Enhancement of CTLs induced by DCs loaded with ubiquitinated hepatitis B virus core antigen

Jian-Hua Chen, Yong-Sheng Yu, Xiao-Hua Chen, Hong-Hong Liu, Guo-Qing Zang, Zheng-Hao Tang

Department of Infectious Diseases, Sixth People’s Hospital Affiliated to Shanghai Jiao tong University, Shanghai 200233, China

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Correspondence to: Dr. Zheng-Hao Tang, PhD, Department of Infectious Diseases, Sixth People’s Hospital Affiliated to Shanghai Jiao tong University, Shanghai 200233, China. tzhhao@hotmail.com

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Abstract

AIM: To investigate whether hepatitis B virus (HBV) could induce a hepatitis B virus core antigen (HBcAg)-specific cytotoxic T lymphocyte (CTL) response in vitro by dendritic cells (DCs) transduced with lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg).

METHODS: Recombinant LV-Ub-HBcAg were transfect ed into highly susceptible 293 T cells to obtain high virus titres. Bone marrow-derived DCs isolated from BALB/c mice were cultured with recombinant granulocyte-macrophage colony-stimulating factor and recombinant interleukin (IL)-4. LV-Ub-HBcAg, lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg), lentiviral vector (LV) or lipopolysaccharide were added to induce DC maturation, and the DC phenotypes were analyzed by flow cytometry. The level of IL-12 in the supernatant was detected by enzyme-linked immunosorbent assay (ELISA). T lymphocytes were proliferated using Cell Counting Kit-8. DCs were cultured and induced to mature using different LVs, and co-cultured with allogeneic T cells to detect the secretion levels of IL-2, IL-4, IL-10 and interferon-γ in the supernatants of T cells by ELISA. Intracellular cytokines of proliferative T cells were analyzed by flow cytometry, and specific CTL activity was measured by a lactate dehydrogenase release assay.

RESULTS: LV-Ub-HBcAg-induced DCs secreted more IL-12 and upregulated the expression of CD80, CD86 and major histocompatibility class II. DCs sensitised by different LVs effectively promoted cytokine secretion; the levels of IL-2 and interferon-γ induced by LV-Ub-HBcAg were higher than those induced by LV-HBcAg. Compared with LV-HBcAg-transduced DCs, LV-Ub-HBcAg-transduced DCs more efficiently stimulated the proliferation of T lymphocytes and generated HBcAg-specific cytotoxic T lymphocytes.

CONCLUSION: LV-Ub-HBcAg effectively induced DC maturation. The mature DCs efficiently induced T cell polarization to Th1 and generated HBcAg-specific CTLs.

Key words: Ubiquitin; Hepatitis B virus core antigen; Lentiviruses; Dendritic cells; Cytotoxic T lymphocytes

Peer reviewer: Elena Vezali, Dr., Department of Hepatology, Hygeia Diagnostic and Therapeutic Center of Athens, Erythrou Staurou 4, Maroussi, Athens 15123, Greece

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INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem, particularly in Asia and South Africa11. Notably,
an effective T cell response is critical for virus clearance, and defective cytotoxic T lymphocytes (CTLs) may lead to persistent HBV infection. Moreover, the defective CTL response was ascribed to the impaired dendritic cell (DC) function. Promoting and improving DC function is a promising approach to combating persistent HBV infection.

Various methods have been attempted to modify DC function, including the use of protein antigens, cytokines, costimulatory molecules, and signalling pathway ligands known to activate the immune response. Nevertheless, these methods may be insufficient to induce a strong antigen-specific immune response. Further enhancement of the immune response to HBV-specific CTL may be more conducive to clear the HBV. Thus, a novel therapeutic approach is needed to activate T cell expansion and induce a strong antigen-specific T cell response.

Ubiquitin (Ub) is a highly conserved small regulatory protein, ubiquitous in eukaryotes, that usually serves as a signal for the target protein that is recognized and degraded in proteasomes. The Ub-mediated processing of antigens is rapid and efficient and stimulates cell-mediated immune responses. Accordingly, Ub-mediated processing of antigens has been widely used in chronic infection and cancer studies to improve immune response. Wang et al. found that an Ub-fused Mycobacterium tuberculosis antigen ESAT-6 DNA vaccine significantly increased the antigen-specific cellular immune response in BALB/c mice. That study confirmed that the Th1-type immune response and CTL activity were enhanced by changing the antigen processing. Zhang et al. reported that Ub-fused melanoma antigens induced antigen proteins to execute proteasome-dependent degradation and created epitopes of major histocompatibility complex (MHC) class I, resulting in the preferential activation of antigen-specific CD8+ T cells.

Retroviral and adenoviral vectors have been the focus of many studies because of their high efficiency. Lentivirus vectors (LVs) transfect both dividing and relatively quiescent cells and have been widely used to modify DCs. The aim of this study was to investigate the capacity of DCs transfected with LVs encoding the ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg-D3) to stimulate lymphocyte proliferation and to generate antigen-specific CTLs. The results may provide effective approaches to the control of persistent HBV infection.

MATERIALS AND METHODS

Animals

BALB/c mice (H-2d), 6-8 wk old, were purchased from the Shanghai Experimental Animal Centre of the Chinese Academy of Sciences and maintained under pathogen-free conditions. 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.

Cell lines

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Gaithersburg, MD, United States) supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, United States), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C in 5% CO2. The H-2d mastocytoma cell line P815/c (expressing the HBV core antigen) was maintained in our lab.

Construction of lentiviral vectors

The plasmid pcDNA3.1(-)-Ub-HBcAg was constructed and maintained in our lab. The Ub-HBcAg gene was amplified by polymerase chain reaction (PCR). The primers used were: Ub-HBcAg: forward: CGTGGGATCTCATGCA GATCTTGTTGAAG, reverse: CGCAGCGTGTCACATTGAATTCCCGAG from plasmid pcDNA3.1(-)-Ub-HBcAg. The purified Ub-HBcAg fragment was cloned into the pWPLXd vector (provided by Prof. Jianming Li, Nanjing, China) using BamHI I and Mlu I restriction sites. The recombinant pWPLXd-Ub-HBcAg plasmid was confirmed by restriction enzyme digestion and DNA sequencing. LV-Ub-HBcAg was derived by a combined transfection of three elements: 10 μg pWPLXd-Ub-HBcAg backbone plasmid, 5 μg psPAX2 packaging plasmid, and 5 μg PMD2.G envelope plasmid. We transiently transfected 293T cells with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States). Two days after the transfection, the viral supernatant was collected and filtered through a 0.45-μm filter. Concentrated vectors for the in vitro studies were prepared by ultracentrifugation at 25 000 rpm and 4 °C for 90 min. Viral pellets were resuspended in 2 mL sterile phosphate-buffered saline (PBS) and stored at -80 °C.

The control plasmid was constructed by inserting the HBcAg fragment into the BamHI I and Mlu I site of the pWPLXd plasmid and named pWPLXd-HBcAg. LV particles (LV-HBcAg) were produced by Lipofectamine transfection into 293T cells.

To determine the titre of the green fluorescent protein (GFP)-expressing vector, 293T cells (1 × 106 cells/well) were infected with serially diluted viral supernatant. On day 2, the infected cells expressing GFP were counted by flow cytometry. The titre was calculated as: transduction units per mL (TU) = the number of infected cells/volume of virus supernatant.

Western blotting

The 293T cells were seeded in six-well plates at 1 × 106 cells/well. LV-Ub-HBcAg, LV-HBcAg or LV was added at an multiplicity of infection (MOI) of 1. In some experiments, a specific inhibitor of proteasomes, MG132, was used at 10 μmol. The cells were harvested 48 h after infection, washed twice with PBS, gently dispersed into a single-cell suspension and homogenised using RIPA lysis buffer. Protein concentrations were determined using the Pierce BCA Protein Assay Reagent kit (Rockford, IL, United States). Homogenates were diluted to the desired protein concentration with 2 × SDS-PAGE loading buffer (Invitrogen). Samples were boiled and loaded onto polyacrylamide mini-gels (Invitrogen) for electrophoresis. Proteins from the gels were transferred to Im-
Dendritic cell generation

Femurs and tibiae of B6F1 mice were removed and purified from the surrounding muscle tissues. Thereafter, intact bones were left in 70% ethanol for 5 min for disinfection and then washed with PBS. Both ends were cut with scissors and the marrow was flushed with PBS using a syringe with a 0.45-mm diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting. Bone marrow cells were cultured at 2 × 10^6 cells/mL in complete RPMI 1640 culture medium (containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin) in the presence of 20 ng/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, United States) and 10 ng/mL murine IL-4 (mIL-4; PeproTech). Nonadherent single cells were gently removed, and fresh medium containing murine GM-CSF and mIL-4 was added on day 3 after beginning culture.

Dendritic cell immunophenotyping

On day 5, immature DCs were cultured for an additional 96 h in the presence of LV-Ub-HBcAg, LV-HBcAg or LV (MOI = 20), and lipopolysaccharide (LPS, 0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, United States) was used as a control group. On day 9, non-adherent and loosely adherent cells were harvested as DCs. The expression of DC surface molecules was analyzed by incubation with allophycocyanin-labelled anti-mouse CD11c, CD80, CD86 and MHC class II (eBioscience, San Diego, CA, United States). The stained cells were analyzed by flow cytometry.

Interleukin-12 production

On day 5, immature DCs were infected with LV-Ub-HBcAg, LV-HBcAg or LV (MOI = 20) for 72 h. On day 8, the IL-12 levels in harvested supernatants of mature DCs were measured using a standard sandwich enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems, Minneapolis, MN, United States) according to the manufacturer’s instructions.

Mixed leukocyte reaction

On day 9, harvested mature DCs were pre-treated with 25 μg/mL mitomycin C and 5% CO_2 for 30 min at 37 °C. Mouse spleens were dissociated on 200-gauge nylon mesh. Splenocytes were collected and treated with lysis buffer to eliminate red cells, washed, and resuspended in RPMI-1640 with 10% FBS. Lymphocytes were derived from splenocytes using nylon wool columns. Single-cell suspensions of lymphocytes (5 × 10^5 cells/well) were grown in 96-well plates. Lymphocytes were co-cultured with mature DCs at different responder/stimulator (T cell/DC) ratios (5:1, 10:1 or 20:1) for 72 h. The cells were incubated in a final volume of 200-μL complete RPMI 1640 for 72 h, and 10-μL Cell Counting Kit-8 solution (Beyotime Institute of Biotechnology, Haimen, China) was added to the plates for 4 h at 37 °C. The absorbance was finally read at 450 nm.

Hepatitis B virus core antigen-specific cytotoxic T lymphocytes activity

The former stimulated splenocytes (5 × 10^6/mL) were used as effectors, and the P815/c cell line was used as target cells. P815/c cells were seeded at a density of 5 × 10^4 cells/well in 96-well plates. Effectors were incubated with P815/c at different effector and target (E/T) ratios (12.5:1, 25:1 or 50:1) at 37 °C under 5% CO_2 for 4 h. The HBcAg-specific CTL activity was measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison WI, United States) for lactate dehydrogenase (LDH) release according to the manufacturer’s instructions. The absorbance values of the supernatants were recorded at optical density 450 nm. Percent cytotoxicity was calculated as follows: [(Experimental release - Effector spontaneous release - Target spontaneous release)/ (Target maximum release - Target spontaneous release)] × 100%.

Statistical analysis

Results were expressed as mean ± SD. Differences between groups was determined using Student’s t test, and the differences between two or more groups were determined using a one-factor analysis of variance. Data were considered statistically significant at P < 0.05.
RESULTS

Construction of lentiviral vectors and transduced dendritic cells

A 780 bp fragment of the Ub-HBcAg gene was cloned into pWPXLd (Figure 1B) and packed into LVs. The HBcAg gene was similarly assembled as a control. After concentration, all vectors in the study achieved a titer of approximately $7.5 \times 10^8$ transducing units/mL.

The construction procedure is shown in Figure 1A. As expected, Ub-HBcAg expression was lower than that of HBcAg and recovered to the same level as that of HBcAg when MG-132 was added to the culture (Figure 1C). The transduction efficiency of LVs into DCs was evaluated using flow cytometry by detecting GFP expression (Figure 2). On day 4 after infection, 56.1% of GFP-expressing DCs were detected.

Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen-induced dendritic cell maturation increased IL-12 production and enhanced lymphocyte proliferation

At the end of the treatment, the amount of DCs (CD11c+) was 75% by fluorescence-activated cell sorting analysis. MHC II, CD80 and CD86 molecules, which are characteristic of DCs, were used to evaluate DC differentiation and maturation. These molecules were highly expressed in DCs transduced with LV-Ub-HBcAg compared with those transduced with the alternatives (Figure 3). DC function was evaluated by IL-12 secretion and promotion of lymphocyte proliferation. DCs transduced with LV-

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**Figure 1** Schematic diagram, electrophoresis of ubiquitinated hepatitis B virus core antigen and HBcAg genes, pWPXLd-Ub-HBcAg digested by BamH I and Mlu I, and HBcAg protein expression (about 21 kDa). A: Schematic diagram of pWPXLd vector; B: Lane 1, ubiquitinated hepatitis B virus core antigen (Ub-HBcAg) polymerase chain reaction (PCR) product (780 bp); lane 2, HBcAg PCR product (552 bp); lane 3, The digested products pWPXLd-Ub-HBcAg by BamH I and Mlu I; lane 4 and lane M1, DNA marker 2000; lane M2, DNA marker 15 000; C: 293T cells were transduced with lentiviral vector (LV), LV-Ub-HBcAg or LV-HBcAg and cultured for 48 h. MG-132 (10 mM) was added for 24 h before harvesting the cells. Cell lysates (10 mg) were analyzed by immunoblotting with an anti-HBc antibody. Relative expression of HBcAg was calculated by a gray value.

**Figure 2** Transduction of dendritic cells with lentiviral vectors expressing green fluorescent protein was evaluated by flow cytometry. Dendritic cells (DCs) were seeded in six-well plates at $1 \times 10^6$ cells/well. Lentiviral vectors ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg), lentiviral vectors hepatitis B virus core antigen (LV-HBcAg) or lentiviral vector (LV) was added at an multiplicity of infection of 20. GFP: Green fluorescent protein.
Figure 3  Percentage of dendritic cell surface molecules. In the lentiviral vectors ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg) group, the expression of major histocompatibility complex (MHC) class II, CD80 and CD86 molecules characteristic of dendritic cells (DCs) was significantly higher than in the lipopolysaccharide (LPS) or empty lentiviral vectors (LV) group. The results express one of three experiments. LV: Lentiviral vector; APC: Antigen-presenting cell.

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Ub-HBcAg showed significantly higher levels of IL-12 production and lymphocyte proliferation than did the others ($P < 0.01$) (Figure 4A and B). Lymphocyte proliferation capacity was enhanced by the T cell/DC ratio.

**Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen boosted cytokine production and CD8$^+$ T cells elicited from proliferative T cells in vitro**

T cells stimulated by DCs transduced with LV-UB-HBcAg showed increased IFN-γ and IL-2 secretion compared with DCs transduced with LV-HBcAg (Figure 5). No significant difference between the two groups was observed for IL-4 and IL-10 production. CTLs were analyzed by intercellular IFN-γ and CD8α$^+$ levels. The levels and intensities of IFN-γ expression were higher in the LV-UB-HBcAg than in the LV-HBcAg samples (Figure 6A and B), suggesting that DCs transduced with LV-UB-HBcAg were effective for inducing CTLs.

**Enhancement of cytotoxic T lymphocyte activity in dendritic cells transduced with Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen**

The LDH relaxation index was determined to evaluate the specific cytotoxicity of T lymphocytes in response to different LV-transduced DCs. HBcAg-specific CTL activities with different effector/target ratios are shown in Figure 7. T lymphocytes from the LV-UB-HBcAg-transduced DCs killed 55.0% ± 4.3% target cells at an effector and target ratio of 50:1, which was significantly higher than that of the LV-HBcAg-transduced DC group (32.4% ± 5.2%) ($P < 0.01$). Accordingly, these results indicated that LV-UB-HBcAg-transduced DCs induced strong specific CTL responses.

**DISCUSSION**

The development of novel immunotherapies has been highly anticipated because HBV infection is one of the
leading causes of cancer or hepatocellular carcinoma-related death. Several studies have demonstrated that the main cause of viral persistence during HBV infection is an inadequate antiviral immune response to the viral antigens[13,14]. The viral-specific CD8 T cell response plays an important role in the process of viral clearance. Patients with chronic hepatitis B (CHB) or therapeutic failure show deficient Th1 immunity associated with inefficient CD8 T cell cytotoxicity[15]. Therefore, induction of CTL responses specific to HBV represents a promising strategy to protect against HBV infection.

DCs are key antigen-presenting cells that induce primary and memory immune responses. Impaired DC function is found in chronic HBV infection, in which patients are generally in an immunocompromised state of immune tolerance[16-18]. Considerable effort has been made to introduce antigens into DCs in the forms of peptides, proteins, or transgenic protein antigens using viral vectors[19,20]. Various viral vectors, including poxvirus and adenovirus, have been used to genetically modify DCs, but low transduction efficiencies have limited their application. We chose LVs as a gene transfer vector because they can transduce non-dividing, monocyte-derived DCs and bone marrow-derived DCs with very high transduction efficiencies[21,22]. Several reports have demonstrated that immunizing mice with LVs by delivering viral or tumor model antigens mice elicited broad and long-lasting specific immune responses. For example, lentiviral transduction of DCs expressing ovalbumin effectively processed and presented the ovalbumin antigens and induced ovalbumin-specific T cell responses[23]. Our results confirmed that lentivirus-mediated gene transfer could offer the unique opportunity to investigate the biologic activity of DCs.

The ubiquitin-proteasome system (UPS) is a highly selective adenosine-5'-triphosphate-dependent proteolytic system present in all eukaryotic cells and plays a key role in antigen presentation[24]. It is well established that short antigenic peptides must be presented on MHC class I molecules of target cells to be recognized by specific CTLs. Proteasomes are responsible for the proteolysis of intracellular proteins, including viral antigens, to generate MHC class I ligands.

Attachment of Ub to a protein is the initial signal for targeted protein degradation. To prevent fusion gene (Ub-HBcAg) cleavage by deubiquitination enzymes, we constructed a pWPXLd vector encoding HBCAg fused with Ub, in which the Ub C-terminal glycine was replaced with alanine[25]. Additionally, HBCAg with a modified N-terminal Met residue was replaced by Arg. By this method, the fusion protein can be quickly recognized by the UPS, resulting in a promotion of HBCAg degradation[26,27]. In our study, Western blotting analyses identified efficient expression of HbAg from the 293T cells transduced with recombinant LVs. Ub-fused HbAg was converted into an excellent substrate for the UPS. We found that the 293T cells transduced with LV-Ub-HbAg showed low levels of protein expression in the absence of MG-132.

Immature DCs expressed low levels of surface MHC molecules, producing almost no expression of CD40, CD80 or CD86. Fully matured DCs showed strong surface expression of MHC class II and co-stimulatory mo-

**Figure 7 Cytotoxic response of proliferative T cells.** Effector cells were incubated with P815/c at different effector/target (E/T) ratios (12.5:1, 25:1 or 50:1) for 4h. Experiments were repeated three times with similar results. Data represent the mean ± SD. *P < 0.01 vs lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group, LV-Ub-HbAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV: Lentiviral vector.
molecules (CD80 and CD86). In our study, the surface molecules CD80, CD86, and MHC class II DCs were markedly upregulated by LV-Ub-HBcAg stimulation, whereas no significant change was observed after LPS or LV-HBcAg stimulation. An important sign of mature DCs is IL-12 secretion. Mature DCs secrete high levels of IL-12 that promote activation of effector cells (e.g., natural killer cells, lymphokine-activated killer cells, tumor-infiltrating lymphocytes and macrophages) and induce a variety of cytokines (e.g., IFN-γ, GM-CSF, IL-2 and IL-8). IL-12 is produced by mature DCs in response to infection by various intracellular pathogens. This response plays a critical role initiating a specific T cell-mediated immune response and drives Th1 cell activation and differentiation. We examined IL-12 production after adding different maturation factors to the culture medium. As expected, IL-12-induced LV-Ub-HBcAg production of DCs was markedly elevated compared with that produced by other treatments. In this study, LV-Ub-HBcAg-transduced DCs not only promoted DC surface molecule expression, but also promoted further secretion of IL-12, which helped stimulate the immune response.

Higher rates of intracellular antigen traffic should increase the number and variety of peptides available for MHC class I binding, which may result in an increase in the cell immune response to the expressed antigen. DCs pulsed with HBV antigens effectively abrogated CTL tolerance in HBV transgenic mice. Chen et al. demonstrated that DCs loaded with HBcAg not only induce the production of HBV-specific T cells but also restore the impaired function of such cells. The DCs generated by transfection of LV-Ub-HBcAg were able to stimulate proliferation of naïve allogeneic T lymphocytes and to increase the number of antigen-specific CD8+ IFN-γ+ T cells in vitro. Th1 cells primarily secrete IL-2 and IFN-γ, whereas Th2 cells secrete type II cytokines IL-4 and IL-10. Th1/Th2 immune balance plays a key role in the outcome of HBV infection. Dominant Th1 cells tend to lead to an acute self-limited HBV infection; dominant Th2 cells tend to occur with a chronic persistent HBV infection. In our study, we observed that the LV-Ub-HBcAg group had higher levels of both IL-2 and IFN-γ in the lymphocyte supernatant compared with those in the LV-HBcAg group. This was further supported by enhanced levels of IFN-γ-producing CD8+ T cells. These results clearly indicate that the immune responses were directed toward a Th1 type rather than a Th2 type. Th1 cells are correlated with the induction of CTL activity, which is beneficial for viral or tumor eradication. In this study, the LV-Ub-HBcAg-transfected DCs stimulated T lymphocytes and generated antigen-specific cytotoxic T lymphocytes more efficiently than those of the LV-HBcAg-transfected DCs. Thus, LVS carrying Ub-fused HBcAg effectively activated antigen-specific CD8+ T cells. Inadequate endogenous antigen presentation by MHC class I molecules to CD8+ T cells is one of the reasons for the failure of the immune system to eliminate pathogens. Patients with CHB or therapeutic failure showed deficient Th1 immunity associated with inefficient CD8+ T cell cytotoxicity. In our study, enhanced antigen presentation increased the number of antigen-specific CD8+ IFN-γ+ T cells in the LV-Ub-HBcAg-transfected DC group. Ub-fused HBcAg was rapidly degraded by the proteasome, resulting in efficient production of a variety of peptides, including many CTL epitopes that may be presented by many types of MHC class I molecules.

In summary, we have successfully transfected murine bone marrow-derived DCs with LVS encoding the Ub-HBcAg fusion gene. The Ub-HBcAg-transfected DCs proliferated and generated HBcAg-specific CTLs more efficiently than did the HBcAg-transfected DCs. Therefore, this novel strategy may have therapeutic value that can be applied to the treatment of infectious diseases.

**COMMENTS**

**Background**

Hepatitis B virus (HBV) infection is a serious public health problem. Defective cytotoxic T lymphocytes (CTLs) may lead to persistent HBV infection, and the defective CTL response was ascribed to the impaired dendritic cell (DC) function. Promoting and improving DC function is a promising approach to combating persistent HBV infection. Ubiquitin (Ub) is a highly conserved small regulatory protein, and the Ub-mediated processing of antigens is rapid and efficient and stimulates cell-mediated immune responses.

**Research frontiers**

Ub-fused melanoma antigens could induce antigen proteins to execute proteasomal degradation in proteasomal degradation in proteasomes, that usually serves as a signal for the target protein that is recognized and degraded in proteasomes.

**Terminology**

Ubiquitin is a highly conserved small regulatory protein, ubiquitous in eukaryotes, that usually serves as a signal for the target protein that is recognized and degraded in proteasomes.

**Peer review**

The topic is novel, with very few articles published in this field till now. The manuscript is well organized with objectives, methods, results being adequately described, and the conclusions are based on the results found. Tables and figures are appropriate. Statistical analysis needs better description by providing P values through the text where comparisons between groups are present.

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