Effects of T cell immunoglobulin and mucin domain-containing molecule-3 signaling molecule on human monocyte-derived dendritic cells with hepatitis B virus surface antigen stimulation *in vitro*

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Abstract. The aim of the present study was to investigate the *in vitro* effects of hepatitis B virus surface antigen (HBsAg) on the immune function of human monocyte-derived dendritic cells (MD-DCs), and the moderating role of T cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) signaling molecule. The monocytes, obtained from healthy adult peripheral blood, were incubated with recombinant human granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4 to induce DCs. DC-associated cell markers were detected using flow cytometry. MD-DCs were treated with HBsAg (5 μg/ml) *in vitro* for 48 h and subsequently, cell markers, lymphocyte stimulatory capacity, signaling protein and downstream cytokines were assessed. In addition, a Tim-3 monoclonal antibody was used to inhibit the Tim-3 signaling pathway, and subsequently the immune responses of MD-DCs to HBsAg stimulation were determined using the aforementioned method. The cell phenotype expressions of MD-DCs were all significantly increased with cluster of differentiation (CD)11c at 70.09±0.57%, human leukocyte antigen-DR at 79.83±2.12%, CD80 at 48.33±7.34% and CD86 at 44.21±5.35%. The treatment of MD-DCs with HBsAg resulted in a CD80 and CD86 enhanced expression, enhanced lymphocyte stimulatory capacity, upregulated expression of Tim-3 and nuclear factor-κB (NF-κB), as well as enhanced cytokine secretion of IL-6, IL-10 and interferon (IFN)-γ. However, a reduced immune response of MD-DCs in response to HBsAg stimulation was observed when the Tim-3 signaling pathway was inhibited prior to stimulation. The expression of NF-κB was decreased and the cytokine secretion level of IL-6, IL-10 and IFN-γ were downregulated. The treatment with HBsAg *in vitro* resulted in an enhanced immune response of MD-DCs, which may be positively regulated by the Tim-3 signaling molecule.

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Abbreviations: BSA, bovine serum albumin; CHB, chronic hepatitis B; DCs, dendritic cells; MD-DCs, monocyte derived DCs; HBV, hepatitis B virus; HBsAg, HBV surface antigen; IFN, interferon; NF-κB, nuclear factor κB; NK, nature killer cell; PBMCs, peripheral blood mononuclear cells; Th1, T helper 1 cells; TH17, T helper 17 cells; SI, stimulation index; Tim-3, T cell immunoglobulin and mucin domain-containing molecules-3.

Key words: dendritic cells, hepatitis B virus surface antigen, immune response, nuclear factor-κB, T cell immunoglobulin, mucin domain-containing molecules-3

Introduction

Hepatitis B virus (HBV) infection, an infection that can cause chronic liver disease, and which raises the risks of mortality from liver cirrhosis and cancer, is a major public health problem worldwide (1). It is estimated that ~2 billion individuals worldwide exhibited serological evidence of current or past HBV infection, and ~350,000,000 individuals are suffering from chronic HBV infection (2). As a non-cytopathic virus, HBV fails to directly destroy the host cells, however, it could influence disease progression and prognosis by activating the innate or adaptive immune response (3). Dendritic cells (DCs), which are powerful antigen-presenting cells *in vivo*, have been shown to form a critical interface between the innate and adaptive immune response (4), and serve as important components of the regulation of immune responses (5). DC dysfunction in patients with chronic HBV (CHB), which can cause inefficient antigen presenting and inadequate special antiviral immune response, was an important reason for persistent infection of HBV (6). In addition, previous studies have suggested that the significantly lower frequency of myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in the umbilical cord blood, compared with that in the peripheral blood of healthy adults, can comprise the mechanism of easily-acquired chronicity among newborns following HBV infection (7).

Large quantities of HBV particles and viral proteins, including the hepatitis B virus surface antigen (HBsAg), were identified in the peripheral blood of HBV-infected

patients. The HBsAg can accumulate up to concentrations of 100 μg/ml (8). In acute HBV infection, the cellular and humoral immune response induced by HBsAg may be important in the control of HBV replication and life cycle (9). In addition, it has previously been suggested that HBsAg can alter the immune response, which may contribute to the establishment of chronic infection; Wang *et al* (10) demonstrated an association between HBsAg and decreased cytokine production, in particular that of interleukin (IL)-12, which may have been induced via the toll-like receptor 2 ligand in peripheral blood mononuclear cells (PBMCs) from patients with CHB. These findings can lead to the understanding of the mechanism through which HBV evades host immunity. It has also been demonstrated that HBsAg may directly interfere with pDC function through the HBsAg-mediated upregulated expression of the suppressor of cytokine signaling 1 and blood DC antigen-2 ligation (11,12).

T cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3), a member of the Tim family, was found to be involved in the pathogenesis of numerous diseases, including virus infections, cancer, autoimmune diseases, and also allergic reactions and transplantation rejection (13). Khademi *et al* (14) first reported the high mRNA expression levels of Tim-3 in human T helper 1 cells (Th1) *in vitro*, therefore Tim-3 was initially considered as a special membrane protein expressed on the Th1 cells (15); however, it was revealed that Tim-3 was also expressed on other cell types, including cluster of differentiation (CD)8+T, Th17, nature killer (NK), mast, endothelial and regulatory cells, as well as monocytes, macrophages, DCs and neurogliocytes (13). A previous study showed that Tim-3 was also expressed in tumor cells and could serve as an independent prognostic factor for cancer (16).

In the present study, human monocyte-derived DCs (MD-DCs) from normal adult peripheral blood were selected in order to investigate the effects of HBsAg on the immune function of MD-DCs *in vitro*, and the possible role of the Tim-3 signaling molecule in the regulation of MD-DCs.

Materials and methods

Generation of MD-DCs. DCs were cultured, as previously described with slight modifications (17). The PBMCs were obtained from healthy adult donors by centrifugation with Ficoll-Paque (Sigma-Aldrich, St. Louis, MO, USA). Written informed consent was obtained from all donors prior to blood donation. The present study was approved by the ethics committee at the Affiliated Taizhou Hospital of Wenzhou Medical University (Linhai, China). The cells were cultured in RPMI-1640 medium in 6-well plates for 2 h at 37˚C. Non-adherent cells were removed by gently swirling the plate and adherent cells (monocytes) were subsequently cultured in RPMI-1640 medium, supplemented with 20% heat-inactivated fetal bovine serum (HyClone™; GE Healthcare Life Sciences, Logan, UT, USA) in 6-well plates with recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; 750 U/ml; R&D Systems, Inc., Minneapolis, MN, USA) and rhIL-4 (800 U/ml; R&D Systems, Inc.). Fresh medium containing GM-CSF and IL-4 was added every 3 days. Phosphate-buffered saline (PBS) was used to replace GM-CSF and IL-4 in the control group.

Flow cytometry. MD-DCs were collected in test tubes, incubated in PBS and stained with fluorescein isothiocyanate (FITC)-phycoerythrin (PE)-allophycocyanin (APC)- or peridinin-chlorophyll-protein (PerCP)-labeled monoclonal antibodies (anti-human CD80, CD86, CD11c and human leukocyte antigen (HLA)-DR, or the relevant isotype control; eBioscience Inc., San Diego, CA, USA), according to the manufacturer's protocol. Following incubation at room temperature in the dark for 15 min, the cells were washed twice with PBS buffer and analyzed using a FACSort cell analyzer (FACSCalibur; Becton-Dickinson, San Jose, CA, USA). The flow cytometry data was analyzed using the FlowJo software, version 7.6.5 (FLOWJO, LLC, Ashland, Oregon, USA).

Treatment of MD-DCs with HBsAg. HBsAg (5 μg/ml; ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel) was added to the medium of MD-DCs on day 7 following culture, while the control group was treated with 0.2% bovine serum albumin (BSA). The cells were treated for 48 h.

Inhibition of the Tim-3 signaling pathway. Initially, the Ultra-Leaf purified anti-human Tim-3 (10 μg/ml; BioLegend, San Diego, CA, USA) was added to the medium of MD-DCs on day 7 following culture to inhibit the Tim-3 signaling pathway, while the control group was treated with BSA (0.2%). After 1 h, HBsAg (5 μg/ml) was added to the medium for 48 h.

Mixed leukocyte reaction. MD-DCs treated with mitomycin C (25 μg/ml) for 30 min, were used as the stimulating cells. Allogenic lymphocytes, which were obtained from other healthy adult donors, as previously described, were distributed at in 96-well plates a density of $1x10^5$ cells/well and were incubated for 96 h in certain ratio of stimulating cells (DC/lymphocytes = 1/5 or 1/10). MTS-phenazine methosulfate (20 μl/well; Promega Corp., Madison, USA) was added and incubated for 4 h at 37˚C, after which the absorbance (A) at 450 nm was determined using a Microplate Reader (CLARIOstar®; BMG LABTECH GmbH, Ortenberg, Germany). The stimulation index (SI) was calculated as follows: $SI = (A_{experimental} - A_{background}) / (A_{control} - A_{background})$ (18).

Western blotting. The proteins from the MD-DCs were collected through progressions of pyrolysis and extraction, and the total protein concentration was determined using a bicinchoninic acid protein detection kit (Sigma-Aldrich). The total protein (40 μg) was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sigma-Aldrich), and the proteins were transferred onto polyvinylidene difluoride membranes (Sigma-Aldrich). The membranes were first incubated for 2 h at room temperature with 1% BSA in PBS, and then overnight at 4˚C with rabbit anti-human Tim-3 polyclonal antibody (dilution, 1:1,000; cat. no. 3808-100; BioVision, Inc., Milpitas CA, USA), and anti-NF-κB (dilution, 1:1,000; cat. no. 1559-1; Epitomics, Inc., Burlingame, CA, USA), and anti-GAPDH monoclonal (dilution, 1:1,000; cat. no. 5632-1; Epitomics, Inc.) antibodies. For signal detection, horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin G (dilution, 1:10,000; cat. no. 5618-1; Epitomics, Inc.) and the enhanced chemiluminescence detection system (Cell Signaling

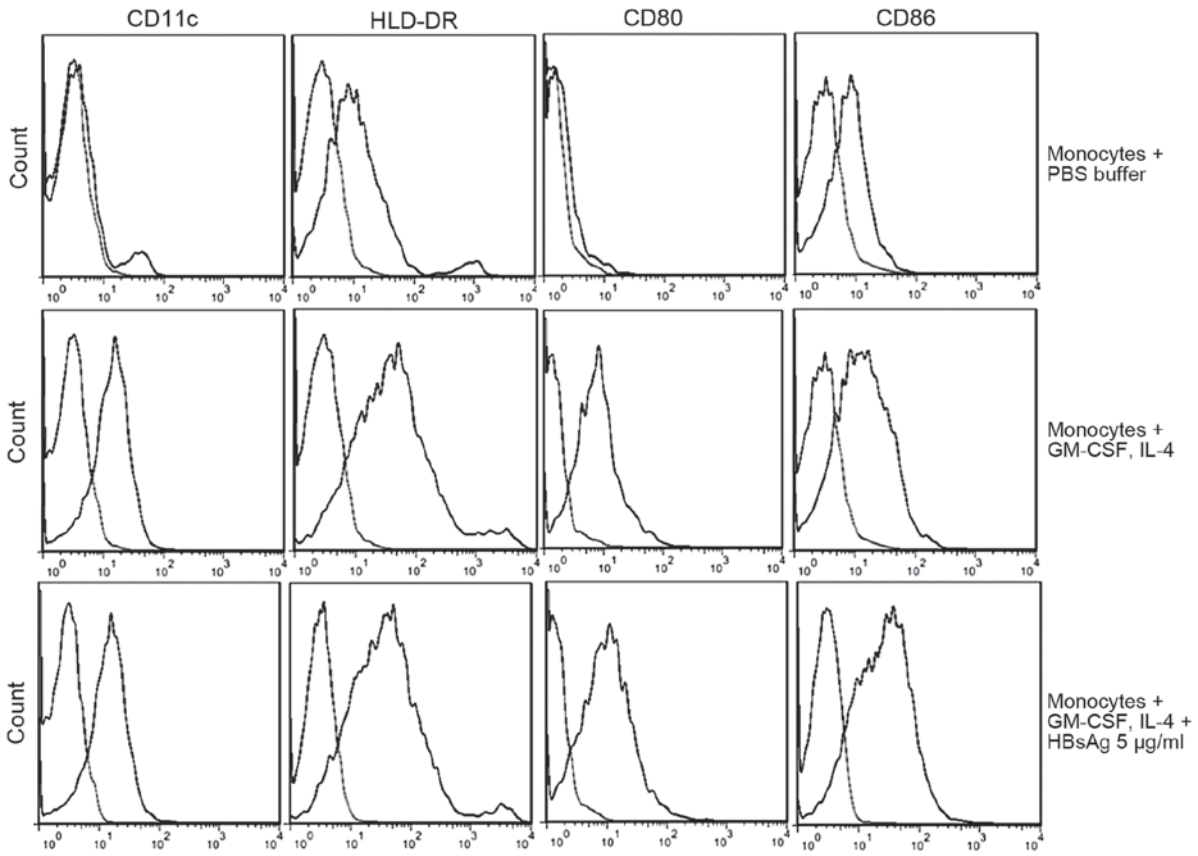

Figure 1. Phenotypic analysis of human monocyte-derived dendritic cells. The phenotypic expression values of monocytes cultured with PBS buffer were as follows: CD11c (9.41±6.38%), HLA-DR (26.13±6.61%), CD80 (3.46±2.00%) and CD86 (6.70±3.21%). The expression of monocytes cultured with GM-CSF and IL-4 was significantly increased (P<0.01): CD11c (70.09±0.57%), HLA-DR (79.83±2.12%), CD80 (48.33±7.34%) and CD86 (44.21±5.35%). In addition, HBsAg treatment with GM-CSF and IL-4, increased the expression of CD80 (t=3.75; P<0.05) and CD86 (t=7.61; P<0.01). The cell phenotypic expression values were as follows: CD11c (69.71±7.42%), HLA-DR (81.60±8.79%), CD80 (68.87±6.01%), CD86 (75.48±4.70%). The statistical differences between the groups were determined using Student's t-test. The graphs were generated using FlowJo software, version 7.6.5. The light regions on the graphs represent the control samples. GM-CSF, granulocyte-macrophage colony-stimulating factor, HLA, human leukocyte antigen; CD, cluster of differentiation; HBsAg, hepatitis B virus surface antigen; IL, interleukin; PBS, phosphate-buffered saline.

Technology, Inc., Danvers, MA, USA) were used. According to the gray value ratio of target protein against GAPDH, the relative expression of the target protein was calculated using the Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), as demonstrated in a previous study (19).

Enzyme-linked immunosorbent assay (ELISA). The concentrations of inflammatory cytokines in the supernatant were determined using the following ELISA kits: Human IL-6 Quantikine ELISA kit, Human IL-10 Quantikine ELISA kit and Human IFN-γ Quantikine ELISA kit (R&D Systems, Inc.). The experiments were performed according to the manufacturer's protocol.

Statistical analysis. Continuous variables are expressed as the mean ± standard deviation. Student's t-test was used to determine the statistical differences between two groups, one-way analysis of variance for multiple groups and the least significant difference t-test was used for multiple comparisons. Statistical analyses were conducted using the GraphPad Prism software, version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

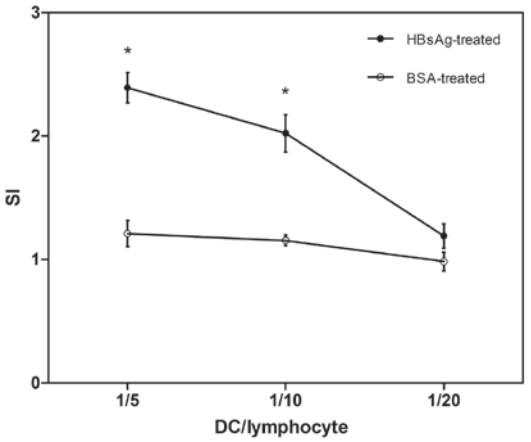

Figure 2. Lymphocyte stimulatory capacity of MD-DCs. A higher SI represented a higher lymphocyte stimulatory capacity. At a 1/5 ratio of DC/lymphocytes, the SI of BSA-treated MD-DCs was 1.21±0.10, while that of HBsAg-treated MD-DCs was 2.39±0.12. *In vitro* HBsAg stimulation enhanced the lymphocyte stimulatory capacity of MD-DCs (t=14.71; *P<0.01). Similarly, the capacity of MD-DCs was enhanced at the 1/10 ratio of DC/lymphocytes (t=11.12; *P<0.01). The SI difference revealed no statistically significant difference when the ratio of DC/lymphocytes was 1/20. Statistical differences between the groups were determined using Student's t-test. Data are presented as the mean ± standard deviation. SI, stimulation index; MD-DCs, human monocyte-derived dendritic cells; HBsAg, hepatitis B virus surface antigen; BSA, bovine serum albumin

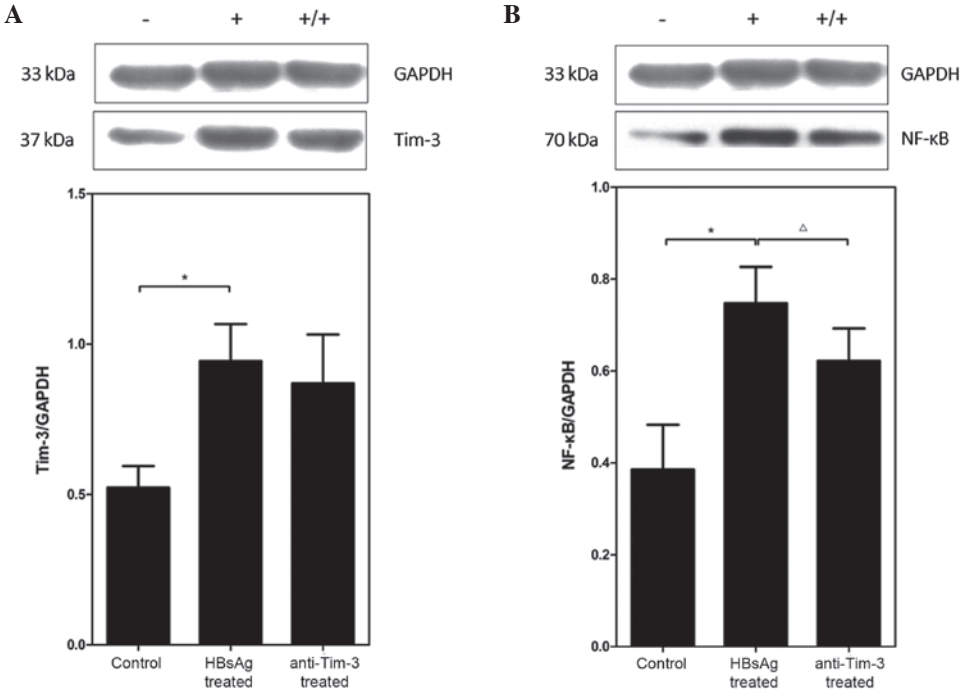

Figure 3. Expression of signaling proteins. The expression of (A) Tim-3 and (B) NF-κB was determined in the three groups. Following HBsAg stimulation *in vitro*, the expression of the signaling proteins Tim-3 and NF-κB was significantly increased in MD-DCs, while a decreased expression of NF-κB was observed when the Tim-3 signaling pathway was inhibited with anti-Tim-3 prior to HBsAg stimulation. The expression of Tim-3 in the MD-DCs revealed no significant decrease when treated with anti-Tim-3, since it was the antagonist of Tim-3 signaling molecule. ($^{\Delta}$P<0.05; *P<0.01). Statistical differences between the groups were determined using one-way analysis of variance and the least significant difference t-test. Data are presented as the mean ± standard deviation. NF-κB, nuclear factor-κB; Tim-3, T cell immunoglobulin and mucin domain-containing molecules-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBsAg, hepatitis B virus surface antigen; MD-DCs, human monocyte-derived dendritic cells; BSA, bovine serum albumin.

Results

Identification of MD-DCs. Following 7 days of incubation with RPMI-1640 medium containing rhGM-CSF and rhIL-4, the majority of the cells aggregated to form suspended colonies, which were subsequently observed using an optical microscope. Electron microscopy revealed that the morphology of the MD-DCs was irregular, with burr cells observed on the surface. These findings were consistent with the typical DC morphology (20). The expression of DC-associated cell phenotypes was revealed to be significantly increased with CD11c 70.09±0.57%, HLA-DR 79.83±2.12%, CD80 48.33±7.34% and CD86 44.21±5.35% (P<0.01; Fig. 1).

Effects of HBsAg on MD-DCs. Compared with the control group, HBsAg treatment resulted in an enhanced expression of co-stimulatory molecules (P<0.05) with CD80 (68.87±6.01%) and CD86 (75.48±4.70%). The changes in the cell phenotype expression of CD11c and HLA-DR revealed no statistically significant difference (P>0.05; Fig. 2). A higher stimulation index (SI) represented a more marked lymphocyte stimulatory capacity. The SI of HBsAg-treated MD-DCs was higher compared with the control BSA treated group, with a 1/5 or 1/10 ratio of DC/lymphocytes (P<0.01; Fig. 2). Treatment of the MD-DCs with HBsAg resulted in the upregulation of NF-κB (t=7.55, P<0.01; Fig. 3B), as well as an enhancement in the cytokine secretion levels of IL-6 (t=6.05, P<0.01), IL-10 (t=7.02, P<0.01) and interferon (IFN)-γ (t=11.69, P<0.01; Fig. 4).

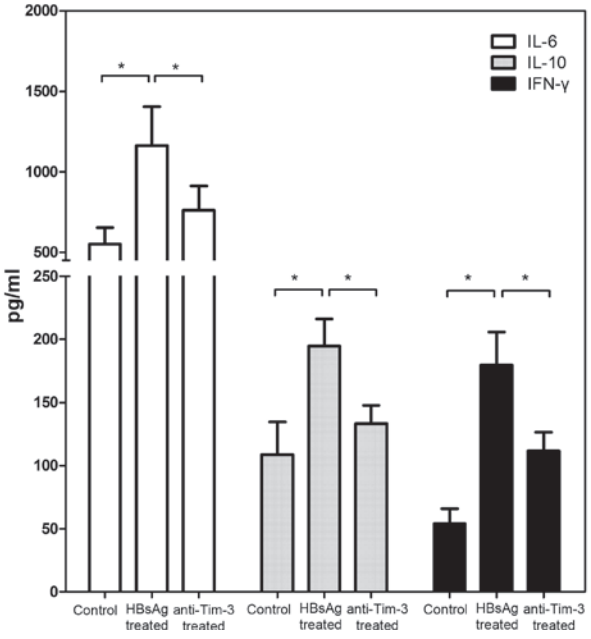

Figure 4. Cytokine secretion levels. Following HBsAg stimulation, *in vitro*, levels of the downstream cytokines, IL-6, IL-10 and IFN-γ, were significantly increased, which indicated an enhanced immune response of MD-DCs to HBsAg stimulation. This immune response, was reduced following inhibition of the Tim-3 signaling pathway, using anti-Tim-3 prior to HBsAg stimulation. The secretion of IL-6, IL-10 and IFN-γ cytokines was significantly downregulated. (*P<0.01). Statistical differences between the groups were determined using one-way analysis of variance and the least significant difference t-test. Data are presented as the mean ± standard deviation. HBsAg, hepatitis B virus surface antigen; IL, interleukin; IFN-γ, interferon-γ; MD-DCs, human monocyte-derived dendritic cells; Tim-3, T cell immunoglobulin and mucin domain-containing molecules-3; BSA, bovine serum albumin.

Role of Tim-3 in the regulation of MD-DCs. HBsAg stimulation *in vitro* increased the expression of Tim-3 (t=5.83, P<0.01) in MD-DCs (Fig. 3A) and enhanced the immune function of MD-DCs; however, reduced the immune response of MD-DCs to HBsAg stimulation was observed when the Tim-3 signaling pathway was inhibited prior to treatment. The NF-κB expression was revealed to be significantly decreased (t=2.61, P<0.05) and the cytokine secretion level of IL-6 (t=3.99, P<0.01), IL-10 (t=5.03, P<0.01) and IFN-γ (t=6.30, P<0.01) were significantly downregulated (Fig. 4).

Discussion

The present study demonstrated that the HBsAg enhanced the immune response of MD-DCs *in vitro*. Previous studies have revealed large quantities of HBV particles and viral proteins in the peripheral blood of HBV-infected patients, which enabled multiple interactions among the virus, its viral proteins and the immunocytes (21). HBsAg enhanced the innate or adaptive immune response found in the mice bone marrow-derived DCs treated with HBsAg *in vitro*, as well as affect the differentiation of T helper cells (22). Different forms of HBsAg elicited various types of B and T cell immunity (23), and both HBV DNA vaccine and HBsAg induced the humoral immunity (24). In the present study, MD-DCs were treated with HBsAg *in vitro*, which resulted in the enhanced expression of co-stimulatory molecules and the lymphocyte stimulatory capacity of MD-DCs, as well as the upregulated secretion of inflammatory cytokines. Consistent with the present study, Jan *et al* (19) also used the MD-DCs from healthy adult donors and reported that HBsAg increases the expression of CD80, CD83, CD86 and major histocompatibility complex class II of MD-DCs. This treatment also increases the production of interleukin IL-12 and IL-10, as well as enhances T cell-stimulatory capacity. In addition, treated with different concentrations of HBsAg, the immature DCs, which were cultured from PBMCs of patients with CHB, differentiate into mature DCs (25); and DCs stimulated with HBsAg more efficiently presented antigen and induced a specific T cell immune response (26). However, Op den Brouw *et al* (21) reported that both HBV particles and purified HBsAg had an immune modulatory capacity and directly contributed to the dysfunction of mDCs in patients with chronic HBV; since the mDCs were directly isolated from PBMCs, the differences between circulating mDCs and MD-DCs in immune function, as well as the exact molecular association between the HBsAg and DCs, remained unclear.

In the present study, *in vitro* HBsAg stimulation increased the expression of the Tim-3 signal molecule and enhanced the immune function of MD-DCs. Tim-3 was expressed in a variety of immune cells, exerts an important role in regulating acquired or innate immune responses, and was revealed to be closely associated with the pathogenesis of infection, cancer and other diseases (27). The interaction between Tim-3 and its natural ligand, galectin-9, induces intracellular calcium efflux, resulting in Th1 cell aggregation and apoptosis, followed by immune tolerance and inhibition of acquired immune response (15). In the innate immune response, Tim-3 acts as a promoter or inhibitor through its interaction with galectin-9 or other ligands. Anderson *et al* (28) reported that Tim-3, expressed on antigen presenting cells, cooperates with Toll-like receptors to promote the secretion of proinflammatory cytokines. Kanzaki *et al* (29) reported that the galectin-9-Tim-3 signaling pathway induces TNF-α production in cultured DCs in a dose-dependent manner; however, Chiba *et al* (30) revealed that the high expression of Tim-3 on tumor-associated DCs inhibited the immune response mediated by nucleic acid. Therefore, different immune environments may lead to different roles of Tim-3-mediated immune response.

The Tim-3 signal molecule may positively regulate the immune response of MD-DCs. As described in the present study, a reduced immune response of MD-DCs to HBsAg stimulation was observed when the Tim-3 signaling pathway was inhibited in advance; however, this conclusion was not consistent with previously published results. Ju *et al* (31) found that the inhibition of the Tim-3 signaling pathway with anti-Tim-3 antibodies or Tim-3-Fc fusion proteins resulted in an increased cytotoxicity for NK92 cells and an elevated IFN-γ production. The increased expression of Tim-3 on PBMCs, circulating NK and CD8+T cells in patients with CHB led to immune response dysfunction. In addition, partial restoration of immune function was observed when the Tim-3 signaling pathway was inhibited prior to treatment (31,32). The opposite regulatory effects of Tim-3 may be attributed to the relatively different immune environment between MD-DCs with simple HBsAg stimulation and patients with chronic HBV infection; however, the exact mechanism remains to be elucidated.

In conclusion, *in vitro* HBsAg treatment resulted in an enhanced immune response of MD-DCs, which may be positively regulated by Tim-3. However, further study is required in order to determine the regulatory role of Tim-3 on the immune function of DCs in the intracorporeal immune environment of patients with CHB and the exact mechanism of Tim-3-mediated regulation.

Acknowledgments

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