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ORIGINAL ARTICLE

B7-DC-silenced dendritic cells induce stronger anti-HBV immunity in transgenic mice

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Abstract Hepatitis B virus (HBV) is a noncytopathic DNA virus and is the pathogen of acute and chronic hepatitis. Interferon and nucleotide analogues such as lamivudine and adefovir are the current treatment strategies of HBV infection; however, it is still a serious disease. Therefore, the development of new therapeutic options against HBV is needed. In the present study, we have investigated whether the vectors carrying short hairpin RNA (shRNA) targeting the murine B7-DC gene could silence the expression of B7-DC and analyzed the function of gene-modified dendritic cells (DCs) by mixed lymphocyte reaction. The results demonstrated that two shRNA vectors efficiently suppressed the expression of B7-DC. The MLR assay showed that shRNA-B7-DC-transfected DCs induced markedly higher allogeneic lymphocyte proliferation than transfected DCs with the vector plasmid pAS and untreated DCs at all dilutions. The most efficient shRNA plasmid vector against B7-DC was then used to silence the expression of B7-DC on DCs, the gene-modified DCs were pulsed with HBV-specific peptides, and HBV transgenic mice were immunized. After three rounds of immunization, the splenocytes were stimulated in vitro and tested for cytotoxicitic T lymphocyte activity, while the sera were used to detect the level of HBsAg and HBV DNA. The data demonstrated that blockade of B7-DC on DCs augmented the cytolytic activity induced by immunization with peptide-pulsed DCs and significantly reduced the concentration of serum HBsAg and HBV DNA, suggesting that silencing of B7-DC is of potential value in DC-based therapy of HBV infection.

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Introduction

Hepatitis B virus (HBV) is a species of the genus *Orthohepadnavirus*, which is likewise a part of the *Hepadnaviridae* family of viruses. HBV infection is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma [18]. Despite the availability of effective vaccines against HBV for many years, this disease remains a major public health problem, leading to around one million deaths per year [12]. It is now widely accepted that viral persistence is associated with a weak or absent specific immune responses to HBV, particularly the cellular immune response. Studies show that CD8+ T cells display poor proliferation and effector functions in chronic HBV-infected patients [12, 33]. Although the exact molecular mechanism responsible for this phenomenon is not completely understood, this observation suggests that enhancement of the responses of virus-specific T-cells may be a way of terminating chronic HBV infection.

Recently, cell-based therapy has been proposed as an approach to manipulating the immune system. Dendritic cells (DCs) have long been recognized as "professional" antigen-presenting cells (APCs) with the potent capacity to initiate primary immune responses [3, 32]. They originate from the bone marrow and are able to capture and present antigens to the cells of the adaptive immune system. After sampling antigens, they migrate to the secondary lymphoid organs, where they initiate and regulate T- and B-cell

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immune responses by expressing high levels of lymphocyte costimulatory molecules and MHC molecules, and secreting biologically active molecules [28, 35]. Because of these properties, DCs are being considered as an efficient means of immunization against viral infections such as HIV, herpes simplex virus, and papillomavirus [22]. Thus, a new strategy for DC-based vaccines against HBV is likely to be effective.

Many studies have revealed that the B7 family of molecules provides signals that are critical for both stimulating and inhibiting T cell activation [19]. B7-DC (also called PD-L2), whose expression is highly restricted to DCs, is the fifth member of the B7 family. It has significant homology to B7-H1 (also called PD-L1) and been identified as a second ligand for programmed death 1 (PD-1), which is a negative regulator of T cell activation [17, 31]. Despite many reports suggesting different functions of B7-DC in inhibiting T cell proliferation, promoting tumor immunity, and maintaining peripheral tolerance, its distinct immunologic role remains to be clarified. However, similar to B7-H1 interacting with PD-1, B7-DC/PD-1 signals have been shown to inhibit T cell proliferation and cytokine production [10, 17, 27]. Based on these studies, blockade of co-stimulatory signals for T cells by interrupting B7-DC/PD-1 interactions is a promising approach to increase antiviral T cell activity.

RNA interference (RNAi) is a novel gene regulatory mechanism that uses dsRNA molecules to shut down gene expression at the posttranscriptional level through mRNA degradation [4]. A short hairpin RNA (shRNA) is a DNA-based siRNA production strategy in which siRNA are produced by intracellular processing of shRNA transcripts. Compared to siRNA, shRNA has several advantages in silencing longevity, delivery options and cost [23, 29]. The successful use of endogenous expression of shRNA to silence the target gene makes the use of this technology a potential rational therapeutic strategy against HBV infection [11, 15, 16]. Therefore, RNA silencing provides a new platform for effectively treating HBV infection, in addition to traditional antiviral therapies. In this study, two shRNA plasmid vectors against murine B7-DC that could generate siRNA inside cells were constructed and used to transfect murine bone-marrow-derived DCs. Its effect on enhancing the allostimulatory activity of DCs and anti-HBV immunity were investigated.

Materials and methods

Peptides

Peptides with a purity of >95% were synthesized using an automatic solid-phase peptide synthesizer (Applied Biosystems, Foster City, CA) by GL Biochem Co. Ltd. (Shanghai, China) and purified by reversed-phase HPLC. The TT-HBsAg$_{28-39}$ peptide consists of amino acids 830–843 of the universal T helper epitope of tetanus toxoid (QYIKANSKFIGITE) and an immunodominant, L^d-restricted CTL epitope located between residues 28 and 39 in the hepatitis B surface antigen (IPQSLDSWWTSL), with a linker consisting of Ala-Ala-Ala. An HIV-1 IIIB gp120 epitope peptide (RGPGRAFVTI,311-320) was used as an irrelevant peptide control. Peptides were dissolved in DMSO at a concentration of 20 mg/ml and stored at −20°C.

Animals

Female C57BL/6 (H-2K^b), BALB/c (H-2K^d) mice, 6–8 weeks old, were purchased from the Animal Experimental Center of the Second Military Medical University (Shanghai, China). The transgenic HBV mice, provided by the Infectious Disease Center of 458 Hospital of PLA, P. R. China, was initially produced on a BALB/c background and has 1.3 copies of the HBV DNA (subtype ayw) integrated in the genome. A high level of HBsAg and HBV DNA in the sera could be detected in the HBV transgenic mice.

Generation of murine bone marrow-derived DCs in vitro

Bone marrow was isolated from the femurs and tibias of normal BALB/c mice. Erythrocytes were lysed by treatment with 0.83% ammonium chloride at 37°C for 5 min. The remaining cells were cultured in a 6-well plate (1×10^6 cells/ml) in 2 ml of RPMI 1640 containing 10% fetal bovine serum, 10 ng/ml recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ) and 1 ng/ml murine IL-4 (PeproTech, Rocky Hill, NJ). All cultures were incubated at 37°C in 5% humidified CO_2. Non-adherent granulocytes were removed after 48 h of culture, and fresh medium was added. At day 5 of culture, the non-adherent cells were harvested. The purity, tested by FACS, was greater than 80%.

Design of shRNA and plasmid preparation

Plasmid vector pAS containing the polymerase-III H1-RNA gene promoter and GFP gene was kindly provided by our team colleague, Dr. Lei Wang. The targeted sequence was designed to be homologous to the murine B7-DC mRNA consensus sequence (GenBank accession number NM_021396). The oligonucleotide sequences of two different shRNAs are shown in Table 1. The shRNA expression cassette contained a 19-nucleotide sequence in the sense strand, followed by a short spacer (TTCAAGAGA), the reverse complement of the sense strand, and five thymidines as an RNA polymerase III transcriptional stop

Table 1 The oligonucleotide sequences of two different shRNAs

shRNA	Forward strand	Reverse strand
shRNA1-B7-DC	5′-GATCCCGCTTCTTACATGAGGATAGT TCAAGAGACTATCCTCATGTAAGAAGC TTTTTGGAAA-3′	5′-AGCTTTTCCAAAAAGCTTCTTACATGAGG ATAGTCTCTTGAACTATCCTCATGTAAGAAGCGG-3′
shRNA2-B7-DC	5′-GATCCCCTTCAGCTGCATGTTCTGGTTC AAGAGACCAGAACATGCAGCTGAAGTTT TTGGAAA-3′	5′-AGCTTTTCCAAAAACTTCAGCTGCATGTTCTGGTC TCTTGAACCAGAACATGCAGCTGAAGGG-3′

signal. Oligonucleotides were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) and annealed with the reverse strands and cloned into the *Hin*dIII and *Bgl*II sites of pAS to produce the resultant plasmids shRNA1-B7-DC and shRNA2-B7-DC. The cloned B7-DC target sequences were all confirmed by automatic sequence analysis.

Plasmid transfection

Approximately, 5×10^5 DCs/well were plated in a 12-well plate in 500 µl of serum-free RPMI 1640 and incubated at 37°C in a 5% CO_2 humidified atmosphere incubator, where they were allowed to grow overnight to 70–80% confluency. Transfection of cells with the plasmids was performed using Lipofectamine reagent (Beyotime, China) according to the manufacturer's instructions. For each well, 0.8 µg of plasmid DNA and 2 µl of Lipofectamine in 35 µl of serum-free medium were mixed gently in a tube and incubated for 15 min at room temperature to form DNA–liposome complexes. Then, the entire complexes were added to 500 µl of DC cell culture as described above. After 4 h of incubation, an equal volume of RPMI 1640 supplemented with 20% FBS was added to the cells. Cells were usually assayed at 48 h post-transfection. Specific silencing was confirmed by at least three independent experiments.

Extraction of total RNA and analysis of B7-DC expression by RT-PCR

Total RNA was extracted from the transfected cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The extracted RNA was used as a template, and first-strand cDNA was synthesized using a RNA PCR kit (TaKaRa, Dalian) with the supplied oligo (dT)16 primer. PCR amplifications for the murine B7-DC with the specific primers (forward primer: 5′-GGAATTCC CTAAAGAAGTGTACACCG-3′; reverse primer: 5′-CCG CTCGAGTTAGACTTTGGGTT-3′) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) with the specific primer (forward primer: 5′-ACGGCAAATTCAACGGCA CAGTCA-3′; reverse primer: 5′-CATTGGGGGTAGGA ACACGGAAGG-3′) were performed under the recommended conditions, and the products were subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized by UV illumination. The GAPDH housekeeping gene was used as a control for normalization.

Western blot

Western blot analysis was performed as described previously [14]. The transfected cells were lysed in lysis buffer (10 mM Tris–Cl, pH 7.4, 1 mM $MgCl_2$, 0.5% NP40, 20 µg/ml DNase I), and cell extracts were separated by 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes, which were probed sequentially with a 1:500 dilution of anti-mouse B7-DC antibody (produced in the authors' lab) and a 1:5,000 dilution of horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and reactive protein bands were visualized using an enhanced chemiluminescence assay with LumiGlo reagents (Cell Signaling).

Mixed lymphocyte reaction (MLR)

T cells were purified from splenocytes of a C57BL/6 mouse using T Cell Enrichment Columns (R&D Systems, Minneapolis, MN) and were used as responders (1×10^5/well). The bone-marrow-derived DCs from BALB/c mice used for genetic modification were prepared as described before. The harvested DCs were transfected with shRNA1-B7-DC, shRNA2-B7-DC and vector plasmid pAS using Lipofectamine reagent and incubated with RPMI 1640 supplemented with 10% FBS, 10 ng/ml recombinant murine GM-CSF and 1 ng/ml murine IL-4 for 40 h in a humidified atmosphere of 5% CO_2 at 37°C. Then, the DCs were treated with 30 µg/ml mitomycin C (Sigma, USA) for 45 min. After being washed 4 times by RPMI 1640, they were used as stimulators.

For the induction of allogeneic MLR, purified T cells were cocultured with transfected or untreated allogeneic DCs at DC/T cell ratios of 1:1, 1:5, 1:10 and 1:20 in triplicate in flat-bottom 96-well microplates. The negative control received T cells and medium. The plates were incubated at 37°C in a humid atmosphere with 5% CO_2, and the result was measured by MTT Cell Proliferation Kit (Beyotime, China). After 72 h, 10 µl of MTT

(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml) was added to each well, and the plate was incubated for a further 4 h. One hundred µl of formazan solvent was added to each well, incubation was continued until all of the formazan was dissolved, and the absorbance was evaluated using a microplate reader at 570 nm. The results were expressed as a stimulation index (SI, SI = OD_{570} of experimental cells/OD_{570} of negative control), which indicates significant proliferation if SI $\geq$ 2.

Peptide pulsing of DCs and immunization

For peptide pulsing, 1×10^6 DCs transfected with shRNA1-B7-DC or vector plasmid pAS and untreated DCs were resuspended in 2 ml of RPMI 1640 containing 20 µg/ml HBV-specific peptide; HIV-1 IIIB gp120 CTL epitope peptide (20 µg/ml) served as irrelevant control. After 3 h of incubation at 37°C with gentle shaking every 30 min, the peptide-pulsed DCs were activated with 100 ng/ml LPS and washed twice with phosphate-buffered saline (PBS) for vaccination of mice.

Twenty-five female HBV transgenic mice, 8–10 weeks old, were divided into 5 groups (shRNA1-B7-DC/DCs + HBsAg, pAS/DCs + HBsAg, DCs + HBsAg, DCs + gp120, PBS), and five mice of each group were immunized three times at 7-day intervals by i.p. injection of 1×10^6 DCs. Mice were killed 7 days after the last immunization, and cell suspensions were prepared from the spleens.

Cytotoxicity assay

Spleens were removed from the immunized mice and were compressed through a sterile stainless steel mesh and then washed twice with RPMI 1640. Erythrocytes were lysed as before, and the splenocytes were resuspended in RPMI 1640 medium containing 10% FBS as the effector cells. The stimulator cells, harvested from naive mice, were pulsed with final concentration of 20 µg/ml of HBV-specific peptide for 4 h at 37°C in 5% CO_2 and then treated with 40 µg/ml mitomycin C at 37°C in 5% CO_2 for another 2 h. The cells were washed extensively with RPMI 1640 medium. The effector cells (8×10^6 cells) were incubated with stimulator cells at an effector-stimulator ratio of 10:1 for 5 days in culture medium containing 20 ng/ml IL-2 at 37°C in 5% CO_2. The target cells were prepared from P815 cells (mouse mastocytoma cell line, Shanghai Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences, China) pulsed with 20 µg/ml HBV-specific peptide for 4 h at 37°C in 5% CO_2.

The lactate dehydrogenase (LDH) release assay was employed to measure the ability of in vitro-stimulated responder cells to lyse P815 target cells. This assay yields results similar to those obtained with the standard chromium release assays but does not require the use of radioisotopes. The assays were performed in triplicate with 1×10^4 target cells/well incubated with effector cells at various effector cell/target cell (E:T) ratios of 50:1, 25:1 and 12.5:1 in 96-well round-bottom plates for 4 h in RPMI 1640 containing 10% FBS. After a 4-h incubation, 100 µl of the supernatant per well was then transferred to new 96-well plates, and lysis was determined by measuring LDH release using a Non-Radioactive Cytotoxicity Assay Kit (Promega, USA). The released LDH converts the added substrate tetrazolium salt into a red formazan product, and the intensity of the color is proportional to the number of lysed cells. The absorbance values from supernatants were recorded at 490 nm on an ELISA microplate reader. The percentage of specific lysis of target cells for a given effector cell sample was calculated using the following formula: % specific lysis = (optical density (OD) of experimental LDH release − OD of effector cell spontaneous LDH release − OD of target cell spontaneous LDH release)/(OD of maximum target LDH release − OD of target spontaneous LDH release) × 100%. All determinations were performed in triplicate.

Serological analysis

Serum was recovered from the immunized mice by eye socket bleeding at the killing time and was examined for HBsAg using an enzyme-linked immunosorbent assay (ELISA) kit (Kehua Bio-Engineering Co. Ltd., China) according to the manufacturer's instructions.

Quantitation of HBV DNA in serum

HBV DNA in serum was detected by the fluorescent quantitative PCR method using an HBV DNA FQ-PCR kit (Fosun High Technology Co. Ltd., Shanghai, China) according to the manufacturer's instructions.

Statistical analysis

Differences between groups were analyzed by Student's *t* test. Results were expressed as the mean ± standard deviation (S.D.). *p* values less than 0.05 were considered statistically significant in all cases.

Results

ShRNA blocks the expression of B7-DC on DCs

The mRNA expression intensities of murine B7-DC in bone marrow-derived DCs inhibited by specific shRNAs against murine B7-DC were analyzed by RT-PCR. The

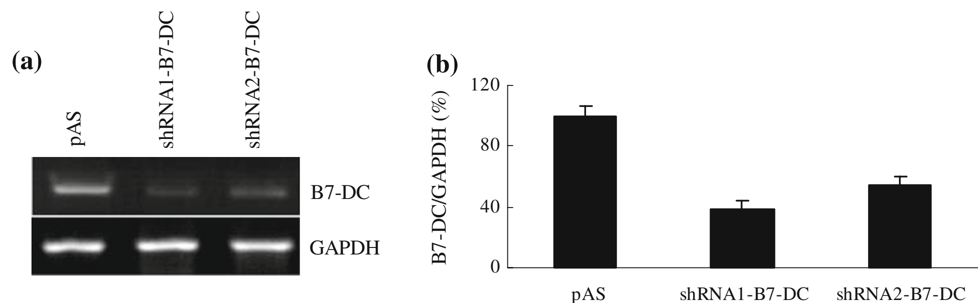

Fig. 1 RNA interference-mediated suppression of cellular mRNA. **a** The mRNA expression of B7-DC in DCs at 48 h post-transfection was examined by RT-PCR. The GAPDH gene served as an internal control. **b** B7-DC/GAPDH ratios from three independent experiments were determined using Image J

mRNA levels were normalized using GAPDH as an internal control. Statistical analysis showed that B7-DC mRNA of DCs was downregulated significantly after transfection either with plasmid shRNA1-B7-DC or with shRNA2-B7-DC when compared with DCs transfected with the vector plasmid pAS (Fig. 1a). The inhibition rates were 61.4 and 45.9% in the shRNA1-B7-DC and shRNA2-B7-DC group, respectively (Fig. 1b). These results suggested that shRNA1-B7-DC could inhibit the expression of B7-DC more strongly than shRNA2-B7-DC. Western blotting analysis gave similar results (data not shown).

ShRNA-B7-DC enhances DCs allostimulatory activities

To address the effect of shRNA-B7-DC on the allostimulatory activity of DCs, purified T cells from C57BL/6 mice were cocultured with plasmid shRNA1-B7-DC, shRNA2-B7-DC, pAS-transfected DCs or untreated DCs from BALB/c mice for 72 h, and T cell proliferation was measured by MTT. The stimulation index is shown in Fig. 2. The results demonstrate that although DCs transfected with vector plasmid pAS and untreated DCs showed similar allostimulatory activities, blocking the expression of B7-DC induced a higher allostimulatory effect than that observed with untreated DCs at DC/T cell ratios of 1:1 and 1:5 ($p < 0.05$). shRNA1-B7-DC-transfected DCs showed somewhat stronger allogeneic proliferation than those of shRNA2-B7-DC. Therefore, plasmid shRNA1-B7-DC was used for further experiments.

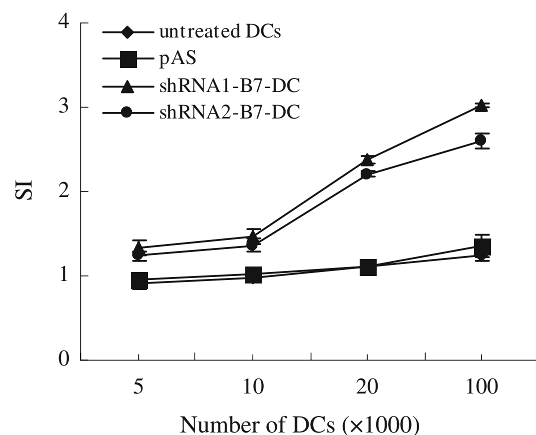

Fig. 2 shRNA-B7-DC enhances allostimulatory activity of DCs. Bone marrow-derived DCs were transfected with shRNA1-B7-DC, shRNA2-B7-DC or vector plasmid pAS for 48 h. Allogenic T cells (1×10^5/well) from C57BL/6 mice were incubated with the indicated numbers of untreated or transfected DCs for 72 h. Proliferation was determined using an MTT Cell Proliferation Assay Kit. The results are representative of two independent experiments

Silencing of B7-DC on DCs induces stronger CTL responses

To examine whether or not immunization of peptide-pulsed DCs transfected with shRNA-B7-DC induces stronger cytolytic activity of lymphocytes, HBV transgenic mice were immunized with either HBV-specific peptide-pulsed DCs transfected with shRNA1-B7-DC (shRNA1-B7-DC/DCs + HBsAg), HBV-specific peptide-pulsed DCs transfected with vector plasmid pAS (pAS/DCs + HBsAg), HBV-specific peptide-pulsed DCs (DCs + HBsAg), gp120 peptide-pulsed DCs (DCs + gp120), or PBS. Splenic cells obtained from mice 7 days after the final immunization were restimulated specifically using naive mouse splenocytes pulsed with HBsAg-specific peptides in vitro for 5 days. P815 cells pulsed with HBsAg-specific peptides were used as target cells. The cytotoxic activity was tested by non-radioactive lactate dehydrogenase (LDH) release assay, and the specific lysis rates are shown in Fig. 3. HBsAg-specific CTL was detectable in mice immunized with HBV-specific peptide-pulsed DCs compared with gp120-specific peptide-pulsed DCs or PBS at the E/T ratio of 50:1 ($p < 0.05$). Importantly, the specific CTL activities were increased by silencing of B7-DC compared with HBV-specific peptide-pulsed DCs transfected with pAS or HBV specific peptide-pulsed DCs at an E/T ratio of 50:1 ($p < 0.05$). These results demonstrate that blocking B7-DC on DCs augments the cytolytic activity induced by immunization with peptide-pulsed DCs.

Peptide-pulsed DCs elicit effective antiviral immunity in HBV transgenic mice

HBV transgenic mice have a high level of HBsAg and a high titer of HBV DNA in their sera before immunization. In order to measure the effect of anti-virus immunity elicited by peptide-pulsed DCs in HBV transgenic mice, the levels of serum HBsAg and HBV DNA were detected by ELISA using an HBsAg ELISA kit and by a fluorescent quantitative PCR method using an HBV DNA FQ-PCR kit, respectively. As shown in Fig. 4a, serum HBsAg from the mice immunized DCs pulsed with HBV-specific peptide decreased markedly compared to the control mice immunized with an irrelevant peptide and negative control ($p < 0.05$). Moreover, B7-DC-silencing DCs produced a lower level of serum HBsAg than non-silencing DCs. The titer of HBV DNA in serum was also detected by fluorescent quantitative PCR (Fig. 4b). The results demonstrated that the titer of HBV DNA in sera was reduced in mice immunized with DCs pulsed with the HBV-specific peptide, and blockade of B7-DC on DCs augmented this effect. These results indicate that DCs pulsed with HBV-specific peptide can elicit anti-HBV immunity in HBV transgenic mice, and silencing the expression of B7-DC on DCs can promote this effect.

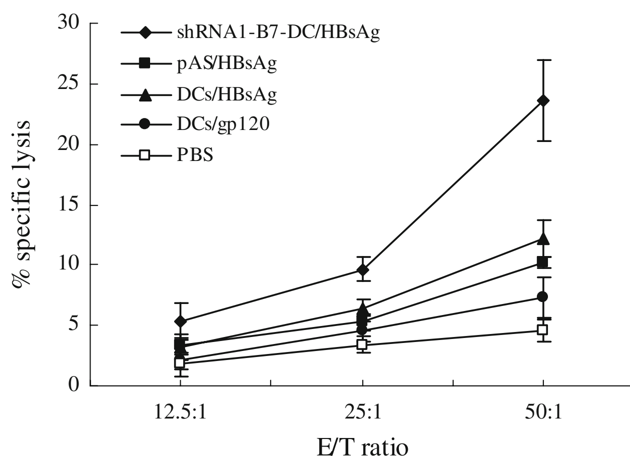

Fig. 3 Cytolytic activity against P815 cells pulsed with an HBV-specific peptide. Splenocytes were taken from mice 7 days after the final injection of DCs pulsed with HBV-specific peptide and transfected with shRNA1-B7-DC, DCs pulsed with HBV-specific peptide and transfected with vector plasmid pAS, DCs pulsed with HBV-specific peptide, DCs pulsed with gp120-specific peptide, or PBS. After the splenocytes were stimulated in vitro for 5 days, P815 cells pulsed with an HBV-specific peptide were used as target cells. Effector cell-target cell ratios are indicated on the abscissa. The percentage of specific lysis is shown on the vertical axis. Data are presented as the mean value of triplicate samples ± SD

Discussion

The use of RNAi is an excellent strategy for specific gene silencing, which was first discovered by Fire and his colleagues in 1998 [9]. Since its discovery, RNAi has rapidly evolved into a powerful tool for studying gene function and treating a variety of diseases. It can be induced in vitro, either by application of synthetic siRNA or by intracellular expression of siRNA or shRNA from vectors introduced by transfection. However, the use of chemically synthesized siRNA is limited by the fact that different sequences have greatly different inhibitory abilities. Furthermore, variable transfection efficiency is also a potential problem [6]. Consequently, plasmid and viral vectors containing shRNA expression cassettes have been developed to overcome these limitations. In the plasmid-based expression vector, the shRNA cassette, including the siRNA sequence and its reverse complement, are transcribed as a single RNA with a short loop of 4–10 bases in the middle. The transcript

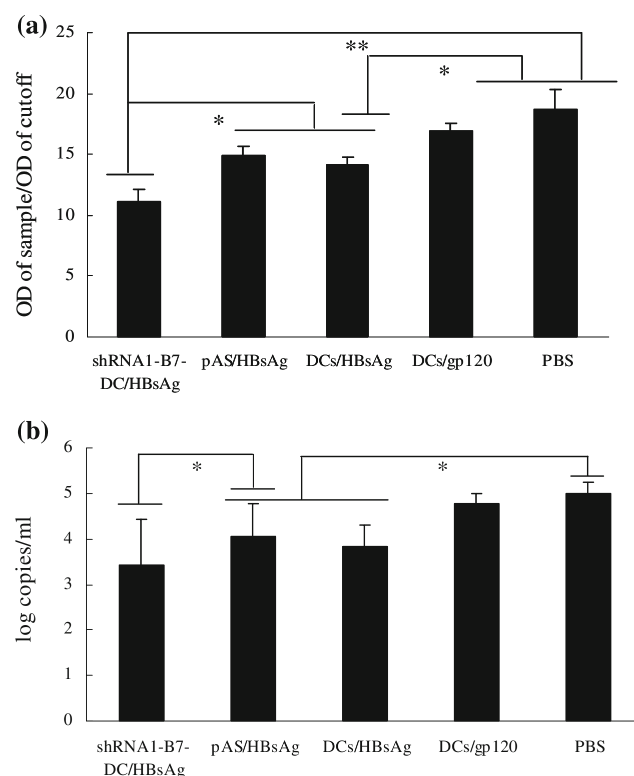

Fig. 4 Serum assay. **a** ELISA analysis of serum HBsAg level. Mice were immunized three times with peptide-pulsed DCs at 7-day intervals and killed 7 days after the final vaccination. The sera were isolated, and HBsAg was measured with an HBsAg ELISA kit according to the manufacturer's instructions. The results were expressed as the ratio OD value of sample/OD value of cutoff. **b** Titer of HBV DNA in sera. HBV DNA in sera was extracted and analyzed by a fluorescent quantitative PCR method using an HBV DNA FQ-PCR kit. The results were expressed as log of copies/ml. *$p < 0.05$, **$p < 0.01$

forms a hairpin structure that can be digested into a functional siRNA by Dicer upon export into the cytoplasm [7, 38]. In this study, we have examined the effects of a vector carrying shRNA targeting murine B7-DC in dendritic cells. Both of the designed shRNAs could act effectively.

It is widely accepted that efficient T cell activation requires not only the TCR-mediated Ag-specific signal but also a costimulatory signal provided by APCs [19]. The costimulatory signal is not Ag-specific. Many molecules on the surface of T lymphocytes may receive a costimulatory signal. The B7 family of molecules provides signals that are the most critical and best characterized in both stimulating and inhibiting T cell activation [8]. B7-1 (also called CD80) and B7-2 (also called CD86) expressed on APCs deliver a co-stimulatory signal to naive T cells via CD28, and a co-inhibitory signal via cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) expressed on activated T cells [20]. PD-1 is related to CD28 and CTLA-4 and it is induced on peripheral CD4+ and CD8+ cells, NK cells, B cells and monocytes, whereas the expression of other members of the CD28 family is restricted to T cells. PD-1-deficient mice develop a variety of autoimmune-like diseases and autoimmune dilated cardiomyopathy, suggesting that this immunoinhibitory receptor plays an important role in tolerance [24, 25]. B7-DC, which belongs to the B7 family, has been identified as the second ligand for PD-1. Similar to other B7 family members, B7-DC has a structural organization that consists of a signal sequence, IgV-like, IgC-like and transmembrane domains, and a short cytoplasmic tail. However, the function of B7-DC in T cell regulation is still being debated. Most studies have suggested that B7-DC serves as a negative regulator of T cell function. Latchman et al. [17] have shown that engagement of PD-1 by B7-DC dramatically inhibits TCR-mediated proliferation and cytokine production by CD4+ T cells. Seo et al. [30] reported that in the alloreactive T-T model, blockade of the B7-H1/PD-1 or B7-DC/PD-1 pathways significantly increased the proliferation and IFN-γ and IL-2 production of alloreative T cells, indicating that T-cell-associated B7-H1 and B7-DC negatively regulate the T-cell response via the T–T interaction. Zhang et al. [37] created a B7-DC-deficient mouse to characterize its function in T cell activation and tolerance. The data suggested that APCs from B7-DC-deficient mice were more potent in activation of T cells in vitro than the wild-type controls, which depended on PD-1. However, there have been reports that B7-DC is a positive costimulatory molecule that costimulates T-cell immunity under certain conditions via an unidentified co-stimulatory receptor other than PD-1. Liu et al. [21] demonstrated that expression of B7-DC on tumor cells promoted CD8 T-cell-mediated rejection of tumor cells; moreover, B7-DC bound to PD-1(−/−) cells and enhanced T cell killing in a PD-1-independent mechanism. In the current study, we found that blockade of B7-DC increased the allostimulatory activity of DCs, which showed that the suppression of B7-DC augmented the function of DCs.

Chronic HBV infections are leading causes of liver cirrhosis and hepatocellular carcinoma worldwide. Despite intensive clinical efforts, a limitation for the combination of interferon and ribavirin therapy exists [13]. A defect in specific T-cell immunity has long been assumed to be the central mechanism of persistent HBV infection [36], and increasing evidence has shown that high-functional-avidity CD8+ T cells can mediate effective immunity to viral infection [2, 34]. B7-DC-PD-1 interactions may be important for controlling antiviral CD8+ T cell responses. Chen et al. [5] reported that blockade of PD-1 ligands on imDCs resulted in enhanced T-cell proliferation, which is perhaps due to the enhancement of IL-2 production from DC-stimulated T cells. Peng et al. [26] demonstrated that PD-1 upregulation in HBV-specific CD8 T cells is involved in the dysfunction of T cells and high viraemia in chronic hepatitis B (CHB) patients, and the antiviral T cell responses could be improved by the blockade of this inhibitory PD-1/B7-H1 pathway. In this study, HBV transgenic mice were immunized with HBV-peptide-pulsed DCs, and the cytolytic activity of lymphocytes was evaluated. The data showed that DCs pulsed with an HBV-specific peptide could induce HBV-specific CTL responses. To address silencing of B7-DC in regulating anti-HBV immunity, shRNA1-B7-DC-transfected DCs were also pulsed with HBV-specific peptide and used to immunize HBV transgenic mice, and the cytolytic activity of lymphocytes was investigated. The data demonstrated that blockade of B7-DC could induce more potent CTL activities, which indicated that silencing the expression of B7-DC may promote the immune response elicited by a DC-based vaccine. The specific lysis rates are low, which is perhaps due to the method we used in the experiment. Compared to standard chromium-release assays, the LDH release assay has advantages in safety and cost, but its sensitivity may be lower.

HBV transgenic mice have been found to be a suitable animal model for the HBV-carrier state because they harbour HBV from the neonatal period and express HBV-related antigens in sera and liver throughout their life [1]. In the present study, HBV transgenic mice, which express a high level of HBsAg and have detectable HBV DNA in their sera, were used to evaluate the immune response elicited by peptide-pulsed DCs. The level of HBsAg and HBV DNA in the sera of mice immunized with HBV-peptide-pulsed DCs was reduced significantly compared with irrelevant peptide control or negative control. Moreover, blockade of B7-DC enhanced the effects, suggesting that blockade of B7-DC may promote the antiviral

immunity of DCs. Therefore, this study provides insight for future research using DC-based strategies for preventing and controlling HBV infection.

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References

1. Akbar SK, Onji M (1998) Hepatitis B virus (HBV)-transgenic mice as an investigative tool to study immunopathology during HBV infection. Int J Exp Pathol 79:279–291
2. Alexander-Miller MA, Leggatt GR, Berzofsky JA (1996) Selective expansion of high-or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc Natl Acad Sci USA 93:4102–4107
3. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–252
4. Bass BL (2000) Double-stranded RNA as a template for gene silencing. Cell 101:235–238
5. Chen C, Qu QX, Huang JA, Zhu YB, Ge Y, Wang Q, Zhang XG (2007) Expression of programmed-death receptor ligands 1 and 2 may contribute to the poor stimulatory potential of murine immature dendritic cells. Immunobiology 212:159–165
6. Chen Y, Cheng G, Mahato RI (2008) RNAi for treating hepatitis B viral infection. Pharm Res 25:72–86
7. Chen Y, Du D, Wu J, Chan CP, Tan Y, Kung HF, He ML (2003) Inhibition of hepatitis B virus replication by stably expressed shRNA. Biochem Biophys Res Commun 311:398–404
8. Coyle AJ, Gutierrez-Ramos JC (2001) The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. Nat Immunol 2:203–209
9. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811
10. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T (2000) Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192:1027–1034
11. Huang WS, Wang JP, Wang T, Fang JY, Lan P, Ma JP (2007) ShRNA-mediated gene silencing of β-catenin inhibits growth of human colon cancer cells. World J Gastroenterol 13:6581–6587
12. Inchauspé G, Michel ML (2007) Vaccines and immunotherapies against hepatitis B and hepatitis C. J Viral Hepat 14:97–103
13. Iwasaki Y, Ikeda H, Araki Y, Osawa T, Kita K, Ando M, Shimoe T, Takaguchi K, Hashimoto N, Kobatake T, Tomita M, Kawaguchi M, Kobashi H, Sakaguchi K, Shiratori Y (2005) Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. Hepatology 43:54–63
14. Jiang WZ, Jin NY, Li ZJ, Zhang LS, Wang HW, Zhang YJ, Han WY (2005) Expression and characterization of Gag protein of HIV-1(CN) in *Pichia pastoris*. J Virol Methods 123:35–40
15. Kanda T, Steele R, Ray R, Ray RB (2007) Small interfering RNA targeted to hepatitis C virus 5' nontranslated region exerts potent antiviral effect. J Virol 81:669–676
16. Kayhan H, Karatayli E, Turkyilmaz AR, Sahin F, Yurdaydin C, Bozdayi AM (2007) Inhibition of hepatitis B virus replication by shRNAs in stably HBV expressed HEPG2 2.2.15 cell lines. Arch Virol 152:871–879
17. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ (2001) PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2:261–268
18. Lavanchy D (2004) Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 11:97–107
19. Lenschow DJ, Walunas TL, Bluestone JA (1996) CD28/B7 system of T cell costimulation. Annu Rev Immunol 14:233–258
20. Linsley PS, Clark EA, Ledbetter JA (1990) T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc Natl Acad Sci USA 87:5031–5035
21. Liu X, Gao JX, Wen J, Yin L, Li O, Zuo T, Gajewski TF, Fu YX, Zheng P, Liu Y (2003) B7DC/PDL2 promotes tumor immunity by a PD-1-independent mechanism. J Exp Med 197:1721–1730
22. Ludewig B, Ehl S, Karrer U, Odermatt B, Hengartner H, Zinkernagel RM (1998) Dendritic cells efficiently induce protective antiviral immunity. J Virol 72:3812–3818
23. McIntyre GJ, Fanning GC (2006) Design and cloning strategies for constructing shRNA expression vectors. BMC Biotechnol 6:1
24. Nishimura H, Nose M, Hiai H, Minato N, Honjo T (1999) Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 11:141–151
25. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, Sasayama S, Mizoguchi A, Hiai H, Minato N, Honjo T (2001) Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 291:319–322
26. Peng G, Li S, Wu W, Tan X, Chen Y, Chen Z (2008) PD-1 upregulation is associated with HBV-specific T cell dysfunction in chronic hepatitis B patients. Mol Immunol 45:963–970
27. Rodig N, Ryan T, Allen JA, Pang H, Grabie N, Chernova T, Greenfield EA, Liang SC, Sharpe AH, Lichtman AH, Freeman GJ (2003) Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. Eur J Immunol 33:3117–3126
28. Saas P, Tiberghien P (2002) Dendritic cells: to where do they lead? Transplantation 73:S12–S15
29. Seibler J, Kleinridders A, Küter-Luks B, Niehaves S, Brüning JC, Schwenk F (2007) Reversible gene knockdown in mice using a tight, inducible shRNA expression system. Nucleic Acids Res 35:e54
30. Seo SK, Seo HM, Jeong HY, Choi IW, Park YM, Yagita H, Chen L, Choi IH (2006) Co-inhibitory role of T-cell-associated B7–H1 and B7-DC in the T-cell immune response. Immunol Lett 102:222–228
31. Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, Shalabi A, Shin T, Pardoll DM, Tsuchiya H (2001) B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. J Exp Med 193:839–846
32. Usharauli D (2005) Dendritic cells and the immunity/tolerance decision. Med Hypotheses 64:112–113
33. Webster GJ, Reignat S, Brown D, Ogg GS, Jones L, Seneviratne SL, Williams R, Dusheiko G, Bertoletti A (2004) Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 78:5707–5719
34. Yang S, Tsang KY, Schlom J (2005) Induction of higher-avidity human CTLs by vector-mediated enhanced costimulation of antigen-presenting cells. Clin Cancer Res 11:5603–5615
35. Yao V, Platell C, Hall JC (2002) Dendritic cells. ANZ J Surg 72:501–506

36. Zhang BJ, Zhou J, Qu D, Siu KL, Lam TW, Lo HY, Lee SS, Wen YM (2004) Selective functional deficit in dendritic cell-T cell interaction is a crucial mechanism in chronic hepatitis B virus infection. J Viral Hepat 11:217–224
37. Zhang Y, Chung Y, Bishop C, Daugherty B, Chute H, Holst P, Kurahara C, Lott F, Sun N, Welcher AA, Dong C (2006) Regulation of T cell activation and tolerance by PDL2. Proc Natl Acad Sci USA 103:11695–11700
38. Zhao J, Wei XL, Jia YS, Zheng JQ (2008) Silencing of herg gene by shRNA inhibits SH-SY5Y cell growth in vitro and in vivo. Eur J Pharmacol 579:50–57