

# Exosomes from Ub-HBcAg-overexpressing dendritic cells induce T-lymphocyte differentiation and enhance cytotoxic T-lymphocyte activity

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**Abstract.** Hepatitis B virus (HBV) infection is a major public health concern. The clearance of HBV may involve cytotoxic T-lymphocyte (CTL) activity and T helper type 1 reactions. Exosomes generated from dendritic cells (DCs) can induce immunological responses capable of eradicating viruses. However, exosomes loaded with antigens have not yet demonstrated therapeutic potential in HBV infection. Therefore, the present study aimed to investigate the antiviral effects of DC-derived exosomes (Dexs) loaded with ubiquitinated HBV core antigen (Dexs-Ub-HBcAg). Murine bone marrow-derived DCs were loaded with a recombinant lentivector encoding the ubiquitinated form of HBcAg. High-purity Dexs were generated using differential velocity centrifugation. Splenic T-lymphocytes were stimulated with Dexs-Ub-HBcAg and the specific T-cell-mediated immune responses were examined. Cytokine expression was analyzed using enzyme-linked immunosorbent assays. T-lymphocyte proliferation was detected using a Cell Counting Kit-8 assay and HBcAg-specific CTL activity was determined using a lactate dehydrogenase release assay. The results revealed that Dexs-Ub-HBcAg effectively stimulated T-cell proliferation and induced the activation of antigen-specific CTLs to exhibit HBcAg-specific CTL immune responses *in vitro*. These results suggest the potential of Dexs-Ub-HBcAg for development as a future therapeutic option for the elimination of HBV.

## Introduction

Hepatitis B virus (HBV) infection is a major public health concern. The World Health Organization estimated in 2015 that >257 million individuals were chronically infected with HBV (1). Long-term HBV infection causes acute and

chronic hepatitis B (CHB) and the development of complications, including cirrhosis and liver cancer (2). T helper type 1 (Th1) reactions and antigen-specific cytotoxic T-lymphocytes (CTLs) appear to be crucial in the clearance of chronic HBV infection (3). The elimination of HBV is mainly dependent on effective and diverse T-cell immune responses (4). However, as the body is unable to destroy affected hepatocytes, chronic HBV infection can persist for an extended period of time (5). Antiviral medications inhibit the replication of HBV; however, they have minimal effects on the ability of the body to restore the function of Th cells or on the role of CTLs (6). Therefore, boosting HBV-specific T-cell reactions may be a potential treatment strategy for patients with CHB.

HBV core antigen (HBcAg) displays distinct immunological characteristics. Patients in which the virus is completely eradicated often have strong CTL reactions that are specific to HBcAg (7). Ubiquitin is a very small protein comprising 76 amino acids that is very well conserved. In the proteolytic process, ubiquitin acts as a signal for the target protein to be identified and broken down in the proteasome (8). Previous studies have indicated that a lentivector encoding the ubiquitinated form of HBcAg (LV-Ub-HBcAg) produced HBV-specific CTLs, promoted dendritic cell (DC) maturation and promoted lymphocyte growth (9-11).

DC-based immunotherapy is a very promising therapeutic strategy; however, the separation and transduction of DCs from patients to generate specific autologous DC vaccines is a costly and time-consuming process, and DCs have a short survival time and stringent preparation requirements (12,13). Exosomes derived from DCs are known as dexosomes (Dexs) and are capable of triggering and increasing antigen-specific T-cell reactions *in vivo*. Dexs express major histocompatibility complex (MHC) class I/II and costimulatory molecules (14). The *in vitro* production process of exosomes utilizing DCs is simple (14). Phase I and II clinical trials in which patients with malignant melanoma and non-small cell lung cancer were treated with Dexs demonstrated the feasibility of using Dexs as an antitumor vaccination (15-17); these trials demonstrated the safety and immunotherapeutic effects of Dex-based vaccines. In the present study, high-purity Dexs were generated from murine DCs loaded with LV-Ub-HBcAg. Splenic T-lymphocytes were then stimulated with Dexs to investigate the HBV-specific T-cell immune reaction.

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**Key words:** dendritic cell-derived exosomes, hepatitis B virus, ubiquitin, cytotoxic T-lymphocyte, lentiviral vector, exosome

## Materials and methods

**Mice.** A total of 20 mice of the C57BL/6 (H-2b) strain, aged between 6 and 8 weeks (weight, 20–24 g) and with an equal number of males and females, were obtained from the Jiangsu University Experimental Animal Center (Zhenjiang, China). All mice were bred in an environment that was free of all pathogens (22–26°C; humidity 50–55%; 12-h light/dark cycle) and allowed access to food and water *ad libitum*. Animals were euthanized by the intraperitoneal injection of an overdose of sodium pentobarbital (200 mg/kg; cat. no. 69020181; Sinopharm Chemical Reagent Co., Ltd.). Death of the mice was verified by the absence of heartbeat, breathing or respiration for  $\geq 5$  min. The Laboratory Animal Ethics Committee of Jiangsu University approved all the experimental methods (ref. no. K-20180031-Y).

**Reagents and cells.** Abcam provided the anti-HBcAg antibody (cat. no. ab8637) that was used in the present study. R&D Systems, Inc. supplied the enzyme-linked immunosorbent assay (ELISA) kits used to measure IFN- $\gamma$  (cat. no. MIF00), IL-2 (cat. no. M2000), IL-4 (cat. no. M4000B) and IL-10 (cat. no. M1000B) levels. The P815/c cell line, which comprises H-2b mastocytoma cells expressing the HBV core antigen, was preserved in the authors' laboratory (11). Briefly, P815 mouse mastocytoma cells ([https://www.cellosaurus.org/CVCL\\_2154](https://www.cellosaurus.org/CVCL_2154)) were transfected with recombinant lentiviruses carrying HBcAg and a puromycin resistance gene. After 48 h of transfection, 2  $\mu$ g/ml puromycin was applied for screening for 10 days. The surviving cells were P815/c cells, which were resistant to puromycin and carried the HBcAg gene. The cells were cultured at 37°C in a humidified environment containing 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. The recombinant lentiviral vectors (LV-Ub-HBcAg and LV) were constructed as previously described (10).

**DC isolation and LV-Ub-HBcAg transfection.** The generation of murine DCs was carried out according to the methodology outlined in the study by Chen *et al.* (18). Briefly, bone marrow cells were obtained from the tibiae and femurs of C57BL/6 mice and erythrocytes were lysed. The bone marrow cells were cultured at 37°C at a concentration of  $2 \times 10^6$  cells/ml in complete RPMI-1640 culture medium (Gibco; Thermo Fisher Scientific, Inc.) containing 20 ng/ml murine granulocyte-macrophage colony-stimulating factor (mGM-CSF; PeproTech, Inc.) and 10 ng/ml murine IL-4 (mIL-4; PeproTech, Inc.). On day 3, after establishing the initial culture, non-adherent single cells were discarded and fresh RPMI-1640 containing mGM-CSF and mIL-4 was added. The transfection of DCs with LVs was performed as previously described (10). In brief, on day 5, the immature DCs (imDCs) and their progenitors were seeded in a 24-well plate containing 0.5 ml complete RPMI-1640 enriched with mIL-4, mGM-CSF and polybrene (2  $\mu$ g/ml). The cells were then transfected with LV-Ub-HBcAg to produce DEXS-UB-HBcAg, or with LV to produce control DEXS (DEXS-Con). Subsequently, the infected cells were cultured at 37°C for 24 h. The supernatant

was then removed and replaced with fresh medium, and the cells were treated with 1 mg/ml lipopolysaccharide (LPS; MilliporeSigma) for 24 h. Untransfected imDCs were stimulated with LPS for 24 h to produce blank DEXS as the blank control (DEXS-Blank). At 5 days post-transduction, green fluorescent protein (GFP) expression was measured to determine the transduction efficiency in the DCs using a fluorescence microscope (Nikon Eclipse TE2000-U; Nikon Corporation) by Image J software (National Institutes of Health). CD11c is a specific DC marker (19), therefore, CD11c<sup>+</sup>GFP<sup>+</sup> cells were sorted for enrichment using a MoFlo<sup>®</sup> High-Performance Cell Sorter (code S2500; Beckman Coulter, Inc.). Trypan blue (MilliporeSigma) labeling was utilized to ascertain whether the DCs were viable, and only those with  $>85\%$  viability were employed.

**Dex isolation and characterization.** DEXS were isolated by differential velocity centrifugation, as previously described (20,21). The culture supernatants of DCs transfected with LV-Ub-HBcAg or LV were retrieved and centrifuged at 300  $\times$  g for 10 min at 37°C. The supernatant was collected and centrifuged at 3,000  $\times$  g for 15 min at 4°C followed by 10,000  $\times$  g for 30 min at 4°C. The supernatant was then filtered using a PVDF membrane (MilliporeSigma) with a pore size of 0.22  $\mu$ m and transferred to an ultracentrifuge tube. In some previous studies, the supernatant was centrifuged at 100,000  $\times$  g for 2 h at 4°C (22,23). However, in the present study, it was considered more appropriate to centrifuge the supernatant at 110,000  $\times$  g for 90 min at 4°C according to another previously described protocol as this method was shorter in time while maintaining a similar extraction efficiency (21). The supernatant was then discarded, and the residual Dex pellets were resuspended in 100  $\mu$ l phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc.). The DEXS were stored at -80°C for use in subsequent experiments.

The total protein content in the DEXS was measured using a Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Inc.). Transmission electron microscopy (TEM) using a JEM-2100 instrument (JEOL, Ltd.) was utilized to visualize the DEXS. The DEXS were processed for TEM following standard experimental methods (24). The size of the DEXS was measured using the ZetaVIEW<sup>®</sup> nanoparticle tracking analysis system (Particle Metrix GmbH) according to the manufacturer's guidelines. Furthermore, western blot analysis was utilized to evaluate the expression levels of Dex marker proteins. RIPA lysis buffer containing a protease inhibitor mixture (Beyotime Institute of Biotechnology) was employed to lyse the DEXS. Protein concentration levels were measured using a BCA protein assay kit. After separation by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 30  $\mu$ l protein lysate/lane was subsequently transferred onto a PVDF membrane (MilliporeSigma). Subsequently, the membrane was blocked with 5% non-fat milk at room temperature for 1 h. The primary antibodies used were rabbit anti-mouse CD63 (1:500; cat. no. ab216130; Abcam), anti-CD9 (1:500; cat. no. ab92726; Abcam) and tumor susceptibility gene 101 (TSG101; 1:500; cat. no. 102286-T38; SinoBiological) monoclonal antibodies. Rabbit anti-human HBcAg antibody (1:1,000; cat. no. ab115992; Abcam) was also used as the primary antibody. All the primary antibodies were employed at 4°C overnight. The secondary

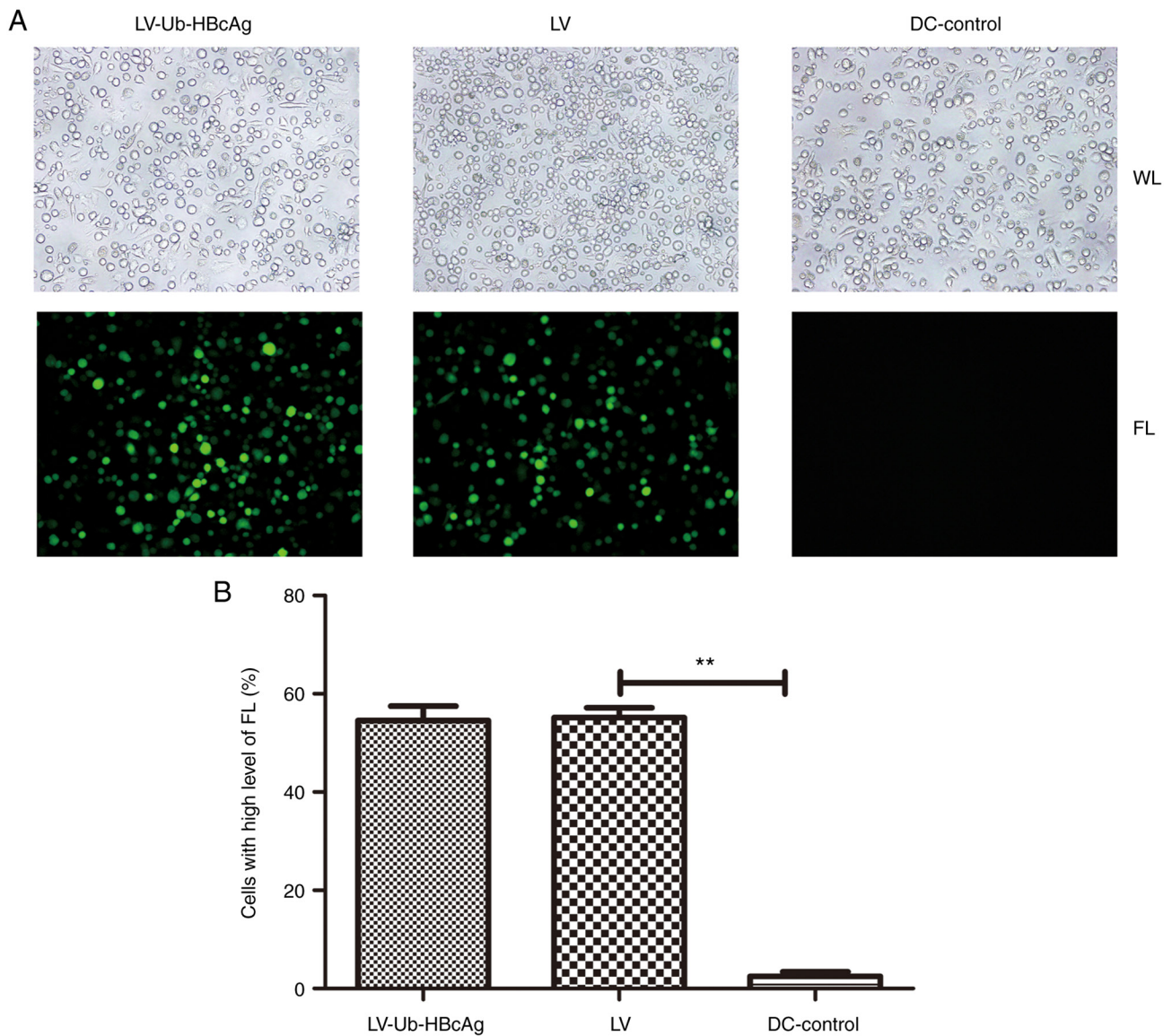


Figure 1. GFP expression in DCs transduced with LVs. (A) GFP expression was observed using a fluorescence microscope. Magnification, x200. (B) The proportion of cells with a high level of fluorescence was quantified. \*\*P<0.01. GFP, green fluorescent protein; DCs, dendritic cells; LV, lentiviral vector; LV-Ub-HBcAg, recombinant LV encoding the ubiquitinated form of the hepatitis B virus core antigen; WL, white light; FL, fluorescence light.

antibody, a horseradish peroxidase-conjugated anti-rabbit immunoglobulin-G antibody (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) was employed at room temperature for 1.5 h. Enhanced chemiluminescence (BeyoECL Plus; cat. no. P0018M; Beyotime Institute of Biotechnology) was used to visualize the protein bands.

**T-lymphocyte generation.** T-lymphocytes were isolated from splenocytes using nylon wool columns (FUJIFILM Wako Pure Chemical Corporation). Single-cell suspensions of the lymphocytes at a concentration of  $2 \times 10^6$  cells/ml were plated on six-well plates with RPMI-1640 containing 10% FBS and allowed to develop at 37°C for 24 h. After labeling with CD3 monoclonal antibody (17A2), phycoerythrin-Cyanine5 (anti-CD3-PE-Cy5; cat. no. 15-0032-82; eBioscience), the extracted T-cells were analyzed using CytoFLEX flow cytometry (Beckman Coulter, Inc.) to measure their purity and only cells with >80% purity were employed.

**Cytokine release assay.** T-lymphocytes were plated at a concentration of  $2 \times 10^6$  cells/ml in RPMI-1640 culture medium with 10  $\mu$ g/ml Dexs-Ub-HBcAg, Dexs-Con, Dexs-Blank or PBS in 24-well plates at 37°C for 72 h. The supernatants were retrieved and the quantities of IFN- $\gamma$ , IL-2, IL-4 and IL-10 were determined using ELISA kits in compliance with the manufacturer's protocol. The results are presented in units of pg/ml.

**T-lymphocyte proliferation assay.** On day 5 of DC isolation, DCs were co-cultured with 10  $\mu$ g/ml Dexs-Ub-HBcAg, Dexs-Con, Dexs-Blank or PBS for 72 h. Subsequently, the DCs were pre-treated with mitomycin C (25  $\mu$ g/ml; MilliporeSigma) for 30 min. Separately, T-lymphocytes ( $2 \times 10^6$  cells/ml) were plated in six-well plates coated with anti-CD3 (cat. no. 14-0032-82; eBioscience) at a concentration of 0.5  $\mu$ g/ml overnight at 4°C. The plates were then maintained at 37°C and supplied with RPMI-1640 culture medium. Subsequently,

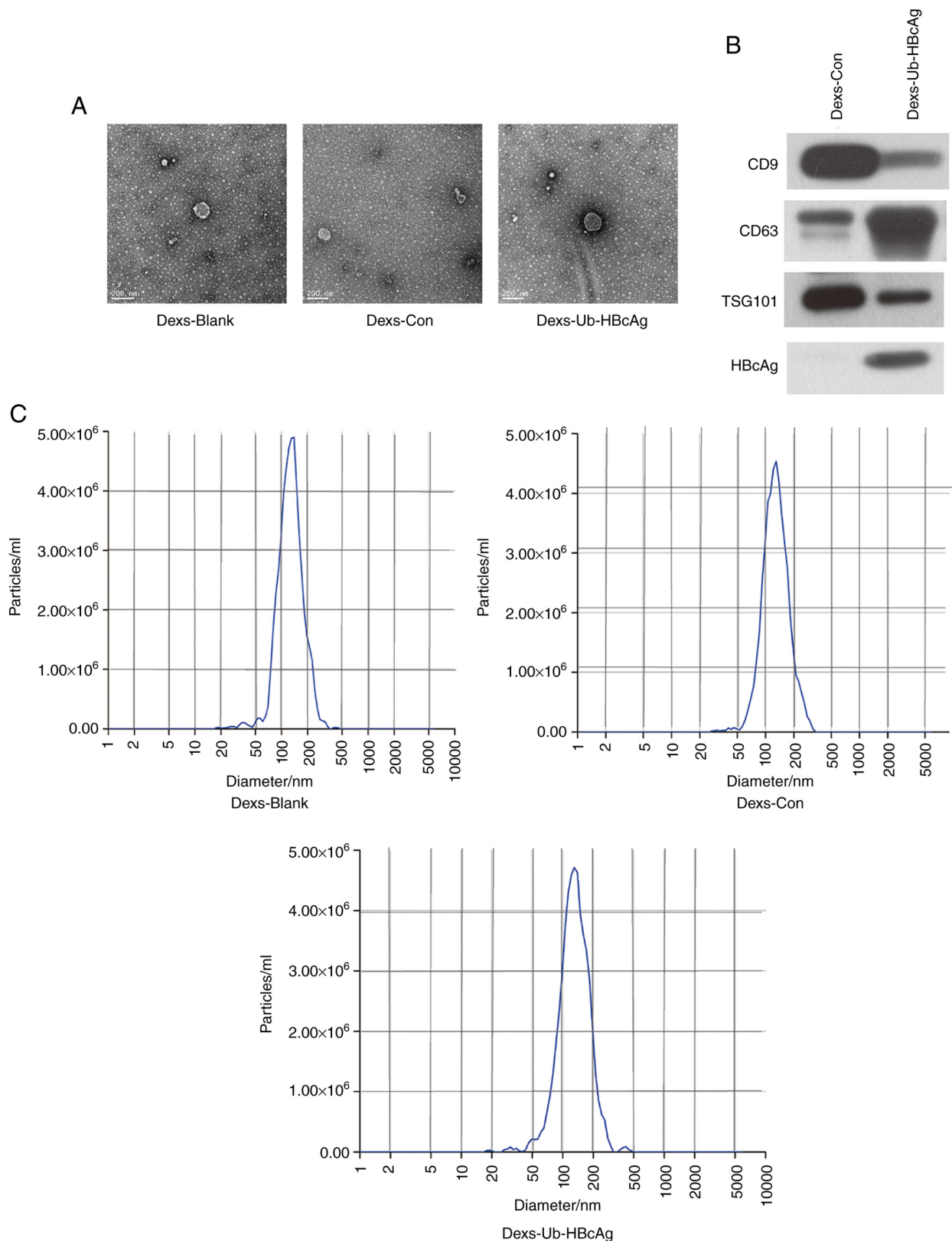


Figure 2. Isolation and identification of Dexs-Ub-HBcAg. (A) Transmission electron microscopy of the mDexs ultrastructure. Scale bar, 200 nm. (B) Expression of the positive exosomal markers CD9, CD63 and TSG101, and of HBcAg was examined via the western blot analysis of DEXS lysates. (C) Size dispersion profile of mDexs evaluated using ZetaVIEW® nanoparticle tracking analysis, indicating a size peak of 123.8 nm. Dexs, dendritic cell-derived exosomes; HBcAg, hepatitis B virus core antigen; Dexs-Ub-HBcAg, Dexs loaded with ubiquitinated HBcAg; Con, control; TSG101, tumor susceptibility gene 101.

anti-CD28 (0.5  $\mu$ g/ml; cat. no. 14-0281-82; eBioscience) was added to activate the T-cells for 24 h at 37°C. The activated T-lymphocytes were then used as responder cells

in a co-culture with the DCs using a responder/stimulator (T-cell/DC) ratio of 20:1. The cells were cultured in a final volume of 100  $\mu$ l for 96 h at 37°C, during which 10  $\mu$ l Cell

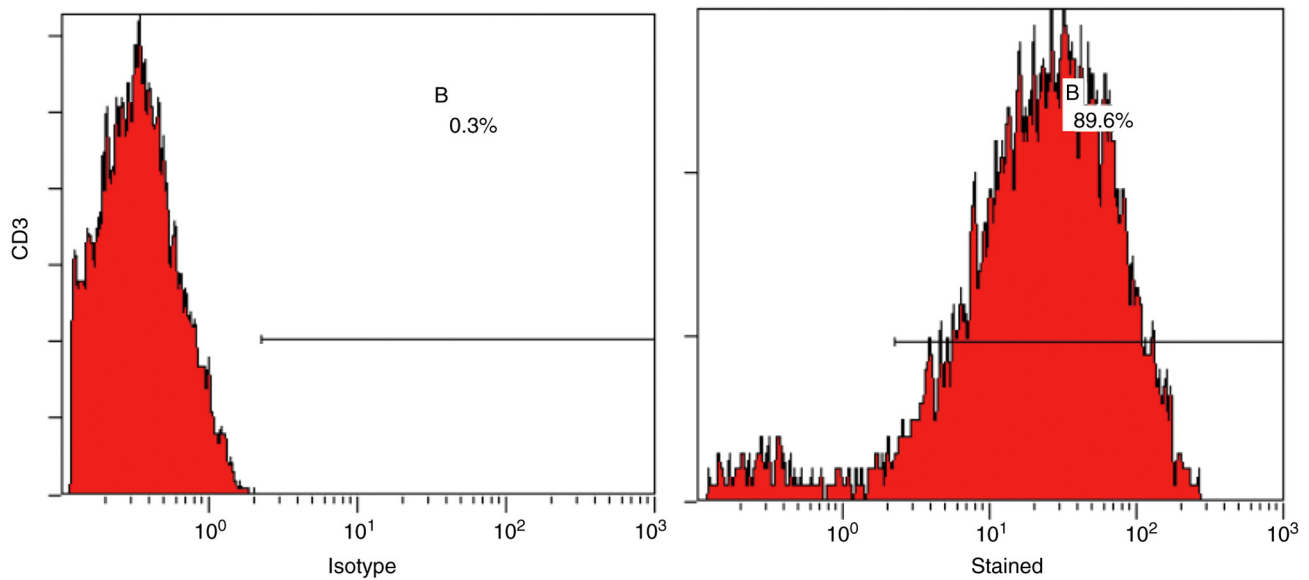


Figure 3. T cells were stained with anti-CD3-phycoerythrin-Cyanine5 and analyzed by flow cytometry. The plots are representative of three independent experiments. B, CD3 positive T cells.

Counting Kit-8 solution (Dojindo Laboratories, Inc.) was added for the final 4 h. The absorbance of the cultures was measured at 450 nm using a Multiskan Ascent microplate reader (Thermo Fisher Scientific, Inc.).

**CTL assay.** T-cells were activated in a humidified environment with 5% CO<sub>2</sub> at 37°C for 72 h with Dexs-Ub-HBcAg (10 µg/ml), Dexs-Con (10 µg/ml), Dexs-Blank (10 µg/ml) or PBS. P815/c cells were plated as the target cells, and previously activated T-lymphocytes were used as the effector cells. The T-lymphocytes were co-cultured with the P815/c cells for 4 h at 37°C in a humidified environment containing 5% CO<sub>2</sub>. Effector/target ratios of 5:1, 10:1 and 20:1 were used. The HBcAg-specific CTL activity was evaluated utilizing a lactate dehydrogenase (LDH) release assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay kit; Promega Corporation), in accordance with the manufacturer's guidelines. The absorbance was measured at 490 nm using a Multiskan Ascent microplate reader. The percentage of cytotoxicity was determined using the following formula: [(Experimental release-effector spontaneous release-target spontaneous release)/(target maximum release-target spontaneous release)] x100 (18,25).

**Statistical analysis.** Data are presented as the mean ± SD of at least three separate experiments. To detect statistically significant differences, the data were analyzed using one-way analysis of variance with Tukey's post hoc test. SPSS 20.0 software (IBM Corp.) was used to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Lentiviral transduction of DCs.** Bone marrow-derived DCs were cultured in RPMI-1640 medium supplemented with mIL-4 and mGM-CSF. On day 5 post-culture, the isolated DCs were transduced with LV-Ub-HBcAg or LV. GFP expression

was measured using a fluorescence microscope to evaluate the transduction efficiency in the DCs. A positivity rate of 50-60% was reached, with higher levels of fluorescence observed in the LV-Ub-HBcAg and LV group compared with DC control group (P<0.01; Fig. 1).

**Dex morphology, size and marker protein expression.** Exosomes were extracted and purified from the culture supernatants via ultracentrifugation and ultrafiltration. TEM images revealed that the Dexs were spherical or ovoid in shape with an envelope-like structure (Fig. 2A). The expression of the exosomal protein markers CD9, CD63 and TSG101 was then measured in the Dexs-Ub-HBcAg and Dexs-Con groups. A protein band for HBcAg was observed in the Dexs-Ub-HBcAg group, indicating that the isolated exosomes expressed HBcAg (Fig. 2B). However, the expression levels of these proteins were not measured in the Dexs-Blank group, which is a limitation of the study. The analysis of the size of the exosomes using nanoparticle tracking analysis revealed a scattered or clustered distribution with a mean particle diameter of 123.8 nm (Fig. 2C).

**Dexs-Ub-HBcAg stimulates the secretion of cytokines.** T-lymphocytes were isolated from mouse splenocytes and analyzed using flow cytometry, which confirmed that they were of adequate purity (>80%) (Fig. 3). The production of the cytokines IFN-γ, IL-2, IL-4 and IL-10 by the T-lymphocytes was examined in the presence of Dexs-Ub-HBcAg (10 µg/ml), Dexs-Con (10 µg/ml), Dexs-Blank (10 µg/ml) or PBS. As shown in Fig. 4A and B, T-lymphocytes from the Dexs-Ub-HBcAg group released larger amounts of IFN-γ and IL-2, a Th1-like cytokine, compared with those from the other groups (P<0.01). However, the secretion of IL-4 and IL-10, a Th2-like cytokine, did not exhibit any significant differences among the groups (Fig. 4C and D). These results indicate that the Th1 immune response was preferentially primed.



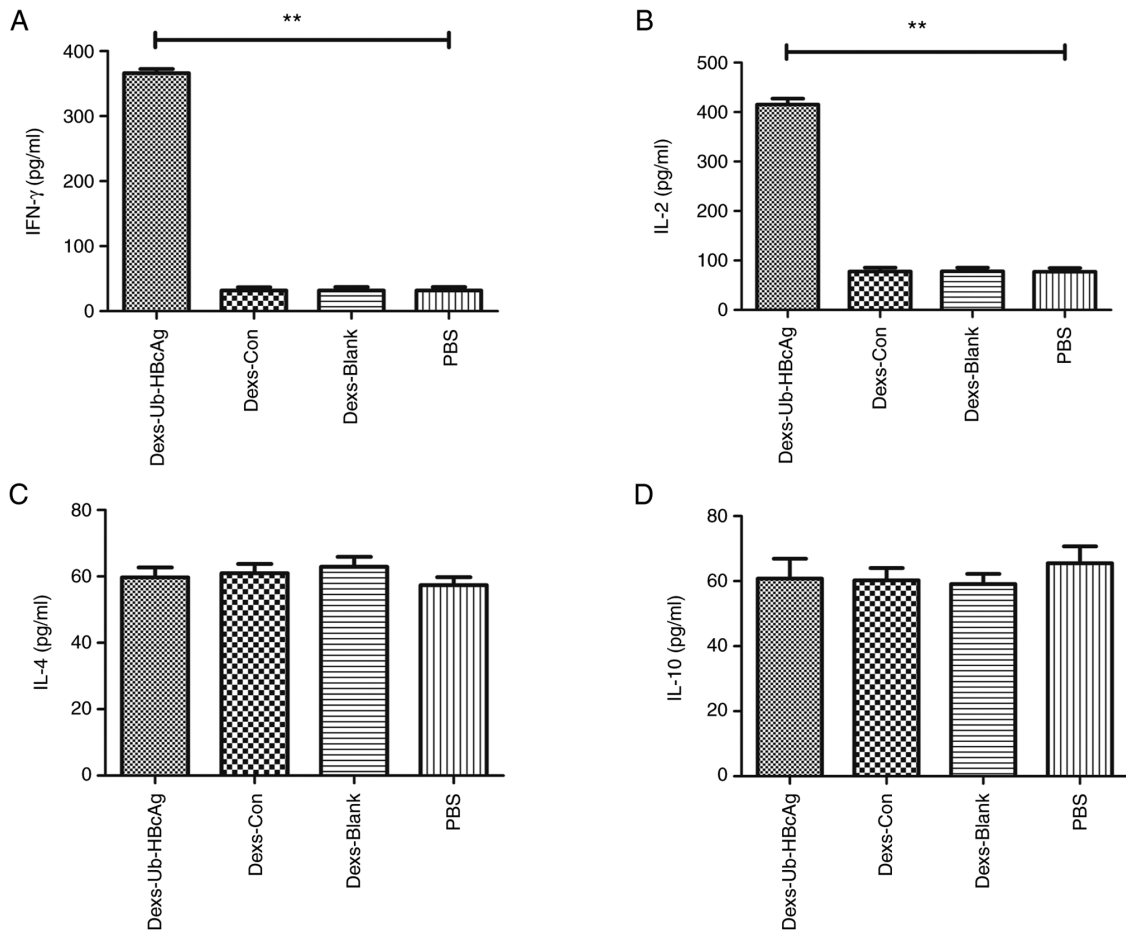


Figure 4. Production of IFN- $\gamma$ , IL-2, IL-4 and IL-10 cytokines. Levels of (A) IFN- $\gamma$  and (B) IL-2 in the supernatants of proliferative T-cells stimulated with various Dexs. IFN- $\gamma$  and IL-2 are significantly most abundant in the supernatants of T-cells from the Dexs-Ub-HBcAg group. \*\* $P < 0.01$ . Release of (C) IL-4 and (D) IL-10 by T-cell supernatants. No statistically significant differences were detected among the groups. The results are presented as the mean  $\pm$  SD from at least three separate experiments. Dexs, dendritic cell-derived exosomes; Dexs-Ub-HBcAg, Dexs loaded with ubiquitinated hepatitis B virus core antigen; Con, control.

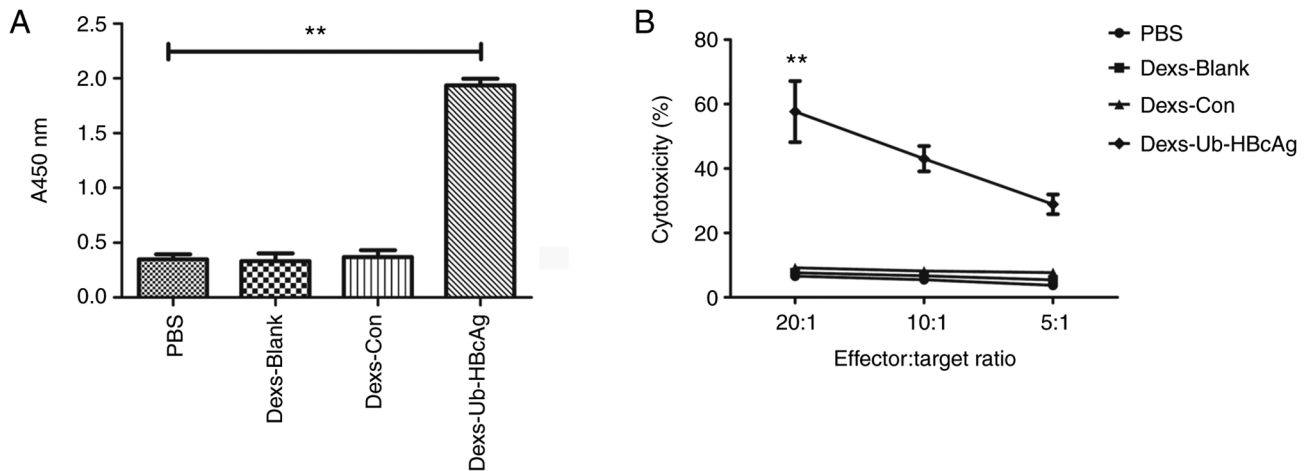


Figure 5. Activation of T-lymphocyte proliferation and CTL evaluation. Cell Counting Kit-8 and lactate dehydrogenase release assays were used to quantify (A) T-lymphocyte growth and (B) specific CTL activity, respectively. CTL activity was assessed as the average proportion of specific lysis (mean  $\pm$  SD) at distinct effector:target ratios (20:1, 10:1 and 5:1). Results are presented as the mean  $\pm$  SD. \*\* $P < 0.01$  vs. the other groups. CTL, cytotoxic T-lymphocyte; PBS, phosphate-buffered saline; Dexs, dendritic cell-derived exosomes; Con, control; Dexs-Ub-HBcAg, Dexs loaded with ubiquitinated hepatitis B virus core antigen; A450 nm, absorbance at 450 nm.

*Dexs-Ub-HBcAg enhances T-cell proliferation.* The growth of T-lymphocytes in the different groups was then evaluated.

The Dexs-Ub-HBcAg group exhibited a significantly greater T-lymphocyte proliferative capacity compared with the

other groups ( $P<0.01$ ), as illustrated in Fig. 5A. This result indicates the markedly higher T-cell proliferation in the Dexs-Ub-HBcAg group.

**Dexs-Ub-HBcAg enhances CTL activity.** The HBcAg-specific CTL activity towards P815/c cells was evaluated using an LDH release assay. As demonstrated in Fig. 5B, the proportions of specific cytolysis in the Dexs-Ub-HBcAg group were  $57.66\pm9.48$ ,  $43.04\pm3.94$  and  $28.89\pm3.07\%$ , respectively, at effector:target ratios of 20:1, 10:1 and 5:1. The Dexs-Ub-HBcAg group generated a significantly greater proportion of specific cytolysis compared with the other groups ( $P<0.01$ ). These results indicate that the HBV-specific CTL activity was enhanced in the Dexs-Ub-HBcAg group.

## Discussion

Patients who have persistent HBV infection have a significant possibility of developing cirrhosis of the liver and hepatocellular carcinoma (26). A weak Th1 immunity combined with the inefficient activation of CD8<sup>+</sup> CTLs leads to therapeutic failure in patients with CHB (25). Host anti-HBV immune responses have been identified as the major determinants during viral replication and clearance (27,28). Several therapeutic vaccination strategies for HBV have recently been developed to enhance the immune response and eliminate the virus (29). DCs are potent antigen-presenting cells with a notable capacity to interact with naive T-cells and trigger immunological reactions (27). The viability of activating Th1 immunity and CTL reactions to remove chronic HBV infection using DC-based therapeutic immunotherapy has already been demonstrated. Specifically, in previous research, it was demonstrated that DCs transduced with LV-Ub-HBcAg preferentially initiated anti-HBcAg Th1 immunity and induced specific CTL activity (10,11). Nonetheless, DC-based vaccines are challenging to prepare and use on a large scale in clinical settings. The implementation of DC-based therapeutic immunotherapy in large populations is costly and dependent on professional competence. Additionally, the long-term storage of DCs and maintenance of their efficacy is difficult (30). DEXs possess the crucial immunostimulatory capacity of DCs. In addition, they may be stored in a frozen state for  $\geq 6$  months due to the stability of the exosomal membranes (31). DEXs have been recommended as a potential solution to a number of technical challenges involved in DC-based immunotherapy (15). As biological agents, DEXs are also more suitable than DCs for preparation using a highly supervised and monitored process. In addition, they do not carry the risks associated with viable cellular or viral therapies, such as *in vivo* replication (15). To date, exosomes have been utilized as medication carriers, vaccination and immunotherapy tools, as well as biomarker transporters (32,33).

Exosome-bound antigens may produce higher antigen-specific anticancer or antiviral immune reactions than those produced by soluble antigens (34,35). In the present study, it was discovered that antigen-modified DEXs stimulated T-lymphocyte growth, cytokine release and CTL development *in vitro*. Exosomes derived from mature DCs have higher surface expression levels of intercellular adhesion molecule-1, MHC and CD86 molecules than imDCs, which may increase their

uptake by DCs and thereby promote T-cell activation (36-38). In contrast to exosomes released from imDCs, these exosomes have a stronger ability to activate T cells (36-39). In the present study, DCs derived from mouse bone marrow cells were loaded with LV-Ub-HBcAg and then stimulated with mIL-4, mGM-CSF and LPS. Following differential velocity centrifugation, very pure exosomes were isolated from mature DCs, which were termed DEXs-Ub-HBcAg. These exosomes were 50-150 nm in diameter with potent immunostimulatory properties.

Previous studies have demonstrated that the responsiveness of patients with CHB to antiviral medication is associated with the predominance of the Th1 immune reaction and elevated CTL function, suggesting that Th1 immunity may be a crucial modulator in the treatment of patients with CHB (27,40). Th1 cells release substantial quantities of type 1 cytokines, including IFN- $\gamma$  and IL-2. By contrast, Th2 cells release substantial amounts of type 2 cytokines, such as IL-4 and IL-10 (6). In the present study, the DEXs-Ub-HBcAg group clearly produced the largest amounts of the Th1-like cytokines IFN- $\gamma$  and IL-2. Furthermore, no significant variations in the IL-4 and IL-10 levels were detected among the groups. These results indicate that anti-HBcAg Th1 immunity was preferentially primed. IL-2 plays a key role in the growth, differentiation and maturation of T-cells, as well as in the growth of Th cells (41). IFN- $\gamma$  is required for the development of Th1 cells, and CTL activity is associated with the stimulation of Th1 immunity. The findings of the present study suggest that the CTL activity of HBV-specific CD8<sup>+</sup> T-cells was increased in the DEXs-Ub-HBcAg group due to the stimulatory effect of cytokines secreted by Th1-type cells. In the present study, DEXs-Ub-HBcAg were found to induce greater CTL cytotoxicity and higher killing potency against P815/c cells compared with the controls. These findings demonstrate that DEXs-Ub-HBcAg enhanced T-cell growth, cytokine production and differentiation into CTLs *in vitro*.

In conclusion, the present study demonstrated that DEXs, a cell-free vaccine that includes ubiquitinated HBcAg and is antigen-presenting, may efficiently promote T-cell growth and activation to develop antigen-specific CTLs that display HBcAg-specific CTL immune reactions *in vitro*. Based on their unique combination of DCs and cell-free vectors, DEXs have great potential as a replacement for DCs in therapeutic vaccines. Additionally, in mice carrying the hepatitis delta virus, antigen-modified DEXs have exhibited beneficial effects on the antiviral immune response (42). This suggests that treatment with DEXs-Ub-HBcAg may provide an effective therapeutic option for the elimination of HBV.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

SD conceived and designed the study. YY, KL and WZ performed the experiments. YY wrote the manuscript. SD, KL, WZ and YY revised and edited the manuscript. YY, KL, WZ and SD confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The Laboratory Animal Ethics Committee of Jiangsu University approved all the experimental methods (approval no. K-20180031-Y).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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