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Original Paper

An Engineered DC-Targeting Lentivector Induces Robust T Cell Responses and Inhibits HBV Replication in HBV Transgenic Mice via Upregulating T Cell Autophagy

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Key Words
Hepatitis B virus • DC-targeting lentivector • Cytotoxic T lymphocyte • Autophagy

Abstract
Background/Aims: Developing engineered dendritic cell (DC)-targeting lentivectors (LVs) have been the target of intense research for their potential to create antigen-directed immunotherapeutics which can be safely administered to patients. In this study, we constructed a DC-directed LV (LVDC-UbHBcAg-LIGHT) as a potential vaccine to induce anti-HBV immune responses. ***Methods:*** Specificity of LVDC-UbHBcAg-LIGHT for DCs *in vivo* was confirmed through live animal imaging studies. The levels of cytokine production in T cells were assessed by flow cytometry. The HBcAg-specific cytotoxic T lymphocyte (CTL) responses and antibody responses induced by direct administration of the LVs were detected by LDH release assay and ELISA respectively. The levels of serum HBsAg and HBV DNA were evaluated by Abbott kits and quantitative PCR respectively. The expression levels of HBsAg and HBcAg in liver tissues of HBV transgenic mice were examined by immunohistochemistry. In addition, molecular mechanism underlying the activation of CD8+ T cells was explored. ***Results:*** Live animal imaging studies showed that following subcutaneous administration of LVDC-UbHBcAg-LIGHT, no obvious luminescence signal was detected at the injection site. Immunization with LVDC-UbHBcAg-LIGHT elicited potent T cell responses in HBV transgenic mice evidenced by increased percentages of IFN-γ, TNF-α and GzmB producing CD8+ T cells as well as IFN-γ producing CD4+ T cells, improved HBcAg-specific CTL activities and antibody responses. Additionally, vaccination with LVDC-UbHBcAg-LIGHT efficiently reduced serum HBsAg, HBV DNA levels and the expression of HBsAg and HBcAg in liver tissues of HBV transgenic mice. More importantly, autophagy was induced in the activated CD8+ T cells, and the induced autophagy noticeably promoted the proliferation of T cells and decreased the frequencies of apoptotic CD8+ T cells by selectively degrading ubiquitinated apoptosis and cell cycle-associated protein aggregates.

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Futhermore, we confirmed the interaction between autophagosomes and ubiquitinated aggregates by confocal microscopy and immunoprecipitation analysis. ***Conclusions:*** These results demonstrated that LVDC-UbHBcAg-LIGHT provided a simple method of eliciting effective antiviral immune responses in HBV transgenic mice and might potentially be used as a therapeutic strategy to eradicate HBV with more safety and efficiency. Moreover, our results revealed a direct role of autophagy in promoting the survival and proliferation of activated CD8+ T cells.

Introduction

Hepatitis B virus (HBV) infection is a major public health concern which is related to many clinical complications, especially chronic liver disease that can progress with time to cirrhosis or even to hepatocellular carcinoma [1]. There is no available treatment which can really lead to a cure in chronic HBV patients. Standard chemotherapeutic drugs including nucleos(t)ide analogs and (pegylated) IFN-α are only partially successful due to the emergence of drug-resistant, the risk of relapse upon treatment discontinuation, and unwanted side effects [2]. As we all know, the effective removal of HBV and the establishment of long-term immune protection by the body mainly depend on HBV-specific cytotoxic T lymphocytes (CTLs) [3]. There is therefore of great clinical interest to develop reliable strategies to induce robust and sustained HBV-specific T cell responses.

As sentinels of the immune system, dendritic cells (DCs) play an essential role in regulating immune responses through antigen capture and presentation [4, 5]. Lentivectors (LVs) have attracted considerable interest for their potential as a vaccine delivery vehicle. Efficient antigen delivery to DCs and their subsequent presentation to prime virus-specific T cells are vital for the success of a LV-based vaccine [6]. Delivering a cargo antigen to DCs by LVs has been demonstrated by many studies through both *ex vivo* and *in vivo* routes [7-9]. The approach of modifying autologous DCs *ex vivo* and transferring them back to the patients is laborious and costly. By contrast, *in vivo* administration of LVs carrying antigen gene has been confirmed to induce equally strong antigen-specific T cell response to that of the LV-modified DCs [10]. However, *in vivo* delivery systems may have multiple limitations including inefficient DC tropism as well as off-target effects in the surrounding cells. A reliable method is to target the delivery of immunogens to DCs *in vivo*.

It has been reported that HBV specific immune responses, especially hepatitis B core antigen (HBcAg) specific CTL responses can inhibit the replication of HBV and control the progress of liver damages [11]. Ubiquitin (Ub) is a small highly conserved protein in eukaryotic cells and it serves as a signal for the target protein to be recognized and degraded into antigen peptides, which can be presented by MHC-I, thereby triggering significantly enhanced antigen-specific immune responses [12, 13]. We have demonstrated that LV-delivered and Ub-fused HBcAg (LV-Ub-HBcAg) could evoke CD8+ cytotoxic T cells and generate therapeutic immunity in HBV transgenic mice [14-16]. LIGHT is a type II transmembrane protein and its expression on DCs has been suggested to provide potent co-stimulatory activity for T cells, resulting in potent T cell immune responses in HBV transgenic mice [17, 18]. To take full advantage of the immuno-stimulatory efficacy of DCs and mitigate adverse effects, we recently synthesized a novel LV (LVDC-UbHBcAg-LIGHT) enveloped with a mutated Sindbis virus glycoprotein (SVGmu) which was engineered to specifically bind to DC-specific ICAM3-grabbing Nonintegrin (DC-SIGN), a relatively DC-restricted surface protein. DC-SIGN has also been explored by many investigators as the target receptor of DCs for protein recognition [19-22].

In our prior studies, the recombinant LVDC-UbHBcAg-LIGHT has been confirmed to exhibit great ability to trigger HBV-specific CTL response *ex vivo* through upregulating autophagy in T cells. We also revealed a direct role of autophagy in promoting the survival and proliferation of T lymphocyte by selectively degrading the proteins associated with apoptosis and cell cycle after TCR stimulation. In the present study we aimed to characterize

the properties of the *in vivo* administered LV-based vaccine with regard to its cellular targets and its ability to generate anti-viral immunity in HBV transgenic mice. The results may be helpful in developing novel strategies with improved efficiency and safety for controlling persistent HBV infection and would shed some light on the activation mechanisms of T cells by the DC-targeting LVs.

Materials and Methods

Mice and ethics statement

Fifty *H-2K*^{d} BALB/c mice (25 males and 25 females, 6-8 weeks old) were purchased from Shanghai Shrek Animal Co., LTD (Shanghai, China). Eighty *H-2K*^{d} HBV-transgenic BALB/c mice (40 males and 40 females, 6–8 weeks old) were obtained from the Key Liver Army Laboratory (The No.458 Hospital, Guangzhou, China). The characterization of the mice has been described in detail before [23, 24]. All mice were bred under specific pathogen-free conditions in the Experimental Animal Centre of the Shanghai Sixth People's Hospital. The mice were cared for and treated in accordance with the guidelines established by the Shanghai Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Cells

The P815/c cells expressing HBcAg were stored in our laboratory. Briefly, P815 cells were transduced by a recombinant lentivirus encoding HBcAg and puromycin resistance gene. 48 h after transduction, 2 μg/ml puromycin was added for screening for 10 days and the surviving P815/c cells were successfully transduced with HBcAg gene. The expression of HBcAg in the cells was further identified by western blot analysis. Human embryonic kidney 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 (Invitrogen, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C in a humidified atmosphere with 5% CO_2.

Recombinant DC-targeting lentivectors preparation

The engineered lentivector LVDC-UbHBcAg-LIGHT was constructed as described previously. Briefly, the recombinant pLOV-UBC-UB-HBcAg-EGFP-P2A-Tnfsf14-3FLAG plasmid was constructed by inserting the EGFP-P2A-Tnfsf14-3FLAG gene into the BamHI/XbaI sites of the CN1043 (pLOV-UBC-Ub-HBcAg-EGFP-3FLAG) which was maintained in our lab [15]. The DC-targeting envelope plasmid (SVGmu) was generated by alterations as described by Yang et al [19].. The packaging of lentiviral particles was accomplished in 293T cells and tittered as described before [15, 16].

In vivo assay of targeting of DCs by the engineered lentivector

H-2K^d BALB/c mice were randomly divided into three groups, with eight mice in each group. The recombinant lentivector LVDC-UbHBcAg-LIGHT or LV-UbHBcAg-LIGHT (5×10^7 TU resuspended in 200 μl PBS; TU is short for Transducing Units) was injected subcutaneously into the left flank of the *H-2K*^{d} BALB/c mice close to an inguinal lymph node (within 1 cm range). phosphate-buffered saline (PBS) was used as a control. 72 h after injection, the left inguinal lymph node and the equivalent lymph node at the opposite site were isolated for size examination. Cells were harvested from the above nodes and their total numbers were counted. The percentage of GFP+ cells within DCs was determined by a Coulter Epics XL flow cytometer (Beckman, Miami, USA).

Live animal imaging

Two lentivectors (LVDC-luc and LV-luc) encoding the imaging gene-luciferase (luc) were constructed to enable us to visualize the *in vivo* transduction of the tissue cells using bioluminescence imaging (BLI). LVDC-luc or LV-luc (5×10^7 TU resuspended in 200 μl PBS) was injected subcutaneously into the left flank of the *H-2K*^{d} BALB/c mice (with eight mice in each group). One month post-immunization, the mice were anesthetized and injected intraperitoneally with D-luciferin (150 mg/kg body weight; Xenogen, Alameda, CA, USA) 10 min prior to imaging. Since we have previously integrated the luc gene into the DNA to express luciferase through the lentivector, luminescence can be generated within a few minutes when exogenous

administration of its substrate luciferin. The luciferase catalyzes the luciferin oxidation reaction in the presence of ATP and oxygen, therefore, luminescence can only be generated in living cells, and the intensity of light is linearly related to the number of transduced cells. We obtained the gray-scale reference image under low-level illumination. Photons emitted from within the animal and transmitted through the tissues were collected using a cooled CCD camera (Xenogen IVIS, Xenogen Corp., Alameda, CA, USA) (IVIS is short for *In vivo* Imaging Systems) and analyzed with Living Imaging Software (Xenogen).

In vivo immunization of the HBV transgenic mice

Transgenic mice were randomly divided into six groups, with twelve mice in each group. The mice were injected subcutaneously in the hind footpads twice at an interval of 2 weeks with 2×10^7 TU recombinant lentiviral vectors (LVDC-UbHBcAg-LIGHT, LV-UbHBcAg-LIGHT, LVDC-UbHBcAg or LVDC; "LVDC" represents "an empty DC-targeting lentiviral vector without HBV antigen), 20, 000 IU (IU is short for International Unit) interferon (IFN)-α (Roche Diagnostics, Basel, Switzerlad), or PBS. The mice were sacrificed at the indicated time points. Splenocytes and livers were collected for detection.

Cytokine release assay

Splenocytes were collected two weeks after the last immunization, then, T cells were separated using the nylon filtration method, as described previously [16, 17] and cultured in 24-well plates (2×10^6 cells/ml) at 37°C in complete RPMI-1640 in the presence of 10 μg/ml HBcAg-specific CTL peptide HBcAg 87–95 (YVNTNMGL, *H-2K*^{d} restricted) (Sangon Biotech Co., Ltd, Shanghai, China). After 72 h of incubation, the supernatant was collected and examined for the concentrations of different cytokines (IFN-γ, TNF-α, IL-2 and IL-6) using ELISA kits (R&D Co.,Ltd., Minneapolis, USA), according to the manufacturer's protocols. The results were expressed as pg/ml.

T lymphocyte generation and measurement of cytokine production by intracellular cytokine staining

Peripheral T lymphocytes were isolated from the spleen of immunized transgenic mice two weeks after the last immunization using the nylon filtration method, as described previously [15]. The isolated cells were first stimulated in the presence of 10 ug/ml HBcAg for 6 h, then, 25 mg/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, USA), 1 mg/ml of ionomycin (Sigma), and 1.7 mg/ml of monensin (Sigma) were added and incubation continued for another 3 h. The stimulated cells were washed twice with PBS and stained with saturating concentrations of perCP-Cy5.5-conjugated anti-CD8 McAb (eBioscience, San Diego, CA, USA) for 30 min. After being fixed with Fix and Perm reagents A and B (eBioscience), the cells were incubated for another 30 min with APC-labeled anti-IFN-γ, anti-granzyme B (GzmB) or anti-TNF-α antibody, followed by washed two times with PBS and analyzed by flow cytometry.

Evaluation of the CTL response

T lymphocytes were used as the effector cells, P815/c cells containing the HBcAg expression were used as target cells, and were co-cultured in 96-well plates at different effector-to-target (E/T) ratios (5:1, 10:1, and 20:1) at 37°C with 5% CO2 for 4 h. The lactate dehydrogenase (LDH) release assay was used to evaluate HBcAg-specific CTL activity (CytoTox 96 Non-Radioactive Cytotoxicity kit; Promega, Madison, USA). The absorbance values of the supernatants were recorded at optical density 490 nm. Cytotoxicity (%) was calculated as follows: [(experimental release-effector spontaneous release-target spontaneous release)/(target maximum release-target spontaneous release)] × 100% [14].

Cell proliferation assay

T lymphocytes were seeded at a density of 2×10^6 cells/ml into 96-well plates in a final volume of 200 μl in the presence of 1 μg concanavalin A (ConA, Sigma) solution for 48 h. Cell proliferation was detected by incubating them with Cell Counting Kit-8 (CCK-8) reagents (Dojindo, Shanghai, China) for 4 h at 37 °C and measuring the absorbance at 450 nm.

Assessment of apoptosis

CD8+ T cells were purified from the spleen of transgenic mice using EasySep negative bead selection (StemCell Technologies) and cultured in 24-well plates at 37°C in complete RPMI-1640 in the presence of HBcAg (10 μg/ml) for 72 h. The CD8+ T cells were stained with Annexin V–FITC and Propidium Iodide (PI) (eBioscience) according to the manufacturer's instructions, then the frequencies of Annexin V+PI- (early apoptosis) and Annexin V+PI+ (late apoptosis) cells were determined by flow cytometry.

Transmission electron microscopy

The purified CD8+ T cells in the LVDC-UbHBcAg-LIGHT group were washed three times with PBS, treated with indicated concentrations of triptolide for 24 h and collected by trypsinization. Cells were fixed with 4% paraformaldehyde at 4 °C overnight, post-fixed in 1% phosphatebuffered osmium tetroxide at room temperature for 1.5 h and dehydrated stepwise with ethanol. The dehydrated pellets were rinsed with propylene oxide at room temperature for 30 min. After being embedded and double-stained with uranyl acetate/lead citrate, the sections were determined under a transmission electron microscope (Hitachi).

Serological analysis

Sera were collected from the mice two weeks after the last immunization and determined for HBsAg by Abbott kits (Abbott Diagnostics, Chicago, IL, USA) and HBV DNA by the quantitative PCR (Terra PCR Direct Polymerase mix, Clontech, CA, USA). Additionally, serum alanine aminotransferase (ALT) and aspartate transaminase (AST) activities were assessed using the ARCHITECT Automatic Biochemistry Analyzer (Abbott Diagnostics).

Detection of anti-HBcAg antibodies

Antibodies to HBcAg were determined by a commercial diagnostic kit (Kehua, Shanghai, China). Approximately 50 μl of serial dilutions of mouse sera was added to the plates which were precoated with HBcAg and incubated for 1 h at 37°C. Bound mouse IgG, IgG1, or IgG2a antibodies were assessed using the corresponding secondary antibodies (HRP-conjugated anti-mouse IgG, IgG1, or IgG2a, 1:10000) (Abcam, Cambridge, UK). The titers of antibodies to HBcAg in the serum were calculated by extrapolating the ELISA values of serially diluted samples and corresponded to the reciprocal values of the highest dilutions that were considered positive [25]. The absorbance was determined at 450 nm.

Liver immunohistochemistry

Liver samples from the immunized transgenic mice were fixed in formaldehyde and embedded in paraffin. For immunohistochemistry, the dewaxed 3-5-mm-thick sections were treated with 0.3% H_2O_2 for 10 minutes to inactivate the endogenous peroxidase and blocked with 2% normal goat serum for 30 minutes at room temperature. Then, a goat anti-HBsAg or anti-HBcAg polyclonal antibody (Novus Biologicals, USA) was added overnight at 4°C. After three washes with PBS, the sections were stained with biotinylated secondary antibody (Boster, China) for 30 minutes at 37°C and incubated with the streptavidin-biotin-peroxidase complex for another 30 minutes. Visualization was performed with diaminobenzidine (DAB, Booster) and hematoxylin.

RNA extraction and quantitative PCR analysis

The purified CD8+ T cells in each group were collected for total RNA extract according to the protocol for Trizol reagent (TaKaRa Bio, Shiga, Japan). 1 μg of RNA was reversely transcribed at 42 °C for 60 min in 20 μl PrimeScriptTM RT reagent Kit (TaKaRa). Relative quantitative real-time PCR was performed using the SYBR Green qPCR Mix kit (TOYOBO, Japan) on an ABI 7500 FAST system (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The PCR primers used were as follows: ATG5, 5′-GACAAAGATGTGCTTCGAGATGTG-3′ (F) and 5′-GTAGCTCAGATGCTCGCTCAG-3′ (R); Pro-caspase-3, 5′-ATGGACAACAACGAAACCTCCGTG-3′ (F) and 5′-CCACTCCCAGTCATTCCTTTAGTG-3′ (R); Pro-caspase-8, 5′-GTGACAAGGGTGTCGTCTATGG-3′ (F) and 5′- GGATGCTAAGAATGTCATCTCC-3′ (R); Bim, 5′- TGATTACCGCGAGGCTGAA-3′ (F) and 5′- ACCAGACGGAAGATAAAGCGTAAC-3′ (R); CDKN1B, 5′-CGCCTGGCTCTGCTCCATTTGAC-3′ (F) and 5′-GACACTCTCACGTTTGACATCTTCC-3′ (R); GAPDH, 5′-AGAAGGCTGGGGCTCATTTG-3′ (F) and 5′-AGGGGCCATCCACAGTCTTC-3′ (R). Primers were observed from Sangon Biotech (Shanghai, China). The cycle profile was 40 cycles at 95 °C for 3 min, 95 °C for 15 s, and 60 °C for 30 s. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis and immunoprecipitation

The above purified CD8+ T cells were harvest to isolate the total protein with RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of proteins from each sample were loaded onto polyacrylamide mini gels (Invitrogen) for electrophoresis. Proteins from the gels were transferred to Immobilon-PVDF membranes (Millipore, Bedford, MA, USA) using a semidry apparatus (Bio-Rad, Hercules, CA, USA). After incubation with corresponding primary and secondary antibodies, the signals were detected with an ECL assay kit (Amersham). Antibodies against ATG-5, LC-3, p62, Beclin, CDKN1B, Pro-caspase-3, Pro-caspase-8 and Bim were purchased from Abcam (Cambridge, UK). Immunoprecipitation was performed according to published protocol with modification [26]. Briefly, CD8+ T cells were lysed with 0.5 ml lysis buffer (30 mM Tris, pH 7.6, 10% glycerol, 150 mM NaCl and 1% Triton supplemented with protease inhibitor cocktail) (Hoffmann-La Roche Inc., Nutley, NJ, USA). Immunoprecipitation was performed using the p62 monoclonal antibody (M01, clone 2C11, Abnova, Walnut, CA, USA) or control IgG to immunoprecipitate the protein. Cell lysates and immunoprecipitates were electrophoresed on SDS-PAGE, and then subjected to immunoblotting analysis using the corresponding antibodies.

Confocal microscopy

CD8+ T cells were adhered to poly-L-lysine-coated cover slips, fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with corresponding primary and secondary antibodies. The primary antibodies used for confocal microscopy assay were as follows: anti-p62 antibody (Abcam), anti-ubiquitin antibody (Millipore, Billerica, MA, USA), anti-Bim antibody (Abcam) and anti-CDKN1B antibody (Abcam). The secondary antibodies used were: rabbit anti-mouse Alexa Fluor 488 (Invitrogen), goat anti-rabbit Alexa Fluor 633 (Invitrogen), donkey anti-goat Alexa Fluor 555 (Invitrogen). At last, cells were counterstained with Hoechst 33342 nuclear dye.

Statistical analysis

The data are presented as the means ± SD. Statistical analysis was performed using Student's t-test and one-way ANOVA for multiple comparisons. Differences were considered statistically significant at $p<0.05$.

Results

Targeting of DCs in vivo by the engineered lentivector

To test whether the engineered lentivectors could transduce DCs specifically *in vivo*, 5×10^7 TU of LVDC-UbHBcAg-LIGHT or LV-UbHBcAg-LIGHT was injected subcutaneously into the left flank of *H-2K*^{d} BALB/c mice. Three days post-transduction, a significant enlargement of the left inguinal lymph nodes close to the injection site was observed in the LVDC-UbHBcAg-LIGHT group (Fig. 1a). The number of cells in these lymph nodes increased by about 10-fold compared to the equivalent lateral lymph nodes (Fig. 1b). However, changes in the size and cell number of left inguinal lymph nodes in the LV-UbHBcAg-LIGHT group were far less pronounced than those in the LVDC-UbHBcAg-LIGHT group (Fig. 1a and b), suggesting that LVDC-UbHBcAg-LIGHT immunization can enhance trafficking of DCs and proliferation of lymphocytes in a nearby draining lymph node. Flow cytometry showed that in the LVDC-UbHBcAg-LIGHT group approximately 4.4% of the total DCs (CD11c+) in the left inguinal lymph node cells were GFP+CD11c+ cells, which had probably migrated from the injection site. By contrast, in the LV-UbHBcAg-LIGHT group only 1.4% of the total DCs were GFP+CD11c+ cells, revealing that LVDC-UbHBcAg-LIGHT had a more specificity for DCs (Fig. 1c).

To further confirm the specificity of the targeting vector for DCs *in vivo*, we constructed two lentivectors (LVDC-luc and LV-luc) encoding the luciferase (luc) (Fig. 1d), which enabled us to visualize the *in vivo* transduction of the tissue cells using BLI. No obvious luminescence signal was detected at any time at the injection site of the mice injected by LVDC-luc, mainly due to targeted transduction of the vector and rare distribution of DCs *in vivo* which is beyond the sensitivity of the current BLI method. In contrast, LV-luc-treated mice had a strong luminescence signal at the injection site (Fig. 1e) indicating that the lentivector bearing engineered SVG envelope had a relatively rigorous target property.

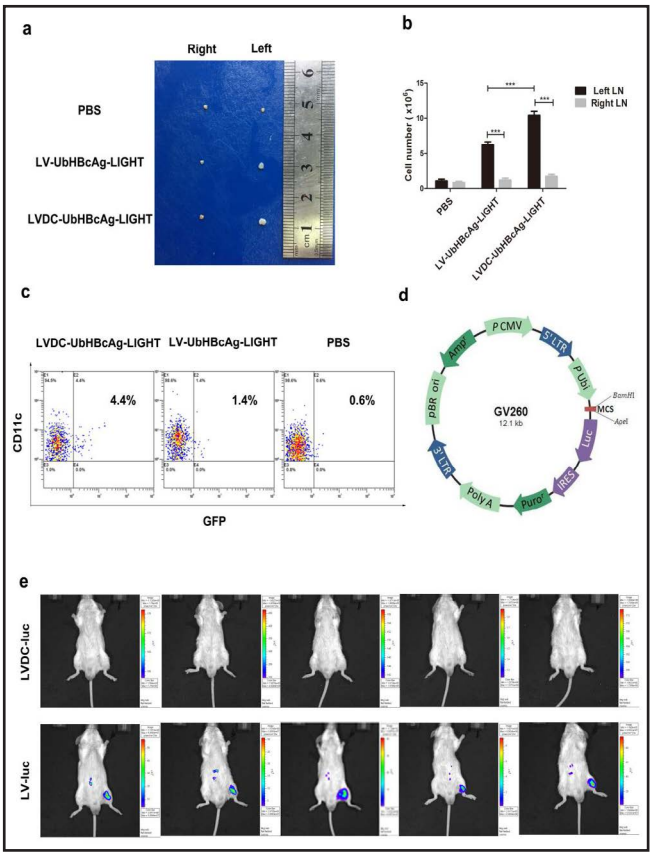

Fig. 1. The engineered lentivector enveloped with a mutant SVG could selectively transduce DCs in vivo. BALB/c mice were injected subcutaneously into the left flank close to an inguinal lymph node (within 1 cm range) with 5×10^7 TU of LVDC-UbHBcAg-LIGHT or LV-UbHBcAg-LIGHT (5×10^7 TU resuspended in 200 µl PBS), mice injected with PBS were included as a control. 3 days later, the left inguinal lymph node and the equivalent lymph node at the opposite site were isolated. (a) Comparison of the representative inguinal lymph nodes close to the injection site (left) and the equivalent lateral lymph nodes (right) (n=8). (b) Determination of total cell number of the indicated lymph nodes. The data are the mean ± SD from eight mice per group. ***p<0.001. (c) Representative flow cytometric analysis of CD11c+ cells from the lymph nodes that are close to the injection sites. The numbers indicate the proportions of GFP+CD11c+ populations (n=8). (d) Schematic representations of the plasmid encoding the imaging gene (luciferase, luc). (e) Bioluminescence imaging of BALB/c mice injected subcutaneously into the left flank close to an inguinal lymph node (within 1 cm range) with 5×10^7 TU of either the DC-specific LVDC-luc lentivector or the non-specific LV-luc lentivector (5×10^7 TU resuspended in 200 µl PBS). Representative images were obtained at day 30 post-injection using IVIS. Data are representative of three independent experiments.

CD8+ and CD4+ T cell responses were elicited by the DC-targeting lentivector in HBV transgenic mice

We evaluated the efficacy of the engineered lentivector for inducing HBV-specific T lymphocyte responses by monitoring the cytokine secretion and intracellular staining. Splenocytes from the immunized HBV transgenic mice were collected two weeks after the last immunization and cytokines (IFN-γ, IL-2, TNF-α, and IL-6) secreted in the supernatants of the T cells were measured. As shown in Fig. 2a, splenocytes from the LVDC-UbHBcAg-LIGHT group produced higher levels of IFN-γ, IL-2, TNF-α and IL-6 than those in other groups.

The intracellular IFN-γ, TNF-α and cytolytic granule production in CD8+ T cells of the immunized mice was measured by flow cytometry. As shown in Fig. 2b, c, d and f, the LVDC-UbHBcAg-LIGHT group displayed a higher percentage of IFNγ+CD8+, TNF-α+CD8+ and GzmB+CD8+ T cells than that in other groups indicating that the method of DC targeting through lentivector immunization can stimulate an enhanced specific CD8+ T cell response compared with the alternatives in *H-2K*^{d} HBV-transgenic mice. We also examined intracellular IFN-γ production in CD4+ T cells of the immunized mice. Similarly, a higher frequency of IFN-γ+CD4+ T cells was observed in the LVDC-UbHBcAg-LIGHT group (Fig. 2e and f).

Induction of CTL responses, antibody responses and antiviral efficacy by direct administration of LVDC-UbHBcAg-LIGHT

At day 14 post-immunization, T cells were harvested from spleens and analyzed for the HBcAg-specific CTL activity against P815/c cells by the LDH release assay. As shown in Fig.

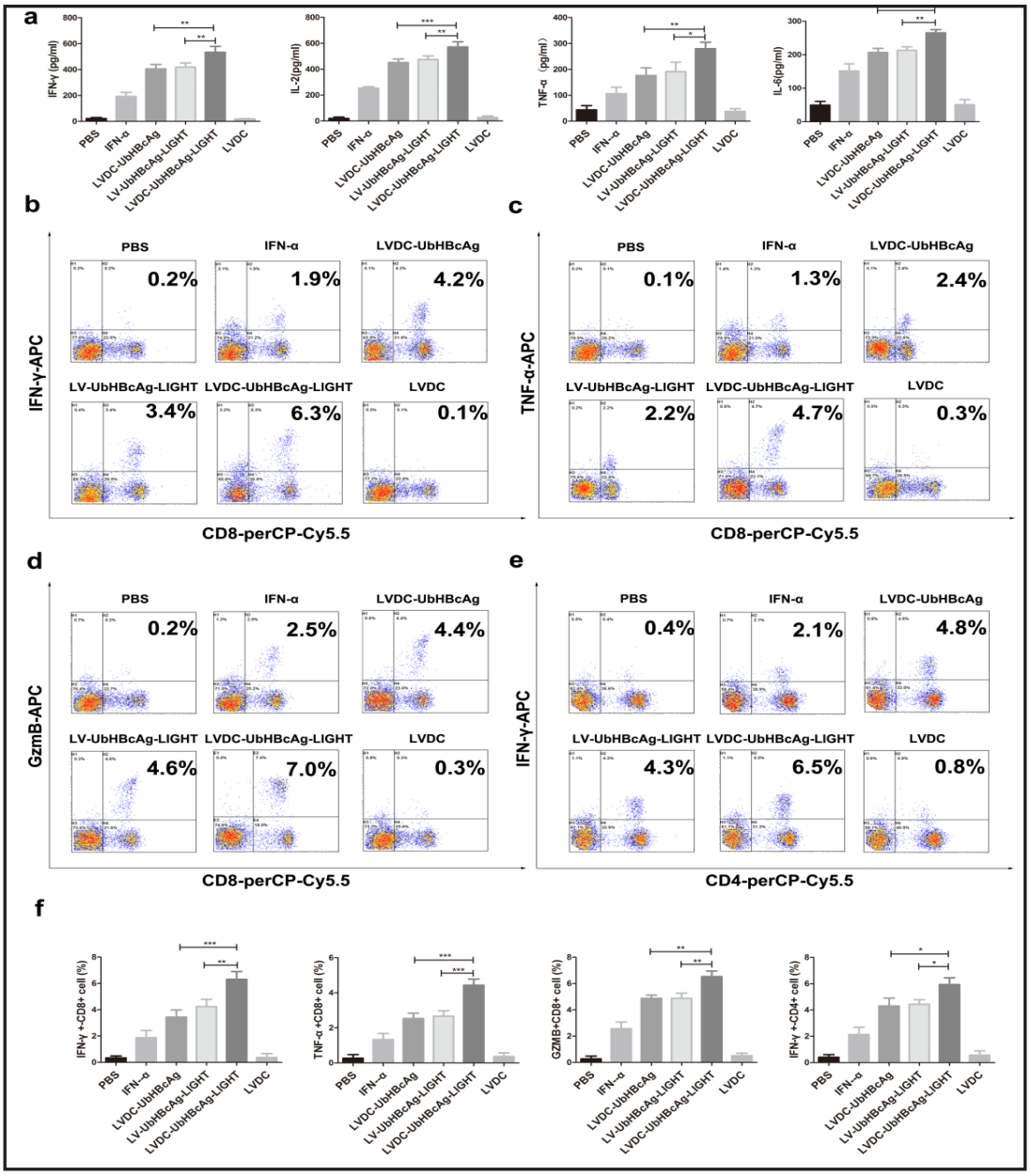

Fig. 2. Potent induction of HBV-specific CD8+ and CD4+ T cell response in HBV transgenic mice by immunization with LVDC-UbHBcAg-LIGHT. The mice were injected subcutaneously in the hind footpads twice at an interval of 2 weeks with 2×10^7 TU recombinant lentiviral vectors (LVDC-UbHBcAg-LIGHT, LV-UbHBcAg-LIGHT, LVDC-UbHBcAg or LVDC), IFN-α was used as a positive control. (a) Splenocytes were harvested one week after the last immunization and cultured in 24-well plates (2×10^6 cells/ml) in the presence of HBcAg-specific CTL peptide HBcAg 87–95 (YVNTNMGL, H-2K^d restricted; 10 μg/ml). After 72 h of incubation, the secretion of IFN-γ, IL-2, IL-6 and TNF-α in the supernatants of T cells from the immunized HBV transgenic mice was examined by ELISA. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p<0.05, **p<0.01, ***p<0.001. (b,c,d) T cells isolated from the immunized HBV transgenic mice were stained with CD8-perCP-Cy5.5, IFN-γ-APC, TNF-α-APC or GzmB-APC antibodies. The frequencies of IFN-γ+CD8+, TNF-α+CD8+ and GzmB+CD8+ T cells were examined by flow cytometry. (e) T cells harvested from the immunized mice were stained with CD4-perCP-Cy5.5 and IFN-γ-APC antibodies. The percentage of IFN-γ+CD4+ T cells was examined by flow cytometry. (f) The statistical analysis of Fig. 2b, c, d and e. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p<0.05, **p<0.01, ***p<0.001.

3a, the percentages of specific cytolysis in the LVDC-UbHBcAg-LIGHT group were higher than other groups. Administration of LVDC or PBS failed to generate HBcAg-specific CTL responses (Fig. 3a).

We further compared the HBcAg specific antibody responses generated by LVDC-UbHBcAg-LIGHT with those generated by other lentivectors (LV-UbHBcAg-LIGHT or LVDC-UbHBcAg) on the 14th day after immunization. The results showed that LVDC-UbHBcAg-LIGHT provided a rather impressive anti-HBcAg IgG antibody response, as a comparison, immunization with the same vector titer of either LV-UbHBcAg-LIGHT or LVDC-UbHBcAg generated less serum IgG (Fig. 3b). The IgG antibody subtypes (IgG1 and IgG2α) were also detected with mouse IgG1- and IgG2α-specific secondary antibodies. The results demonstrated that no significant difference in IgG1 antibodies levels was observed between LVDC-UbHBcAg-LIGHT, LV-UbHBcAg-LIGHT and LVDC-UbHBcAg group (Fig. 3c). By contrast, all groups had higher levels of IgG2α antibodies than IgG1 and the IgG2α antibody titer was

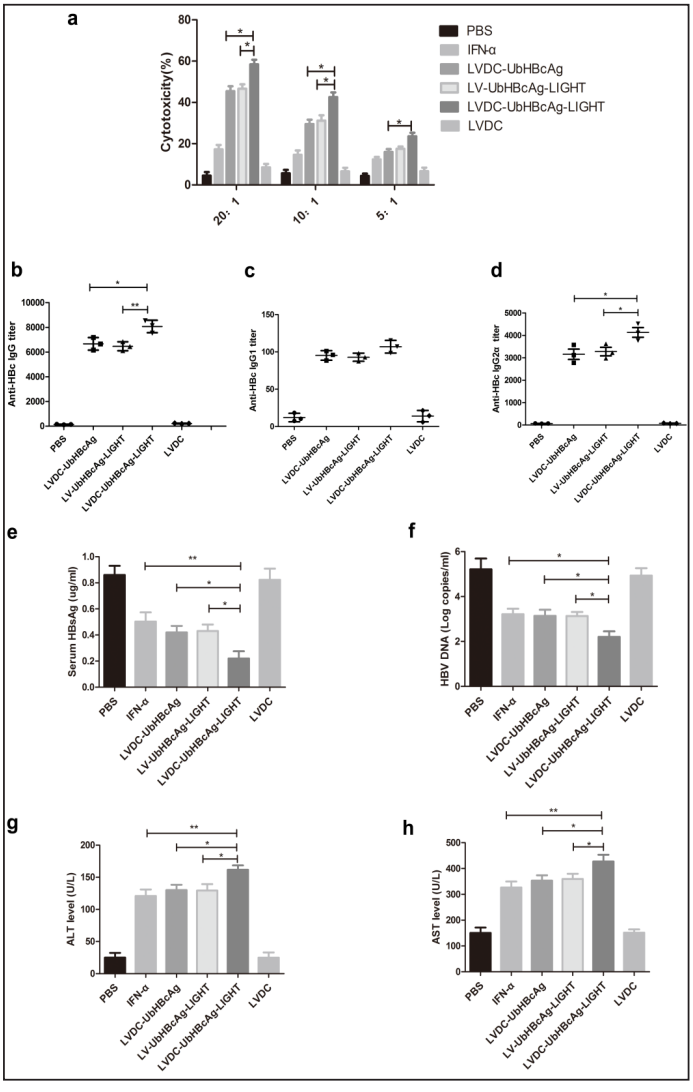

Fig. 3. In vivo stimulation of HBV specific CTL and antibody responses in HBV-transgenic mice and serum HBsAg, HBV DNA, ALT and AST levels. (a) T cells were isolated two weeks after the last immunization and the specific CTL activity was detected with LDH release assay. CTL activity was indicated as the mean percentage of specific lysis at different effector: target (E:T) ratios (20:1, 10:1, and 5:1). Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p<0.05. (b) Sera were collected on day 14 post-immunization and were analyzed for the titer of anti-HBcAg IgG using ELISA. The titer values of a given serum sample were defined as the reciprocal value of the highest dilution yielding a positive result. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p<0.05, **p<0.01. (c,d) The titers of anti-HBcAg IgG subtypes IgG1 (c) and IgG2α (d) were measured. The symbols represent serum titers from the indicated mice. The horizontal lines represent the means (n = 6). *p<0.05. (e,f) Serum HBsAg and HBV DNA analysis. (e) Detection of serum HBsAg level. The results were expressed as the concentration of HBsAg (μg/ml). Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p<0.05, **p<0.01. (f) Detection of the titer of HBV DNA in sera. The results were expressed as log of copies/ml. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p< 0.05. (g,h) The levels of ALT and AST in immunized HBV transgenic mice. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p<0.05, **p< 0.01.

highest in the LVDC-UbHBcAg-LIGHT group, suggesting that LVDC-UbHBcAg-LIGHT could effectively induce IgG2α-dominant HBcAb responses (Fig. 3d). These results indicated that *in vivo* administration of the DC-targeting lentivector could be a powerful method to elicit both cellular and humoral immune responses against the delivered antigen.

Serum was collected from the immunized H-2Kd HBV-transgenic mice two weeks after the last immunization with the purpose of identifying whether treatment with LVDC-UbHBcAg-LIGHT could achieve therapeutic effects of the viral clearance in HBV transgenic mice. As shown in Fig. 3e and f, the level of serum HBsAg and the titer of HBV DNA in LVDC-UbHBcAg-LIGHT group decreased significantly when compared with other groups indicating that LVDC-UbHBcAg-LIGHT had a potent antiviral efficacy. By contrast, the AST and ALT levels in LVDC-UbHBcAg-LIGHT group increased significantly compared with those in LV-UbHBcAg-LIGHT, LVDC-UbHBcAg, and IFN-α group (Fig. 3g and h).

Immunohistochemistry of the liver

Liver tissues were obtained from the immunized HBV mice two weeks after the last immunization. To further confirm the therapeutic effects of recombinant LVs in HBV transgenic mice, immunohistological analysis was performed in livers from the above various treatment groups. The results suggested that a large number of HBsAg in the cytoplasm and HBcAg in the nuclei of hepatocytes were observed (stained brownish yellow) in mice treated with PBS and LVDC, however, LV immunization, especially LVDC-UbHBcAg-LIGHT, significantly reduced the expression of HBsAg and HBcAg (Fig. 4a and b).

Autophagy was induced in the CD8+ T cells of mice immunized by LVDC-UbHBcAg-LIGHT and was related to enhanced proliferation

In our prior study, we have confirmed that autophagy can be obviously induced in T

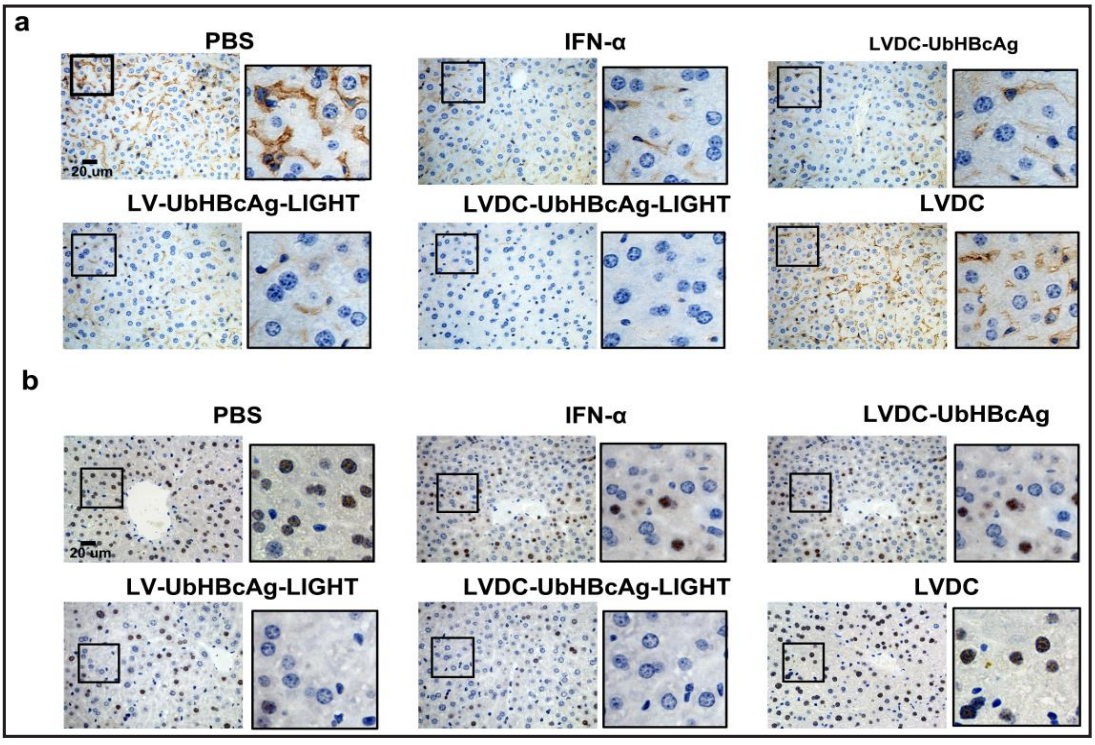

Fig. 4. Immunohistochemical analysis of HBsAg and HBcAg in liver sections of HBV transgenic mice. (a,b) Liver sections were subjected to immunohistological analysis of HBsAg (a) and HBcAg (b). Representative photographs are shown and the right panels show enlarged regions of the left panels in each group (original magnification: × 400). The scale bars: 20 μm.

cells co-cultured with LVDC-UbHBcAg-LIGHT loaded DCs. To explore whether autophagy was induced in CD8+ T cells of HBV-transgenic mice immunized by the lentivectors, CD8+ T cells from all groups were restimulated with the 87–95 amino acid peptide of HBcAg and examined for protein levels of autophagy-related proteins as that of LC3-II, Beclin and p62. Western blot results showed that LC3-II expression increased obviously in the CD8+ T cells of mice treated with LVDC-UbHBcAg-LIGHT, LV-UbHBcAg-LIGHT and LVDC-UbHBcAg (Fig. 5a and b). Furthermore, the LVDC-UbHBcAg-LIGHT group displayed the highest LC3-II expression when compared with other groups. Similar results were observed for the changes in Beclin protein expression, however, the expression of p62 varied in opposite manners (Fig. 5a, c and d). Transmission electron microscopy analysis was also performed to further verify the induction of autophagy in CD8+ T cells of mice immunized with LVDC-UbHBcAg-LIGHT. As expected, a lot of characteristic double membrane autophagic vesicles that contained engulfed organelles were observed in the LVDC-UbHBcAg-LIGHT group (Fig. 5h).

We have reported in our previous *ex vivo* experiments that the induced autophagy could selectively degrade CDKN1B, the main negative cell-cycle regulator, thus facilitating the transition from G1- to S-phase in T cells after TCR stimulation. Consistent with the previous experimental results, in the present study western blot analysis demonstrated the most significant reduction of CDKN1B expression in the LVDC-UbHBcAg-LIGHT group, which exactly had the most obvious induction of autophagy (Fig. 5a and e). T cell proliferation in all groups was determined by CCK-8. As shown in Fig. 5g, the capacity of T cell proliferation in the LVDC-UbHBcAg-LIGHT group apparently enhanced compared with other groups and the results coincided well with the lowest protein level of CDKN1B in this group. However, we did not detect any difference between all groups for CDKN1B mRNA expression (Fig. 5f).

The induced autophagy contributed to decreased apoptosis by selectively degrading the proteins associated with apoptosis in the activated CD8+ T cells of HBV transgenic mice

Two weeks after the last immunization, CD8+ T cells were harvested from spleens of the transgenic mice immunized by LVDC-UbHBcAg-LIGHT or LV-UbHBcAg-LIGHT, CD8+ T cells from the mice treated by PBS were used as a control. To investigate the effect of induced autophagy on T cell apoptosis, CD8+ T cells from the LVDC-UbHBcAg-LIGHT-immunized mice were transduced with ATG5 siRNA for 24 h or pretreated with autophagy inhibitors 3-Methyladenine (3-MA, 10 mM) for 1 h before restimulated with the 87–95 amino acid peptide of HBcAg. The interference efficiency of ATG5 siRNA was determined by real-time PCR and western blot (Fig. 6a and b). CD8+ T cells in all groups were subjected to western blot for detecting expression of the proteins associated with apoptosis. As shown in Fig. 6c and d, the protein levels of Bim slightly increased in LVDC-UbHBcAg-LIGHT and LV-UbHBcAg-LIGHT groups when compared with the control. However, there was no statistically significant difference between the two groups. It is noteworthy that the protein levels of Bim increased considerably in the CD8+ T cells of LVDC-UbHBcAg-LIGHT-immunized mice upon autophagy inhibition with 3-MA or ATG5 siRNA. Similar results were observed for the changes in Pro-caspase-3 and Pro-caspase-8 protein level indicating that the induced autophagy in the primed CD8+ T cells significantly promoted the degradation of apoptosis-related proteins. In comparison, no significant difference was detected between all groups for mRNA expression of all the above targets (Fig. 6e). Consistent with the western blot results, slightly higher frequencies of apoptotic CD8+ T cells were observed in LVDC-UbHBcAg-LIGHT and LV-UbHBcAg-LIGHT groups by flow cytometry analysis. Similarly, autophagy inhibition significantly increased the frequencies of apoptotic CD8+ T cells (Fig. 6f and g).

Bim or CDKN1B was found in protein aggregates containing p62 and ubiquitin in CD8+ T cells of HBV transgenic mice immunized by LVDC-UbHBcAg-LIGHT

CD8+ T cells harvested from the LVDC-UbHBcAg-LIGHT group were pretreated with chloroquine (CQ, 3 μM) for 1 h and restimulated with 10 μg/ml of HBcAg peptide for another 72 h. CQ is a lysosomotropic agent which prevents endosomal acidification, thus inhibiting lysosomal enzymes and causing accumulation of autophagic vesicles that contained engulfed

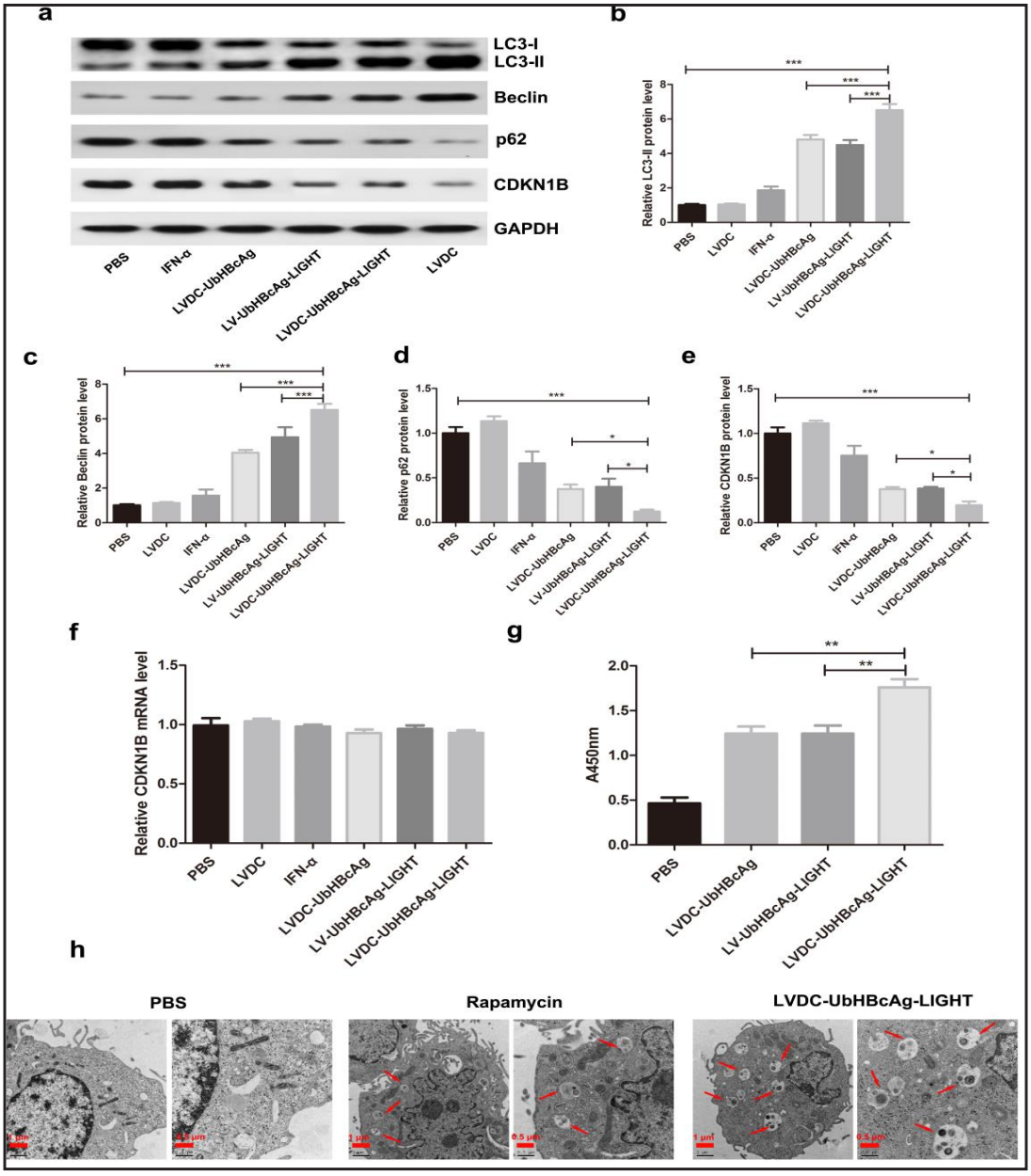

Fig. 5. The upregulated autophagy in the CD8+ T cells of mice immunized by LVDC-UbHBcAg-LIGHT contributed to enhanced proliferation. (a-e) CD8+ T cells from the immunized HBV transgenic mice were isolated by negative selection. The protein expression in each group was determined by western blot using antibodies against LC3, Beclin, p62 and CDKN1B. The fold change of LC3 only refers to LC3-II. (a) Representative immunoblots. (b-e) Densitometric analysis. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p<0.05, ***p<0.001. (f) Total RNA in the above CD8+ T cells was extracted and analyzed by real-time PCR for CDKN1B. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). (g) T lymphocytes were seeded into 96-well plates (2 × 10^6 cells/ml) in the presence of 1 mg ConA solution for 48 h. Lymphocyte proliferation activity was detected by CCK-8. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). (h) CD8+ T cells from the mice immunized by LVDC-UbHBcAg-LIGHT were subjected to transmission electron microscopy analysis. Rapamycin (150 nM) added for 4h was included as the positive control. The right panels show enlarged regions of the left panels. The arrows indicate autophagosomes. The scale bars: 1 μm (left) and 0.5μm (right), respectively. Original magnification: × 10000 (left) and × 20000 (right), respectively. Representative images from six mice per group.

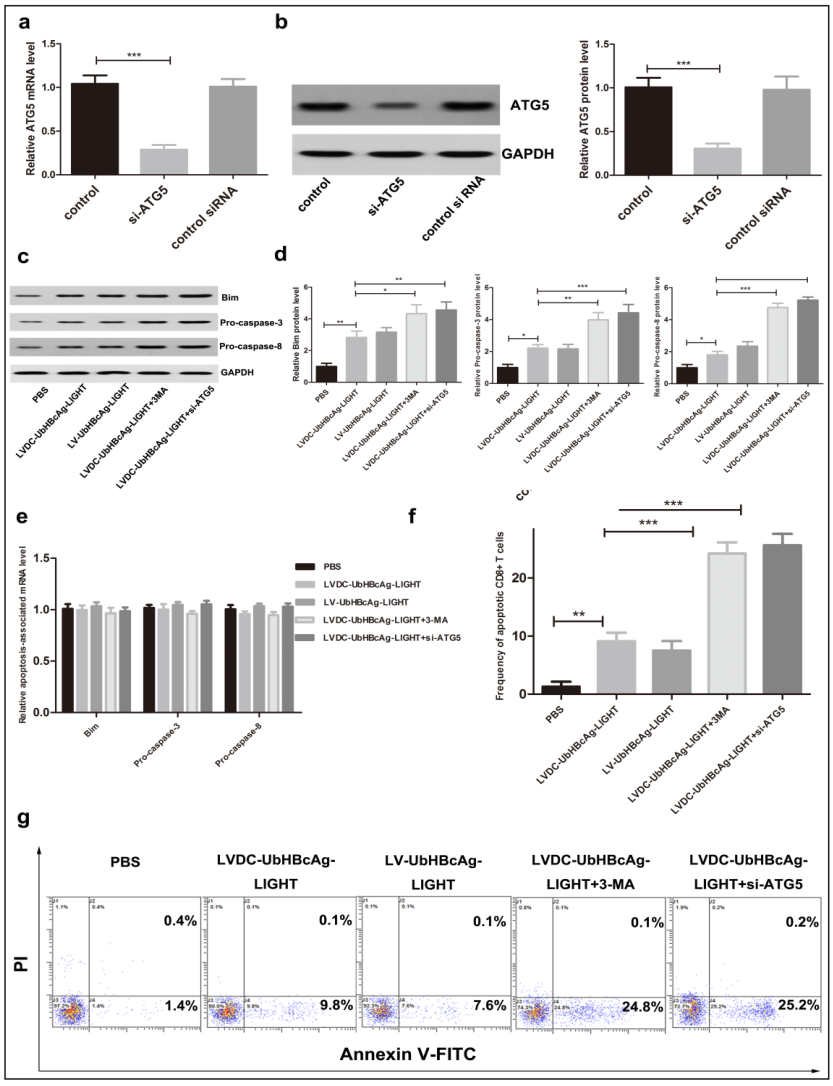

Fig. 6. The induced autophagy inhibited apoptosis by selectively degrading the apoptosis-related proteins in CD8+ T cells of the immunized HBV transgenic mice. (a,b) The interference efficiency of ATG5 siRNA was assessed by real-time PCR and western blot. (a) Total RNA was extracted from CD8+ T cells and detected by real-time PCR for ATG5. Data are shown as the mean ± SD from three independent experiments, which were run in triplicate. ***p<0.001. (b) Total protein was extracted from CD8+ T cells and subjected to western blot analysis for ATG5. Left: representative immunoblots. Right: densitometric analysis. Data are shown as the mean ± SD from three independent experiments, which were run in triplicate. ***p<0.001. (c,d) CD8+ T cells were harvested from spleens of the transgenic mice immunized by LVDC-UbHBcAg-LIGHT or LV-UbHBcAg-LIGHT. CD8+ T cells from the mice treated by PBS were used as the control. In some experiments, CD8+ T cells from the mice immunized by LVDC-UbHBcAg-LIGHT were transduced with ATG5 siRNA for 24 h or pretreated with autophagy inhibitors 3-MA (10 mM) for 1 h before restimulated with 10 μg/ml of HBcAg peptide. The stimulated CD8+ T cells were subjected to western analysis of Bim, Pro-caspase-3 and Pro-caspase-8. (c) Representative immunoblots. (d) Densitometric analysis. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). *p<0.05, **p<0.01, ***p<0.001. (e) Total RNA was extracted from the above CD8+ T cells in each group and analyzed by real-time PCR for Bim, Pro-caspase-3 and Pro-caspase-8. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). ***p<0.001. (f,g) The apoptosis of the above CD8+ T cells was analyzed by flow cytometry. CD8+ T cells derived from the immunized mice were stained with Annexin V-FITC and PI. (g) Representative flow cytometric analysis. Numbers indicate the percentage of cells in each region. (f) Densitometric analysis. Data are representative of three independent experiments and are presented as the mean ± SD (n = 6). **p<0.01, ***p<0.001.

organelles. The results showed that Bim co-localized with p62 in large protein complexes in CD8+ T cells of transgenic mice immunized by LVDC-UbHBcAg-LIGHT (Fig. 7a). The co-localization of CDKN1B and p62 in the primed T cells was also detected with a confocal microscopy (Fig. 7b). In addition, we observed that a lot of these protein aggregates were

also positive for ubiquitin (Fig. 7a and b). However, we did not detect co-localization of the above proteins in the CD8+ T cells of mice treated with PBS. Immunoprecipitation was performed to further confirm that Bim and CDKN1B were involved in autophagy-mediated degradation. As expected, Bim and CDKN1B were found to be co-immunoprecipitated with p62 in the CD8+ T cells of transgenic mice immunized by LVDC-UbHBcAg-LIGHT, indicating that there was a direct interaction between autophagy and the above mentioned proteins (Fig. 7c and d). Pro-caspase-3 and Pro-caspase-8, however, could not be detected in these immunoprecipitates (data not shown). Collectively, these data verified that the induced autophagy selectively degrading the protein aggregates associated with apoptosis and cell cycle in the CD8+ T cells of HBV transgenic mice immunized by LVDC-UbHBcAg-LIGHT, resulting in increased proliferation and decreased apoptosis.

Discussion

Although effective prophylactic vaccines available for HBV have been developed for a long time, there is still no therapeutic vaccine against HBV infection. Patients with chronic HBV infection are at increased risk of developing hepatocellular carcinoma and would benefit greatly from the availability of a therapeutic vaccine. Cytotoxic T lymphocytes play a key role in controlling the outcome of HBV infection by participating in the clearance of persistent infection and mediating protective immunity [27]. Some investigations have showed that individuals with self-limited acute HBV infection boost a strong, polyclonal, multispecific CTL response, whereas these responses are low to undetectable in chronically

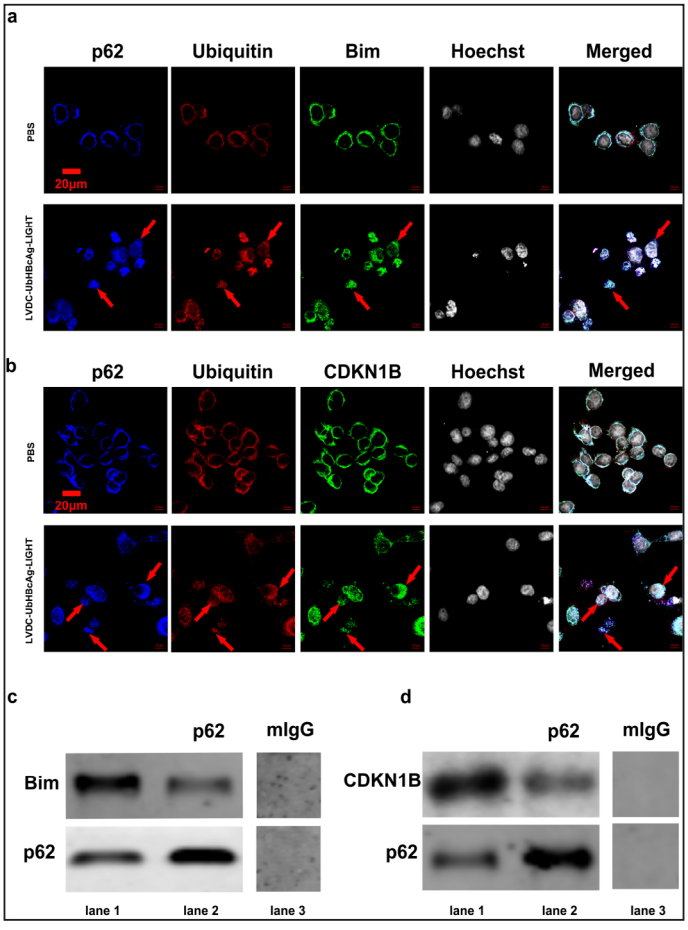

Fig. 7. Bim or CDKN1B was found in protein aggregates containing p62 and ubiquitin. (a,b) CD8+ T cells harvested from the LVDC-Ub HBcAg-LIGHT group were pretreated with CQ (3 μM) for 1 h and re-stimulated with 10 μg/ml of HBcAg peptide for another 72 h. Then the cells were stained with antibodies, as indicated in materials and methods. Confocal images were obtained, arrows pointing to aggregates containing (a) Bim or (b) CDKN1B. CD8+ T cells from the mice treated by PBS were used as the control. Representative images from six mice per group (original magnification: × 600). The scale bars: 20 μm. (c,d) The lysates of CD8+ T cells from the LVDC-UbHBcAg-LIGHT group were immunoprecipitated with anti-p62 antibodies (lane 2) or control IgG (lane 3), then subjected to western blot with antibodies against Bim or CDKN1B. Lysates equal to 1/20 of the amount used for immunoprecipitation was used as control (lane 1). Representative images from six mice per group.

infected patients who usually exhibit immune tolerance to the targeted antigens [28]. Based on these findings, therapeutic vaccination has been presented as a promising strategy to break cytotoxic T lymphocyte tolerance and eliminate persistent HBV infection [29, 30].

We have constructed a novel and highly efficient DC-targeting LV (LVDC-UbHBcAg-LIGHT) which could target genes of antigenic proteins to DCs by virtue of pseudotyping the LV with a mutated DC-tropic SVG. The engineered lentivector has been demonstrated to achieve high efficiency in delivering the vectored genes and be capable of stimulating strong HBV-specific CTL responses *ex vivo*. In the present study, we further explored whether LVDC-UbHBcAg-LIGHT could target the delivery of HBV immunogens to DCs *in vivo* and elicit antiviral immune responses in HBV transgenic mice.

By direct injection of the engineered LV subcutaneously into the mice, we could observe a rapid enlargement of the lymph node near the injection site within 3 days. It would appear that dermal DCs were transduced and stimulated by the vector, then became mature and migrated to the lymph node, approximately 10 millimeters from the injection site. Thus, the more DCs transduced by the vectors at the injection site, the larger lymph nodes can be observed. In this study, we showed that LVDC-UbHBcAg-LIGHT immunization produced a distinctly larger node as well as more transduced DCs than LV-UbHBcAg-LIGHT indicating that the engineered LV immunization could enhance trafficking of DCs and stimulate lymphocyte proliferation in a nearby draining lymph node. To further confirm the target specificity of the engineered vector for DCs, mice immunized with LVDC-luc or LV-luc were subjected to BLI analysis. At no time were we able to detect a luminescence signal in the LVDC-luc group verifying the lack of non-specific transduction in the animals. We speculated that the engineered LVs specifically transduced DCs and the transduced DCs were scatteredly distributed *in vivo*, therefore, the luminescence signals within DCs were beyond the sensitivity of the current BLI method.

We focused our subsequent studies on the efficacy of the DC-targeting LV for eliciting T cell responses in HBV transgenic mice. The mice were injected subcutaneously in the hind footpads twice at an interval of 2 weeks with recombinant lentivectors, then the presence of HBV-specific T cells were monitored by measuring cytokine secretion and intracellular double staining. Our results demonstrated that the secretion levels of IFN-γ, IL-2, TNF-α, and IL-6 markedly increased in LVDC-UbHBcAg-LIGHT group, accompanied by increased percentages of IFN-γ, TNF-α or GzmB producing CD8+ T cells in the spleen. We also found a higher percentage of IFN-γ+CD4+ T cells in the LVDC-UbHBcAg-LIGHT group, however, stimulation of CD4+ cells was not as pronounced as that of CD8+ cells, presumably due to the less efficient presentation of endogenous antigen peptides to the MHC class II molecules. In addition, our findings showed that LVDC-UbHBcAg-LIGHT significantly improved the HBV-specific CTL activity and IgG antibody responses two weeks after the last immunization. The observations suggested that LVDC-UbHBcAg-LIGHT immunization could also elicit obvious secretion of antigen-specific antibodies by B cells, presumably due to cross-presentation of the endogenous antigen peptides to the MHC class II molecules.

HBV transgenic mice express HBV-related antigens and replicate HBV DNA in hepatocytes, but they cannot develop chronic hepatitis because of their immune tolerance to viral antigens [23]. Thus, HBV transgenic mice can be represented as a chronic HBV infection model suitable for assessing the antiviral efficacy of therapeutic vaccines. In this study, we discovered that serum HBV DNA and HBsAg levels decreased significantly in the LVDC-UbHBcAg-LIGHT group compared with other groups, however, the amounts of AST and ALT were obviously higher than other groups presumably due to serious damage of hepatocytes by the strong HBcAg-specific CTL activity. These results correlated well with immunohistological analysis which revealed significantly reduced expression of cytoplasmic HBsAg and nuclear HBcAg in LVDC-UbHBcAg-LIGHT group. From the above experimental results we could find that LVDC-UbHBcAg-LIGHT induced robuster cytotoxic T lymphocyte responses than the traditional envelope-pseudotyped LV (LV-UbHBcAg-LIGHT) in HBV transgenic mice. This was in contradiction with our previous *ex vivo* experiments in which LV-UbHBcAg-LIGHT exhibited the same ability to elicit HBV-specific CTL responses as

LVDC-UbHBcAg-LIGHT. Although exact mechanisms behind the discrepancy are still under investigation, it seems likely that the percentage of DCs in BMDCs used for *ex vivo* study is about 70 % or more, far exceeding the low percentage of DCs that are resident in the dermis of HBV transgenic mice, therefore, the DC-targeting advantages of LVDC-UbHBcAg-LIGHT cannot be fully demonstrated in the *ex vivo* research.

Autophagy has been previously reported to regulate the homeostasis of organelles, such as the ER and mitochondria, and promote T cell survival [31, 32]. It has been discovered that the primary immune response of CD8+ T cells against the Listeria monocytogens (LM) infection was severely impaired in the absence of autophagy because the T cell population could not expand after infection both *in vitro* and *in vivo* [33]. In consistent with our *ex vivo* experiment, we observed that autophagy was notably induced in the CD8+ T cells of mice immunized by LVDC-UbHBcAg-LIGHT and the capacity of T cell proliferation enhanced gradually with the increased induction of autophagy. CDKN1B is the main negative cell cycle regulator and its expression remains higher during the early to middle of the G1 phase to prevent cell cycle progression [34, 35]. We and others have shown that the degradation of CDKN1B after TCR stimulation facilitated entry into the S phase and massive proliferation of T cells, however, CDKN1B was accumulated in autophagy-deficient T cells after TCR stimulation accompanied by weakened proliferation capacity and compromised T cell function [36]. In the current study, we found that CDKN1B protein levels in CD8+ T cells of HBV transgenic mice decreased gradually with the increased induction of autophagy suggesting autophagy may be involved in maintaining homeostatic levels of CDKN1B, thereby regulating the proliferation and activation of CD8+ T cells.

Autophagy plays an indispensable role in the function of T lymphocytes and is essential for effector CD8+ T cell survival [36, 37]. We found that the protein levels of many pro-apoptotic machineries, such as Pro-caspase-3, Pro-caspase-8 and Bim slightly increased in LVDC-UbHBcAg-LIGHT and LV-UbHBcAg-LIGHT groups when compared with the control. Corresponded to our *ex vivo* observations, we found a drastic increase in the expression of these proteins, but not mRNA levels upon autophagy inhibition, in the CD8+ T cells of mice immunized by LVDC-UbHBcAg-LIGHT, which was in line with massive apoptosis in these cells. Our results supported the idea that the slightly increased expression of the pro-apoptotic machineries was a critical self-limiting mechanism for activated CD8+ T cells, at the same time, the degradation of apoptosis-related proteins by autophagy was a self-protection mechanism for activated CD8+ T cells and autophagy blockade may sensitize the CD8+ T cells to both Bim and caspases-mediate cell apoptosis which resulted in compromised T cell function.

Selective autophagy can degrade specific targets. Several autophagy receptor proteins have been confirmed to facilitate the delivery of targets into autophagosomes. The receptor molecule p62 (SQSTM1) is one of these autophagy receptors [38, 39]. It has been shown that clearing of ubiquitinated protein aggregates by autophagy can be facilitated by p62, which contains an ubiquitin-binding domain and an LC3-interacting region, thus as a bridge between autophagosomes and ubiquitinated proteins [40, 41]. As expected, we found co-localization of ubiquitin, p62 and Bim or CDKN1B in the CD8+ T cells of mice immunized by LVDC-UbHBcAg-LIGHT and speculated that recruitment of CDKN1B or Bim into these protein aggregates might be achieved via p62. Consistent with this idea, CDKN1B or Bim was found to be immunoprecipitation with P62 further confirming the involvement of autophagy in regulating cell cycle and apoptosis associated proteins. However, we did not detect the presence of Pro-caspase-3 and Pro-caspase-8 in these protein aggregates indicating the existence of some autophagy-independent pathway for the regulation of T cells activation.

There is much left to learn about this DC-targeting lentivector. For instance, we did not perform the phenotypic analysis of the induced antigen-specific T cells to determine whether they were the typical effector memory cells which is important for therapeutic vaccination. More work is needed to quantify the efficiency of the DC-targeting lentivector to generate central memory T cells, which is essential for protective vaccination. Furthermore, we will consider the use of ATG5 knockout mice to fully understand the important role of autophagy

in T cell activation since neither 3-MA nor si-ATG5 can completely abolish the expression of ATG5 gene in T lymphocytes. There are still many problems to be solved before applying the lentivector directly to patients with chronic HBV infection. Firstly, the HBV load in patients with chronic hepatitis B usually far exceeds the HBV load in the mouse model used in this study, and even if the serum HBsAg and HBV DNA titer have decreased significantly in the mouse model, the degree of decline is still not sufficient to eradicate the virus in human body. Additionally, when conducting immunotherapy in clinical practice, we must also seriously consider the issue of safety. The ultimate goal of HBV immunotherapy is to completely eliminate HBV on the basis of no obvious harm to the liver by cytotoxic CD8+ T cells. As we all know, if the concentration of intrahepatic virus antigen is high, and a strong CD8+ T cell antiviral response is generated at this time, there will be great risks of hepatocellular necrosis. The decline in viral load may be a prerequisite for successful immunotherapy. Therefore, with a combination of antiviral drugs during immunotherapy, we could minimize the immunopathology of the liver by cytotoxic CD8+ T cells via reducing the viral load and the percentage of infected hepatocytes. Taken together, this study provided experimental basis for the antigen-directed immunotherapeutics that may be safely administered to patients as a therapeutic strategy to combat persistent HBV infection in the future. Meanwhile, our observations shed some light on the activation mechanism of CD8+ T cells by the DC-targeting lentivectors, and established a crucial role of autophagy in promoting the survival and proliferation of T lymphocyte by selectively degrading the ubiquitinated apoptosis and cell cycle associated protein aggregates.

Acknowledgements

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Disclosure Statement

The authors declare no conflict of interests.

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