.

Keywords: gp96, HSP70, HBV DNA vaccine, immunotherapy

About 350 million people worldwide are chronically infected with hepatitis B virus (HBV)[1]. Chronic HBV infection is a serious global health problem as it is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC)[2]. HBV persistence in chronic hepatitis B (CHB) is associated with a weak or undetectable antiviral immune response, especially the HBV-specific T cell responses[3-5]. Several molecular mechanisms have been shown to be involved in T cell impairment, tolerance and exhaustion in CHB, including upregulation of co-inhibitory molecule programmed death (PD)-1, the cytotoxic T lymphocyte antigen-4 (CTLA-4) or proapoptotic protein Bcl2-interacting mediator (Bim) on viral specific CD8+ T cells[6-8], and enhanced activities of regulatory T cells (Tregs) and the tolerogenic nature of the liver microenvironment in liver[9]. Tregs are a subset of CD4+ T cells with high expression of IL-2R α chain (CD25) and the forkhead/winged helix transcription factor (Foxp3). They suppress viral specific T cell responses and play an important role in inducing immune tolerance to HBV[10-12].

Most of the therapeutic HBV vaccines have been designed to stimulate or generate HBV-specific T cells, or target inhibitory signals on exhausted T cells[13-15]. In addition, strong Th1- or Th2-inducing adjuvants might substantially increase the efficiency of therapeutic vaccination[16-21]. HBV DNA vaccines encoding envelope proteins (preS1/S2/S) or other viral proteins including core, polymerase and X protein, in combination with antiviral therapy lamivudine to reduce HBV load, have been initiated in clinical trials for treatment of CHB[22-26]. Despite their safety and immunological effectiveness, the anti-HBV effect has been relatively modest in these clinical trials, indicating that immunosuppressive mechanisms may enter to play in the long-term established infection.

Heat shock proteins (HSPs), including HSP70 and gp96, have the ability to bind with antigenic peptides, and activate specific CD8+ and CD4+ T cells by presentation of bound peptides to MHC class I and class II molecules[27-30]. In our current study we choose the main HBV structure proteins core (HBcAg) and surface (HBsAg) protein as the target antigens, along with gp96 or HSP70 as adjuvant to analyze and compare HBV-specific T-cell responses and antiviral effects in HBV transgenic mice. The results could provide a promising and cost-effective approach for the development of HSP-based therapeutic DNA vaccines against HBV infection.

1 Materials and methods

1.1 Plasmid DNA constructs and analysis of gene expression

The human gp96, HBs and HBc gene were inserted into a mammalian expression vector

pcDNA3.1, and designated as pcDNA3.1-gp96, pcDNA3.1-HBs and pcDNA3.1-HBc, respectively. pCMV-HSP70 (provided by Ye Xin, Institute of Microbiology, Chinese Academy of Sciences) was taken as the template to amplify the HSP70 sequence, and HSP70 gene was inserted into pcDNA3.1 and designated as pcDNA3.1-HSP70. All constructs were confirmed by enzyme digestion and DNA sequencing. Plasmids were expanded in *Escherichia coli* and purified using Plasmid Maxprep Kit (Vigorous Biotechnology, Beijing, China).

The expressions of pcDNA3.1-gp96 and pcDNA3.1-HSP70 were verified by western blotting analysis of gp96 and HSP70 in transfected Chang liver cells. The expressions of pcDNA3.1-HBs and pcDNA3.1-HBc were verified by ELISA in transfected Chang liver cells.

1.2 Mice immunization

Female HBV transgenic Balb/c mice (6–8 weeks old) were purchased from Transgenic Engineering Lab, Infectious Disease Center, Guangzhou, China. The HBV transgenic Balb/c mice were generated with a viral DNA construct pHBV 1.3, containing 1.3 copies of the HBV genome (genotype D). All transgenic mice were detected positive for serum HBsAg and virus DNA, and HBcAg in hepatocytes in liver. These animals have normal serum alanine aminotransferase (ALT) levels. The mice were allocated into four groups, and each group contained at least five transgenic mice. The mice were injected with 20 μg of plasmid pcDNA3.1-HBs and pcDNA3.1-HBc with or without pcDNA3.1-gp96 and pcDNA3.1-HSP70 as adjuvant in 100 μL PBS in the tibialis anterior muscle of the hind leg. Mice immunized with the empty vector pcDNA3.1 served as control. Immediately after DNA administration, a pair of electrodes (TERESA-EPT-I Drug Delivery Device, Shanghai Teresa Healthcare Sci-Tech Co., Ltd., Shanghai, China) was placed over the injection site. Electroporation was applied with six electric pulses at a voltage of 36 V. All injections were performed at weeks 1, 3 and 6, respectively.

1.3 ELISAs

Quantitation of mouse anti-HBs and anti-HBc antibody was performed by ELISA as previously described[31]. HBsAg level in serum was determined by commercial enzyme immunoassay kits (Shanghai Kehua Bio-engineering Co., Ltd. shanghai, China).

1.4 IFN-γ enzyme-linked immunospot (ELISPOT) assay

HBV transgenic mice were sacrificed two weeks after the third immunization. Splenocytes and liver-infiltrating lymphocytes were harvested from individual mouse. ELISPOT assay was performed as previously described[31]. In brief, 96-well PVDF plates were precoated with the coating antibody overnight at 4°C. Purified splenocytes or liver-infiltrating lymphocytes (5×10^5 cells/well) and HBsAg or HBcAg, or control protein BSA as negative control, or PMA/ionomycin as positive control were added to the well in triplicate and incubated at 37 °C for 36 h. The spots were counted and analyzed with the ELISPOT Reader (Biosys, Germany).

1.5 Flow cytometry

Splenocytes were stained with various fluorochrome-conjugated antibodies against interested surface markers. FITC-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD8, PerCP-Cy5.5-conjugated anti-mouse CD3 and PE-conjugated anti-mouse CD25 and CD69 were purchased from eBioscience. Foxp3 cytokine staining was performed using the CytoWx/Cytoperm kits (eBioscience). Samples were analyzed using FACSCalibur and CellQuest software (BD Biosciences) after staining.

1.6 Immunohistochemistry

The detection of HBcAg expression and CD8+ T cell infiltration in liver tissues was performed by immunohistochemical staining as previously described[31].

1.7 Real-time quantitative PCR assay

HBV DNA levels in serum and levels of HBV DNA replicative intermediates in hepatocytes were determined by Real-time quantitative PCR using the SYBR Green Premix reagent (TaKaRa Bio Inc., Shiga, Japan). HBV DNA levels in hepatocytes were normalized to those for the single copy PLAT gene[32].

1.8 Statistical analysis

The results are presented as $\bar{x} \pm s$. Student's *t*-test was used for comparison between groups.

2 Results

2.1 Immunization with plasmids expressing HBsAg, HBcAg and HSPs induces antigen-specific T cell responses in HBV transgenic mice

Female HBV transgenic mice (6–8 weeks old) were immunized three times with HBsAg and HBcAg DNA constructs or the empty plasmid pcDNA3.1 as control through intramuscular injection plus electroporation, along with gp96 or HSP70 as adjuvant. Two weeks after the third immunization, fresh splenocytes were collected from mice and HBV-specific T cells were quantified by IFN-γELISPOT assay. A control protein BSA was used for evaluation of the background of ELISPOT. As seen in Fig. 1A, only very low level of HBs- and HBc-specific T cell responses was observed in control mice, indicating that the HBV transgenic mice are immunotolerant to HBV. Immunization with HBsAg and HBcAg induced 3-fold and 4.7-fold increase of HBs- and HBc-specific T cell levels in mice, respectively, compared to control. Moreover, compared to immunization with HBsAg/HBcAg alone, gp96 treatment significantly increased HBs- and HBc-specific T cell response (both $P<0.05$), while HSP70 treatment only significantly increased HBs-specific T cell level.

As CD69 is one of the earliest activation marker expressed on the surface of T lymphocytes [33-34], we also measured the proportions of CD4+ and CD8+ T lymphocytes expressing CD69. Similar to the results in ELISPOT assay, significant difference in frequency of CD69+CD8+ (Fig. 1B) and CD69+CD4+ (Fig. 1C) T cells were observed between HBsAg/HBcAg immunized mice treated with and without gp96 (both $P<0.05$).

Next, we examined HBV-specific T cells in liver. Liver infiltrating lymphocytes were isolated from liver tissues of HBV transgenic mice and analyzed by ELISPOT assay. Compared to control mice, immunization with HBsAg/HBcAg DNA effectively elicited antigen specific T cells in liver (1.6-fold increase in mice immunized with gp96 and HBsAg/HBcAg, and 1.4-fold increase in mice immunized with HSP70 and HBsAg/HBcAg), as seen in Fig. 1D. Notably, mice treated with gp96 as adjuvant exhibited a significant increase in antigen-specific T cells, compared to no treatment ($P<0.05$).

In the meantime, CD8+ T cells in liver were detected by immunohistochemistry staining. As seen in Fig. 1E , immunization with gp96 adjuvanted DNA vaccine caused a dramatic increase in the number of liver infiltrating CD8+ T cells compared to HBsAg/HBcAg alone and control (both $P<0.01$). These data indicate that HBsAg/HBcAg DNA effectively induces HBV-specific T cell responses in HBV transgenic mice, and gp96 could greatly enhance the T-cell responses.

2.2 Immunization with plasmids expressing HBsAg, HBcAg and HSPs induces humoral immune responses

The levels of HBs- and HBc-specific IgG, IgG1 and IgG2a antibodies were determined by ELISA. No anti-HBsAg antibody response was observed in all immunization groups compared to control (Fig. 2A), which is probably due to the pre-existence of large amount of HBsAg produced in HBV transgenic mice. However, DNA vaccination with HBs/HBc combined with gp96 or HSP70 significantly

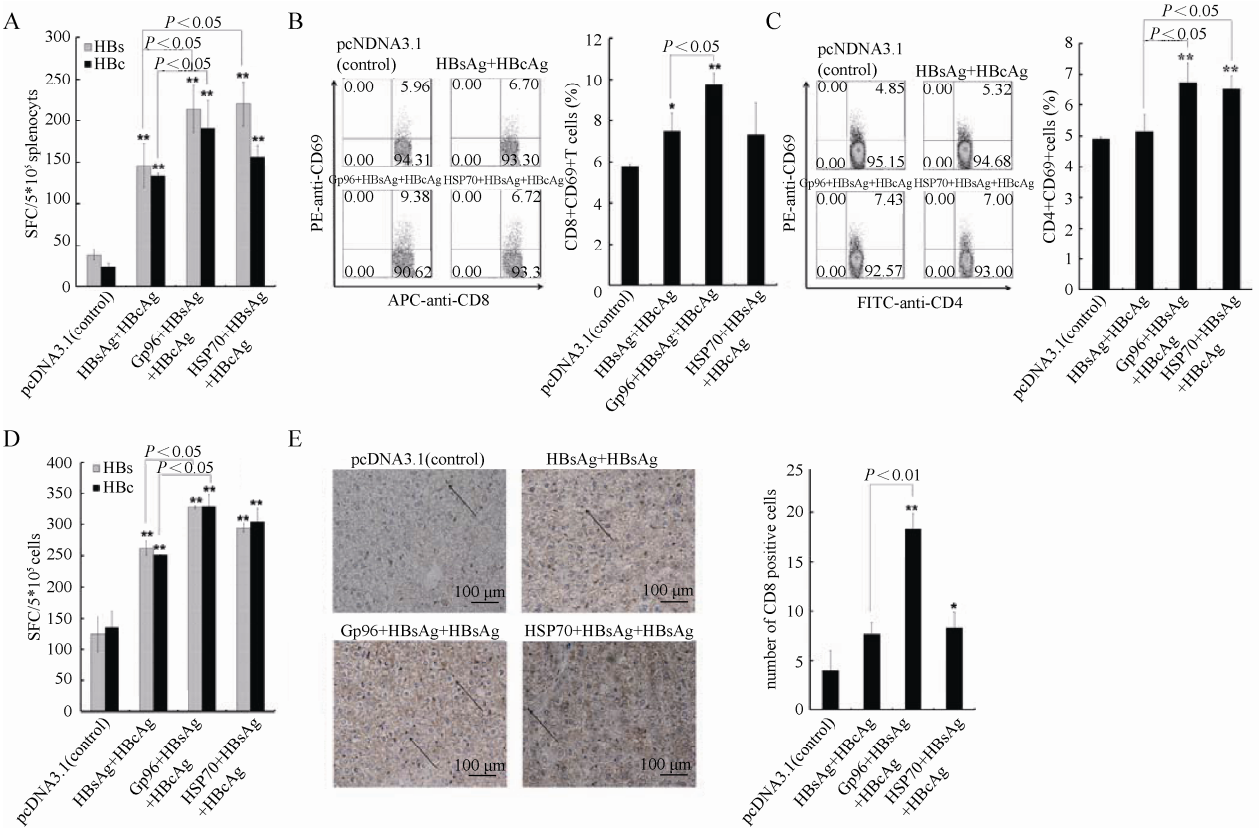

Fig.1 T cell responses induced by DNA vaccination in HBV transgenic mice. Mice were immunized with DNA constructs of HBsAg/HBcAg, HBsAg/HBcAg+gp96, HBsAg/HBcAg+HSP70, or the empty vector pcDNA3.1 as a control at weeks 1, 3 and 6, respectively. Mice were sacrificed at week 8 and splenocytes and liver-infiltrating lymphocytes were collected. (A) IFN-γ Elispot assay. Splenocytes were stimulated with HBsAg or HBcAg, or BSA for background evaluation. (B, C) FACS analyses were performed to quantify CD69+CD8+ (B) and CD69+CD4+ (C) T cell populations of splenoctyes. (D) Liver-infiltrating lymphocytes were stimulated with HBsAg, HBcAg or BSA as negative control for background evaluation, and analyzed by Elispot assay. (E) Immunohistochemistry analysis of CD8+ T cell infiltration in liver. Data show $\bar{x} \pm s$ of 5 mice. *P< 0.05, **P< 0.01 compared with control. Data are representative of two independent experiments.

enhanced production of total IgG and IgG2a against HBcAg, compared to control mice (P < 0.05 or 0.01) (Fig. 2B). In contrast, no significant difference of IgG1 levels was observed between HBs/HBc combined with gp96 or HSP70 DNA immunized mice and control mice. The results suggest that HSPs as adjuvant more likely induce Th1-type immune response against HBV.

2.3 Immunization with HSPs as adjuvant downregulates CD4+CD25+Foxp3+ Treg cells in transgenic mice

As CD4+CD25+Foxp3+ Treg cells inhibit both the induction and effector function of T cells and play an important role in host immune tolerance in HBV infection, we then determined if HSPs immunization could affect Tregs by FACS analysis (Fig. 3A). There were no significant differences in the proportions of Tregs in spleen between the control mice and mice immunized with HBsAg/HBcAg DNA alone (Fig. 3B). However, the percentage of Tregs decreased significantly in mice treated with gp96 or HSP70 compared to control (both P < 0.05). Vaccination with gp96 or HSP70 as adjuvant resulted in reduction of Treg populations by

21% or 20%, respectively. The result indicates that downregulation of Tregs by gp96 and HSP70 may contribute to their enhancement of HBsAg- and HBcAg-specific T cell responses.

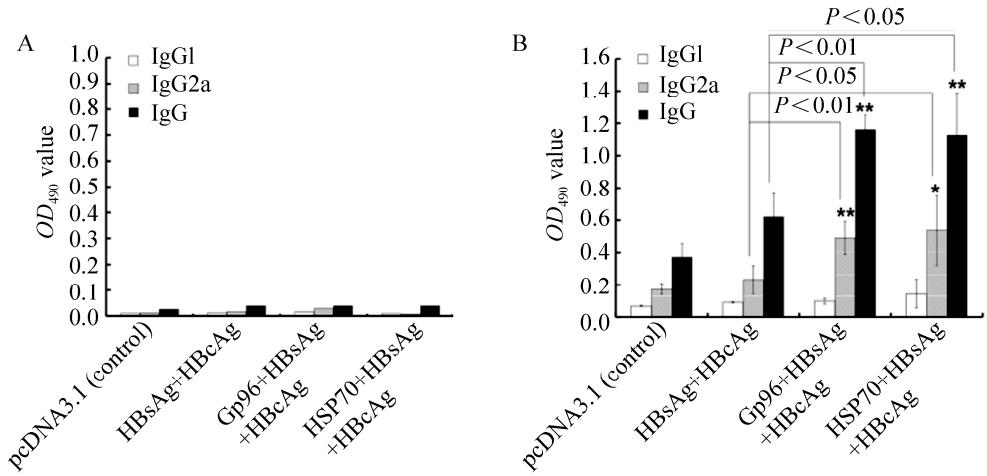

Fig.2 Humoral immune responses by DNA vaccination. HBs-specific (A) and HBc-specific (B) IgG, IgG1 and IgG2a antibodies titers were determined by ELISA in immunized HBV transgenic mice. Data show $\overline{x} \pm s$ of five mice. *P<0.05, **P<0.01 compared with control (pcDNA3.1). Data are representative of two independent experiments.

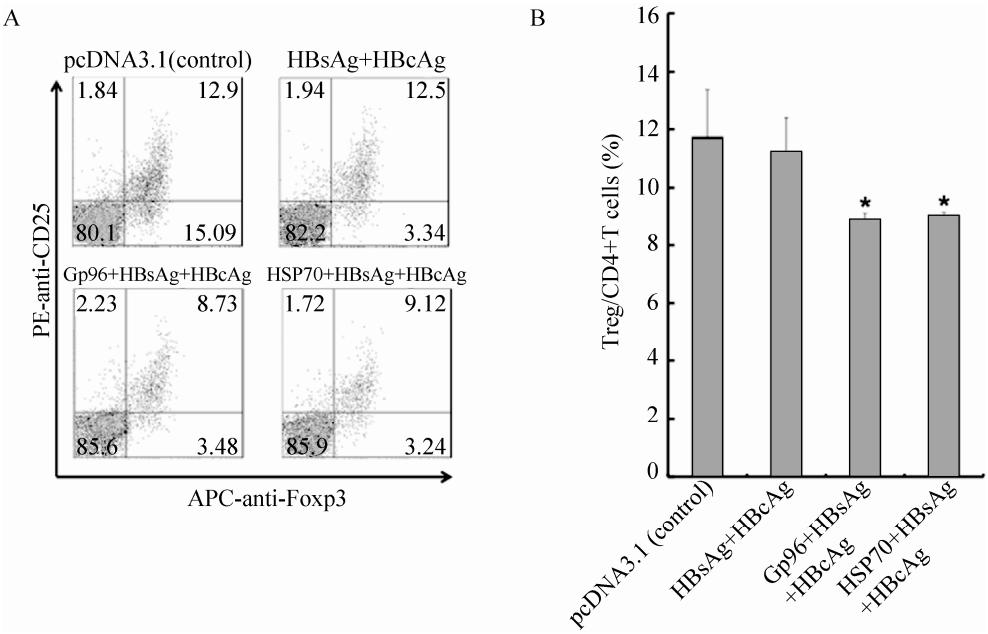

Fig.3 HSPs immunization downregulates CD4+CD25+Foxp3+Treg cells. (A) Splenocytes from immunized HBV transgenic mice were stained with antibodies against CD3, CD4, CD25 and intracellular Foxp3 and analyzed by flow cytometric analysis. (B) Comparisons of the frequencies of CD4+CD25+Foxp3+Treg cells among mice immunized with different DNA formulations. Each group contained at least 5 mice. *P <0.05, **P <0.01 compared with control group (pcDNA3.1). These experiments were repeated three times with comparable results.

2.4 Vaccination with HSPs as adjuvant caused pronounced antiviral responses

Serum HBsAg levels in HBV transgenic mice were detected weekly after the first immunization. As shown in Fig. 4A, the HBsAg levels began to decrease from week 6 in all immunized groups. Mice

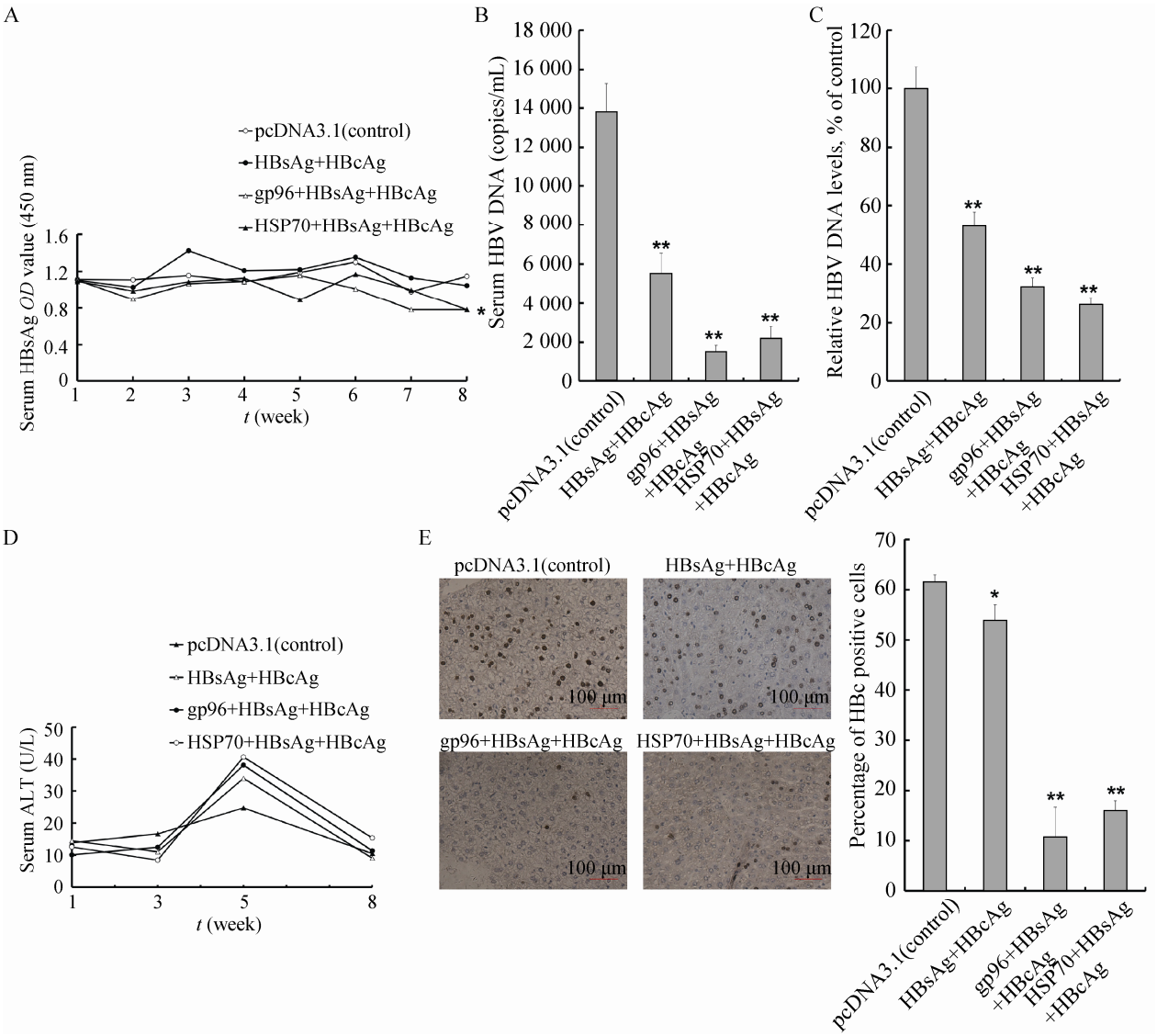

Fig.4 Inhibition of HBV replication by DNA vaccination in HBV transgenic mice. Mice were immunized for three times with HBsAg/HBcAg, HBsAg/HBcAg+gp96, HBsAg/HBcAg+HSP70, or the empty vector pcDNA3.1 as a control at weeks 1, 3 and 6, respectively. (A) Serum HBsAg levels were determined weekly after the first immunization by ELISA. * P <0.05 compared with HBsAg levels at week 1. (B, C) HBV DNA levels in the serum (B) and viral DNA intermediates in hepatocytes (C) were determined by real-time PCR at week 8. (D) Serum ALT levels were detected at the indicated times. (E) Immunohistochemistry analysis of HBcAg expression in liver at week 8. Data show $\bar{x} \pm s$ for five mice. *P< 0.05, **P< 0.01 compared with control. The data are representative of two independent experiments.

treated with gp96- or HSP70-adjuvanted DNA vaccine displayed a decrease of around 30% in HBsAg levels at week 8 ($P < 0.05$), compared to HBsAg levels at week 1 before treatment. Interestingly, a moderate decrease of HBsAg levels was also observed in the control group at week 7. A possible interpretation is that moderate immunoactivation against HBV in mice may, partly, if not all, cause the HBsAg repression as the ALT levels began to rise at week 3 and peaked at week 5 in control mice (Fig. 4D). HBV DNA concentrations in serum were detected by quantitative PCR at week 8. Compared with control, immunization with HBsAg/HBcAg along with gp96 or HSP70 induced 8-fold or 5-fold decrease in serum HBV DNA levels (Fig. 4B) and 2.1 fold or 2.8 fold reduction of HBV DNA intermediates in hepatocytes (all $P < 0.01$) (Fig. 4C). Meanwhile, the serum ALT levels in gp96- and HSP70-immunized mice peaked at week 5 and return to normal level at week 8 (Fig. 4D), suggesting that vaccination-induced immune responses caused transient liver damage.

We further detected the HBcAg expression in mice liver by immunohistochemistry (Fig. 4E). Consistent with the results of viral DNA levels, HBcAg expression in liver of gp96 or HSP70 immunized mice was decreased by 4.7-fold or 2.8-fold, respectively, compared to control mice. Taken together, immunization with HSP-based DNA vaccine could greatly inhibit HBV replication and lead to viral clearance.

3 Discussion

In this study, we investigated the efficiency of the therapeutic HBV DNA vaccines of combined HBsAg and HBcAg formulation along with HSPs as adjuvant by electroporation. Vaccination with gp96- or HSP70-based vaccine initiated potent cellular and antibody responses towards HBsAg and HBcAg in HBV transgenic mice. Notably, a marked decrease of Tregs was observed under co-administration of gp96 or HSP70, indicating the novel immunomodulatory property of HSPs in breaking immune tolerance. Compared with nonimmunized transgenic mice, immunization with gp96 or HSP70 adjuvanted DNA vaccine dramatically reduced serum HBsAg and viral DNA levels, and HBcAg expression in liver. The current work may therefore help to elucidate the T cell activation mechanism of HSPs, and to design HSPs-based therapeutic HBV DNA vaccines against HBV infection.

Electroporation has been shown to enhance DNA uptake in tissues and increase the immunological potency of DNA vaccines[35-37]. Our previous study showed that gp96 adjuvanted protein vaccine is able to potentiate anti-HBV humoral and T cell responses, however, the DNA vaccine is not as effective[31]. In this study, we assessed of using electroporation as an effective strategy for improving DNA vaccine-induced viral specific cellular immunity and anti-HBV efficiency in HBV transgenic mice. Our results demonstrated that electroporation treatment of HSP-based DNA vaccine produced high antigen-specific cellular and antibody immune responses, which provides a rationale to enhance immunogenicity of HBV DNA vaccine.

Co-administration with adjuvant molecules is an effective approach to increase the immunogenicity of HBV DNA vaccines. The use of potent Th1-inducing adjuvants, including Fms-like tyrosine kinase 3 ligand[16], cationic liposomes and non-coding DNA complexes[17,19], CpG-enriched plasmids[18], and Levamisole[21], has been shown to induce robust humoral and cellular immune responses against HBV. Our previous study showed that immunization with HSP70 or gp96 as adjuvant could induce significant increase of T-cell and antibody immunity in BALB/c mice[38]. In this study we further investigated the ability of gp96 and HSP70 to initiate anti-HBV immune responses and viral inhibition in HBV transgenic mice which are

generally immunotolerant to HBV. The results show that T-cell responses induced by gp96 or HSP70 could dramatically suppress HBV expression and replication, validating the therapeutic effect of HSP-based DNA vaccines against HBV. A combination of two major HBV structural proteins, HBsAg and HBcAg, were chosen as the target antigens. Compared with HSP70, immunization with gp96 as adjuvant induced stronger antiviral immunity against HBsAg and HBcAg (Fig. 1 and Fig. 2). Unlike other HSPs, including the cytosolic HSP70, gp96 may be directly involved in antigen presentation to MHC class I and class Ⅱ molecules due to its location within the endoplasmic reticulum (ER) [39]. Although treatment with gp96 and HSP70 led to a similar decrease of Treg populations (around 20%), higher T cell responses were observed under gp96 treatment (Fig. 1). This may be probably due to the unique capability of gp96 to prime and induce CD8+ T cell response by cross-presentation of antigenic peptides to MHC class I molecules[40-41]. Indeed, similar CD4+ T cells levels but quite different CD8+ T cells levels were found between mice treated with gp96 and HSP70. This deserves further investigation. In addition, gp96 binds both hydrophobic and hydrophilic peptides, whereas HSP70 associates with peptides containing a hydrophobic core or only larger polypeptides[42-43]. The broad peptide-binding property makes gp96 a universal and powerful adjuvant for the generation of T-cell responses.

We observed that the frequency of Tregs declined by more than 20% in the spleen of HBV transgenic mice under gp96 or HSP70 treatment. 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[10,12,44]. Conceivably, down-regulation of Treg may be one of the contributing factors for the highly effective gp96 adjuvanted HBsAg/HBcAg DNA vaccine, as Treg may suppress HBV specific T cell responses which play critical role in viral elimination after infection. More studies are needed to understand the immunomodulatory role of gp96 in various states of HBV infection, which may help to optimize the efficiency of gp96-based vaccines against HBV infection.

REFERENCES

[1] Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat, 2004, 11(2): 97–107.
[2] Lok AS. Chronic hepatitis B. N Engl J Med, 2002, 346(22): 1682–1683.
[3] Penna A, Chisari FV, Bertoletti A, et al. Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. J Exp Med, 1991, 174(6): 1565–1570.
[4] Bertoletti A, Chisari FV, Penna A, et al. Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein. J Virol, 1993, 67(4): 237–2380.
[5] Thimme R, Wieland S, Steiger C, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol, 2003, 77(1): 68–76.
[6] Fisicaro P, Valdatta C, Massari M, et al. Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. Gastroenterology, 2010, 138(2): 682–693, 693 e681–684.
[7] Schurich A, Khanna P, Lopes AR, et al. Role of the coinhibitory receptor cytotoxic T lymphocyte antigen-4 on apoptosis-Prone CD8 T cells in persistent hepatitis B virus infection. Hepatology, 2011, 53(5): 1494–1503.
[8] Lopes AR, Kellam P, Das A, et al. Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection. J Clin Invest,

2008, 118(5): 1835–1845.
[9] Maini MK, Schurich A. The molecular basis of the failed immune response in chronic HBV: therapeutic implications. J Hepatol, 2010, 52(4): 616–619.
[10] Koay LB, Feng IC, Sheu MJ, et al. Hepatitis B virus (HBV) core antigen-specific regulatory T cells confer sustained remission to anti-HBV therapy in chronic hepatitis B with acute exacerbation. Hum Immunol, 2011, 72(9): 687–698.
[11] Otano I, Suarez L, Dotor J, et al. Modulation of regulatory T-cell activity in combination with interleukin-12 increases hepatic tolerogenicity in woodchucks with chronic hepatitis B. Hepatology, 2012, 56(2): 474–483.
[12] Manigold T, Racanelli V. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. Lancet Infect Dis, 2007, 7(12): 804–813.
[13] Michel ML, Deng Q, Mancini-Bourgine M. Therapeutic vaccines and immune-based therapies for the treatment of chronic hepatitis B: perspectives and challenges. J Hepatol, 2011, 54(6): 1286–1296.
[14] Obeng-Adjei N, Choo DK, Saini J, et al. Synthetic DNA immunogen encoding hepatitis B core antigen drives immune response in liver. Cancer Gene Ther, 2012, 19(11): 779–787.
[15] Kosinska AD, Johrden L, Zhang E, et al. DNA prime-adenovirus boost immunization induces a vigorous and multifunctional T-cell response against hepadnaviral proteins in the mouse and woodchuck model. J Virol, 2012, 86(17): 9297–9310.
[16] Zhou Q, Wang F, Yang F, et al. Augmented humoral and cellular immune response of hepatitis B virus DNA vaccine by micro-needle vaccination using Flt3L as an adjuvant. Vaccine, 2010, 28(5): 1357–1362.
[17] Cote PJ, Butler SD, George AL, et al. Rapid immunity to vaccination with woodchuck hepatitis virus surface antigen using cationic liposome-DNA complexes as adjuvant. J Med Virol, 2009, 81(10): 1760–1772.
[18] Chen Z, Cao J, Liao X, et al. Plasmids enriched with CpG motifs activate human peripheral blood mononuclear cells *in vitro* and enhance th-1 immune responses to hepatitis B surface antigen in mice. Viral Immunol, 2011, 24(3): 199–209.
[19] Morrey JD, Motter NE, Chang S, et al. Breaking B and T cell tolerance using cationic lipid--DNA complexes (CLDC) as a vaccine adjuvant with hepatitis B virus (HBV) surface antigen in transgenic mice expressing HBV. Antiviral Res, 2011, 90(3): 227–230.
[20] Yin Y, Wu C, Song J, et al. DNA immunization with fusion of CTLA-4 to hepatitis B virus (HBV) core protein enhanced Th2 type responses and cleared HBV with an accelerated kinetic. PLoS ONE, 2011, 6(7): e22524.
[21] Zhang W, Du X, Zhao G, et al. Levamisole is a potential facilitator for the activation of Th1 responses of the subunit HBV vaccination. Vaccine, 2009, 27(36): 4938–4946.
[22] Im SJ, Yang SH, Yoon SK, et al. Increase of plasma IL-12/p40 ratio induced by the combined therapy of DNA vaccine and lamivudine correlates with sustained viremia control in CHB carriers. Immune Netw, 2009, 9(1): 20–26.
[23] Hoa PT, Huy NT, Thu le T, et al. Randomized controlled study investigating viral suppression and serological response following pre-S1/pre-S2/S vaccine therapy combined with lamivudine treatment in HBeAg-positive patients with chronic hepatitis B. Antimicrob Agents Chemother, 2009, 53(12): 5134–5140.
[24] Yang FQ, Yu YY, Wang GQ, et al. A pilot randomized controlled trial of dual-plasmid HBV DNA vaccine mediated by *in vivo* electroporation in chronic hepatitis B patients under lamivudine chemotherapy. J Viral Hepat, 2012, 19(8): 581–593.
[25] Cavenaugh JS, Awi D, Mendy M, et al. Partially randomized, non-blinded trial of DNA and MVA therapeutic vaccines based on hepatitis B virus surface protein for chronic HBV infection. PLoS ONE, 2011, 6(2): e14626.
[26] Senturk H, Tabak F, Ozaras R, et al. Efficacy of pre-S-containing HBV vaccine combined with lamivudine in the treatment of chronic HBV infection. Dig Dis Sci, 2009, 54(9): 2026–2030.
[27] Robert J, Ramanayake T, Maniero GD, et al.

Phylogenetic conservation of glycoprotein 96 ability to interact with CD91 and facilitate antigen cross-presentation. J Immunol, 2008, 180(5): 3176–3182.

[28] Li H, Zhou M, Han J, et al. Generation of murine CTL by a hepatitis B virus-specific peptide and evaluation of the adjuvant effect of heat shock protein glycoprotein 96 and its terminal fragments. J Immunol, 2005, 174(1): 195–204.

[29] Liu Z, Li X, Qiu L, et al. Treg suppress CTL responses upon immunization with HSP gp96. Eur J Immunol, 2009, 39(11): 3110–3120.

[30] Wald A, Koelle DM, Fife K, et al. Safety and immunogenicity of long HSV-2 peptides complexed with rhHsc70 in HSV-2 seropositive persons. Vaccine, 2011, 29(47): 8520–8529.

[31] Wang S, Qiu L, Liu G, et al. 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. Vaccine, 2011, 29(37): 6342–6351.

[32] Hosel M, Quasdorff M, Wiegmann K, et al. Not interferon, but interleukin-6 controls early gene expression in hepatitis B virus infection. Hepatology, 2009, 50(6): 1773–1782.

[33] Risso A, Smilovich D, Capra MC, et al. CD69 in resting and activated T lymphocytes. Its association with a GTP binding protein and biochemical requirements for its expression. J Immunol, 1991, 146(12): 4105–4114.

[34] Hamann J, Fiebig H, Strauss M. Expression cloning of the early activation antigen CD69, a type II integral membrane protein with a C-type lectin domain. J Immunol, 1993, 150(11): 4920–4927.

[35] Chen H, Wen B, Deng Y, et al. Enhanced effect of DNA immunization plus *in vivo* electroporation with a combination of hepatitis B virus core-PreS1 and S-PreS1 plasmids. Clin Vaccine Immunol, 2011, 18(11): 1789–1795.

[36] Liu KH, Ascenzi MA, Bellezza CA, et al. Electroporation enhances immunogenicity of a DNA vaccine expressing woodchuck hepatitis virus surface antigen in woodchucks. J Virol, 2011, 85(10): 4853–4862.

[37] Kim CY, Kang ES, Kim SB, et al. Increased *in vivo* immunological potency of HB-110, a novel therapeutic HBV DNA vaccine, by electroporation. Exp Mol Med, 2008, 40(6): 669–676.

[38] Wang YZ, Wang SF, Zhang XJ, et al. Enhancement of cellular and humoral immune responses of HBV DNA vaccine by HSP70 and gp96. Chin J Biotech, 2011, 27(5): 790−798 (in Chinese).
王彦中, 王赛锋, 张小俊, 等. 热休克蛋白HSP70和gp96增强乙肝DNA疫苗的细胞和体液免疫应答. 生物工程学报, 2011, 27(5): 790−798.

[39] Kropp LE, Garg M, Binder RJ. Ovalbumin-derived precursor peptides are transferred sequentially from gp96 and calreticulin to MHC class I in the endoplasmic reticulum. J Immunol, 2010, 184(10): 5619–5627.

[40] Singh-Jasuja H, Toes RE, Spee P, et al. Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis. J Exp Med, 2000, 191(11): 1965–1974.

[41] Arnold D, Faath S, Rammensee H, et al. Cross-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with the heat shock protein gp96. J Exp Med, 1995, 182(3): 885–889.

[42] Demine R, Walden P. Testing the role of gp96 as peptide chaperone in antigen processing. J Biol Chem, 2005, 280(18): 17573–17578.

[43] Javid B, MacAry PA, Lehner PJ. Structure and function: heat shock proteins and adaptive immunity. J Immunol, 2007, 179(4): 2035–2040.

[44] Stross L, Gunther J, Gasteiger G, et al. Foxp3+ regulatory T cells protect the liver from immune damage and compromise virus control during acute experimental hepatitis B virus infection in mice. Hepatology, 2012, 56(3): 873–883.

(本文责编 陈宏宇)