Plasmacytoid Dendritic Cell Function and Cytokine Network Profiles in Patients with Acute or Chronic Hepatitis B Virus Infection

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Abstract

Background: Plasmacytoid dendritic cells (pDCs) and cytokines play an important role in occurrence and recovery of hepatitis B virus (HBV) infection. The aim of this study was to explore the frequency and function of pDC and serum cytokine network profiles in patients with acute or chronic HBV infection.

Methods: The healthy individuals (HI group), hepatitis B envelope antigen (HBeAg)-positive chronic HBV patients in immune tolerance (IT) phase (IT group), HBeAg-positive chronic HBV patients (CHB group), and acute HBV patients (AHB group) were enrolled in this study. The frequency of cluster of differentiation antigen 86 (CD86) + pDC and the counts of CD86 molecular expressed on surface of pDC were tested by flow cytometer. The quantitative determinations of cytokines, including Fms-like tyrosine kinase 3 ligand (Flt-3L), interferon (IFN)-α2, IFN-γ, interleukin (IL)-17A, IL-6, IL-10, transforming growth factor (TGF)-β1 and TGF-β2, were performed using Luminex multiplex technology.

Results: In this study, there were 13 patients in HI group, 30 in IT group, 50 in CHB group, and 32 in AHB group. Compared with HI group, HBV infected group (including all patients in IT, CHB and AHB groups) had significantly higher counts of CD86 molecular expressed on the surface of pDC (4596.5 ± 896.5 vs. 7097.7 ± 3124.6; P < 0.001). The counts of CD86 molecular expressed on the surface of pDC in CHB group (7739.2 ± 4125.4) was significantly higher than that of IT group (6393.4 ± 1653.6, P = 0.043). Compared with IT group, the profile of cytokines of Flt-3L, IFN-γ, and IL-17A was decreased, IFN-α2 was significantly increased (P = 0.012) in CHB group. The contents of IL-10, TGF-β1, and TGF-β2 in AHB group were significantly increased compared with IT and CHB groups (all P < 0.05).

Conclusions: This study demonstrated that the function of pDC was unaffected in HBV infection. The enhanced function of pDC and IFN-α2 might involve triggering the immune response from IT to hepatitis active phase in HBV infection. Acute patients mainly presented as down-regulation of the immune response by enhanced IL-10 and TGF-β.

Key words: Acute Hepatitis B Virus Infection; Chronic Hepatitis B Virus Infection; Interferon-α2; Interleukin-10; Plasmacytoid Dendritic Cells

Introduction

Recovery from Hepatitis B virus (HBV) infection depends on the complex immune response, and the activities and phenotype of immune cells are affected by other cells or cytokines environment. In HBV infection, dendritic cells (DCs) are the starters and undertakers of the immune response, and closely related with the persistent HBV infection.1,2 Previous studies have shown that the functions of plasmacytoid DC (pDC), including responses...
to stimulation with toll-like receptor 9 ligand (TLR9-L) and TLR7-L, interferon (IFN)-α secretion, and natural killer (NK) cell activation are impaired in patients with chronic HBV infection.[3-5] However, several studies have also indicated that the pDC function in patients with chronic HBV infection was similar to that of health people.[6,8] Until now, it is not fully understood “why” patients with acute hepatitis can recover from the infection, “who and how” trigger the immune response during immune tolerance (IT) phase transition to immune clearance phase in chronic HBV infected patients.

pDC link innate and adaptive immunity and produce different cytokines and chemokines, especially Type I IFN, however, its function could be regulated by other cytokines. The clinical outcome in patients infected with HBV is dependent on the types of T helper type 1 (Th1) and Th1 immune response. Many cytokines are involved in the development of chronic HBV infection. IFN-α is an important antiviral active cytokine and widely used for the treatment of chronic HBV infection.[7,9] IFN-γ is the most important cytokine of cytotoxic T lymphocyte for controlling HBV replication or eradicating HBV in a noncytolytic manner.[10] Fms-like tyrosine kinase 3 ligand (Flt-3L) is also an essential growth factor for DC cells and NK cells homeostasis in vivo.[11] Interleukin (IL)-10 has primary effects on inhibition of pro-inflammation cytokines, regulation of humoral immunity, termination of inflammation, and is considered for sustaining HBV replication, initiating chronic HBV infection, and directing fibrogenesis through inhibition of IFN-γ secretion.[12,13] The pivotal function of transforming growth factor (TGF)-β in the immune system is to induce tolerance through the regulation of lymphocyte proliferation, differentiation, and survival,[14,15] with IL-6, IL-21, and IL-1β inducing the differentiation of type 17 helper T cells (Th17).[16,17] IL-17A is a pro-inflammatory cytokine with dual effects on immune responses and may play an important role in the development of HBV-related liver diseases.[18] In this study, the frequency and function of pDC and cytokine profiles were explored in patients with acute and chronic HBV infection.

**Methods**

**Ethical approval**

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Review Committee of Beijing Ditan Hospital. Informed written consent was obtained from all participants before their enrollment in this study.

**Subjects**

In this study, patients from February 2014 to December 2016 in Liver Diseases Center, Beijing Ditan Hospital were consecutively enrolled. This study included healthy individuals (HI group) and HBV infected group. The HBV infected group included HBeAg-positive chronic HBV patients in IT phase (IT group), patients with HBeAg-positive chronic HBV infection (CHB group), and patients with acute HBV infection (AHB group). Acute HBV infection was diagnosed as no HBV infection 6 months ago, positive for hepatitis B surface antigen (HBsAg), positive or negative HBeAg and HBV DNA, abnormal alanine aminotransferase (ALT) level, and anti-HBe-IgM titer >1:1000 or diagnosed by liver histological examination. HBeAg-positive chronic HBV infection in IT phase was defined as HBSAg positive for >6 months, high levels of HBeAg and serum HBV DNA load >10^7 U/ml, and persistently normal ALT levels (female <19 U/L, male <30 U/L), and/or liver mild inflammation by histological examination. HBeAg-positive chronic HBV infection in this study was diagnosed as patients with HBSAg positive for >6 months, HBeAg positive, serum detectable level of HBV DNA, ALT abnormal lasted more than 3 months and at least one time >200 U/L and/or with obvious inflammation in the liver histological examination.[19-21] Exclusion criteria included co-infection with other virus infections, such as hepatitis C virus, hepatitis D virus, and human immunodeficiency virus infections; with other liver diseases (alcohol liver disease, fatty liver, autoimmune liver disease, metabolic liver disease, or liver cancer), or with fibrosis and cirrhosis of the liver which was determined by transient elastography.[22] HIs were defined as negative for HBV and other virus infection with normal ALT values (female <19 U/L, male <30 U/L).

**Biochemical and virological detection**

The parameters of liver and kidney functions, including ALT, aspartate aminotransferase, total bilirubin, albumin, creatinine, and blood urea nitrogen, were measured by Hitachi 7600 automatic biochemical analyzer (Hitachi 7600-11; Hitachi, Tokyo, Japan). Serum HBV DNA was quantitated using a Roche CobasAmpliPrep/CobasTaqMan 96 real-time fluorescence quantitative polymerase chain reaction detection reagent (with a lower limit of 20 U/ml; Roche, Pleasanton, CA, USA). The levels of HBsAg, anti-HBs, HBeAg, and anti-HBe were tested using an Abbott Architect i2000 detection reagent (Abbott Diagnostics, Abbott Park, IL, USA); the HBSAg dynamic range was 0.05–250.00 U/ml. Samples with HBsAg levels >250.00 U/ml were automatically re-tested at 1:500 dilution. HBsAg negative was defined as <0.05 U/ml.

**Frequency and molecular expression of plasmacytoid dendritic cell**

In this study, the peripheral blood mononuclear cells (PBMCs) percentage of peripheral blood leukocytes, frequency of pDC in PBMC, frequency of cluster of differentiation antigen 86 (CD86) + pDC, and the counts of CD86 molecular expressed on surface of pDC were measured by four-color flow cytometry (FACS Caliburflow Cytometer; Becton-Dickinson, USA); and the steps to measure the expression of pDCs were as follows: the 100 μl of whole peripheral blood samples were incubated with monoclonal antibodies (mAbs) of Lin1-fluorescein isothiocyanate, human leukocyte antigen DR (HLA-DR)-peridinin chlorophyll protein, CD123-human antigen presenting cells, and
CD86 - phycoerythrin (PE; all provided by BD Biosciences, Cowley, UK) inappropriate tubes at room temperature in the dark for 20 min, then added 2 ml of fluorescence activating cell sorter (FACS) Lysing solution and incubated at room temperature in the dark for 5 min, centrifuged at 300 × g for 5 min, aspirated the supernatant, then added 2 ml of phosphate buffer saline (PBS) and vortex gently, centrifuged at 300 × g for 5 min and aspirated the supernatant again, vortex gently and re-suspended with 200 μl PBS; finally analyzed using the FACS flow cytometer. 

pDCs were identified as mononuclear cells that the Lin1-(CD3-CD14-CD16-CD19-CD20+)/HLA-DR+/CD123+, the activation/maturity state of pDC was assessed by the co-stimulatory molecules CD86 in Lin1-CD123+ HLA-DR+cells.

Plasma cytokine quantitation
Peripheral venous blood was collected; plasma was separated and stored in an −80°C refrigerator for cytokine detection. Eight cytokines, including Filt-3L, IFN-α2, IFN-γ, IL-10, IL-17A, IL-6, TGF-β1, and TGF-β2, were measured. Each sample was assayed in duplicate, and cytokine standards supplied by the manufacturer were run on each plate. Data were acquired by Luminex assay and analyzed using FlexMap 3D analyzer (Austin, TX, USA) according to manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Serum HBV DNA levels and HBsAg concentrations were logarithmically transformed for analysis. Continuous variables with normal distribution were expressed as the mean ± standard deviation (SD). One-way analysis of variance was used in the comparisons between two groups. Continuous variables with abnormal distribution were expressed as median (Q1, Q3), and Mann-Whitney U test and Kruskal-Wallis test were used for comparisons between groups or among multiple groups. Categorical variables were expressed as absolute and relative frequencies, and comparisons between groups were used Chi-square or Fisher’s exact tests. A \( P < 0.05 \) was considered as statistically significant.

### Results

#### Demographics and clinical characteristics of subjects

In this study, 125 subjects (65 males and 60 females) were collected, including 13 HIs, 30 patients in IT group, 50 in CHB group and 32 in AHB group, with mean age of 32.7 ± 10.3 years. Of 50 patients in CHB group, 29 cases were liver function exacerbation (ALT >200 U/L), and 10 were hepatitis flare with ALT level of 3–5 times the upper limit of normal values (40 U/L), and other 11 had obvious inflammation in the liver histological examination. There were significant differences in HBV DNA load, HBsAg levels, HBeAg contents, and ALT levels among IT, CHB, and AHB groups. The demographic and clinical characteristics of all patients are summarized in Table 1.

#### Frequency and molecular expressions of plasmacytoid dendritic cell

Compared with HI group, HBV infected group had significantly higher counts of CD86 molecular expressed on the surface of pDC (4596.5 ± 896.5 vs. 7097.7 ± 3124.6; \( t = 6.463, P < 0.001 \); Figure 1). The percentage of PBMC in peripheral blood leukocytes in AHB group was 42.46 ± 8.17%, which was significantly higher than those in IT group (37.66 ± 8.99%) and CHB group (37.56 ± 9.23%, all \( P < 0.05 \)). However, the pDC frequency in PBMC in AHB group (0.22 ± 0.09%) was significant lower than that of IT group (0.28 ± 0.14%, \( t = 3.422, P = 0.042 \)), and slightly lower than that of CHB group (0.24 ± 0.11%) without significant difference. While, the frequencies of CD86+ pDC in AHB (17.74 ± 7.28%) and CHB groups (18.74 ± 6.44%) slightly decreased compared with IT group (20.28 ± 9.23%), without significant differences. But compared with IT group (6393.4 ± 1653.6), the counts of CD86 molecular expressed on surface of pDC increased in AHB (6775.8 ± 2120.6) and CHB groups (7739.2 ± 4125.4), and the difference between CHB group and IT group was significant (\( t = 3.422, P = 0.043 \); Figure 2).

#### Cytokines concentration

Compared with HI group, HBV infected group had significant lower levels of Filt-3L (median: 108.42 pg/ml vs. 41.62 pg/ml, \( P = 0.002 \)), IFN-γ (66.48 pg/ml vs. 33.99 pg/ml, \( P = 0.028 \)), and IL-17A (35.96 pg/ml vs. 15.17 pg/ml, \( P = 0.005 \)), and had slightly higher concentrations of IFN-α2

### Table 1: Demographics and clinical characteristics of all subjects in this study

| Characteristics | HI group \( (n = 13) \) | IT group \( (n = 30) \) | CHB group \( (n = 50) \) | AHB group \( (n = 32) \) | Statistical values | \( P \) |
|-----------------|----------------|----------------|----------------|----------------|----------------|-------------|
| Male/female, \( n \) | 2/11 | 14/16 | 28/22 | 21/11 | 10.025* | 0.018 |
| Age (years), mean ± SD | 26.1 ± 1.9 | 29.9 ± 7.3 | 32.0 ± 7.7 | 38.7 ± 14.6 | 7.196* | <0.001 |
| ALT (U/L), mean ± SD | 10.48 ± 2.68 | 34.44 ± 17.66 | 304.10 ± 268.18 | 1187.71 ± 858.28 | 39.739* | <0.001 |
| HBV DNA (log_{10}, U/ml), mean ± SD | NA | 8.11 ± 0.48 | 7.05 ± 1.23 | 4.61 ± 1.40 | 78.578* | <0.001 |
| HBsAg (log_{10}, U/ml), mean ± SD | NA | 4.66 ± 0.52 | 3.81 ± 0.69 | 2.55 ± 1.40 | 41.405* | <0.001 |
| HBeAg (PEU/ml), median (Q1, Q3) | NA | 1606.36 (1556.53, 1679.44) | 933.34 (467.38, 1316.67) | 5.93 (0.92, 59.31) | 105.444* | <0.001 |

*Chi-square test; †One-way analysis of variance; ‡Comparison among IT, CHB and AHB groups. HI group: Health individuals; IT group: HBeAg-positive chronic HBV patients in immune tolerance phase; CHB group: HBeAg-positive chronic HBV patients; AHB group: Acute HBV patients. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B envelope antigen; NA: Not applicable; SD: Standard deviation.
and IL-10 (all $P > 0.05$; Table 2). Compared with IT group, CHB group had significant increased IFN-α2 level (median: 16.74 pg/ml vs. 39.53 pg/ml, $P = 0.012$), decreased Flt-3L level (median: 59.96 pg/ml vs. 25.45 pg/ml, $P = 0.014$) and slightly decreased concentrations of IFN-γ and IL-17A (all $P > 0.05$). The levels of IL-10 (median: 22.77 pg/ml vs. 6.80 pg/ml, $P = 0.001$), TGF-β1 (10,447.00 pg/ml vs. 4713.50 pg/ml, $P = 0.007$) and TGF-β2 (782.28 pg/ml vs. 419.01 pg/ml, $P = 0.001$) in AHB group elevated significantly compared with IT group. Furthermore, AHB
group had significant higher IL-10 (median: 22.77 pg/ml vs. 8.66 pg/ml, \( P = 0.011 \)), TGF-β1 (10,447.00 pg/ml vs. 3,755.50 pg/ml, \( P = 0.001 \)), and TGF-β2 levels (782.28 pg/ml vs. 482.87 pg/ml, \( P = 0.005 \)) than CHB group [Table 2].

**DISCUSSION**

The 90% of HBV infection in infants will develop chronic infection; conversely, more than 95% of HBV infection in adults will recover from infection.[23] It is considered that the immaturity of the newborn immune system contributes to the development of chronic infection.[24] In chronic HBV patients, it was demonstrated that HBV and its antigens can induce Treg cells to impact or directly impair the maturation and functions of pDC and myeloid DC (mDC).[25-28] However, it was unclear why and how the immune response be triggered in chronic HBV patients; even though, they are in the condition of high levels of HBV DNA, HBeAg, and HbsAg. In this study, frequency and stimulation functional molecular expression of pDC in AHB, CHB and IT groups were investigated. The results of this study showed that the frequency of pDC in PBMC was significantly lower, however stimulation functional CD86 molecular expressed on pDC surface was significantly higher in HBV infected group, compared with HI group; compared with IT group, the frequency of pDC in PBMC was slightly decreased in CHB group, and significantly decreased in AHB group, but the stimulation functional CD86 molecular expressed on pDC surface was elevated in CHB and AHB groups. These results in this study suggested that chronic and acute HBV infection during inflammation period could reduce pDC frequency, but the function of pDC was not impaired, as demonstrated in other studies.[29,30]

The activation and inhibition of immune cell function often depend on cytokines or cytokine environment. In HBV infection, IFN-α can inhibit virus replication by inducing antiviral protein and activate NK, HBV specific CD4+ and CD8+ T cells to kill the infected hepatic cells.[31] IFN-α has been used widely in treatment of chronic HBV infection to achieve the immune control to HBV, and the immune control was defined as HBeAg seroconversion or HbsAg loss.[32-33] HBV and viral antigens can induce Treg,[13,36-39] secreting IL-10 to down-regulation of other immune cells, thus leading to infection persistence. In this study, we detected the cytokines in AHB, CHB, and IT groups to explore the cytokine network profiles in hepatitis occurrence.

In CHB group, although the contents of immune stimulating factor as Flt-3L was essential growth factor for DCs, and had the action of inducing the proliferation of NK cells,[31,40-42] the immune effect factors such as IFN-γ and IL-17A were decreased slightly compared with IT group, and the CD86 molecular expressed on pDC surface and IFN-α2 content increased significantly. Our study suggested that the activated pDC and the elevated IFN level could trigger the immune response against HBV in chronic hepatitis development from IT, and it might be the basis for those patients in immune active phase receiving PEG-IFN therapy to achieve a higher rate of HBeAg seroconversion and sustained response.[7,43,44] With the increasing of IL-10 and TGF-β2 contents in chronic hepatitis patients, it might limit virus clearance and lead low rate of spontaneous HBeAg seroconversion in nature history.[45]

**Table 2: The cytokine levels of all subjects in this study (pg/ml)**

| Cytokines | HI group (\( n = 13 \)) | HBV infected group | CHB group (\( n = 50 \)) | AHB group (\( n = 32 \)) |
|-----------|-----------------|---------------------|-----------------|-----------------|
| Flt-3L    | 108.54 (30.68, 643.12) | 41.62 (3.41, 119.63) | 59.96 (29.90, 205.50) | 25.45 (0.16, 83.99) |
| IFN-α2    | 15.35 (5.70, 67.65) | 32.35 (9.12, 74.19) | 16.74 (4.36, 331.18) | 39.53 (20.47, 74.19) |
| IFN-γ     | 66.48 (24.31, 342.43) | 33.99 (14.15, 88.65) | 45.48 (20.44, 191.01) | 33.58 (9.38, 86.39) |
| IL-17A    | 35.96 (12.22, 272.99) | 15.17 (5.11, 48.05) | 19.34 (12.32, 68.76) | 12.30 (3.23, 47.54) |
| IL-6      | 3.59 (0.69, 132.93) | 3.50 (1.49, 139.91) | 3.33 (1.45, 19.99) | 2.57 (1.06, 9.98) |
| IL-10     | 3.73 (2.98, 11.92) | 9.47 (4.70, 20.95) | 6.80 (3.13, 13.31) | 8.66 (4.83, 19.26) |
| TGF-β1    | 4842.00 (2787.50, 5635.00) | 4790.00 (3140.00, 9790.00) | 4713.50 (3638.25, 8294.50) | 3755.50 (2879.00, 7079.00) |
| TGF-β2    | 440.18 (339.99, 485.87) | 499.84 (368.61, 593.02) | 491.09 (311.80, 562.83) | 482.87 (358.44, 751.19) |

All data are shown as median (Q1, Q3). HI group: Health individuals; IT group: HBeAg-positive chronic HBV patients in immune tolerance phase; CHB group: HBeAg-positive chronic HBV patients; AHB group: Acute HBV patients. HBV: Hepatitis B virus; Flt-3L: Fms-like tyrosine kinase 3 ligand; IFN: Interferon; IL: Interleukin; TGF: Transforming growth factor.
In this study, the cytokine profiles in AHB group mainly showed significant elevation of IL-10 and TGF-β contents, slightly decrease of IFN-γ, compared with IT and CHB groups. It was reported that 95% of acute HBV patients were adulthood and recovered from the infection. As an anti-inflammation cytokine, high levels of IL-10 can help to limit liver inflammation and damage to prevent deterioration of liver function. The previous study reported that strong immune response to the viral envelope antigen in acute HBV patients has been established in the early stage of infection. 

There are several limitations in our study. First, the sample size of this study was relatively small. Second, the occurrence of acute and chronic HBV infection is often sporadic. In our study, the participants were consecutively enrolled. Thus, it was difficult to pair some baseline factors and enrol the large and the same number of participants among four groups during the study period, which might lead to bias. Therefore, the randomized controlled trials with large samples are required to further resolve these issues in the future.

In summary, this study demonstrated that the function of pDC was unaffected in HBV infection. The enhanced function of pDC and IFN-α2 might involve triggering the immune response from IT to hepatitis active phase in HBV infection. Acute patients mainly presented as down-regulation of the immune response by enhanced IL-10 and TGF-β.

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Conflicts of interest

There are no conflicts of interest.

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