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Original Article

Hepatitis B virus e antigen (HBeAg) may have a negative effect on dendrtic cells maturation

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Abstract: Hepatitis B e antigen (HBeAg) is believed to be required for the establishment of chronic HBV infection, but the mechanisms remain unknown. Dendritic cells (DCs) are the most potent antigen-presenting cells that play an essential role in the initiation of immune response. And only mature DCs are capable of priming and activating T lymphocytes to induce protective immunity. The aim of this study was to investigate the effects of HBeAg on the maturation of lipopolysaccharide (LPS) stimulated murine bone marrow-derived DCs (BM-DCs) and how HBeAg affect DCs' function. We found that HBeAg down-regulated the expression of immunologically important cell surface molecules (CD86 and MHC-II) on DCs and exerted a suppressive function which was partially mediated by IL-10 secretion on DCs' T cell stimulatory activity in vitro. Additionally, HBeAg down-regulated the IL-12 production from DCs by inhibiting the phosphorylation of p38. In conclusion, HBeAg may play a negative role in the activation of DCs, which may be necessary to better understand the immunoregulation of HBeAg and helpful to the HBV immunotherapy.

Keywords: HBeAg, regulatory dendritic cells, immune regulation, chronic hepatitis B, p38MAPK pathway

Introduction

Chronic hepatitis B (CHB) is an ongoing worldwide health problem, affecting more than 350 million people globally [1]. And the persistent infection of hepatic B virus (HBV) is a major cause of liver cirrhosis, hepatic failure and hepatocellular carcinoma. Although considerable insight has been developed into the mechanism by which HBV escapes immunity, the exact mechanisms have not yet been fully elucidated. Therefore, improving our knowledge of the pathogenesis of CHB is critical in order to develop novel treatment strategies to terminate persistent viral infection.

It is widely accepted that the immune tolerance may account for CHB. One or more antigens of HBV may utilize diverse pathways to suppress the anti-HBV immune response and induce HBV-specific tolerance. Hepatitis B e antigen (HBeAg), a secretory form of the nucleocapsid antigen, is translated from the preC transcript, sharing an overlapping reading frame with hepatitis B core antigen (HBcAg) [2]. Although HBeAg is not necessary for infection or replication of HBV [3], it is believed to be required for the establishment of chronic infection and probably responsible for the immunomodulation of host immune responses during CHB [4, 5]. HBV establishes chronic hepatitis mainly by vertical transmission from HBeAg-positive mothers to neonates, which with a significantly higher chronicity rate (up to 90%), whereas in HBV infected adolescents or adults, 5%-10% lead to a chronic carrier state [6]. Interestingly, babies infected perinatally with an HBeAg-negative mutant form of HBV experience an acute or fulminant acute course of infection rather than a persistent infection [7]. Thus, the tremendous difference in chronicity rate is supposed to be closely related to HBeAg.

As the most potent antigen presenting cells (APCs), dendritic cells (DCs) play a key role in the initiation and maintenance of specific T-cell

immunity [8-10]. And only mature DCs are capable of priming and activating T lymphocytes to induce protective immunity. Studies have shown that defect in DC function is an important factor in the host-specific T-cell immune tolerance to viral infection, rather than functional defects in T-or B-cells [11, 12]. The hepatits B virus and/or its antigens may be responsible for the impaired function of DCs in chronic HBV patients compared with healthy individuals. So far, it has been proven that HBeAg may have a negative effect on dendritic cell generation [13]. However, the potential effects of HBeAg on the maturation and function of DCs have not been rigorously elucidated.

In the present study, we investigated the effects of HBeAg on bone marrow derived DCs (BMDCs) from mice. The result showed that HBeAg may play a negative role in the maturation of LPS-stimulated DCs in regard with phenotype and function and HBeAg may suppress the secretion of IL-12 by inhibiting the phosphorylation of p38. These findings provide further understanding of the impact of HBeAg on DCs and may ultimately benefit the development of new DC-based immunomodulatory strategies for chronic hepatitis B.

Materials and methods

Drugs and chemicals

HBeAg was purchased from Beijing Kewei clinical diagnostic reagents Co, Ltd., Beijing, China. All reagents and chemicals were procured from local suppliers and were of analytical grade.

Animals

C57BL/6 (B6) and BALB/c mice (6-8 weeks old, weighing 18-25 g) were purchased from Shanghai Slac Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were all housed in specific pathogen-free facilities and maintained under constant temperature (20-22°C), controlled humidity (45%-55%) and a 12-h light/dark cycle (lights on from 08:00 to 20:00). All experimental procedures were approved by the Institutional Animal Committee of Wenzhou Medical University and all mice received care throughout the experiment in accordance with "Guide for the Care and Use of Laboratory Animals".

Generation of bone marrow-derived dendritic cells and cell cultures

BM-DCs were prepared according to established protocols with minor modification [14, 15]. Briefly, cells were gathered from C57BL/6 bone marrow and cultured at a density of 2×10^6 cells/ml in 6-well plates in RPMI1640 (Gibico, USA), supplemented with 10% fetal calf serum (Gibico, USA), 1% penicillin/streptomycin, 1 ng/ml of recombinant mouse IL-4 and 10 ng/ml GM-CSF (PeproTec, London, United Kingdom) at 37°C, 5% CO_2. On day 6, non-adherent and loosely adherent immature DCs (iDCs) were harvested and sorted by CD11c magnetic microbeads and a MiniMACS separator (Miltenyi Biotec, Germany) to obtain highly purified DCs. The cell viability after magnetic beads sorting was assayed with trypan blue staining. The percentage of CD11c positive cells was detected by flow cytometry and the morphology was identified under a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan).

Experimental design

To investigate the influence of HBeAg on the maturation of LPS-stimulated DC, the purified DCs were then co-cultured with 5 μg/ml HBeAg for 24 h, and 100 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich; USA) was added for additional 24 h. In some experiments, SB203580 (Beyotime Institute of Biotechnology, Haimen; China) for 1 hour in advance, then followed with LPS stimulation. DCs were then purified from the co-cultures using flow cytometry for further analysis. The supernatants were collected, centrifuged to remove cell debris, and stored at -80°C for ELISA.

Flow cytometry analysis

To characterize and compare the phenotype of DC populations, flow cytometry was performed. DCs were harvested and washed. The cells were then incubated in cold buffer and subsequently stained for 30 min with the following PE-labeled monoclonal antibodies or appropriate isotype controls: MHC-II, CD86(eBioscience, San Diego, USA). Stained cells were analyzed in an Elite flow cytometer (Coulter, Hialeah, FL).

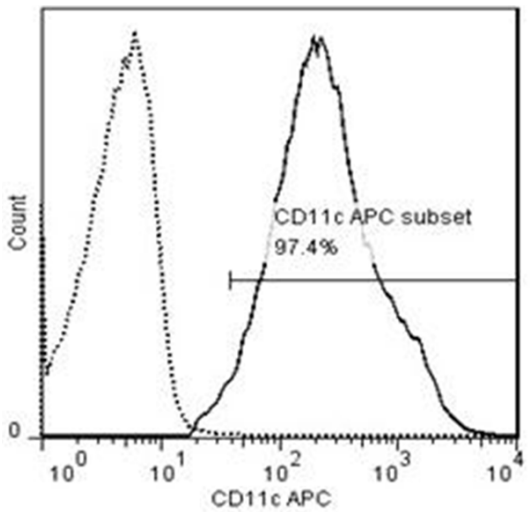

Figure 1. CD11c positive cell percentage after magnetic bead sorting. Purified cells were stained with anti-CD11c and analyzed using flow cytometry (dotted line, isotype control; solid line, specific mAbs). The value shown in the flow cytometry profile is the percentage of CD11c positive cells after magnetic beads sorting.

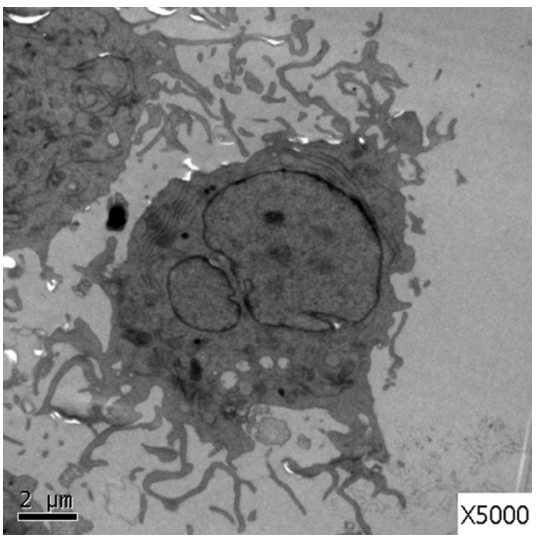

Figure 2. Optical photomicrographs of dendritic cells that were cultured for 6 days (×5000. Scale bars equal 2 μm transmission electron microscope).

Allogeneic-mixed lymphocyte reactions (MLRs)

T-cells were isolated and purified from healthy BALB/c spleen by nylon wool columns and CD4+ T isolation kit (Miltenyi Biotec, Germany), according to the manufacturer's instructions. Primary MLRs were performed as previously described. Briefly, graded concentrations of DCs from different culture conditions were co-cultured in U-bottom 96-well plates with constant number of allogeneic T-cells (1×10^5 cells/200 μl) at different stimulator/responder (DC/T-cell) rations (1:5, 1:10, 1:20) for 96 h. The T-cell stimulatory activity of DC populations in MLR was expressed as stimulation index (SI) value and measured using CCK-8 cell proliferation array kit (Beyotime Institute of Biotechnology, Haimen, China), in accordance with the manufacturer's instruction. In neutralizing cultures, anti-IL-10 antibodies (R&D Systems, Minneapolis, MN) were added at the beginning of the MLR.

Cytokine production

The concentration of IL-12p70 in culture supernatants was estimated by ELISA using the corresponding enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN).

Western blotting

Expression of p-p38, p38 and GAPDH were determined by western blot according to standard protocols. The rabbit anti-p-p38 (Cell Signaling, USA, 1:1000), rabbit anti-p-38 (Cell Signaling, USA, 1:1000) and a horse peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (Biosharp, China, 1:5000) were used. Rabbit anti-GAPDH (Hangzhou Xianzhi Biological Technology Co. Ltd., China) was used as a loading control. Visualization was achieved by chemiluminescence (ECL).

Statistical analysis

All data were reported as mean ± standard deviation (S.D.). Statistical analysis was performed by Student *t* test or one-way ANOVA followed by post-hoc tests (using LSD-t or Dunnett's T3) for multi group comparisons with SPSS18.0 program (SPSS, Chicago). All *P* values <0.05 were considered significant.

Results

Cell purified results

A large number of dendritic cells was generated by culture of murine bone marrow cells in the presence of GM-CSF and IL-4. The percentage of viable DCs detected with trypan blue staining was not significantly changed after purifica-

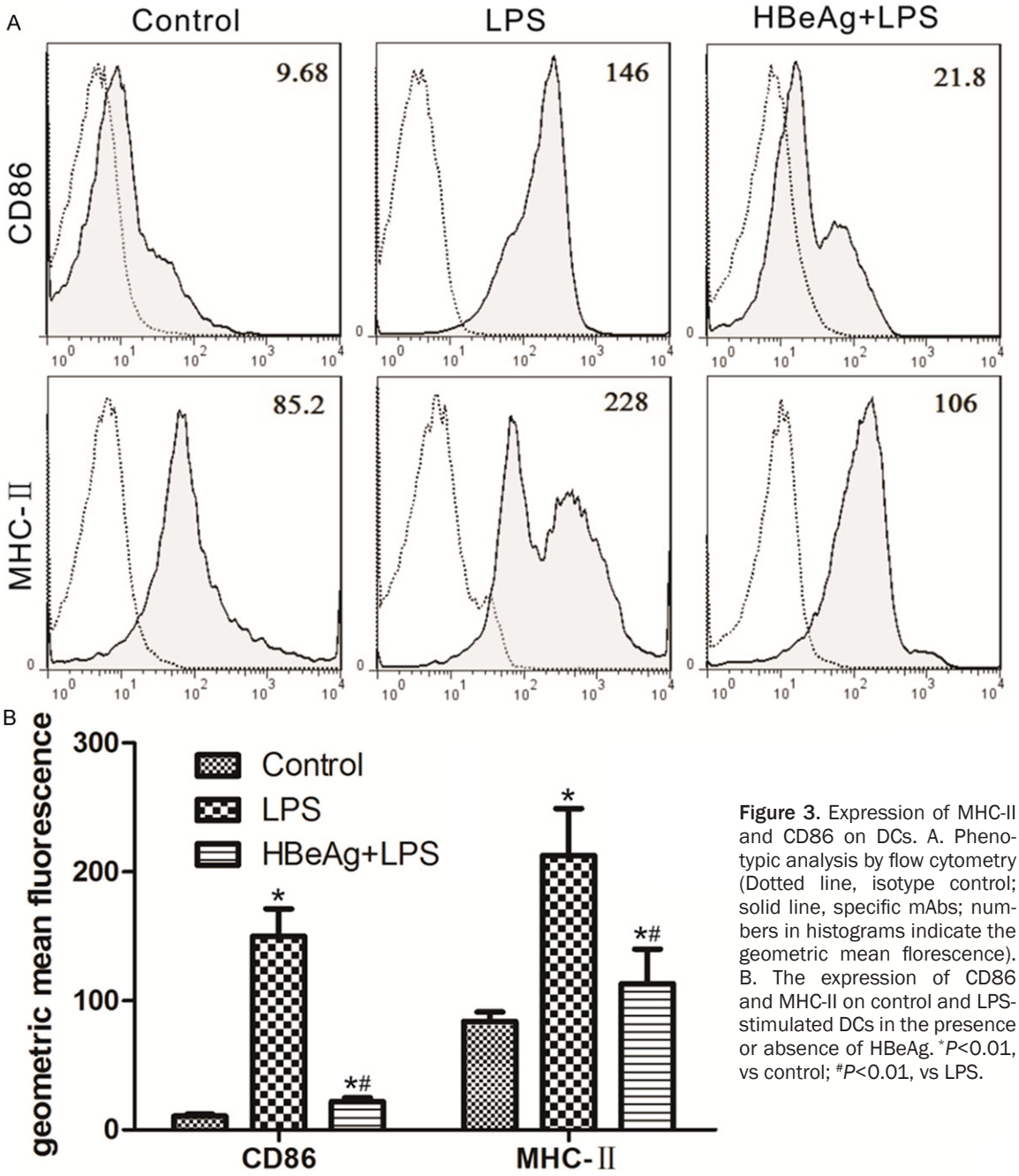

Figure 3. Expression of MHC-II and CD86 on DCs. A. Phenotypic analysis by flow cytometry (Dotted line, isotype control; solid line, specific mAbs; numbers in histograms indicate the geometric mean florescence). B. The expression of CD86 and MHC-II on control and LPS-stimulated DCs in the presence or absence of HBeAg. $^{*}P<0.01$, vs control; $^{\#}P<0.01$, vs LPS.

tion compared with before [(95.2±2.4)% vs (96.1±1.9)%, t=0.5093, $P>0.05$]. And more than 95% cells were positive for CD11c as assessed by flow cytometry (**Figure 1**). Transmission electron micrograph showed the bone marrow derived cells had obvious eccentric nuclei, long dendrites, and rich mitochondria in cytoplasm (**Figure 2**). The results showed that the cells prepared were ready for the follow-up study.

HBeAg can down-regulate the expression of CD86 and MHC-II on DCs

DC's surface molecules, including MHC-II and the costimulatory molecules (CD80/CD86), all of which will be greatly up-regulated during the DC maturation. To clarify the role of HBeAg in regulation of the phenotype of DCs, the cells were collected to test the expression of CD86 and MHC-II by flow cytometry. Control DCs were

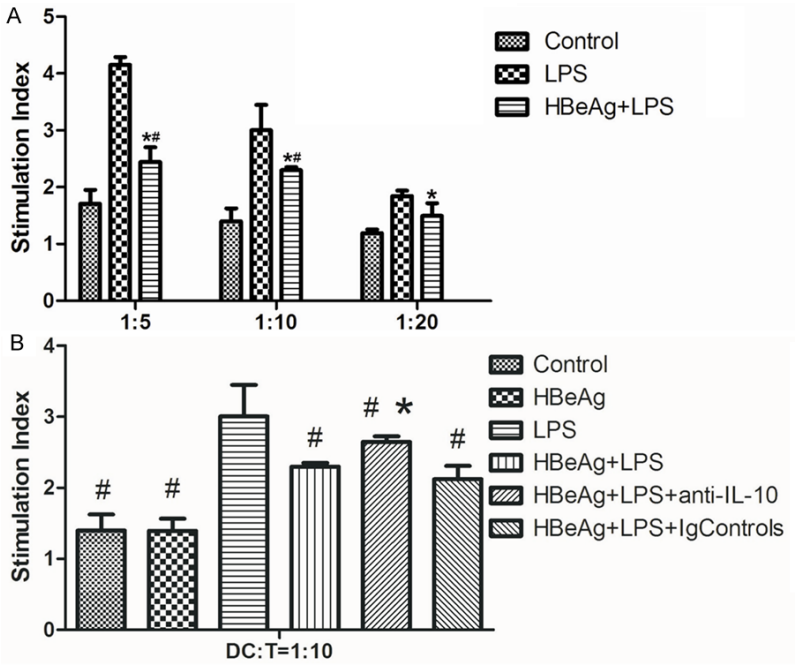

Figure 4. Mixed lymphocyte reaction. Mean ± S.D. of stimulation index of 5 separate experiments are shown. A. DCs function was analyzed in a MLR by incubating DC obtained under different culture conditions with allogeneic T lymphocytes from BALB/c mice at the indicated ratios. B. At a DC: T ratio of 1:10, the T-cell stimulatory capacity of the DC was significantly reduced by HBeAg and anti-IL-10 blocking antibodies partially inhibited the suppressive activity of HBeAg. $^{*}P<0.05$, vs HBeAg+LPS; $^{\#}P<0.05$, vs LPS.

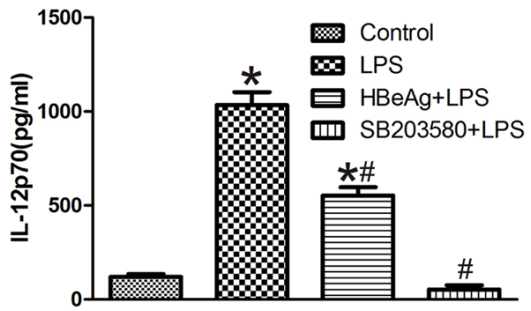

Figure 5. The level of IL-12 in cell culture supernatants. $^{*}P<0.01$, vs control; $^{\#}P<0.01$, vs LPS.

cultured in medium alone. There were dramatic enhancement in the expression of CD86 and MHC-II on LPS-stimulated DCs. We asked whether HBeAg could inhibit the expression of these two surface molecules even when DCs were stimulated with LPS. To answer this question, immature bone marrow-derived DCs co-cultured for 24 h with HBeAg was then added with LPS to induce co-stimulatory molecule expression for an additional 24 h. While adding HBeAg before the LPS stimulation, the result showed a significant reduction in the expression of CD86 and MHC-II when compared with the LPS group ($P<0.01$). The data demonstrated that HBeAg could inhibited DCs maturation (**Figure 3**).

HBeAg treated DCs show reduced allogeneic T-cell stimulatory capacity

Functional DCs are capable of priming and activating T lymphocytes to initiate immune response. To further determine the functional properties of DCs, we analyzed their T-cell stimulatory capacity in an allogeneic mixed lymphocyte reaction (MLR). The T-cell stimulatory activity of DC populations in MLR was expressed as stimulation index (SI) value, which is the ratio between the proliferative response (optical absorbance, OD) of T cells in the presence and the absence of DCs in the cultures at a T cell/DC ratio of 5:1, 10:1 and 20:1, respectively. LPS stimulated DCs showed a stronger capacity to stimulate allogeneic T cell proliferation. While adding HBeAg, the ability of stimulating T cells was reduced compared with LPS-treated group at a ratio of 1:5 and 1:10 ($P<0.01$). But when DC/T was 1:20, there was no significantly difference between the LPS-treated group and HBeAg plus LPS group ($P>0.05$). The results showed that DC incubated with HBeAg tended to have a lower T-cell stimulatory activity ($P<0.05$) (**Figure 4A**).

IL-10, secreted by many kinds of cells with regulatory functions, can induce T-cell unresponsiveness when present during T-cell activation [19]. To further address the suppression mechanism induced by HBeAg, the role of IL-10 was examined. Results showed that at a DC: T ratio of 1:10, blockade of IL-10 by anti-IL-10 antibody partially inhibited the suppressive activity of HBeAg (**Figure 4B**).

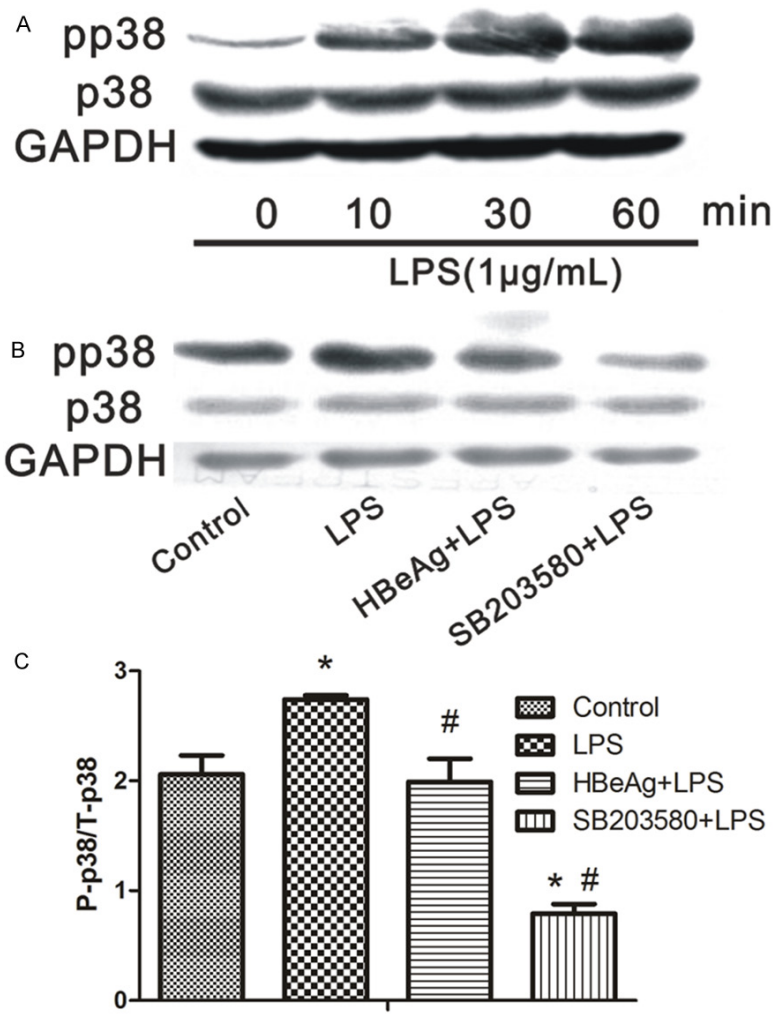

Figure 6. Western blot analysis of phospho-p38 in DCs. A. LPS induces p38 kinase phosphorylation in DCs in a time dependent manner. B. Western blot analysis of the expressions of phospho-p38 in DCs of the each group. C. Western blot analysis of phospho-p38. Results are normalized to total Akt. Each bar represents mean ± S.D., $^{*}P<0.05$ vs the control group and $^{\#}P<0.05$ vs the LPS group.

HBeAg inhibited IL-12 production by DCs

Production of IL-12 by DC in particular is critical for the development of cell-mediated immunity during the immune response to infection. To further address the role of HBeAg on DC activation, LPS-induced secretion of IL-12 was analyzed by ELISA. As expected, LPS-induced mature DCs could secrete high level of IL-12. However, when HBeAg-treated DCs stimulated with LPS, the increase secretion of IL-12 was impaired (**Figure 5**). In addition, we employed SB203580, the antagonists of p38MAPK signaling to the cultures. In concordance with previous study [16], pretreatment of p38MAPK inhibitor (SB203580) could reduce the LPS-induced IL-12 production.

p38MAPK plays a crucial role in HBeAg-mediated IL-12 inhibition

Previous study has reported that p38MAPK signal exerts negative regulation on IL-12 transcription [16]. To investigate this signaling mechanism involved, we detected the expression level of phosphorylated p38 (p-p38) by western blot and found that the expression level of p-p38 was up-regulated in a time dependent manner within 60 minutes in DCs treated with LPS (**Figure 6A**). Compared with the LPS group, the level of phosphorylated p38 in both the HBeAg+LPS and the SB203580+LPS groups reduced significantly (**Figure 6B**, **6C**).

Discussion

Recent studies have shown some new mechanisms of HBV escaping immune responses. Lang et al. [17] found that HBeAg may contribute to the pathogenesis of CHB infection via interacting and co-localizing with TLR proteins, thus suppressing TIR-mediated activation of both NF-κB and IFN-β. Purvina et al. [18] demonstrated that HBeAg inhibited T lymphocyte proliferation through the IL-1 signaling, which in turn aids the establishment of chronic infection. Another study reported that HBeAg could modulate immune responses by down-regulating IL-18 mediated expression of IFN-γ which had direct antiviral effects against HBV [19]. The role of HBeAg in the CHB pathogenesis is becoming more and more apparent. And a latest paper has reported that HBeAg had a negative effect on the

generation of DCs [13]. However, researches about both the HBeAg and DCs are still few, with the effect of HBeAg on DCs' maturation and function remaining incompletely elucidated. We hypothesized that HBeAg could impact on the establishment of chronic HBV infection by inducing DCs' functional deficiency. So, we conducted experiments in order to better understand the role of HBeAg played on DCs. We used immune magnetic beads to sort DCs as the research objective, then further analyze how HBeAg affect the phenotype and function of DCs which play a considerable role in the immunity. Our results indicate that HBeAg has potent inhibitory functions on bone marrow-derived DCs including suppression of CD86 and MHC-II expression; reduction of T-cell stimulatory capacity of DCs and the inhibition of IL-12 production.

The phenotypic maturation is necessary for DCs to exert a central role in the induction of the immune response. The high expression of CD80, CD86 and MHC-II was the marker of DC maturation. Consistent with previous studies, LPS-treated DCs exhibited an apparently mature phenotype ($CD86^{high}$, $MHC\text{-}II^{high}$). When DCs were first treated with HBeAg and then matured by LPS, the increasing extent of the expression of these two cell surface molecules was lower then the LPS group obviously. Thus, HBeAg seems to be able to inhibit DCs maturation phenotypically.

Apart from the immunologically important cell surface molecules, another feature of DCs is their ability to promote T-cell proliferation. Thus, MLR was conducted to determine this capacity of HBeAg-treated DCs. A clearly reduced T-cell proliferation compared to mDCs was observed in HBeAg pre-incubated DCs. Thus, HBeAg is able to impair the T-cell stimulatory activity of DCs. Furthermore, we found that anti-IL-10 antibodies partially inhibited the suppressive function of HBeAg on DCs. Our results indicate that HBeAg can act as a potent inhibitor of MLR with a partially IL-10-dependent regulatory function.

HBeAg can affect key cytokine production during the establishment of CHB [19]. IL-12 is produced by DCs early in immune response and exerts its biological function by driving Th1 cell activation and differentiation [20]. Here, we observed that LPS-induced mature DCs secreted high level of IL-12. While after the stimulation of LPS, the IL-12 production of HBeAg-treated DC increased, but not to the same magnitude. p38MAPK signal pathway has been reported to play a central role in the response of DC to environmental stimuli, including the regulation of IL-12 secretion [16, 21]. So, in order to identify the possible mechanisms for the impacts of HBeAg on the cytokine production of DCs, the phosphorylation level of phosphor-p38 was measured by western blot. Additional SB203580, the specific inhibitor of p38MAPK pathway, was used as an internal positive control to explore the potential mechanism of IL-12 regulation. Pretreatment of DCs with SB203580 could significantly reduce LPS-induced IL-12 production. Western blot analysis showed that, compared with the control group, the intracellular p-p38 protein expression of LPS-treated DCs was up-regulated obviously. While HBeAg and SB203580 all led to significant reductions in expression level of p-p38 compared with LPS-stimulated group. These data suggest that p38MAPK pathway is likely to be involved or at least played a partial role in the HBeAg caused down-regulation of IL-12 production of DCs.

In summary, our study found that HBeAg can down-regulate the expression of CD86 and MHC-II and exert a suppressive function which is partially mediated by IL-10 secretion on DCs' T-cell stimulatory activity. Additionally, HBeAg can inhibit the production of IL-12 through the suppression of p38MAPK pathway. But the underlying processes of HBeAg acting on DCs remain elusive. Nevertheless, we could demonstrate the a negative immunomodulatory role of HBeAg on DC maturation, which in turn aided viral immune escape. Our findings provide novel information for the current recognition of the formation of persistent HBV infection and may cause some meaningful implications.

Acknowledgements

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Disclosure conflict of interest

None.

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