Original Article
High-dose hepatitis B E antigen drives mouse bone marrow-derived dendritic cells to differentiate into regulatory dendritic cells in vitro


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Received October 2, 2015; Accepted November 22, 2015; Epub May 1, 2016; Published May 15, 2016

Abstract: Dendritic cells (DCs) from patients with chronic hepatitis B virus (HBV) infection are functionally deficient, giving rise to more tolerogenic rather than immunogenic responses, which may conduce to viral persistence. Tumor microenvironment can educate DCs to differentiate into regulatory DCs (DCregs), by which tumor cells escape immunity. We wondered whether HBV could induce the similar DCregs, contributing to viral immune evasion. HBeAg is required for the establishment of chronic infection. However, few studies explored the effect of HBeAg or HBcAg on DCs. Additionally, Th1/Th2 imbalance is another important factor of chronic HBV infection. p38 mitogen-activated protein kinase (p38MAPK) signaling pathway is a positive regulator of interleukin 12 (IL-12), whereas phosphoinositide kinase-3 (PI3K)-Akt signaling pathway suppresses p38 activity. Therefore, the present study attempted to investigate functional changes of mouse bone marrow-derived dendritic cells (BMDCs) under the stimulus of HBeAg or HBcAg and explore the effect of PI3K-Akt and p38MAPK signaling pathway in vitro. We found high-dose HBeAg (5 μg/ml) caused high IL-10 secretion but low IL-12 secretion (significantly reduced IL-12/IL-10 ratio) in DCs through PI3K-Akt signaling pathway, which was probably achieved by inhibiting p38 activation. On the other hand, high-dose HBeAg effectively reduced antigen-specific T-cell stimulatory capacity of DCs. The regulatory function of high-dose HBeAg-induced DCs mainly depended on IDO which was positively regulated by PI3K-Akt signaling pathway. Thus, high-dose HBeAg may propel DCs to differentiate into DCregs with reduced antigen-specific T-cell stimulatory capacity and imbalance of Th1/Th2 cytokines, which in turn facilitate persistent HBV infection.

Keywords: Chronic hepatitis B, dendritic cells, HbeAg, HbcAg, regulatory dendritic cells

Introduction

Being a major cause of cirrhosis and hepatocellular carcinoma, hepatitis B virus (HBV) causes more than 350 million people with chronic infection worldwide. Due to an ineffective antiviral immune response towards the virus, individuals, persistently infected with high viral loads, always develop a prolonged immunotolerant to hepatitis B virus [1-3]. It has recently been supposed that through evasion of the innate immune system and subsequent preventing maturation of the adaptive immune system, HBV establishes chronic infection [4, 5]. In this phase, the HBV-specific CD8+ cytotoxic T lymphocytes (CTLs) and B-cells responses are characteristically weak, transient and undetectable [6-9]. Additionally, Th1/Th2 imbalance is one of important factors of chronic HBV infection. The exact mechanism by which HBV escapes immunity is still not known.

Dendritic cells (DCs) play a central role in antiviral immunity and have unique capacity to bridge innate and adaptive immunity [10]. It has also been described that DCs contribute significant
polarizing influences on T helper cell differentiation and regulate the Th1/Th2 balance in vivo [11]. However, DCs may be responsible not only for priming but also for tolerance. Studies have confirmed that the stromal microenvironment of the spleen and liver can educate DCs or hemopoietic progenitors to differentiate into regulatory DCs (DCregs) with high secretion of interleukin 10 (IL-10), TGF-β and IDO, but less IL-12 [12-14]. Thus, we wondered if there were also regulatory DC subsets involved in the tolerance induction during the establishment of chronic HBV infection.

Hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg) are two structural forms of nucleoproteins existing in the hepatitis B virus. They are translated from 2 distinct RNA species that are sharing an open reading frame and just have different 5’ initiation sites [15, 16]. CD8+ CTLs and CD4+ T-cells can cross-reactively target and recognize both HBcAg and cytosolic HBeAg [17]. However, HBeAg is thought to associate directly with, and probably response for immunomodulation of host immune responses during chronic HBV infection via an unknown mechanism, although it is not required for viral assembly, infection, or replication [18]. Ashley Mansell and co-workers recently observed that HBeAg suppresses the activation of the Toll-like receptor (TLR) signaling pathway in HuH7, HEK293, and HEK293T cells [1]. Milich et al. showed that HBeAg is a superior T-cell tolerogen compared with the intracellular HBcAg [18]. As one of the most important members of innate immune system, dendritic cells have been intensively studied aiming at exploring the mechanism of chronic HBV infection. But the effect of HBeAg or HBcAg on DCs during chronic HBV infection should remain be clarified.

To date, some evidence show that p38 mitogen-activated protein kinase (p38MAPK) signaling pathway positively regulates the secretion of IL-12 by DCs [19, 20]. In contrast, phosphoinositide kinase-3 (PI3K)-Akt signaling pathway suppresses p38 activity [21]. Accordingly, in the present study, we aimed to study the mechanism of CHB through investigating functional changes of mouse bone marrow-derived dendritic cells (BMDCs) under the stimulus of HBeAg or HBcAg and exploring the effect of PI3K-Akt and p38MAPK signaling pathway in vitro.

**Materials and methods**

**Mice**

Seven-week-old male C57BL/6 and BALB/c mice were purchased from Shanghai Slac Laboratory Animal Center, Chinese Academy of Science (Shanghai, China). Two kinds of mice were fed separately in polycarbonate cages in a temperature-controlled room (23±1°C) with a 12 h light/dark cycle in a pathogen-free animal housing facility at Wenzhou Medical University. All animals received humane care, and study protocols were in compliance with the institution's guidelines. The animal experimental board of Wenzhou Medical University approved the study.

**Isolation of bone marrow-derived dendritic cells and splenic T lymphocytes**

The C57BL/6 mice were used to isolate bone marrow cells from femora and tibiae. Cells were cultured in 6-well low-adherence plates (Costar, Corning, NY) in RPMI 1640 (Gibco Invitrogen, Carlsbad, CA, USA) complete medium containing 10% heat-inactivated fetal calf serum (Gibco Invitrogen, Carlsbad, CA, USA), 1% penicillin/streptomycin, recombinant murine granulocyte macrophage colony-stimulating factor (rmGM-CSF) and rmIL-4 (10 ng/mL and 2 ng/mL respectively; PeproTec, London, United Kingdom) at 37°C, 5% CO₂. Half of the medium was removed and replaced with fresh medium every other day, the cells continued to be cultured on at least 5 days without passage. Then, all cells were purified using CD11c+ microbeads of a commercial DC isolation kit (Miltenyi Biotec, Germany) as bone marrow-derived dendritic cells (BMDCs). Flow cytometry analysis demonstrated all sorted cells were of purity above 90% and met the requirement for further experiments. The T lymphocytes from BALB/c or C57BL/6 mice spleen single-cell suspension were harvested by using nylon wool columns and CD4+ T cells isolation kit (Miltenyi Biotec, Germany), according to the manufacturer’s instructions. Cells were also cultured in 6-well low-adherence plates in RPMI 1640 complete medium without rmGM-CSF and rmIL-4.

**Stimulating BMDCs with HBcAg or HBeAg in vitro**

HBcAg and HBeAg were purchased from Beijing Kewei clinical diagnostic reagents Co. Ltd.,
Beijing, China. According to different interventions, all the purified BMDCs were classified into five groups randomly. The low HBcAg intervention (lo-HBcAg) group and high HBcAg intervention (hi-HBcAg) group cells were co-cultured with HBcAg in low (50 ng/ml) and high (5 μg/ml) concentrations for 24 hours, respectively, whereas the low HBeAg intervention (lo-HBeAg) group and high HBeAg intervention (hi-HBeAg) group cells were incubated for 24 hours in the presence of two different concentrations with HBeAg, respectively. As a control group, an equivalent amount of RPMI 1640 complete medium containing 50 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich, USA) was added to the cell-culture medium to generate mature DCs. Additionally, a PI3K-specific inhibitor LY294002 (100 nM, Beyotime Institute of Biotechnology, Haimen, China) was added to the hi-HBeAg group for 1 hour before adding the stimuli, in order to study the signaling pathways.

Cytokines production

The concentrations of IL-12 and IL-10 in the culture supernatants were measured with ELISA kits (R&D Systems, Minneapolis, MN). Absorbance was measured on an automatic plate reader. The sensitivity of the assays was 10 pg/ml.

T-cell stimulatory capacity of DCs

T-cell stimulatory capacity of DCs was determined in an allogeneic mixed lymphocyte reaction (MLR). BALB/c spleen T lymphocytes were used as responding cells, whereas the stimulation cells were DCs coming from five groups. All DCs were pretreated with 25 mg/L mitomycin for inactivation. And then co-cultured with T lymphocytes in 96-well U-bottomed plates (Costar, Corning, NY) at the ratio of 1:5, 1:10 and 1:20 for 96 hours. 20 μl CCK-8 (Beyotime Institute of Biotechnology, Haimen, China) was added to each well for 4 hours. Simultaneously, the simple BALB/c spleen T lymphocytes cultivation regarded as the negative control. The data were expressed as a stimulation index. The level of proliferation in control culture was considered to be background proliferation and expressed as a stimulation index of 1.0. Additionally, we optimized the protocol for assessment of antigen-specific T-cell proliferation with slight modifications [14]. T cells (1×10^5) from C57BL/6 mice were cultured with LPS (50 ng/ml)-loaded DCs (control group), HBcAg (50 ng/ml)-loaded DCs (lo-HBcAg group) and HBeAg (50 ng/ml)-loaded DCs (lo-HBeAg group) (1×10^4) at ratio of 1:10, respectively. Then the high concentration HBeAg-treated DCs (hi-HBeAg-DCs) were added to each DC/T coculture system (final DC/T ratio was 1:5) to assess antigen-specific T-cell proliferation. The coculture supernatants were collected for further cytokines detection including TGF-β (R&D Systems, Minneapolis, MN) and IDO (Antibodies-online GmbH, Atlanta, GA). In some experiments, blocking reagent such as the indoleamine dioxygenase inhibitor 1-methyltryptophan (1-MT, Sigma-Aldrich, St Louis, MO) and neutralizing antibodies for IL-10 and TGF-β were used.

Western blot analysis

The phosphorylation levels of Akt and p38 in control group, hi-HBeAg group and LY294002+hi-HBeAg group were determined by Western blot using standard protocols. The primary antibodies were: (1) rabbit anti-Akt (Abcam, UK, 1:1000), (2) rabbit anti-p-Akt (Cell Signaling, USA, 1:1000), (3) rabbit anti-p38 (Abcam, UK, 1:1000), rabbit anti-p-p38 (Abcam, UK, 1:1000). A horse peroxidase (HPR)-conjugated secondary goat anti-rabbit antibody (Biosharp, China) was used. Visualization was achieved by enhanced chemiluminescence (ECL). Finally, the pixel density was calculated with Gel-Pro analyzer version 4.0 software.

Statistical analysis

Data are shown as a mean ± SD of 3 or more independent experiments. Statistical analysis for comparison of different groups was performed using the Student t test or one-way ANOVA followed by post-hoc tests (using Least Significant Difference test, LSD-t) where appropriate. Each P<0.05 was considered significant. Statistical calculations were performed using SPSS (version 17.0) statistical computer program.

Results

Both HBcAg and low-dose HBeAg induce high IL-12 secretion in DCs, but high-dose HBeAg causes high IL-10 secretion in DCs

To study the effect of HBcAg and HBeAg on the cytokine expression profile of murine DCs, levels of IL-12 p70 and IL-10 were determined by
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**Figure 1.** Effects of HBCAg and HBeAg on the productions of IL-12 and IL-10 of DCs. A, B. Expressions of IL-12p70 and IL-10 by DCs of each group were measured by ELISA. C. The expressions of IL-12p70 and IL-10 by DC were used for calculating the IL-12/IL-10 ratio. Each column represents the mean ± SD of five independent experiments. Statistical significance was calculated by t-test (*P<0.05, **P<0.01 versus control group; #P<0.01 versus lo-HBeAg and hi-HBCAg groups).

**Figure 2.** T-cell stimulatory capability of DCs in each group. The T-cell stimulatory capacity of DCs was determined in an allogeneic MLR by incubating DCs obtained under different culture conditions with T lymphocytes at the indicated ratios. The levels of T cell proliferation are shown as the stimulation index (SI). Data of five separate experiments are shown, with means and standard deviations. **P<0.01 compared to other four corresponding groups.

IL-10 compared to that of other two groups (**Figure 1A, 1B**). Then, we analyzed the IL-12/IL-10 ratio of each experiment of all groups, and found that the ratios of HBCAg groups were increased dose-dependently, but high-dose of HBeAg significantly reduced the IL-12/IL-10 ratio compared to other groups (**Figure 1C**). Interestingly, this implied that since the concentration changed from low to high, the effect of HBeAg, but not HBCAg, on cytokines production of DCs was almost adverse.

HBCAg and low-dose HBeAg-treated DCs effectively prime T cell proliferation, whereas high-dose HBeAg-induced DCs have opposite effect via an antigen-specific manner.

ELISA after 24 h stimulation in the presence or absence of two different doses of HBCAg or HBeAg. We observed that the DCs of lo-HBCAg and lo-HBeAg groups secreted higher levels of IL-12 and IL-10 compared with that of control. Furthermore, the level of IL-12 was higher in lo-HBCAg group than that in lo-HBeAg group. In high dose condition, HBCAg-stimulated DCs secreted more IL-12 and IL-10 in contrast to that of control group, while HBeAg-treated DCs excreted significantly lower IL-12 and higher IL-10 compared to that of other two groups (**Figure 1A, 1B**). Since IL-12 acts as a key cytokine in T-cell activation by DCs, then the effects of HBCAg and HBeAg on the T-cell stimulatory capacity of DCs were determined in an allogeneic MLR. The data showed that the impact of DCs on T cell proliferation was almost dose-dependent. Both low-dose HBCAg and HBeAg-treated DCs effectively primed proliferation of T cells compared to the control group. However, the stimulating T cell proliferation capability of high-dose HBeAg-induced DCs was significantly diminished and
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no statistical difference between 1:10 ratio and 1:20 ratio, whereas the high-dose HBcAg-stimulated DCs potently facilitated T cell amplification (Figure 2). These results indicated that the influence of high-dose HBeAg on the T-cell stimulatory capacity of DCs was negative.

As the high-dose HBeAg-induced DCs secreted high levels of IL-10, which was known as an important immunosuppressive cytokine secreted by various kinds of cells with immunoregulatory functions. Thus, we investigated whether the high-dose HBeAg-induced DCs exerted
Mouse bone marrow-derived dendritic cells differentiate immune regulatory function. As shown in Figure 3A, when the high-dose HBeAg-educated DCs were added to three different DC/T coculture systems, the DC-initiated T cell proliferation of all coculture systems in vitro were suppressed profoundly. Moreover, the suppression rate of high-dose HBeAg-educated DCs on the T cell proliferation of lo-HBeAg group was the highest (about 57.52% ± 5.33), whereas the lo-HBcAg group was the second (about 48.46% ± 2.88) and the LPS group was the third (about 33.27% ± 4.54) (Figure 3B). The data indicated that the inhibitory function of high-dose HBeAg-educated DCs was antigen-specific.

**IDO is responsible for the regulatory function of high-dose HBeAg-educated DCs**

To illustrate the mechanism of the regulatory function of high-dose HBeAg-educated DCs, we examined the cytokines in above three coculture systems with or without adding high-dose HBeAg-educated DCs. We found that the concentrations of TGF-β and IDO were much higher in the presence of high-dose HBeAg-educated DCs. Particularly, IDO in HBeAg (low-dose)-loaded DC/T coculture system with adding high-dose HBeAg-educated DCs was the highest and in HBcAg (low-dose) coculture system was the second (Figure 4). Since IL-10, TGF-β and IDO are reported to be referred to the immunosuppression and tolerance induction, we blocked IL-10, TGF-β and IDO in the coculture systems. And we detected that IDO inhibitor 1-MT could significantly reduce the suppression rate in the lo-HBeAg group and even eliminated the statistical differences of suppression rate among three groups, but blockade of TGF-β and IL-10 almost equally reduced suppression rate of three groups without changing statistical difference (Figure 5). These data demonstrated that IDO is account mainly for the regulatory function of high-dose HBeAg-educated DCs, whereas TGF-β and IL-10 may not participate in this process.

**PI3K-Akt signaling pathway regulates IL-12, IL-10 and IDO secretions of murine DCs in the presence of high-dose HBeAg, but not affects TGF-β secretion**

We examined the phosphorylation levels of Akt and p38 in control group, hi-HBeAg group and LY294002+hi-HBeAg group. Through Western Blot analysis, we found the phosphorylation level of Akt was higher in hi-HBeAg group compared with that of control group, whereas the result of p38 phosphorylation level was converse. The data indicated that LY294002 significantly decreased IL-10 secretion and increased IL-12 secretion with enhanced p38 activation. In addition, the expression of IDO, not TGF-β, was also diminished by LY294002 (Figure 6).
Discussion

The importance of DCs in the clearance of viral infection has been shown in HBV infection. Patients who spontaneously clear HBV infection exhibit a strong antigen specific CD4+ and CD8+ T cell response that probably reflects the efficient capacity of DCs to prime and activate antiviral T cells [22, 23]. Due to the central role of DCs in the antiviral immune response, virus can target DCs as one of strategies to exercise their immune evasion via evading the pathogen recognition and elimination properties of the DCs, which in turn causes persistent infection [3]. However, the relation between HBV and DCs during chronic infection phase is still largely unknown. In this study, we demonstrated that high concentration HBeAg in vitro can cause imbalance of Th1/Th2 cytokines secretion of murine BMDCs and suppress their T-cell stimulatory capacity in an antigen-specific manner.

Inflammatory or regulatory cytokines produced by DCs during antigen presentation have major impact on T-cell differentiation [24]. IL-12, which is deemed as a critical factor in T-cell polarization, instructs naive T cell to shift toward a Th1 cells, a T-cell subtype required for elimination of transformed tumor cells and intracellular pathogens such as viruses, whereas IL-10 causes a Th2 response against helminthes [25, 26]. Due to CD4 T-cell-mediated antiviral responses critically rely on production of Th1 cytokines, imbalance of Th1 and Th2 appears to be one of reasons for chronic viral infections [27-29]. Beckebaum et al. had reported that HBV infection weakened the antigen-presenting function of monocyte-derived dendritic cells with concomitant impairment of Th1 responses, and this might contribute to viral immune escape leading to chronic HBV infection [30]. What’s more, this reduced T helper cell induction by autologous dendritic cells in patients with chronic HBV infection could be restored by exogenous IL-12 [31]. HBeAg and HBcAg are important targets for antiviral immunity. They share an overlapping reading frame, but HBeAg has a leading peptide sequence and different conformational structure. HBeAg acting as a tolerogen may preferentially activate Th2 cells immune activity, promote Th2 cytokines production, while inhibiting Th1 immune cells and depleting Th1 cytokines. Furthermore, exquisite studies administrated by Milich and colleagues using mouse models illustrated that HBeAg may be involved in immune regulation through depletion or anergy of HBeAg- and HBcAg-specific Th1 cells, as a means of establishing chronic infection [18, 32, 33]. In present study, we
Mouse bone marrow-derived dendritic cells differentiate high concentration HBeAg promotes Th2 cytokine (IL-10) production of murine BMDCs while suppressing Th1 cytokine (IL-12) production. However, HBCAg and low concentration HBeAg both promote IL-12 production and inhibit IL-10 production. These discoveries are consistent with previous findings and may mean that HBeAg performs as an immunogen in low concentration and as a tolerogen in high concentration.

Recently, Cao and his co-workers verified that the stromal microenvironment of the spleen and liver can educate DCs and hemopoietic progenitors to differentiate to regulatory DCs (DCregs) with high secretion of IL-10, TGF-β and IDO but less IL-12 [12-14]. These DCregs always own identified phenotypes and can inhibit T cell proliferation and also induce Treg cell generation via different mechanisms. Studies reveal that persistent viruses may target immunosuppressive enzymes in DCs to energetically suppress anti-viral T cell immune responses. The tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), is considered as a central factor of the suppressive function of DCs. DC-mediated IDO secretion has been associated with restraint of T cell proliferation and function [34]. When directly exposed to HIV, DCs induces IDO resulting in the suppression of CD4+ T cell proliferation in vitro [35]. HIV also increases IDO activity in pDCs, leading to polarizing naive T cells differentiation into Tregs with suppressive function [36]. Moreover, Chen et al. reported that enhanced IDO activity was observed in patients infected with HBV [37]. Other largely studied immunosuppressive cytokines are TGF-β and IL-10, which are both excessively produced in many advanced tumors. TGF-β causes the efficacy reduction of dendritic cells to stimulate T lymphocytes [38, 39] and accelerates CD4+ T cells toward Th2 polarization rather than Th1 cells receding the efficacy of anti-tumor immune response [40]. Similar to TGF-β, tumor derived IL-10 can differentiate DCs into regulatory phenotype, as well as behaves in an autocrine circuit. The tolerogenic function of IL-10-induced DCregs was confirmed by induction of CD4+ and CD8+ T cells anergy [41]. Thus, previous studies give us clues to investigate whether IDO, TGF-β or IL-10 are responsible for the regulatory function of high-dose HBeAg-induced DCs. Notably, we found that high concentration HBeAg, mainly depending on IDO, decreased T-cell stimulatory capacity of murine BMDCs in vitro via an antigen-specific manner. Therefore, we speculated that high concentration HBeAg in vitro can educate antigen-specific DCregs, performing their regulatory function primarily via IDO, which maybe a new possible immune escape mechanism of hepatitis B virus.

Up to now, there is a sizeable body of evidence that MAPK pathways regulate IL-12 secretion of DC in response to commonly-studied receptors such as TLR4. IL-12 is positively regulated by p38MAPK [19, 42]. In contrast, phosphoinositide kinase-3 (PI3K) negatively regulates IL-12 expression by diminishing p38 activation [21]. Our data also indicated that high concentration HBeAg may activate PI3K-Akt signaling pathway and thereby elicit the imbalance of Th1/Th2 cytokines secretion on DCs. Simultaneously, we also found that the inhibitor of PI3K (LY294002) can reduce IDO secretion, which is consistent with the recent study [43] that the IDO expression is regulated by the c-KIT-PI3K-Akt pathway.

In conclusion, we have shown that high concentration HBeAg gives rise to DCs with a significantly reduced immunogenic function as demonstrated by the reduced antigen-specific T-cell stimulatory capacity and imbalance of Th1/Th2 cytokines secretion. The regulatory function of high-dose HBeAg-induced DCs mainly depend on IDO which is positively regulated by PI3K-Akt signaling pathway. We presumed that these induced DCs represent one kind of identified DCregs, which are similar to those existing in tumor microenvironment. Furthermore, these HBeAg-induced DCregs may polarize naive T cells differentiation into Tregs, contributing to viral persistence by suppressing virus-specific cytotoxic T lymphocytes (CTLs) responses.

Acknowledgements

This work was supported by the Natural Science Foundation of Zhejiang Province (No. LY-12H03003; No. Y2110768).

Disclosure of conflict of interest

None.

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