

Vaccination with ubiquitin-hepatitis B core antigen-cytoplasmic transduction peptide enhances the hepatitis B virus-specific cytotoxic T-lymphocyte immune response and inhibits hepatitis B virus replication in transgenic mice

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Abstract. Chronic hepatitis B virus (HBV) infection is characterized by functionally impaired type 1 T-helper cell (Th1) immunity and poor HBV-specific T-cell responses. Ubiquitin (Ub), a highly conserved small regulatory protein, commonly serves as a signal for target proteins that are recognized and degraded in proteasomes. The rapid degradation of Ub-mediated antigens results in efficient stimulation of cell-mediated immune responses. Thus, the Ub-HBcAg-CTP fusion protein was designed for specific delivery of a foreign modified antigen to the cytoplasm of antigen-presenting cells. HBV transgenic mice were used to determine whether Ub-HBcAg-CTP would restore HBV-specific immune responses and anti-viral immunity in these animals. The results demonstrated that synthesized Ub-HBcAg-CTP not only significantly increased the levels of interleukin-2 and interferon (IFN)- γ compared with those in the HBcAg-CTP, IFN- α , Ub-HBcAg, HBcAg and phosphate-buffered saline groups, but additionally induced the highest IFN- γ ⁺ CD8⁺ T-cell numbers and HBV-specific cytotoxic T lymphocyte (CTL) responses, indicating a strong immune response. In addition, enhancement of specific CTL activity provoked by the fusion protein reduced hepatitis B surface antigen (HBsAg) and HBV DNA serum levels and diminished the expression of HBsAg and HBcAg in liver tissue of HBV transgenic mice, suggesting that there was a therapeutic effect. In conclusion,

the present study provided evidence that Ub-HBcAg-CTP activated the Th1-dependent immunity, triggered functional T cell responses and subsequently inhibited viral replication in HBV transgenic mice. These observations suggested that the fusion protein may represent an innovative and promising candidate for active immunotherapy during chronic and persistent HBV.

Introduction

Hepatitis B virus (HBV) infection remains to be a serious health problem worldwide (1). Carriers of HBV are at increased risk of developing cirrhosis and hepatocellular carcinoma (HCC), with an estimated 563,000 mortalities annually worldwide from cirrhosis and HCC (2). Increasing evidence suggests that host-immune responses are important in determining the outcome of HBV infection (3). Acutely infected individuals typically develop a strong, multispecific cytotoxic T-lymphocyte (CTL) response and a polyclonal T-helper (Th) cell response to the virus (4,5), while these responses are weak or undetected in patients with chronic HBV infection (6). Therefore, novel immunotherapeutic approaches are required in order to alter the T-cell response into a predominant Th1 pathway and enhance HBV-specific CTL responses, which may facilitate the eradication of chronic HBV infection.

HBV core antigen (HBcAg) is a peptide that induces strong immune responses characterized by clear T-cell activity in natural and recombinant forms (7,8). Despite the high immunogenicity of exogenous HBcAg, HBV-specific CTL responses induced by simple exogenous HBcAg are commonly weak, predominantly due to restricted intracellular antigen delivery by the lipophilic and selectively permeable biological membranes (9). The cytoplasmic transduction peptide (CTP) was specifically designed to ensure the efficient delivery of CTP-fused biomolecules into the cytoplasm of cells (10). Increasing evidence has demonstrated that HBV-specific CTL responses are able to become robust, polyclonal and multispecific reactions when exogenous HBcAg is combined with CTP; such fusion leads HBcAg

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through cellular membranes and into the antigen-presenting cell (APC) cytoplasm (9,11).

The 76-residue polypeptide ubiquitin (Ub) is highly conserved among eukaryotes (12) and covalently binds the majority of proteins destined for degradation by the ubiquitin-proteasome system (UPS) (13). This is critical for the generation of the majority of peptides presented by class I major histocompatibility complex (MHC) molecules (14,15). The modification of antigens by ubiquitin or ubiquitin-like proteins remodels their surface, triggering rapid degradation, thus resulting in increased *in vivo* CTL responses to the conjugated antigen (16,17). Therefore, Ub has been suggested to be a potential therapeutic target for HBV treatment (18).

There is a possibility for exogenous HBcAg, with the assistance of CTP, to enter the cytoplasm of APCs. When covalently attached to exogenous Ub, HBcAg is rapidly degraded and presented by MHC-I molecules, allowing fast and efficient action of CTL against viral infections (19). Consequently, a novel recombinant fusion protein, Ub-HBcAg-CTP, was developed in the present study. Of note, Ub-HBcAg-CTP was able to enter the cytoplasm of dendritic cells (DCs) and elicit robust specific HBV immune responses *in vitro* (data not shown). The present study aimed to assess whether the Ub-HBcAg-CTP fusion protein were able to improve HBV-specific CTL immune responses and anti-viral immunity in HBV transgenic mice.

Materials and methods

Fusion proteins and cell culture. The plasmid pcDNA3.1(-)-Ub-HBcAg was constructed and maintained in the Laboratory Centre of The Sixth Hospital affiliated to Shanghai Jiao Tong University (Shanghai, China) (11). Briefly, the Ub-HBcAg cDNA sequence was generated via polymerase chain reaction (PCR) to obtain an 820 bp PCR product. The Ub-HBcAg-CTP gene and other control genes, Ub-HBcAg and HBcAg-CTP, were amplified via PCR and inserted into the pMAL-c2X prokaryotic expression vector (Invitrogen Life Technologies, Carlsbad, CA, USA), respectively. The constructed plasmids were further identified by restriction enzyme digestion and bidirectional DNA sequencing (New England Biolabs, Ipswich, MA, USA). Subsequently, the recombinant plasmids were transformed into the host *Escherichia coli* BL21 (DE3) cells (Tiangen, Beijing, China), which were induced to express the recombinant fusion proteins. All fusion proteins were analyzed via western blot analysis.

HBV transgenic mice and immunization. HBV transgenic mice (n=42; weight, 22–28g), BALB/c-HBV1.3 (ayw subtype), which contained the 1.3-fold over-length HBV genome, were purchased from the Key Liver Army Laboratory (458 Hospital, Guangzhou, China). The mice were maintained in 12 h light/dark cycles at a temperature of 20–25°C, with free access to food and water. The detailed characterization of these mice has been described previously (20). Groups of mice in all experiments were matched for age (6–8 weeks; female) and all animals were housed in the experimental animal centre of the Sixth Hospital affiliated to Shanghai Jiao Tong University (Shanghai, China) under

specific pathogen-free conditions. All experiments were approved by the Laboratory Animal Ethics Commission of Shanghai Jiao Tong University (Shanghai, China). Mice were allowed 1 week of adaptation and randomly divided into six groups (n=7). Subsequently, the animals were immunized intramuscularly in the tibialis anterior muscle three times at 1-week intervals with Ub-HBcAg-CTP (50 µg), HBcAg-CTP (50 µg), Ub-HBcAg (50 µg) and interferon (IFN)-α (20,000 IU; Roche Diagnostics, Basel, Switzerland), HBcAg (50 µg; CalBioReagents, Inc., San Mateo, CA, USA), or phosphate-buffered saline (PBS; 50 µl; Youkang, Beijing, China). At 1 week subsequent to the last immunization, the mice were sacrificed by cervical dislocation following anesthesia with 3% pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA), via intramuscular injection, and serum samples, splenocytes and livers were collected.

Intracellular cytokine staining in splenic lymphocytes. HBV transgenic mouse spleens were extracted for splenocyte collection, by grinding of the spleens. T-lymphocytes were obtained from splenocytes using nylon wool columns (Polysciences Europe GmbH, Eppelheim, Germany). To evaluate the percentage of IFN-γ-secreting cells in mouse splenocytes, single-cell suspensions of harvested T cells were analyzed by flow cytometry (Beckman Coulter, Inc., Brea, CA, USA). Following incubation with 10 µg/ml HBcAg for 3 h, 25 µg/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), 1 µg/ml ionomycin (Sigma-Aldrich) and 1.7 µg/ml monensin (Sigma-Aldrich) were added to spleen T lymphocytes for another 3 h (21). The cells were then washed with PBS and stained with saturating concentrations of phycoerythrin (PE)/Cy5-conjugated anti-CD3 mouse monoclonal antibody (0.2 mg/ml; cat. no. 15-4888; eBioscience, Inc., San Diego, CA, USA) and fluorescein isothiocyanate-conjugated anti-CD8α mouse monoclonal antibody (0.2 mg/ml; cat. no. 11-0081; eBioscience, Inc.) for 30 min at 25°C. Subsequent to fixation/permeabilization with Fix and Perm reagent A and B (BD Biosciences, Franklin Lakes, NJ, USA), cells were incubated with PE-labeled anti-IFN-γ mouse monoclonal antibody (0.2 mg/ml; cat. no. 12-7311; eBioscience, Inc.) for 30 min at 25°C, washed twice with PBS and analyzed by flow cytometry on the Epics XL Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA) using Expo 32-ADC software (Beckman Coulter).

ELISA. For detection of interleukin (IL)-2 and IFN-γ, spleen T lymphocytes (2×10⁶ cells/ml) harvested from immunized transgenic mice were cultured in 24-well plates (1×10⁶ cells/well) at 37°C in the presence of 10 µg/ml HBcAg. Subsequent to 72-h incubation, the cytokine levels in cell supernatants were assessed using commercial mouse cytokine (IL-2 and IFN-γ) ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturers' instructions. Results were expressed in pg/ml.

Enzyme-linked immunospot (ELISPOT) assay. The ELISPOT assay (22) was used to evaluate HBcAg-specific IFN-γ secretion in splenocytes. Spleen T lymphocytes (1×10⁵ cells/well) pulsed with 10 µg/ml HBcAg were seeded in triplicate and incubated at 37°C for 20 h. A positive control (phytohemag-

glutinin; 15 mg/ml; Dakewe Biotech Co., Ltd., Shenzhen, China) and a 'non-peptidic' negative control were included in all assays (i.e. wells containing only medium). Following incubation, cells were removed and ImmunoSpot plates (Dakewe Biotech Co., Ltd., Shenzhen, China) pre-coated with the anti-IFN- γ monoclonal antibody (BD Biosciences) were processed according to the manufacturer's instructions. The number of spots was counted using the Bioreader 4000 PRO-X (Bio-Sys GmbH, Karben, Germany). Exclusively brown-colored spots with 'fuzzy borders' were scored as spot-forming cells.

CTL assay. P815/c cells (Laboratory Centre of The Sixth Hospital affiliated to Shanghai Jiao Tong University, Shanghai, China) used as target cells, were seeded at a density of 5×10^4 cells/well in 96-well plates. The spleen T lymphocytes (5×10^6 cells/well) were used as effector cells and were co-cultured with P815/c cells at effector/target (E/T) ratios of 5:1, 10:1 or 20:1, at 37°C in a humid environment containing 5% CO₂ for 4 h. The HBcAg-specific CTL activity was measured by lactate dehydrogenase release using a CytoTox 96® Non-Radioactive Cytotoxicity kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The absorbance values of supernatants were recorded at 490 nm on an MK3 Multiscan (Thermo Labsystems, Waltham, MA, USA). The cytotoxicity was calculated as follows: [(Experimental release - effector spontaneous release - target spontaneous release) / (target maximum release - target spontaneous release)] x100% (23).

Serology. Venous blood was collected from transgenic mice at 1 week following the second and third immunizations, respectively. Serum HBV DNA and HBsAg levels were assessed using quantitative PCR (Terra PCR Direct Polymerase mix) and Abbott kits (Abbott Diagnostics, Chicago, IL, USA) separately, as described previously (24). The inhibitory rate of HBV DNA and HBsAg induced by the fusion protein was assessed. In addition, serum alanine aminotransferase (ALT) activities were measured at 7 days subsequent to the third injection using the ARCHITECT Automatic Biochemistry Analyzer (Abbott Diagnostics).

Histology and immunohistochemistry of liver tissues. De-paraffinized 5- μ m sections were stained with hematoxylin-eosin (Beyotime Institute of Biotechnology, Shanghai, China). For immunohistochemical analysis, slides were incubated for 1 h at 60°C and de-paraffinized. Following antigen retrieval using citrate buffer (pH=6.0; Beyotime Institute of Biotechnology), endogenous peroxidase was quenched by 3% H₂O₂ (Bioworld, Nanjing, China) in deionized H₂O for 5 min and non-specific binding blocked by 2% normal goat serum (Novus Biologicals LLC, Littleton, CO, USA) for 3 h at room temperature. Samples were then incubated with goat anti-HBsAg polyclonal antibody (Novus Biologicals LLC, Littleton, CO, USA) and goat anti-HBcAg polyclonal antibody (Novus Biologicals LLC) at 1:500 dilutions and 4°C overnight, respectively, followed by addition of biotinylated secondary antibody (Wuhan Boster Biological Technology, Ltd., Wuhan, China) at 37°C for 30 min with streptavidin-biotin-peroxidase complex (Biorbyt,

Cambridgeshire, UK). Detection was conducted using diaminobenzidine (Sigma-Aldrich) and cells were counterstained with hematoxylin (20).

Statistical analysis. Values are expressed as the mean \pm standard deviation and all analyses were performed with SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and the post-hoc least significant difference test were used in order to determine statistical significance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ub-HBcAg-CTP elicits a robust and functional specific cellular immune response. The specific CD8⁺ T-cell responses induced by the fusion proteins were assessed by intracellular IFN- γ staining of T cells isolated 1 week subsequent to the last immunization. Ub fused with HBcAg-CTP, which induced a clear T cell immune response, displaying the largest number of CD8⁺ IFN- γ ⁺ T lymphocytes in the spleens, as detected by flow cytometry. The CD8⁺ IFN- γ ⁺ T cells represented up to 2.2% of the total spleen T lymphocytes in mice immunized with Ub-HBcAg-CTP (Fig. 1), compared with 1.7, 1.4, 1.1, 0.3 and 0.1% identified in mice that received HBcAg-CTP, IFN- α , Ub-HBcAg, HBcAg and the PBS control, respectively. These observations suggested that delivery of ubiquitin and HBcAg was mediated via CTP-enhanced HBcAg-specific CTL generation in HBV transgenic mice.

Ub-HBcAg-CTP boosts IFN- γ and IL-2 production. One week subsequent to the third intramuscular injection of different fusion proteins, mice exhibited distinct levels of Th1 cell-derived cytokines as detected by ELISA. Fig. 2A and B illustrates the levels of IL-2 and IFN- γ secreted by T cells upon re-stimulation with HBcAg. The highest production of IL-2 (480.9 ± 33.7 pg/ml) and IFN- γ (288.2 ± 16.93 pg/ml) was observed in mice immunized with Ub-HBcAg-CTP.

Ub-HBcAg-CTP enhances the specific CTL response. ELISPOT assays were conducted to quantify the IFN- γ -producing lymphocytes. A higher percentage of T cells harvested from Ub-HBcAg-CTP-immunized animals produced IFN- γ (Fig. 2C), in comparison with that in the control group. In general, these results were in agreement with the above flow cytometry results for IFN- γ expression in CD8⁺ T cells. Overall, these observations indicated that Ub-HBcAg-CTP enhanced HBcAg-specific CTL responses *in vivo*.

To verify the role of Ub-HBcAg-CTP in cell-mediated immune responses, the cytolytic activity of HBcAg-specific CD8⁺ T cells was determined by their ability to kill peptide-loaded target cells (P815/c). As presented in Fig. 2D, values of 49.09 ± 1.44 , 29.55 ± 2.01 and $18.2 \pm 3.26\%$ for specific cytotoxicity were obtained for Ub-HBcAg-CTP-immunized mice at E/T ratios of 20:1, 10:1 and 5:1, respectively. The percentage of specific lysis was significantly higher in mice treated with Ub-HBcAg-CTP than that in animals immunized with other control proteins ($P < 0.05$). These observations

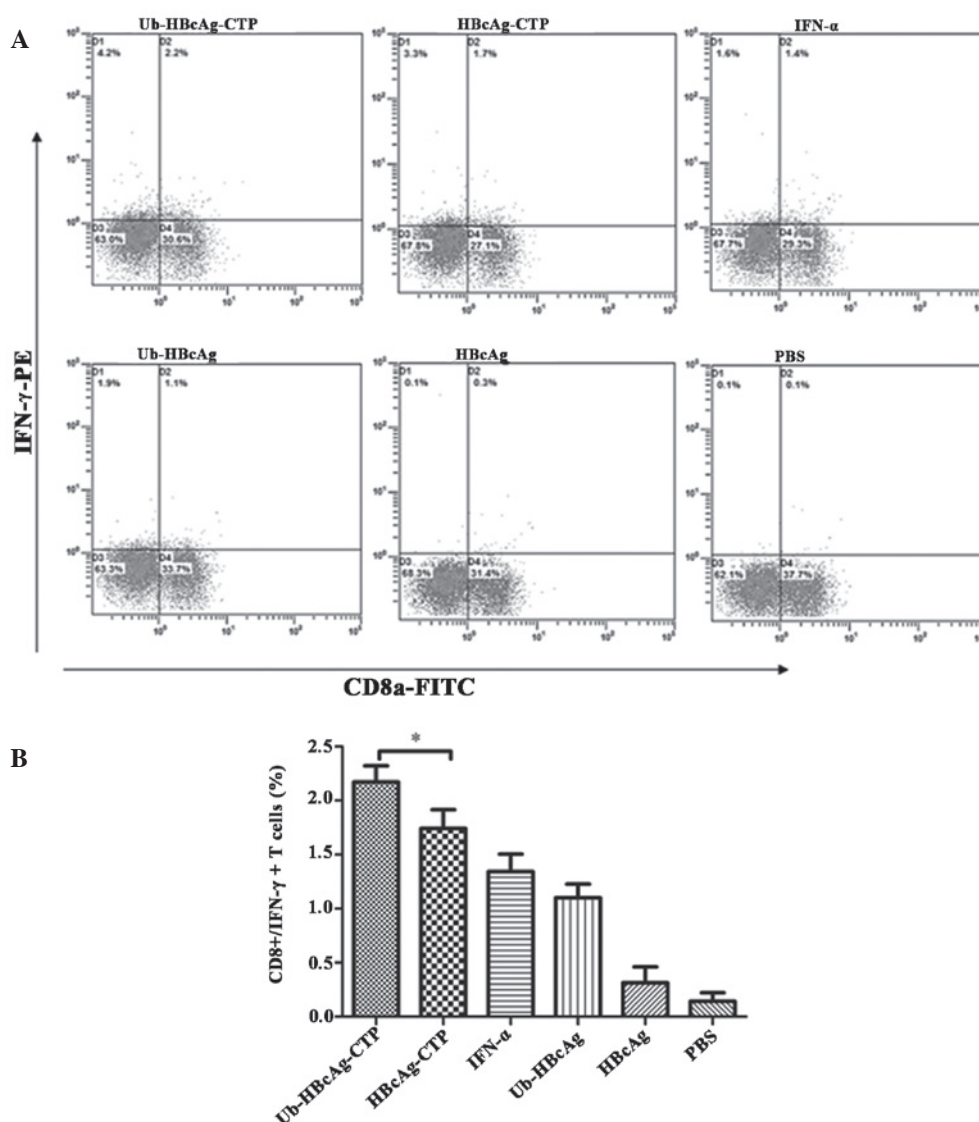


Figure 1. Intracellular cytokine expression in splenic lymphocytes of transgenic mice. (A) One week subsequent to the third immunization, mice were euthanized and splenic lymphocytes were isolated and re-stimulated *in vitro* by HBcAg for 3 h, followed by addition of 25 μ g/ml PMA, 1 μ g/ml ionomycin and 1.7 μ g/ml monensin for an additional 3 h. Re-stimulated T cells were stained with fluorescent FITC-CD8 α and PE-IFN- γ antibodies, then the doubly stained cells were counted and analyzed by flow cytometry. (B) Values are expressed as the mean \pm standard deviation (n=7) and are representative of a minimum of three individual experiments. *P<0.05. HBcAg, hepatitis B core antigen; PMA, phorbol 12-myristate 13-acetate; FITC, fluorescein isothiocyanate; PE, phycoerythrin; IFN, interferon; Ub, ubiquitin; CTP, cytoplasmic transduction peptide; PBS, phosphate-buffered saline.

suggested that Ub-HBcAg-CTP induced specific CTL activity, in accordance with high IFN- γ levels in CD8 $^{+}$ T cells.

HBsAg, HBV DNA and ALT in serum samples from HBV transgenic mice. Subsequently, the virus clearance following immunization was evaluated using the fusion proteins. The serum HBV DNA load of transgenic mice was determined by quantitative PCR to assess the curative effect of HBV-derived epitope-specific CD8 $^{+}$ T cells at 1 week subsequent to the second and third immunizations, respectively. In parallel, serum HBsAg levels were detected at the same time-points. Of note, serum HBsAg levels were markedly reduced in the Ub-HBcAg-CTP group compared with those in the other groups, including HBcAg-CTP, Ub-HBcAg and IFN- α , as presented in Fig. 3A (*P<0.05). Likewise, serum HBV DNA titers were significantly reduced in mice immunized with Ub-HBcAg-CTP, compared with those in the HBcAg-CTP,

Ub-HBcAg and IFN- α groups (Fig. 3B; *P<0.05). These results suggested that specific CD8 $^{+}$ T cells induced by Ub-HBcAg-CTP produced IFN- γ , mediating the inhibition of HBV replication in HBV transgenic mice. Significant elevations in ALT levels were observed in serum samples from mice treated with Ub-HBcAg-CTP (Fig. 3C; *P<0.05).

Histopathological alterations. Liver tissue sections were evaluated primarily in terms of hepatocyte degeneration and necrosis in addition to lymphocyte infiltration. A larger number of lymphocytes appeared in the livers from mice immunized with Ub-HBcAg-CTP (Fig. 4A), compared with that in the other groups. To further confirm the therapeutic effects of the fusion proteins in transgenic mice, immunohistochemical analysis was conducted. While high quantities of HBsAg and HBcAg were observed (brownish yellow stain) in the cytoplasm of hepatocytes from PBS-treated mice,

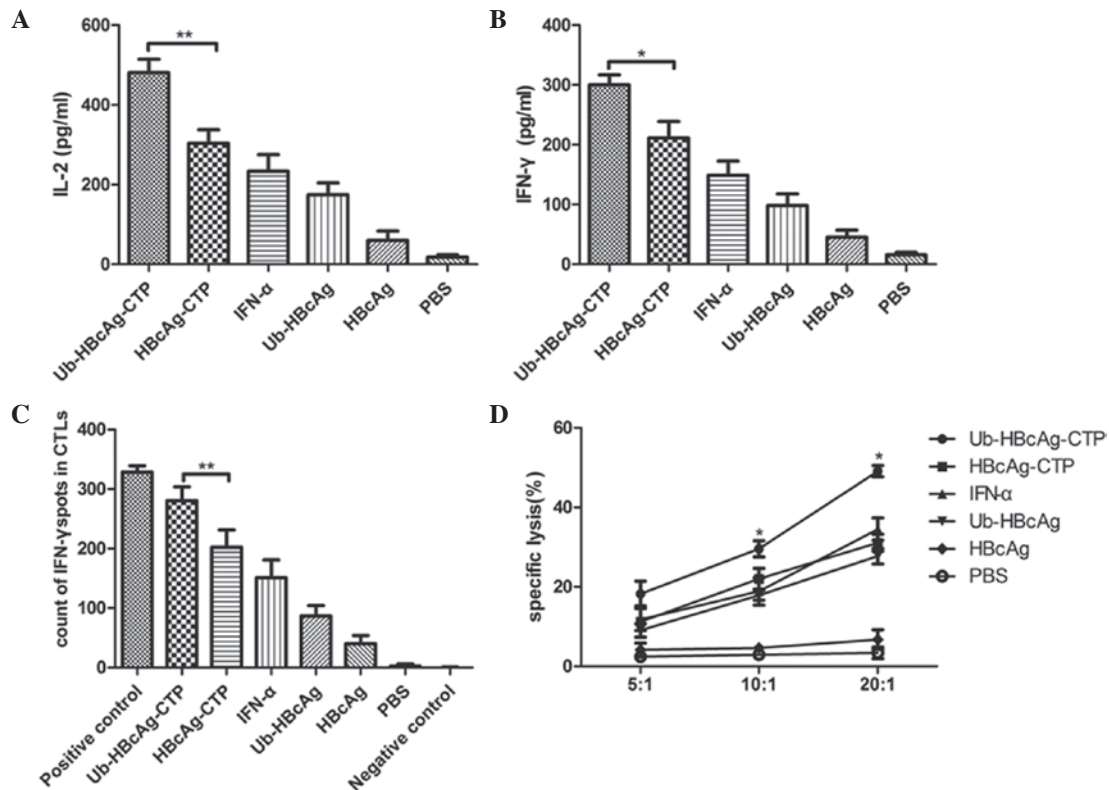


Figure 2. (A) IL-2 and (B) IFN-γ were measured in splenic lymphocyte culture supernatants by ELISA. In addition, IFN-γ spot numbers in CTLs were detected by an enzyme-linked immunospot assay. (C) The number of IFN-γ spots from hepatitis B virus transgenic mice. (D) Specific CTL activity was measured by lactate dehydrogenase release. CTL activity is indicated as the mean percentage of specific lysis at different effector-to-target ratios. Values are expressed as the mean ± standard deviation (n=7) and are representative of a minimum of three individual experiments. *P<0.01; *P<0.05, compared with the HBcAg-CTP. IL-2, interleukin-2; IFN-γ, interferon-γ; CTL, cytotoxic T-lymphocyte; HBsAg, hepatitis B core antigen; Ub, ubiquitin; CTP, cytoplasmic transduction peptide; PBS, phosphate-buffered saline.

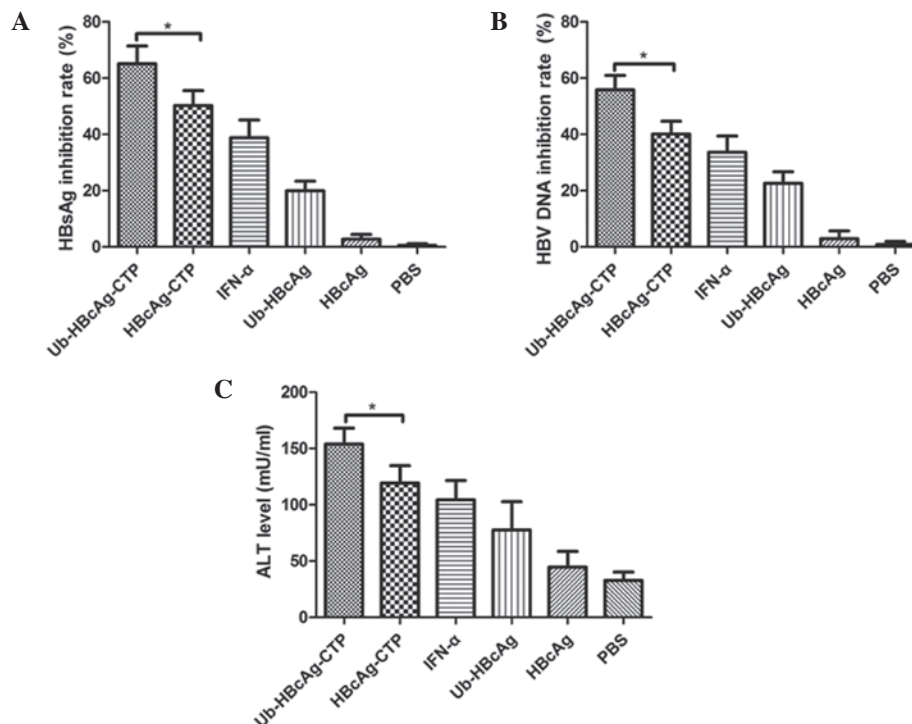


Figure 3. *In vivo* inhibitory effects on HBsAg, HBV DNA and ALT in HBV transgenic mice immunized with different fusion proteins. Mice were bled at 1 week following the second and third immunizations, respectively, and the levels of HBsAg and HBV DNA were quantified. (A) HBsAg and (B) HBV DNA inhibitory rates in serum samples from mice treated with different fusion proteins. (C) Mean levels (mU/ml) of sALT in the six groups of mice were examined at 7 days subsequent to the third injection. Values are expressed as the mean ± standard deviation (n=7) and are representative of a minimum of three individual experiments. *P<0.05. HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; sALT, serum alanine aminotransferase; IFN-α, interferon-α; Ub, ubiquitin; CTP, cytoplasmic transduction peptide; PBS, phosphate-buffered saline.

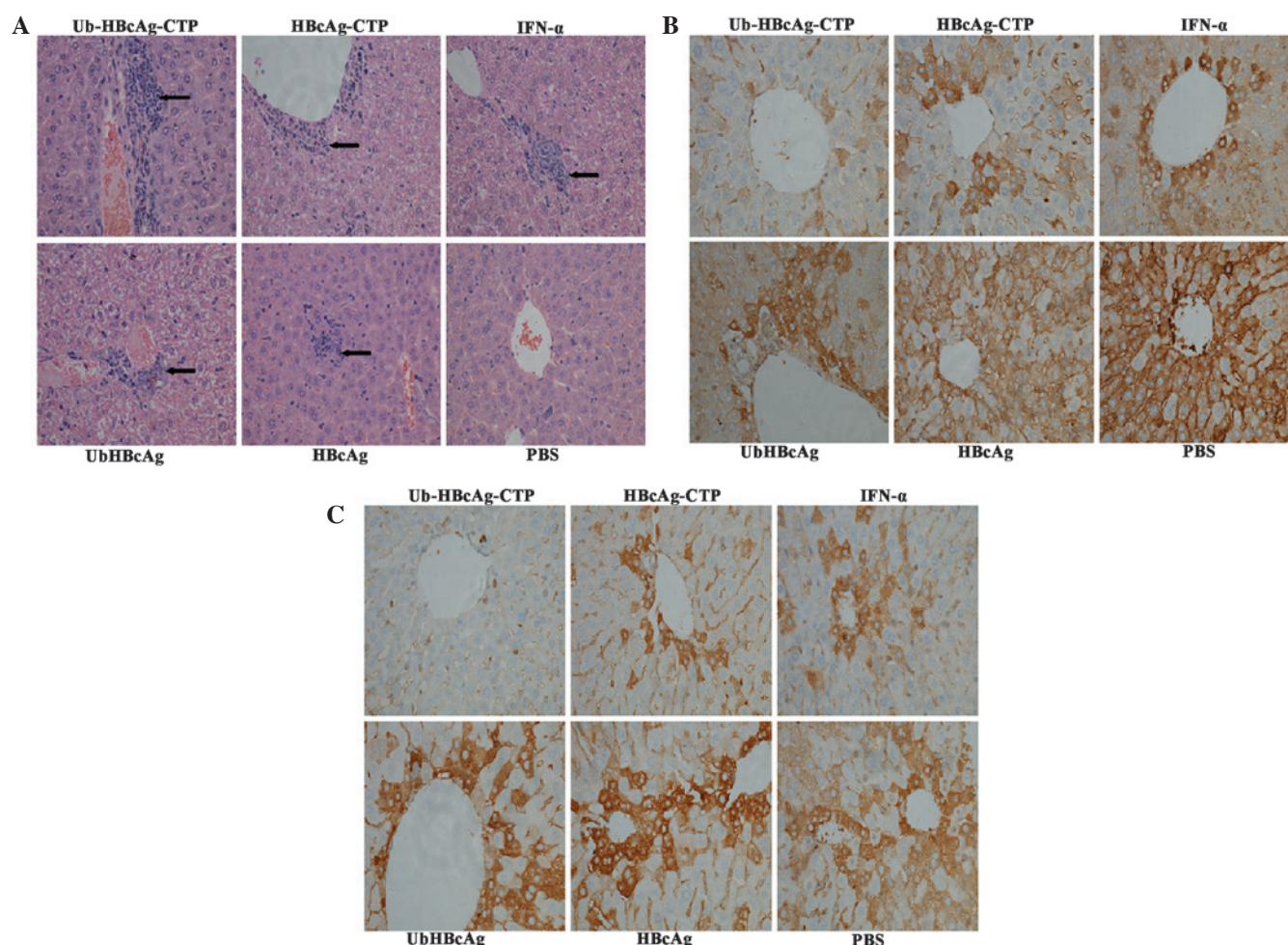


Figure 4. Histopathological alterations and immunohistological analysis of HBsAg and HBcAg in transgenic mouse livers. (A) Histological analysis of liver-infiltrating lymphocytes. Liver sections were stained with hematoxylin and eosin and were examined by light microscopy. A small number of lymphocytes appeared in mouse livers (indicated by arrows). Liver sections were subjected to immunohistological analysis for (B) HBsAg and (C) HBcAg detection, respectively (magnification, x400). HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; IFN- α , interferon- α ; Ub, ubiquitin; CTP, cytoplasmic transduction peptide; PBS, phosphate-buffered saline.

they were not apparent in the Ub-HBcAg-CTP-treated group (Fig. 4B and C). Therefore, it was suggested that immunization with Ub-HBcAg-CTP reduced HBsAg and HBcAg levels, to a certain extent.

Discussion

Antigen-based immunotherapy (vaccine therapy) is considered a promising therapeutic strategy for HBV infections, controlling HBV replication in chronic hepatitis B by inducing Th1 immunity and enhancing HBV-specific immune responses (25,26). Therefore, a novel therapeutic vaccine (Ub-HBcAg-CTP) was designed for effective activation of Th1 immunity and the specific CD8⁺ T cell response, which should compensate for the deficient HBV-specific anti-viral immunity and would not be subject to functional exhaustion during chronic HBV infections.

In the present study, HBV transgenic mice whose hepatocytes replicated the virus at levels comparable to those observed in the livers of patients with chronic hepatitis and who had no evidence of cytopathology were used. This model enabled the assessment of the effects of viral and host factors on HBV pathogenesis and replication, and the evaluation of

the anti-viral potential of pharmacological agents and physiological processes, including the immune response (20).

Efficient T-cell responses require CD8⁺ T cells to recognize antigenic peptides presented by MHC I molecules on the surface of APCs (predominantly DCs) (27). However, patients with chronic hepatitis B commonly display an immunocompromised immune tolerance with impaired DC function (28,29). CTP, a novel transduction carrier, has been demonstrated to be efficient in the delivery of antigens into the cytoplasm of DCs (30). Therefore, CTP was used in the present study as a tool for efficient delivery of Ub and HBcAg, thus boosting DC antigen-presenting capacities and inducing a greater number of HBV-specific CD8⁺ T cells.

Defects in cytokine secretion of antigen-specific CD8⁺ T cells have been previously associated with chronic HBV infection (31). Therefore, the induction of a strong, polyclonal, potent multifunctional HBV-specific CD8⁺ T-cell response is highly desirable, due to the fact that this would be able to trigger cytokine secretion. As mentioned above, CD8⁺ T cells induced by Ub-HBcAg-CTP vaccination were highly functional. Mice immunized with Ub-HBcAg-CTP exhibited significantly higher IFN- γ and IL-2 (Th1-like) secretion and

HBcAg-specific CD8⁺/IFN- γ ⁺ T cells in the spleen, compared with those in the other treatment groups. These observations indicated that the Th1 dominant responses were associated with significant enhancement of CTL activity following Ub-HBcAg-CTP vaccination. Th1 immunity appears to be crucial for the induction of CTL leading to cytolytic effects, which are beneficial for viral or tumor eradication (32). Furthermore, the *in vivo* cytotoxicity data from the present study demonstrated that the higher magnitude of CTL response induced by Ub-HBcAg-CTP was correlated with an increase in cell death of HBcAg-derived peptide-loaded target cells (P815/c). Therefore, a complete response to anti-viral treatment was correlated with predominant Th1 responses accompanied with enhanced CTL activity in patients with chronic HBV or transgenic mice (33). This implied that activation of Th1 immunity accompanied by efficient CTL activity subsequent to vaccination therapy is a common immune mechanism for successful treatment of hepatitis B (34).

It has been previously demonstrated that inadequate endogenous antigen presentation by MHC class I molecules to CD8⁺ T cells is one mechanism by which the immune system fails to eliminate HBV (35). Therefore, efficient elimination of virus-infected target cells by CTLs is only able to occur by rapid MHC class I antigen presentation of viral epitopes on the cell surface (36). Ub-HBcAg-CTP, with a modification for ubiquitin conjugation of antigen protein, exposes its N-terminal residue ('the N-end rule') (37,38), which further facilitates ubiquitination. In this manner, a polyubiquitin chain is synthesized, which targets the protein for rapid degradation by the UPS (39). It was identified in the present study that the enhancement of Ub-antigen presentation increased the number of HBcAg-specific CD8⁺/IFN- γ ⁺ T cells in Ub-HBcAg-CTP immunized mice. This may be due to the fact that increased Ub-fused HBcAg is rapidly degraded by the UPS, which results in efficient production of a variety of peptides, including numerous CTL epitopes, which may be presented by multiple MHC class I molecules. Additional studies have demonstrated that rapidly ubiquitinated antigens are more rapidly presented on class I molecules and/or highly induced (40,41).

The immunization of transgenic mice with Ub-HBcAg-CTP induced an efficient specific immune response and led to the control of viral replication. An increasing number of studies have demonstrated that secreted cytokines and activated CTLs may effectively downregulate HBV gene expression and additionally may control HBV replication (23,42). At 1 week subsequent to the last immunization to HBV transgenic mice, the hepatocytes appeared to swell and exhibited hyperemia and lymphocyte infiltration in liver tissues from Ub-HBcAg-CTP animals. The inflammatory reactions in the livers were consistent with specific CTL activity induced by Ub-HBcAg-CTP. In addition, the results of the present study indicated that the fusion peptide significantly reduced serum HBsAg and HBV DNA levels in addition to the expression of HBcAg and HBsAg in the liver. This reduction was closely associated with Ub-HBcAg-CTP, suggesting that the observed therapeutic effects were associated with the enhanced immune responses. These observations demonstrated the potential of Ub-HBcAg-CTP to induce a stronger CTL response in transgenic mice than control vaccinations. Akbar *et al* (43) reported that the strong immunomodulatory capabilities of HBcAg may

be due to an establishment of an inflammatory hepatic micro-environment, the induction of HBcAg-specific CTL in the liver and the activation of host DCs. To further confirm the specific CTL activity in the liver, the levels of ALT were measured. In contrast with control animals, greater ALT levels were observed in Ub-HBcAg-CTP immunized mice, indicating that infected hepatocytes were eliminated by vaccination-induced cytotoxic T cells. Although the activation levels of HBcAg-specific CTL in the liver were not assessed in the present study, the histological and serological alterations observed provided indirect support for the hypothesis that an anti-inflammatory hepatic microenvironment may be established in the Ub-HBcAg-CTP group. However, this remains to be confirmed in future studies.

In conclusion, the present study demonstrated that vaccination with Ub-HBcAg-CTP activated Th1 immunity, induced a robust and multifunctional HBcAg-specific T-cell response and provided a therapeutic effect in HBV transgenic mice. Therefore, a combination of Ub-HBcAg-CTP may be used as a potential therapeutic strategy for the treatment of chronic hepatitis B viral infections.

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