

# Ubiquitin-modified hepatitis B virus core antigen effectively facilitates antigen presentation and enhances cytotoxic T lymphocyte activity via the cytoplasmic transduction peptide *in vitro*

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**Abstract.** Cluster of differentiation (CD)8<sup>+</sup> cytotoxic T lymphocytes (CTLs) have a key role in the elimination of hepatitis B virus (HBV)-infected cells. Ubiquitin (Ub) functions as a marker for protein degradation, which may promote the generation of peptides appropriate for major histocompatibility complex class I presentation, while the HBV core antigen (HBcAg) possesses marked immunogenic properties. However, it remains to be elucidated whether Ub-modified HBcAg is able to effectively elicit significant CD8<sup>+</sup> CTL activity. In order to address this issue, a prokaryotic vector was constructed to express the Ub-HBcAg-cytoplasmic transduction peptide (CTP). The fusion protein was successfully expressed and subsequently pulsed into bone-marrow-derived dendritic cells (DCs). It was confirmed that with assistance from the cell-penetrating properties of CTP, the fusion protein was able to directly penetrate into the cytoplasm of DCs. The results revealed that the Ub-HBcAg-CTP fusion protein not only increased the expression of surface molecules in DCs and cytokine secretion from proliferating T cells, but also induced T cells to differentiate into specific CTLs and enhanced their antiviral ability. In conclusion, the Ub-HBcAg-CTP fusion protein promoted DC maturation, enhanced the presentation of targeting antigens and efficiently induced HBcAg-specific CTL immune responses *in vitro*.

## Introduction

Hepatitis B virus (HBV) infection remains a significant public health concern globally. Approximately 350 million individuals worldwide are chronically infected with HBV, and such infections may lead to the development of liver cirrhosis or hepatocellular carcinoma (1,2). Although various types of antiviral drugs, including nucleotide/nucleotide analogue and interferon, have been used to eradicate this virus in recent years, no significant progress has been achieved (3). Increasing evidence has demonstrated that patients acutely infected with HBV usually develop marked, multispecific cytotoxic T lymphocyte (CTL) responses to the virus, whereas chronically infected individuals exhibit weak responses (4,5). Therefore, the development of immunotherapeutic strategies to improve weak virus-specific T cell responses is critical.

Dendritic cells (DCs) are considered the most potent antigen-presenting cells (APCs); and as such, are able to initiate immune responses against invading pathogens (6) and have been observed to be responsible for the cross-presentation of antigens *in vitro* and *in vivo* as well as the stimulation of naïve cluster of differentiation (CD)8<sup>+</sup> T cell proliferation and maturation (7). A previous study revealed that defective CTL responses may be attributed to impaired DC function (8). Therefore, promoting and improving the functions of DCs may comprise an efficient treatment strategy for persistent HBV infections.

Ubiquitin (Ub) is a highly conserved small regulatory protein (9), and a centrally significant component of the Ub-proteasome system (UPS), which attaches covalently to numerous cellular proteins through a highly regulated process (10,11). Major histocompatibility complex (MHC) class-I antigen presentation is strictly dependent on the supply of appropriate peptides, mediated by the UPS, with which to efficiently prime CD8<sup>+</sup> T cells and initiate an adaptive immune response (12,13). The HBV core antigen (HBcAg) is a highly immunogenic subviral particle that in natural and recombinant forms may induce marked immune responses characterized by acute T-cell activity (14). These hypotheses prompted the

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scanning (Quanta 450) and transmission (Tecnai 12) electron microscopy (FEI Company, Eindhoven, Netherlands). The samples were treated according to the standard experimental methods (18). The day five DCs were cocultured with the aforementioned proteins at a concentration of 20  $\mu\text{g/ml}$  for 24 h. Following washing with PBS, the cells were fixed in 100% pre-chilled methanol (Keygentec). Following an additional three washes with PBS, the cells were permeabilized with 0.3% Triton X-100 (Keygentec) and blocked with 10% normal goat serum (Wuhan Boster Biological Technology, Ltd.). The cells were subsequently incubated overnight with an anti-HBcAg mAb (1:500 dilution) at 4°C. The cells were then further incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-Immunoglobulin G (Wuhan Boster Biological Technology, Ltd.) for 1 h at room temperature. Following washing with PBS and DAPI-staining (Keygentec), the cells were visualized with a LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and analyzed using LSM image examiner software (Carl Zeiss). The mean fluorescence intensity (MFI) of the entire view was calculated from five sites (the four corners and center of each section).

Day five DCs, which were treated as described above, were also cocultured with or without the specific proteasome inhibitor MG-132 (10  $\mu\text{mol}$ ; Sigma-Aldrich). Following 24 h of incubation, the DCs were harvested to analyze the level of HBcAg via western blotting.

**Western blot analysis.** Following a 24 h incubation the DCs were harvested and washed twice with PBS. The cells were then gently dispersed into a single-cell suspension and homogenized using radioimmunoprecipitation assay lysis buffer (Keygentec). Protein concentrations were determined using Pierce Bicinchoninic Acid Protein Assay Reagent kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The homogenates were diluted to the desired protein concentration using 2X SDS-PAGE loading buffer (Invitrogen Life Technologies). The samples were then boiled and loaded onto polyacrylamide mini-gels (Invitrogen Life Technologies) for electrophoresis. The protein samples were then transferred to polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA) using semi-dry apparatus. The membranes were then incubated with HBcAg monoclonal human anti-mouse antibody at 4°C overnight, followed by an incubation with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody at room temperature for 2 h. GAPDH was used as the control (GAPDH antibody, 1:1,000, 4°C overnight; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Image-Pro Plus (version, 6.0; Media Cybernetics, Inc, Bethesda, MD, USA) was used to visualize and quantify the blots. Gray value was used to compare the differences between the groups. Gray value=gray value of HBcAg/gray value of GAPDH.

**IL-12p70 production, DC immunophenotypic analysis and T cell proliferation.** Day 5 DCs were cocultured with the aforementioned proteins for 72 h, and the IL-12p70 concentrations in the supernatants were measured using a standard sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The concentrations were calculated and expressed as pg/ml. The surface

molecules of day eight DCs were analyzed following incubation with phycoerythrin (PE)-labeled monoclonal antibodies against mouse CD11c (1:50 dilution), CD80 (1:50 dilution), CD83 (1:100 dilution), CD86 (1:100 dilution) and MHC class-I (1:40 dilution) (eBioscience, San Diego, CA, USA) at 4°C in the dark for 30 min. Fluorescence analyses were performed on a COULTER EPICS XL Flow Cytometer (Beckman Coulter, Miami, FL, USA) using Expo32-ADC software (Beckman Coulter).

Day five DCs were cocultured with Ub-HBcAg-CTP, Ub-HBcAg, HBcAg-CTP and HBcAg (20  $\mu\text{g/ml}$ ; Abcam). After 72 h, day eight DCs were pretreated with 25  $\mu\text{g/ml}$  mitomycin C (Sigma-Aldrich) for 30 min. T cells were sorted from splenocytes of allogeneic naive mice using nylon wool columns (Polysciences, Inc.) and grown as responder cells in coculture with DCs at various responder/stimulator (T cell/DC) ratios (5:1, 10:1 and 20:1). The cells were incubated in a final volume of 100  $\mu\text{l}$  for 96 h, during which 10  $\mu\text{l}$  Cell Counting Kit-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added for 4 h. The absorbance values of the cultures were read at a wavelength of 450 nm (Multiskan Ascent; Thermo Fisher Scientific).

**Intracellular cytokine analysis of proliferative T cells.** Proliferative T cells were stimulated for 6 h in the presence of 25  $\mu\text{g/ml}$  phorbol 12-myristate 13-acetate (Sigma-Aldrich), 1  $\mu\text{g/ml}$  ionomycin (Sigma-Aldrich) and 1.7  $\mu\text{g/ml}$  monensin (Sigma-Aldrich) (19). The cells were subsequently stained with a PE-Cy5-conjugated anti-CD3 mAb (eBioscience) and a FITC-conjugated anti-CD8 $\alpha$  mAb (eBioscience) for 15 min at room temperature. Following fixation and permeabilization using the Fix/Perm reagents A and B (BD Biosciences, San Jose, CA, USA) for 15 and 5 min respectively, the cells were incubated with a PE-labeled anti-interferon (IFN)- $\gamma$  mAb (eBioscience) for 30 min. Fluorescence analyses were performed on a COULTER EPICS XL flow cytometer (Beckman Coulter) with Expo 32-Advanced Digital Compensation software (Navios™ Cytometer, version 1.0; Beckman Coulter).

**Cytokine secretion and CTL assay.** T cells were cocultured with mature DCs in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C for four days at a T cell/DC ratio of 10:1. The concentrations of various cytokines (IFN- $\gamma$  and IL-2) in the supernatants were measured using mouse cytokine ELISA kits (R&D Systems). The concentrations were expressed as pg/ml.

The P815/c cells were seeded as the target cells, and previously stimulated T cells were used as the effector cells. The T cells were cocultured with the P815/c cells at effector/target ratios of 5:1, 10:1 and 20:1 for 4 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. HBcAg-specific CTL activity was measured using a lactate dehydrogenase release assay (CytoTox 96® non-radioactive cytotoxicity kit; Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. The absorbance values were recorded at a wavelength of 490 nm (Multiskan Ascent). The percentage cytotoxicity was calculated as follows: [(experimental release - effector spontaneous release - target spontaneous release) / (target maximum release - target spontaneous release)] x 100 (20).

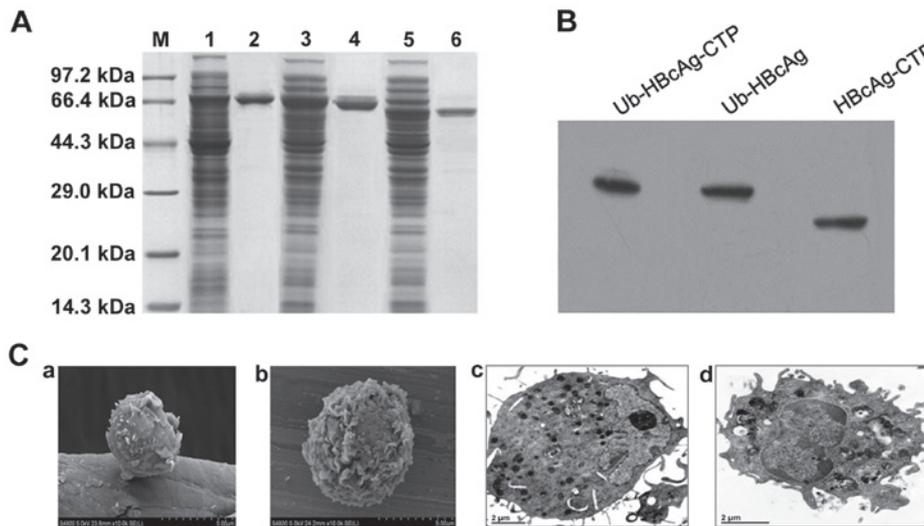


Figure 1. Fusion protein expression, purification, western blot analysis and DC morphology. (A) Expression and purification of fusion proteins by SDS-PAGE. Lanes: M, molecular weight standards; 1 and 2, expression and purification of MBP-Ub-HBcAg-CTP recombinant protein, respectively; 3 and 4, expression and purification of MBP-Ub-HBcAg recombinant protein, respectively; 5 and 6, expression and purification of MBP-HBcAg-CTP recombinant protein, respectively. (B) Purified fusion proteins were analyzed via western blotting using anti-HBcAg monoclonal antibody. (C) Ultrastructures of day five and day eight DCs were observed using (a and b) scanning electron microscopy (magnification,  $\times 5,000$ ) and (c and d) transmission electron microscopy (magnification,  $\times 20,000$ ), respectively. HBcAg, hepatitis B virus core antigen; DC, dendritic cell; CTP, cytoplasmic transduction peptide; MBP, maltose binding protein; Ub, ubiquitin.

**Statistical analysis.** Each value in the present study was obtained from a minimum of three independent experiments and data are expressed as the mean  $\pm$  standard deviation. The differences between groups were determined using one-way analysis of variance. Statistical data analyses were performed with SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Fusion protein expression, purification and western blotting.** The Ub-HBcAg-CTP, Ub-HBcAg and HBcAg-CTP fusion gene constructs were 813, 780 and 585 bp in size, respectively, as determined by PCR amplification. All the target prokaryotic expression plasmids were successfully constructed and identified by restriction endonuclease analysis and bidirectional DNA sequencing (data not shown). The purified fusion proteins were identified to be  $\sim 80\%$  pure via SDS-PAGE analysis (Fig. 1A) and the products were further confirmed by western blot analysis (Fig. 1B).

**DC maturation, cytoplasmic localization and western blotting.** On the DC surfaces, increased numbers of ruffles and rough dendrites were observed by scanning electron microscopy. Similarly, the morphological characteristics of mature DCs were detected by transmission electron microscopy, and identified larger nuclei, as well as increased numbers of mitochondria and ribosomes (Fig. 1C).

Immunofluorescent staining and confocal microscopy were used to characterize the intracellular localization of the various proteins in DCs. The HBcAg protein was identified in the cytoplasm of DCs treated with Ub-HBcAg-CTP and HBcAg-CTP via the presence of green fluorescence (FITC-labeled HBcAg) (Fig. 2A). This fluorescence was

clearly distinguished from the nuclear-specific counterstain, DAPI. Green fluorescence was observed to aggregate primarily on the DC cytomembranes in the Ub-HBcAg and HBcAg-treated groups (Fig. 2A), demonstrating the potent transduction potential and cytoplasmic localization mediated by the CTP peptide. The green fluorescence intensity detected in the Ub-HBcAg-CTP group was weaker than that in the HBcAg-CTP group, indirectly demonstrating the lower HBcAg levels in the former group (Fig. 2B). Furthermore, the levels of HBcAg were detected in the Ub-HBcAg-CTP and HBcAg-CTP groups using western blot analysis to determine the rates of intracellular degradation. As expected, HBcAg levels in Ub-HBcAg-CTP-group were lower than those in the HBcAg-CTP-group, but recovered to the same level as those of HBcAg following the addition of MG-132 (Fig. 2C).

**Ub-HBcAg-CTP leads to increased IL-12 production and surface molecule expression.** IL-12 is considered a vital cytokine, produced by DCs, and is a key cytokine involved in the induction of Type-1 immune responses (21). The IL-12p70 concentrations were assayed in the various protein-treated groups. The results demonstrated that DCs cocultured with Ub-HBcAg-CTP secreted significantly greater quantities of IL-12p70 ( $84.38 \pm 9.625$  pg/ml) than those of the other groups ( $P < 0.05$ ; Fig. 3B). However, no differences were identified among the other groups. Additionally, following treatment, the quantity of DCs ( $CD11c^+$ ) in the culture was  $\sim 85\%$  (flow cytometric analysis) and the surface molecules CD80, CD83, CD86 and MHC class-I were highly expressed on the Ub-HBcAg-CTP-treated DCs (Fig. 3A).

**T-cell proliferation.** T cells were cocultured with DCs, which had been pulsed with various proteins in order to analyze their T-cell proliferation capacities. It was identified that the T-cell proliferation capacity was markedly higher in the

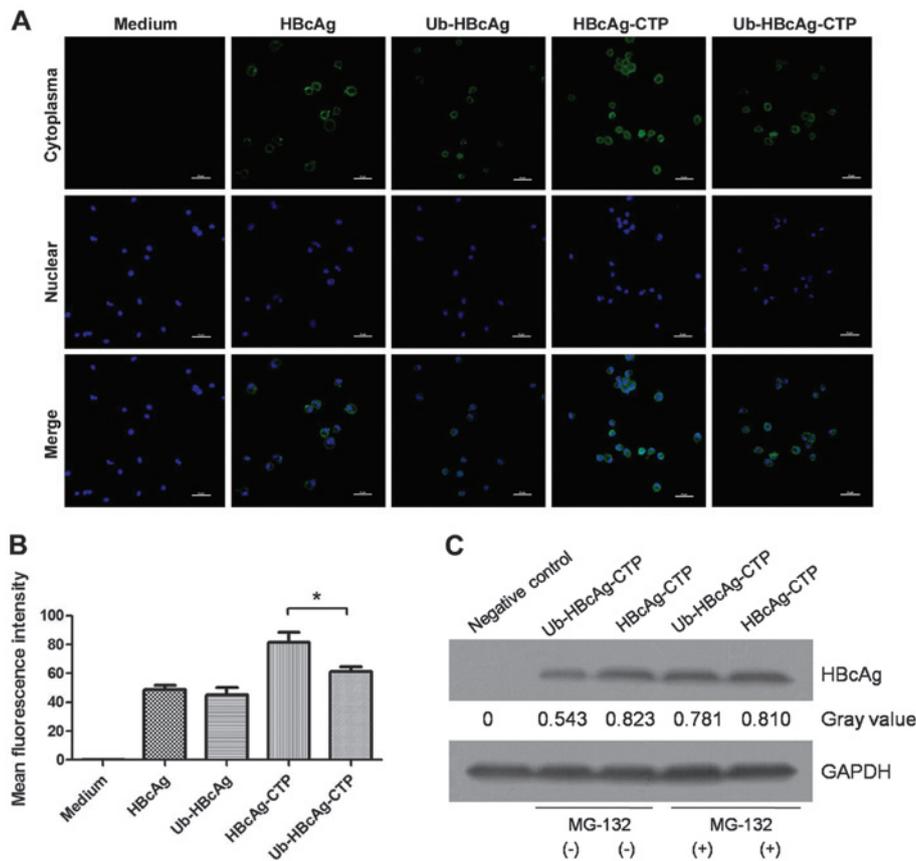


Figure 2. Intracellular localization and western blot analysis. (A) Confocal microscopic analysis of DCs treated with various proteins. Nuclei were stained with DAPI (blue). Fluorescein isothiocyanate-labeled HBcAg (green fluorescence) was detected mainly in the cytoplasm (magnification, x200). (B) Analysis of mean fluorescence intensity was performed in the cytoplasm of DCs treated with various fusion proteins via confocal microscopy. Data is expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$ . (C) Detection of the levels of HBcAg in the cytoplasm of DCs treated with Ub-HBcAg-CTP and HBcAg-CTP. MG-132 (10  $\mu$ mol) was added for 24 h. Cell lysates were analyzed using western blotting. Relative expression of HBcAg was calculated using a gray value. HBcAg, hepatitis B virus core antigen; DC, dendritic cell; CTP, cytoplasmic transduction peptide; MBP, maltose binding protein; Ub, ubiquitin.

Ub-HBcAg-CTP group ( $P < 0.05$ ; Fig. 3C), and was enhanced by lower T cell/DC ratios.

*Ub-HBcAg-CTP leads to increased IFN- $\gamma$  production by CD8<sup>+</sup> T cells in vitro.* The levels of CD8<sup>+</sup> T cell-produced IFN- $\gamma$  were measured by intracellular staining and flow cytometry. The cells were stained with an anti-CD3 mAb to identify T cells within the cell populations, and the samples were subsequently subjected to intracellular staining. As shown in Fig. 4A, the percentage of specific IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells was greater in the Ub-HBcAg-CTP group than in the other groups ( $P < 0.05$ ), suggesting that Ub-HBcAg-CTP-treated DCs may enhance the generation of specific CTLs.

*Ub-HBcAg-CTP induces the enhancement of cytokine production and CTL activity.* Subsequently, the levels of IFN- $\gamma$  and IL-2 secreted by T cells were measured. The Ub-HBcAg-CTP group produced higher levels of IFN- $\gamma$  (348.8 $\pm$ 24.78 pg/ml) and IL-2 (476.5 $\pm$ 20.81pg/ml) than those in the other groups ( $P < 0.05$ , Fig. 4B). Clearer CTL responses were detected at differing effector/target ratios in the Ub-HBcAg-CTP group, compared with those in the other groups (Fig. 4C). These results suggested that Ub-HBcAg-CTP may induce marked specific CTL responses, which was consistent with the high level of IFN- $\gamma$  expressed in CD8<sup>+</sup> T cells.

## Discussion

Various immunotherapeutic strategies have been developed to eliminate HBV; however, these strategies have not had a substantial impact (22). The host antiviral immune response, particularly CTL activity, to HBV-antigens has been established as the main determinant in the processes of viral replication and clearance (23,24). Therefore, the induction of HBV-specific CD8<sup>+</sup> T cells is a current goal in the development of effective immune-based therapeutic interventions for the treatment of HBV infection, intended to enhance antigen presentation in order to induce broad CTL responses.

CD8<sup>+</sup> T cell priming requires the direct and/or cross-presentation of antigenic peptides on MHC class I molecules by APCs (25). DCs are among the most potent APCs and possess a unique capacity to interact with naïve T cells and consequently induce immune responses (26). Patients with chronic HBV infections generally exist in an immune-compromised state, comprising immune tolerance with impaired DC function (27,28). Therefore, efforts should be made to enhance the antigen presenting capacities of DCs.

CTP, which is derived from PTD, was verified to facilitate specific cytoplasmic localization and possess potent membrane transduction potential (16). Accordingly, cytoplasmic functional molecules may be more efficiently targeted

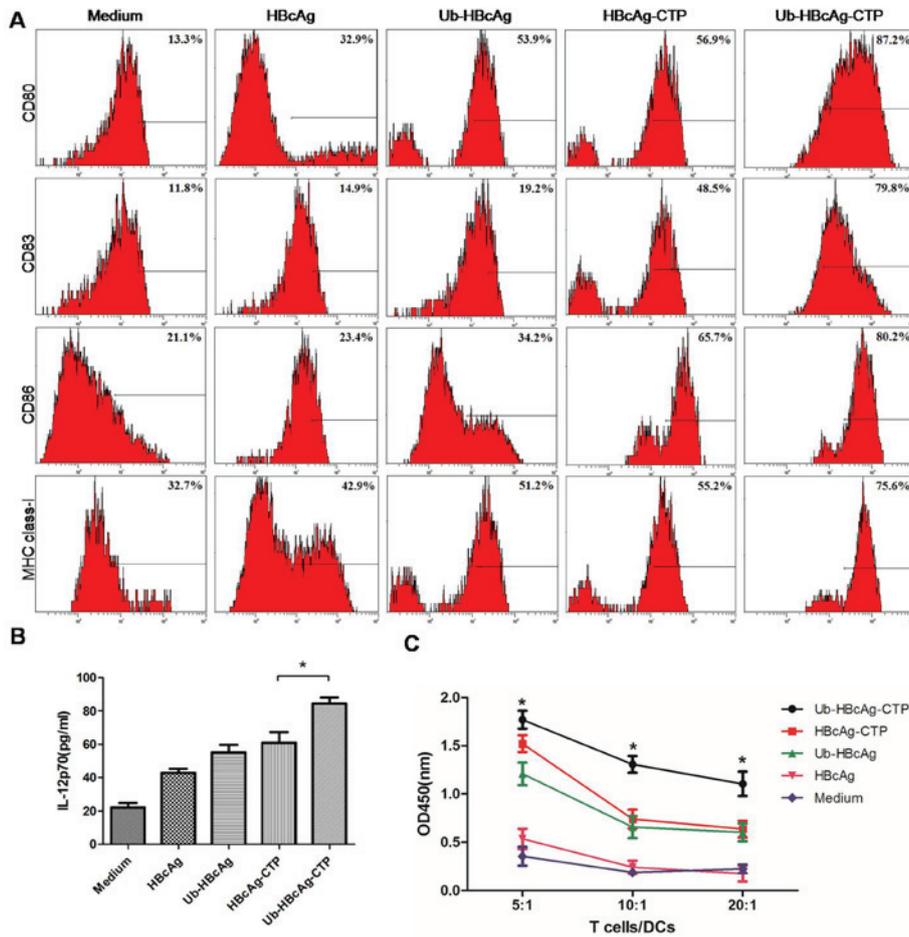


Figure 3. DC immunophenotypic analysis, detection of IL-12p70 production and T-cell proliferation. (A) Surface molecules on DCs (CD80, CD83, CD86, and major histocompatibility complex class-I) were detected using flow cytometry. Surface molecule expression was significantly higher in the Ub-HBcAg-CTP group than in the other groups. \* $P < 0.05$ , \*\* $P < 0.01$ . (B) IL-12p70 concentrations in supernatants of DCs were measured via ELISA. Data is expressed as the mean  $\pm$  SD. \* $P < 0.05$  vs. HBcAg-CTP group. (C) T-cell proliferation capacities at various responder/stimulator (T cell/DC) ratios. Data is expressed as the mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$  vs. HBcAg-CTP group. HBcAg, hepatitis B virus core antigen; DC, dendritic cell; CTP, cytoplasmic transduction peptide; MBP, maltose binding protein; Ub, ubiquitin; SD, standard deviation; IL, interleukin; CD, cluster of differentiation; OD, optical density.

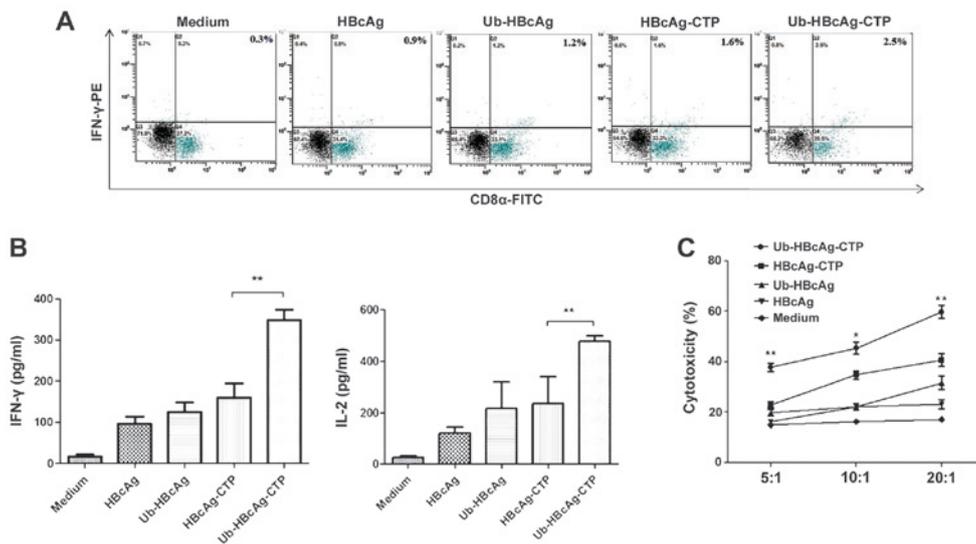


Figure 4. Intracellular cytokine analysis of proliferative T cells, cytokine production and CTL assay. (A) Specific IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells were detected by flow cytometry. (B) Levels of IFN- $\gamma$  and IL-2 in the supernatants of proliferative T cells stimulated by various protein-treated DCs. Data is expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$ . (C) Specific CTL activity was measured by lactate dehydrogenase release assay. The proliferative T cells were incubated with target cells at various effector/target ratios (5:1, 10:1 and 20:1). CTL activity was indicated as the mean percentage of specific lysis (mean  $\pm$  standard deviation). \* $P < 0.05$ , \*\* $P < 0.01$ , compared with control. CTL, cytotoxic T lymphocytes; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; CD, cluster of differentiation; DC, dendritic cell; HBcAg, hepatitis B virus core antigen; CTP, cytoplasmic transduction peptide; Ub, ubiquitin; FITC, fluorescein isothiocyanate.

by CTP-mediated delivery (16,29). In the present study, a Ub-HBcAg-CTP-expressing vector was constructed in order to achieve increased transduction into cells. Following transduction, the Ub-HBcAg-CTP fusion protein was detected via confocal microscopy, in order to characterize the intracellular localization. It was revealed that CTP-containing fusion proteins remained primarily in the cytoplasm of DCs, whereas proteins without CTP were detected primarily on the cell surface. This result confirmed that target proteins were able to be transported efficiently into the DC cytoplasm via CTP, thus providing a basis for the further verification of protein immunogenicity.

Ub is a well-conserved and ubiquitously expressed protein that is conjugated to target proteins (30). It is best known for its role as a marker for protein destruction by the UPS (31). In the present study, the MFI of Ub-HBcAg-CTP-transduced DCs was weaker than that of the HBcAg-CTP group, thus indirectly demonstrating the degradation of HBcAg. Furthermore, HBcAg was quantitatively measured by western blot analysis. It was identified that the Ub-tagged HBcAg protein was at a lower level in the absence of MG-132 (specific proteasome inhibitor), indicating rapid recognition of the fusion protein by the UPS, leading to HBcAg degradation. Ub-mediated antigen processing has been widely used to enhance immune responses in infectious disease and cancer studies (32,33). A previous study confirmed that Ub-fused proteins may improve CTL activity by promoting the introduction of the encoded protein into the MHC class I pathway (34). HBcAg, which possesses unique immunological features, elicits prominent immune responses during chronic HBV infection (35). Therefore, a Ub-modified HBcAg gene was amplified.

In the present study, the immunomodulatory effects of these proteins on bone marrow-derived DC (BMDC) development *in vitro* was investigated. It was identified that Ub-HBcAg-CTP-treated BMDCs expressed higher levels of surface molecules. Additionally, the associated mature DCs secreted higher levels of IL-12 in response to various intracellular pathogenic infections. This response has a key role in the initiation of specific T cell-mediated immune responses and the promotion of T-helper (Th)1 cell activation and differentiation (21). Accordingly, IL-12p70 production was evaluated. As desired, the DC production of IL-12p70 was markedly increased in response to Ub-HBcAg-CTP treatment, compared with that in the other treatment groups. The DC morphology in the Ub-HBcAg-CTP group was also observed to include numerous mitochondria, as well as increased quantities of ribosomes and rough endoplasmic reticulum. The results demonstrated that the Ub-modified HBcAg protein may facilitate DC maturation and increase MHC class-I expression.

Th1 cells are known to produce large quantities of Type-1 cytokines, including IFN- $\gamma$  and IL-2, whereas Th2 cells produce large quantities of Type-2 cytokines, including IL-4 and IL-10 (36). In the current study, the secretion of Type-1 cytokines (IFN- $\gamma$  and IL-2) was demonstrated to be significantly increased in the Ub-HBcAg-CTP group, which indicated that Ub-HBcAg-CTP-pulsed DCs had a tendency to promote Th1 polarization and activate cell-mediated immunity. In addition, in order to determine whether this maturation sufficiently modulated CD8<sup>+</sup> T cells, the production of IFN- $\gamma$  was measured via intracellular cytokine

staining. The cells in the Ub-HBcAg-CTP group were found to induce a greater quantity of IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells and more robust specific CTL activity. The specific cytotoxic response assay confirmed the enhancing effect of Ub-HBcAg-CTP on specific CTL responses. The results demonstrated that Ub-HBcAg-CTP fusion proteins may enhance the capacity of T cells to proliferate, secrete cytokines and develop into CTLs *in vitro*. Based on these findings, it is possible that the role of the Ub-HBcAg-CTP fusion protein in the induction of specific CTLs may be attributed to the fact that UPS-degraded antigenic peptides are presented by cell-surface-expressed MHC class-I molecules and are, therefore, specifically recognized by CTLs.

In conclusion, the present study demonstrated that Ub-HBcAg-CTP fusion proteins do not only increase the expression of MHC class-I molecules, but also enhance the presentation of antigenic peptides and elicit robust HBcAg-specific CTL immune responses *in vitro*. Although the data presented in the present study may not be fully translatable to studies in humans, these findings may suggest a potential therapeutic strategy for chronically infected HBV patients.

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