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ORIGINAL ARTICLE

Quantative HBsAg level correlates dendritic cells maturation in chronic hepatitis B patients

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KEYWORDS
Chronic Hepatitis B;
Dendritic Cells;
HBsAg

Summary
Background: In order to better understand the role of Dendritic cells (DCs) in Chronic Hepatitis B (CHB), we investigated the frequencies and maturation markers on DCs in CHB patients and its change during entecavir treatment.
Methods: Twenty-six CHB patients on anti-virus treatment for 48 weeks were included in this study. Patients' blood samples were collected on every 3 months since starting treatment. Samples on baseline and after 48 weeks treatment were examined using flow-cytometry to investigate frequencies and maturation markers of DCs.
Results: The frequencies of myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) were lower in CHB patients than healthy controls on baseline. pDCs frequencies and mDCs maturation markers expression were increased after entecavir (ETV) treatment. Patients with higher baseline HBsAg levels showed a poorer maturation status than those with low baseline HBsAg levels, regardless of changes in HBsAg levels after treatment.
Conclusions: Entecavir treatment could restore the decreased DCs frequencies in CHB patients and improve DCs maturation levels. Baseline HBsAg level is an important factor that affecting DCs.

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Introduction

Chronic hepatitis B virus (HBV) infection is a significant public health problem that affects approximately 240 million people in the world [1,2]. During an acute HBV infection, vigorous virus-specific T cell response is induced to achieve effective viral clearance. However, such responses are generally decreased in chronically infected patients and could be a major factor that leads to eradication failure of HBV infection [3,4].

Dendritic cells (DCs) play a central role in of the process of inducing specific T-cell responses. They are the most potent Antigen presenting cells (APCs). DCs could be further divided into CD11c+ myeloid DCs (mDCs) and CD123+ plasmacytoid DCs (pDCs). DCs can interact with innate branch of immunity as well. It efficiently links the innate and the adaptive immune response and affects the immune response to HBV infection. Previous studies had demonstrated HBV could alter pDCs functions to disrupt interactions between pDCs and NK cells and further reduce immune control of HBV [5].

DCs in resting status are thought to be ''immature'' but they are primed to recongnize antigens. After sensing of microbial stimulation, immature DCs undergo a modification in morphology, which is called ''maturation'' [6]. The maturation characteristics of DCs including up-regulation of costimulatory molecules (CD80 and CD86), phagocytic capacity reduction, elevated antigen processing and presentation, improved migration to lymphoid tissues, and increased capacity to stimulate B and T cells [7]. The costimulatory molecules (CD80, CD86) on DCs are critical for them to establish stable and long-lasting contacts with T cells. Such contact contributes to T cells expansion and T cells' differentiation into memory and effector T cells [7]. There are some previous studies that aim at investigating the frequency and maturation status of DC in chronic HBV infection, but the results are conflicting [5,8—11]. Also, most of these studies included HBV DNA and ALT levels as potential factors influencing patients' DC status. However, HBsAg levels have not been well studied. Because HBsAg quantification has only recently been used to stratify patients.

In the present study, we evaluated the frequencies and phenotypes of DCs in CHB patients. We also determined whether entecavir treatment affects the characteristics of DCs in CHB patients, and used HBsAg quantification level to stratify patients in order to explore its relationship with DCs status.

Materials and methods

Study design

Adult (> 18 years old) patients with chronic hepatitis B were considered eligible for this study. Exclusion criteria included: patients who had been on anti-virus treatment (either nucleos(t)ide analogue or interferon), patients infected with HAV (hepatitis A virus), HCV (hepatitis C virus), HDV (hepatitis D virus), HEV (hepatitis E virus), HIV (human immunodeficiency virus) and patients with liver cirrhosis, HCC, cardiovascular disease, diabetes, kidney disease, pregnancy or autoimmune disease. The study was conducted in accordance with the Helsinki Declaration and approved by the Institutional Review Board of the Third Affiliated Hospital of Sun Yat-sen Univeristy. The written informed consent was taken from all subjects. Patients were treated with ETV (0.5 mg per day) for 48 weeks.

Flow cytometry analysis

Heparinized peripheral blood samples were obtained from all CHB patients ($n = 26$) and 13 healthy volunteers for virological analysis and isolation of peripheral blood mononuclear cells (PBMC) as described previously [12,13]. PBMCs were stained with anti-lin(linage markers)-FITC, anti-CD14-PECF594, anti-CD11c-PEcy7, anti-CD123-APC, anti-human leucocyte antigen D-related (HLA-DR)-PB (eBioscience, San Diego, CA,USA),anti-CD80-PE and anti-CD86-PE (BD Biosciences,San Jose, CA, USA) for flow cytometry analysis.

Clinical and serological parameters

CHB patients' baseline (before treatment) serum was tested for HBsAg, anti-HBsAg, HBeAg and anti-HBeAg using commercial kits (Abbott Laboratory, North Chicago, IL). Quantitative HBsAg was measured by the commercial kits (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Serum HBV DNA level was measured by the Roche COBAS Ampliprep/COBAS TaqMan HBV test v2.0 (Roche Molecular Diagnostics, Branchbug, NJ).

Statistical analysis

Wilcoxon rank sum test was use to compare parameters between CHB patients and healthy control and Wilcoxon paired rank sum test was used to compare the changes of parameters in baseline and after treatment. Kruskal-Wallis test and Dunn's multiple comparisons test were used to compare multiple groups. All the graphs were created with GraphPad Prism 7 and statistical tests were performed with the R software version 3.2.2.

Results

Characteristics of the patients

Twenty-six treatment-naive patients with chronic HBV infection (21 male and 5 female) were included into this study. Patients' clinical characteristics at baseline were shown in Table 1. Twenty-six CHB patients were divided into two groups in accordance with HBsAg levels: $HBsAg^{high}$ (HBsAg > = 10000 IU/mL) and $HBsAg^{low}$ (HBsAg < 10000 IU/mL) groups [14].

Frequencies and maturation levels of mDCs and pDCs in peripheral blood were lower in CHB patients

The mDCs is identified as Lin-CD14-HLA-DR + CD11c+ cells and pDCs as Lin-CD14-HLA-DR + CD123+ cells (Fig. 1A). We first compared the frequencies of mDCs and pDCs in PBMC

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Table 1 Baseline characteristics of patients.

	CHB	$HBsAg^{high}$	$HBsAg^{low}$
Age (years)	32.04 ± 6.508	29.38 ± 5.875	36.3 ± 5.229
Gender	26	16	10
Male	21	13	8
Female	5	3	2
ALT (U/l)	192.7 ± 144.2	186.1 ± 161.2	203.1 ± 119.4
Log(HBV DNA)(IU/ml)	7.823 ± 0.7446	8.106 ± 0.249	7.369 ± 1.032
HBeAg			
Postitive	23	16	7
Negative	3	0	3
HBsAg (IU/ml)	27174 ± 21475	41755 ± 13215	3845 ± 2708

Values are expressed as mean ± SD.

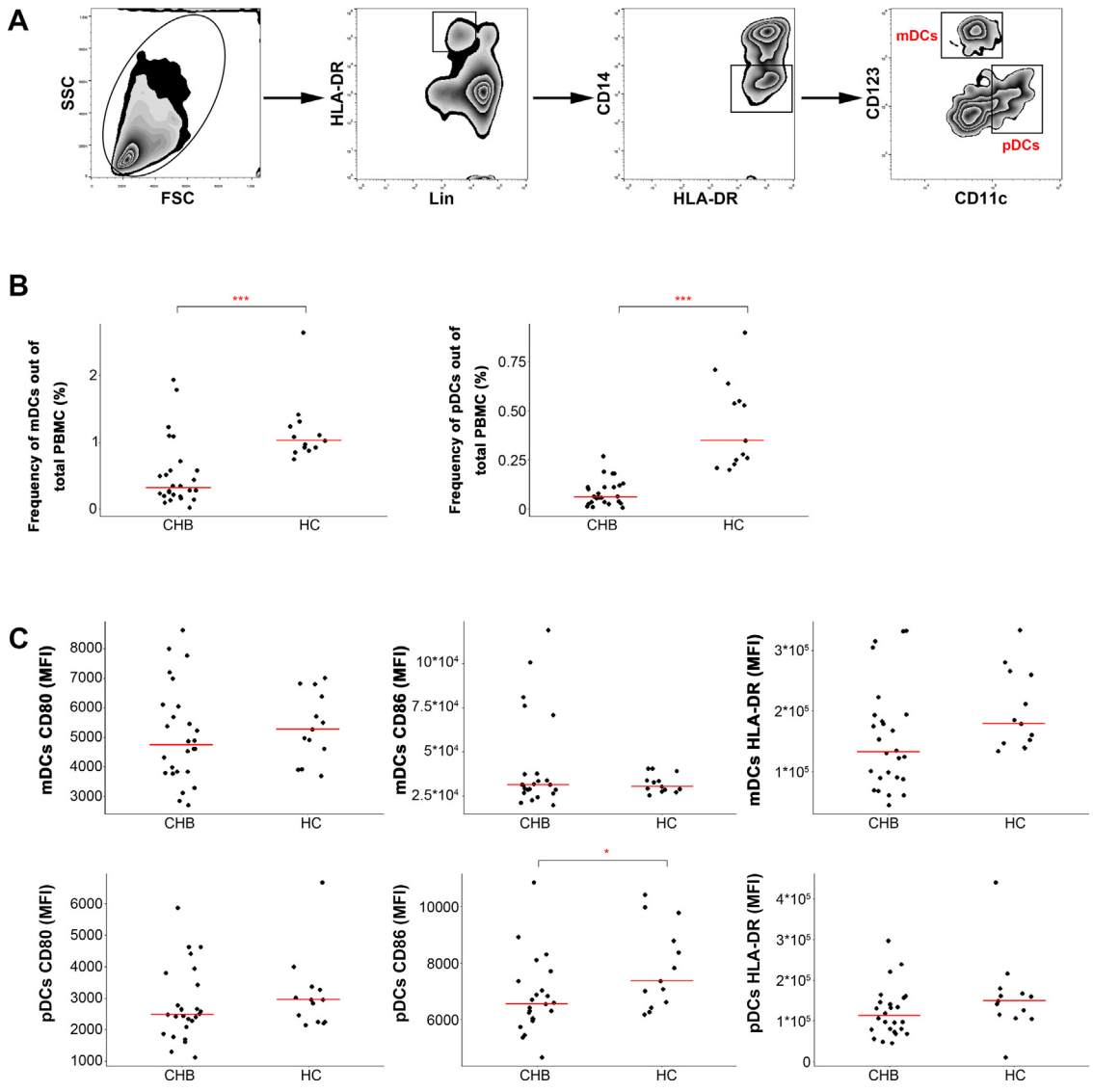

Figure 1 DCs frequency and maturation status on baseline. Gating strategies of DCs. mDCs and pDCs were identified within PBMC after gating on $Lin^{-}CD14^{-}HLA\text{-}DR^{+}$ cells. mDCs were then identified as CD11c+ cells and pDCs as CD123+ cells. The frequency of total mDCs and pDCs were shown in both CHB patients group (CHB) and healthy controls group (HC). Expression of maturation markers CD80, CD86 and HLA-DR on mDCs and pDCs. Expression levels were shown as Mean Fluoresecence Intensity (MFI).

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between treatment-naive CHB patients (n = 26) and healthy controls (*n* = 13) (Fig. 1B). Both the frequencies of circulating mDCs and pDCs were lower in CHB patients than healthy controls, and the maturation marker CD86 on pDCs was also lower in CHB patients. Other maturation markers (e.g. CD80, CD86 and HLA-DR) expression levels on both mDCs and pDCs were comparable in CHB subjects and HC (Fig. 1C).

Frequencies of pDCs and maturation levels of both pDCs and mDCs increased after 48 weeks entecavir treatment

To determine whether entecavir treatment could influence the frequencies and maturation status of mDCs and pDCs in chronic HBV patients, we examined patients' PBMC samples collected before treatment (baseline) and after 48 weeks ETV treatment. As expected, CHB patients' HBsAg, HBV DNA and ALT levels showed a significant decrease after entecavir treatment for 48 weeks (SFig. 1). The frequencies of pDCs significantly elevated after treatment (Fig. 2B). And the CD80 (Fig. 3 A and B) and HLA-DR (Fig. 3 E and F) expressions on both pDCs and mDCs subset elevated significantly after treatment. These results indicate that anti-virus treatment could restore the down-regulated maturation level of DCs in CHB patients.

Baseline HBsAg level is an important factor that affects DCs status

Responses to anti-virus treatment are various in different individuals. Several factors might be involved in this process, such as gender, age, baseline viral load and host immune status. Since patients' HBsAg levels decreased and DCs maturation elevated on 48 weeks' anti-virus treatment, we further analyzed the relationship between HBsAg and DCs status. Though both $HBsAg^{high}$ and $HBsAg^{low}$ patients showed a comparable and significant biochemical and virological response on 48 weeks ETV treatment, $HBsAg^{low}$ patients had a better mDCs maturation than those in $HBsAg^{high}$ group at both baseline and the end of treatment (sFig. 3 A—D). Among $HBsAg^{high}$ patients, 67.85% patients (11 out of 16) had an HBsAg level < 10000 IU/mL at week 48 ($HBsAg^{high}$(Decrease) patients). Noticeably, the mDCs maturation levels in these $HBsAg^{high}$(Decrease) patients at week 48 were lower than $HBsAg^{low}$ patients, and did similar to the $HBsAg^{high}$ patients whose HBsAg level still > 10000 IU/mL at week 48 (Fig. 4 C and D), implying that mDCs maturation did not change along with HBsAg titer reduction along treatment (Fig. 4).

Moreover, during the 1-year therapy, patients' HBV DNA level, ALT and HBsAg level had a dramatic decrease by first 12 weeks (SFig. 2). Early (at week 12 or 24 on treatment) HBsAg reduction of 1 $\log_{10}$ after nucleostide analogue (NA) treatment is pivotal to predict HBeAg seroconversion or HBsAg loss [15—17]. We compared DCs maturation in early significant HBsAg reduction patients (SR), but no early significant HBsAg reduction patients (NSR). However, these patients' achieving a significant early decrease in HBsAg titer seemed not affecting DCs characteristics (Fig. 5).

Discussion

Dendritic cells play a central role in initiating adaptive antiviral immunity. Thus, deficiencies of DCs could be accounted for impaired HBV-specific T cell immunity. In the present study, we found pDCs and mDCs frequencies declined in CHB patients, and anti-virus treatment with entecavir could restore the decreased frequency of DCs as well as up-regulation of the maturation level of DC. We also found that the serum HBsAg level is a vital factor that influences DCs status.

Our data showed that baseline mDCs and pDCs frequencies were lower compareing to healthy controls, which are consistent with previous reports [10,15,16]. The apoptosis-inducing effect of viral particles of HBV in DCs and migration to inflamed liver may contribute to reduction of DCs frequencies [15,17—19]. Reduced peripheral DCs may be a reflection of organ resident DCs decrease and T cell stimulation failure [20]. We also observed an increased pDCs frequency in total PBMC after the entecavir treatment, which further support that HBV could down-regulate pDCs frequency. It might be the consequences of either virus replication suppression or liver inflammation regression. One thing needs to be taken into consideration is that both mDCs and pDCs frequencies are very low in PBMC (about 2% for mDCs and less than 0.5% for pDCs), so the explanation of the above differences should be very careful.

CHB patients in current study before initiating ETV treatment had high viremia and low DCs maturation (comparing to HC). Maturation is a complex process that DCs sensing microbial stimuli causes these cells undergo a modification in morphology and functions profoundly. It increases the expression of co-stimulatory molecules (e.g CD80 and CD86) and MHC molecules (e.g. HLA-DR), and leads to the secretion of a wide variety of inflammatory cytokines and chemokines [6]. And as the result, cross-presentation function was increased [21]. The HBV viremia in treatment-naive CHB patients (1.8×10^5—1.7×10^7 IU/mL) failing to stimulate the co-stimulation molecular and HLA-DR on DCs to elevate (comparing to HC) might be the result of the inhibitory effect of HBV. Several previous studies of DCs maturation status in CHB were using in vitro-generated monocyte-derived DCs (moDCs) rather than directly examine in vivo DCs. However, differences between moDCs and the myeloid DCs present in vivo [22] exists, which make the conclusions derived from moDCs less valuable. The direct examination of maturation molecules on in vivo periphery DCs here could better reflect the status of DCs under the condition of chronic HBV infection.

The connection between serum HBsAg level and DCs status showed in our study further suggest a distinct role of HBsAg as a factor influencing treatment outcome. HBsAg quantification was not included in most previous studies and has only recently been used to stratify patients[99]. Previous ex vivo experiments demonstrated that HBV/HBsAg could directly diminish the maturation of DCs induced by other virus like herpes simplex virus —1 and influenza virus [23]. The high HBsAg level seem to have a long-term effect to DCs. Despite some $HBsAg^{high}$ patients showed an increased DCs maturation after treatment, these patients still have a lower maturation level of periphery DCs than those low

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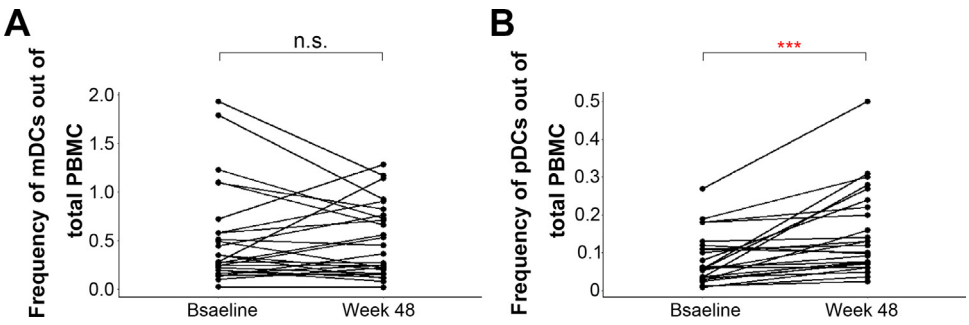

Figure 2 Comparison between DCs frequencies on baseline and end of treatment. The frequencies of mDCs of each CHB patients on baseline (before ETV treatment) and end of treatment (48 weeks ETV treatment). The frequencies of mDCs and pDCs of each CHB patients on baseline (before ETV treatment) and end of treatment (48 weeks ETV treatment). The p values in figures are demonstrated as n.s. > = 0.05; *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

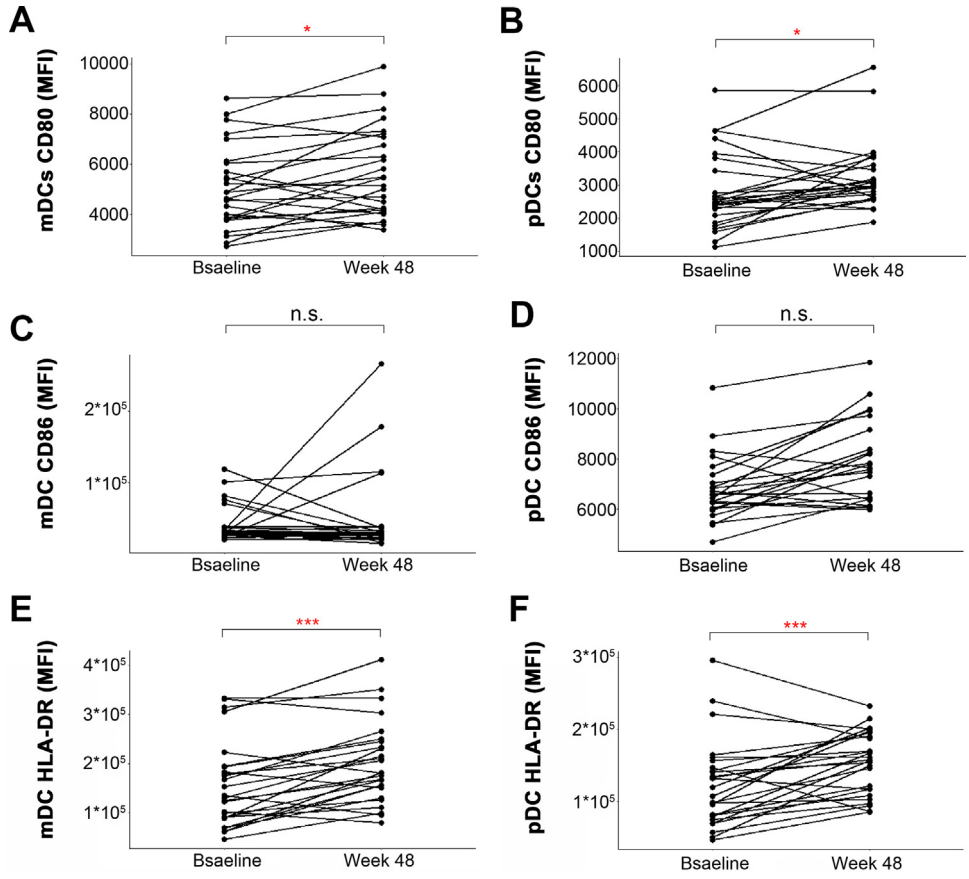

Figure 3 mDCs and pDCs maturation levels on baseline and end of treatment. Expression of maturation markers CD80 (A), CD86 (B) and HLA-DR (C) on mDCs on baseline (before ETV treatment) and end of treatment (48 weeks ETV treatment). Expression of maturation markers CD80 (D), CD86 (E) and HLA-DR (F) on pDC baseline (before ETV treatment) and end of treatment (48 weeks ETV treatment). Expression levels were shown as Mean Fluoresecence Intensity (MFI). The *P*-values in figures are demonstrated as n.s.> = 0.05; *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

pre-treatment HBsAg patients. And those patients with low pre-treatment HBsAg had a higher maturation level and comparable to health controls. Liu et al. [14] reported that low HBsAg level is one of the most important predictor of spontaneous HBV DNA undetectability. They found that a baseline HBsAg level of 10000 IU/mL divided HBeAg positive patients into two distinct groups, between which the HBeAg seroclearance rate and HBV DNA undetectability are different. Besides baseline HBsAg level, the dynamic change of HBsAg level during anti-virus treatment is also an important predictor for HBeAg seroconversion [24,25] or HBsAg loss [26], especially a 1 $\log_{10}$ decrease of HBsAg level in early stage of starting treatment [27]. However, there is no significant effect of such early HBsAg decrease on DCs status

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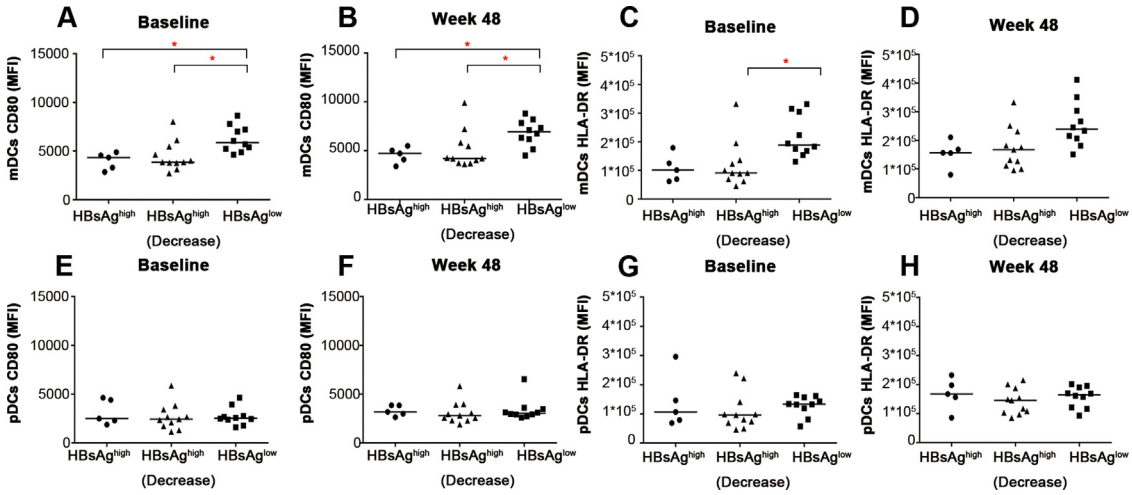

Figure 4 Maturation levels of DCs in patiens with different baseline HBsAg levels. Expression of CD80 on mDCs in $HBsAg^{high}$, $HBsAg^{high}$(Decrease) and $HBsAg^{low}$ patients on baseline (A) and end of 48 weeks' treatment (B). Expression of HLA-DR on mDCs in $HBsAg^{high}$, $HBsAg^{high}$(Decrease) and $HBsAg^{low}$ patients on baseline (C) and end of 48 weeks' treatment (D). Expression of CD80 on pDCs in $HBsAg^{high}$, $HBsAg^{high}$(Decrease) and $HBsAg^{low}$ patients on baseline (E) and end of 48 weeks' treatment (F). Expression of HLA-DR on pDCs in $HBsAg^{high}$, $HBsAg^{high}$(Decrease) and $HBsAg^{low}$ patients on baseline (G) and end of 48 weeks' treatment (H). $HBsAg^{high}$(Decrease) is a special subgroup of $HBsAg^{high}$ whose subjects' HBsAg decreased below 10000 IU/ml after treatment. The p values in figures are demonstrated as $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$.

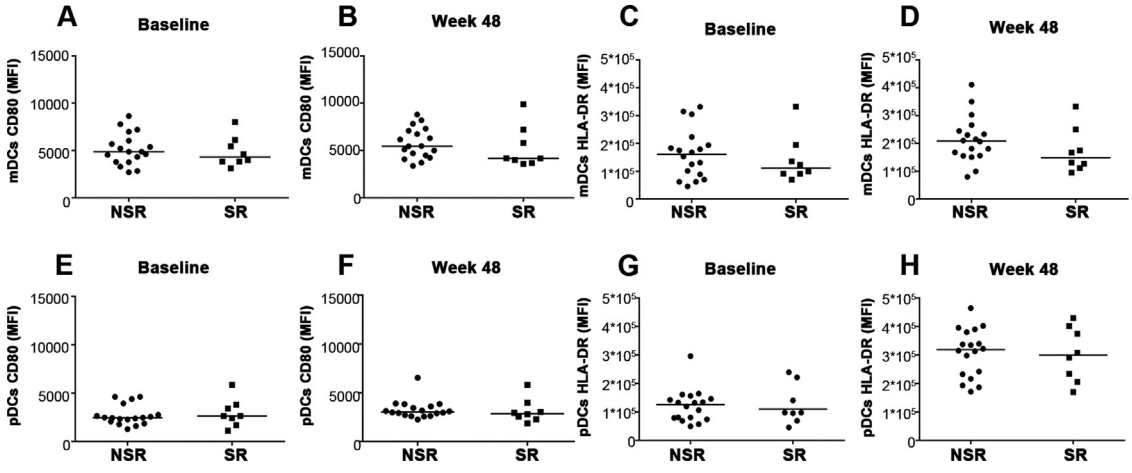

Figure 5 Influence of Early Significant HBsAg reduction on Maturation levels. Expression of CD80 on mDCs in NSR (No Significant Reduction) and SR (Significant Reduction) patients on baseline (A) and end of 48 weeks' treatment (B). Expression of HLA-DR on mDCs in NSR and SR patients on baseline (C) and end of 48 weeks' treatment (D). Expression of CD80 on pDCs in NSR and SR patients on baseline (E) and end of 48 weeks' treatment (F). Expression of HLA-DR on pDCs in NSR and SR patients on baseline (G) and end of 48 weeks' treatment (H). NSR: patients whose HBsAg did not saw a 1 log decrease at week 12 or 24 (no early significant decrease). SR: patients whose HBsAg did not saw a 1 log decrease (early significant decrease) at week 12 or 24. The p values in figures are demonstrated as $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$.

observed in our study. It is reasonable to speculate that high HBsAg level has a long-term effect on host immunity.

Since many patients have a high HBsAg level even after anti-virus treatment, new strategy is needed to antagonize the adverse effect of HBsAg. Recently, many researchers committed to develop new techniques to improve the therapy of CHB. The increasing understanding of immunoregulatory function of DCs attracts attention about using DCs-based therapy as a new strategy to cure cancer and chronic viral infection disease. In 2010, US FDA approved Provenge, a DCs-based immunotherapy, for patients with advanced prostate cancer [28]. The ex vivo matured DCs have the potential to initiate appropriate adaptive immune response (e.g. T cell immunity) after transfused to patients.

Besides serum HBsAg level, we also noticed that whether patients had a vertical transmission history may influence its DC maturation status after treatment. As shown in SFig 5, patients without vertical transmission history seen to have a higher CD86 expression on mDC after treatment. One explanation is that patients with vertical transmission history have worse immune response and treatment outcome. Takano et al. [29] also reported that HBeAg seroconversion

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rates were higher in-patient with horizontal transmission than in those with vertical transmission. But these data should not be over interpretated.

In this study, we focus on the in vivo frequencies and phenotypes of periphery mDCs and pDCs in CHB patients and its correlation with serum HBsAg quantification. Question remains whether the HBsAg level affect the cytokine secretion and antigen presenting function of DCs.

In conclusion, we demonstrated that the frequencies of mDCs and pDCs in total PMBC decreased in CHB patients. Anti-virus treatment by entecavir could restore the decreased frequencies of DCs in CHB patients and improve their DCs maturation levels. Moreover, the HBsAg level plays an important role to serve a long-term inhibitory effects on DCs.

Authors' contributions

XYL, LZ, and YHH designed the research study, acquired and analyzed the data and wrote the paper; XYL, LZ, and LG analyzed and interpreted the data; YRG and LBC revised the paper and language wrongs. All authors read and approved the final manuscript.

Disclosure of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.clinre.2019.07.016.

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