Effect of viral load on T-lymphocyte failure in patients with chronic hepatitis B

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Abstract

AIM: To investigate peripheral T-lymphocyte subpopulation profile and its correlation with hepatitis B virus (HBV) replication in patients with chronic hepatitis B (CHB).

METHODS: Distribution of T-lymphocyte subpopulations in peripheral blood was measured by flow cytometry in 206 CHB patients. HBV markers were detected with ELISA. Serum HBV DNA load was assessed with quantitative real-time polymerase chain reaction (PCR). The relationship between HBV replication and variation in peripheral T-cell subsets was analyzed.

RESULTS: CHB patients had significantly decreased CD3⁺ and CD4⁺ cells and CD4⁺/CD8⁺ ratio, and increased CD8⁺ cells compared with uninfected controls (55.44 ± 12.39 vs 71.07 ± 4.76, 30.92 ± 7.48 vs 38.94 ± 3.39, 1.01 ± 0.49 vs 1.67 ± 0.33, and 34.39 ± 9.22 vs 24.02 ± 4.35; P < 0.001, respectively). Univariate analysis showed a similar pattern of these parameters was significantly associated with high viral load, presence of serum hepatitis B e antigen (HBeAg) expression, liver disease severity, history of maternal HBV infection, and young age at HBV infection, all with P < 0.01. There was a significant linear relationship between viral load and these parameters of T-lymphocyte subpopulations (linear trend test P < 0.001). There was a negative correlation between the levels of CD3⁺ and CD4⁺ cells and CD4⁺/CD8⁺ ratio and serum level of viral load in CHB patients (r = -0.68, -0.65 and -0.75, all P < 0.0001), and a positive correlation between CD8⁺ cells and viral load (r = 0.70, P < 0.0001). There was a significant decreasing trend in CD3⁺ and CD4⁺ cells and CD4⁺/CD8⁺ ratio with increasing severity of hepatocyte damage and decreasing age at HBV infection (linear trend test P < 0.01). In multiple regression (after adjustment for age at HBV infection, maternal HBV infection status and hepatocyte damage severity) log copies of HBV DNA maintained a highly significant predictive coefficient on T-lymphocyte subpopulations, and was the strongest predictor of variation in CD3⁺, CD4⁺, CD8⁺ cells and CD4⁺/CD8⁺ ratio. However, the effect of HBeAg was not significant.

CONCLUSION: T-lymphocyte failure was significantly associated with viral replication level. The substantial linear dose-response relationship and strong independent predictive effect of viral load on T-lymphocyte subpopulations suggests the possibility of a causal relationship between them, and indicates the importance of viral load in the pathogenesis of T cell hyporesponsiveness in these patients.

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Key words: Hepatitis B virus; Chronic hepatitis B virus infection; Hepatitis B virus DNA; T lymphocyte subpopulation; Immune function

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INTRODUCTION

Hepatitis B virus (HBV) is one of the most prevalent...
viral pathogens in humans, with almost a third of the world population having evidence of infection, and about 350 million chronically infected patients. Chronic hepatitis B (CHB) is characterized by inflammatory liver disease of variable severity and is associated with a significantly increased risk of cirrhosis, liver failure and hepatocellular carcinoma. Seventy-five percent of patients with CHB are Asian. In China, > 120 million people are chronic carriers of HBV, and 40%-60% catch HBV infection from their mothers. For neonates and children younger than 1 year who acquire HBV infection perinatally, the risk of the infection becoming chronic is 90%. The pathogenesis of persistent viral infection and hepatitis B is very complex. Both viral factors, as well as the host immune response, have been implicated in the pathogenesis and clinical outcome of HBV infection. Apart from direct biological effects of viral variants, there is a growing consensus that the host immune response, especially the virus-specific T cell response, is the key determinant influencing the course of disease and the onset of liver disease. Many investigators suggest the chronicity of HBV infection is caused by a deficient cellular immune function, but the mechanism has not been defined. For a non-cytopathic virus like HBV to persist, it must either overwhelm or not induce an effective antiviral immune response, or it must be able to evade it. Hepatitis B e antigen (HBeAg) may play an important role in the interaction of the virus with the immune system. Data from transgenic mice indicate neonatal tolerance to HBeAg is a crucial mechanism responsible for the lack of an antiviral immune response following mother to infant transmission. Milich et al have further demonstrated an immunomodulatory role of HBeAg in antigen presentation and recognition by CD4 cells.

The relationship between HBV specific T-cell response, HBV viral load and HBeAg expression in CHB is complicated by their close correlation and remains unclear. In China, where vertical perinatal transmission is the main route of transmission, most patients with CHB have become infected in the early years of life. The influence of age at infection and maternal HBV infection status on T-cell immune status and HBV replication is still not settled. The aim of the present study was to evaluate the peripheral blood T lymphocyte subpopulation profile and its correlation with HBV replication.

**Materials and Methods**

**Enrollment of study subjects**

Two hundred and six consecutive patients with CHB who were admitted to the department of Infectious Diseases of the First Affiliated Hospital of Kunming Medical University, the Third Municipal People’s Hospital of Kunming and the Yunnan General Hospital of The Chinese People’s Armed Police Forces, between January 2006 and May 2007, were prospectively enrolled into the study. The clinical diagnosis was based on liver function tests, hepatitis virus markers, ultrasonography and histopathology. The diagnosis of all the patients was confirmed according to the criteria for viral hepatitis.

The following criteria were fulfilled by all patients: (1) steady positivity for hepatitis B surface antigen (HBsAg) in the serum for at least 12 mo, to establish CHB; and (2) exclusion of other concomitant causes of liver disease (hepatitis C, D and HIV infection and alcohol consumption > 60 g/d), relatively rare liver disease (autoimmune hepatitis and metabolic liver disease), and treatment with immunosuppressive therapy or antiviral therapy for HBV infection within the past 12 mo before entry. None of the patients was a drug user, or exposed to hepatotoxin. Those who had liver cirrhosis were also excluded since their long history of treatment and terminal disease state may have complicated the interpretation of the results.

One hundred individuals who were free of HBsAg were identified from those attending the outpatient service for a health check-up; 61 of the participants were male, 39 were female; mean age, 33.24 (SD 10.28) year. These served as the control group for comparison of T-lymphocyte subpopulations with those who had HBV infection.

**Serological liver function tests and HBV marker evaluation**

Serum alanine aminotransferase (ALT), aspartate transaminase (AST) and total bilirubin were tested with routine automated techniques (upper limit of normal; 40 U/L, 40 U/L and 17.1 μmol/mL, respectively) (AU2700, Japan). HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, Anti-HBcAb IgM) were measured at a virological laboratory with the use of ELISA (Anthos 2010, Austria). The experimental methods followed those specified within the reagent kit package insert (Sino-American Biotech Co., Ltd, Shanghai, China).

**Quantitative measurement of HBV DNA (viremia)**

Serum HBV DNA load in patients was assessed with the real-time fluorescent quantitative polymerase chain reaction method (real-time PCR) using a Lightcycler PCR system (FQD-33A, Bioer, Hongzhou, China) with a lower limit of detection of about 1000 viral genome copies/mL. The handling procedures were performed in strict accordance with the reagent kit package insert (Shenzhen PG Biotech Co., Ltd., Shenzhen, China). The primer was provided in the kit, the reaction volume was 40 μL, and the reaction conditions were 37°C for 5 min, 94°C for 1 min, then 40 cycles at 95°C for 5 s and 60°C for 30 s. Results were considered abnormal when HBV DNA was > 1000 copies/mL.

**Peripheral blood T lymphocyte subsets measurement**

Blood samples were collected in heparinized vacutainer tubes. Samples were analyzed with a Muti-Q-Prep processor (Coulter, USA) and thereafter Epics-XL flow cytometry (FCM) (Coulter). Lymphocytes were analyzed using a gate set on forward scatter versus side scatter. Anti-human monoclonal antibodies CD3-PE-CY5/CD4-FITC/CD8-PE were purchased from Immunotech (USA). For each sample, detection was carried out using CELLQuest software (Coulter). The results were expressed as the percentages of CD3, CD4 and CD8 cells found to be positive for the marker antigen in the total T cell.
Table 1  Characteristics of patients with chronic hepatitis B

| Characteristics                      | All patients (n = 206) | Various courses of chronic hepatitis B                     |   |
|--------------------------------------|------------------------|-----------------------------------------------------------|---|
|                                      | Mild-CHB (n = 101)     | Moderate-CHB (n = 54)                                     | Severe-CHB (n = 51) |
|                                      |                        |                                                          | P-value    |
| Sex (male/female)                    | 143/63                 | 68/33                                                     | 36/18      | 39/12       | > 0.05    |
| Mean age (yr)                        | 29.61 ± 9.38           | 29.06 ± 9.88                                             | 29.72 ± 9.16 | 30.59 ± 8.67 | > 0.05    |
| Patients with MH, n (%)              | 104 (50.5)             | 45 (44.6)                                                | 23 (42.6)  | 36 (70.6)   | < 0.01    |
| Age at HBV-infection (yr), n (%)     |                        |                                                          |            |             |
| ≤ 8                                  | 86 (41.7)              | 34 (33.7)                                                | 22 (40.7)  | 30 (58.8)   |           |
| 8-20                                 | 71 (34.9)              | 39 (38.6)                                                | 22 (40.7)  | 10 (19.6)   |           |
| > 20                                 | 45 (21.8)              | 28 (27.7)                                                | 8 (14.8)   | 9 (18.0)    |           |
| unknown                              | 4 (1.9)                | 0                                                        | 2 (3.7)    | 2 (3.9)     |           |
| Serum ALT (IU/L)                     | 163.75 ± 140.41        | 69.91 ± 17.36                                            | 152.04 ± 19.62 | 379.65 ± 186.44 | < 0.001 |
| Serum AST (IU/L)                     | 116.69 ± 107.97        | 52.61 ± 14.13                                            | 96.26 ± 21.27 | 265.25 ± 125.12 | < 0.001 |
| Serum total bilirubin (μmol/mL)      | 20.79 ± 14.02          | 15.18 ± 2.42                                             | 19.20 ± 4.51 | 33.59 ± 23.22 | < 0.001 |
| HBV DNA positive, n (%)              | 149 (72.3)             | 51 (50.5)                                                | 47 (87.0)  | 51 (100.0)  | < 0.001   |
| Serum HBV DNA (copies/mL), n (%)     |                        |                                                          |            |             |
| ≤ 1.0 × 10^3                         | 57 (27.7)              | 50 (49.5)                                                | 7 (13.0)   | 0           |           |
| > 1.0 × 10^3-1.0 × 10^4              | 32 (15.5)              | 14 (13.9)                                                | 18 (33.3)  | 0           |           |
| > 1.0 × 10^4-1.0 × 10^5              | 31 (15.0)              | 11 (10.9)                                                | 11 (20.4)  | 9 (17.6)    |           |
| > 1.0 × 10^6                         | 86 (41.7)              | 26 (25.7)                                                | 18 (33.3)  | 42 (82.4)   |           |
| HBV DNA load (Log, copies/mL)        | 5.84 ± 2.22            | 4.77 ± 2.11                                              | 5.91 ± 1.83 | 7.91 ± 1.17 | < 0.001   |
| HBeAg positive, n (%)                | 96 (46.6)              | 25 (24.8)                                                | 31 (57.4)  | 40 (78.4)   | < 0.001   |

HBV: Hepatitis B virus; CHB: Chronic hepatitis B; MH: Maternal HBV-infection status. *Date are expressed as mean ± SD; †Chi-square test P-value; ‡ANOVA P-value.

Population. The handling procedures were performed in strict accordance with the manufacturer’s instructions.

**Maternal HBV infection status (MH)**

MH was confirmed according to the maternal presence of serum HBV markers and/or HBV DNA, documented on at least two occasions, at least 3 mo apart; or documented maternal death from HBV-related liver diseases such as CHB, HBV-related liver cirrhosis and/or hepatocellular carcinoma.

**Age at HBV infection**

In the past three decades in China, all children have been obliged to be tested for HBV markers when they first go to kindergarten and elementary school. Subsequent obligatory tests are carried out when they apply for university or a job. The results of these tests were obtained from medical records and interviews. We classified the age of the first positive test as < 8 years, 8-20 years and > 20 years old.

**Statistical analysis**

Initial sample size calculation came up with 50 subjects positive for HBV DNA positive and the same number negative. This provided the study with a statistical power of 80% at the 0.025 level of significance to detect a difference in T-cell variation values of 33 versus 38. However, to cover the problem of being potentially confounded by other variables, and to have enough subjects for stratifying levels of HBV DNA load to examine a dose-response relationship, 206 CHB patients and 100 controls were recruited.

Descriptive statistics were used to examine the age, gender, serum HBV load, HBeAg status, ALT, AST, total bilirubin, age at HBV infection and maternal HBV infection status. The levels of T-lymphocyte subpopulation in normal individuals (HBsAg-negative) were summarized as means ± SD, to serve as a control reference. Effects of various independent demographic, clinical and serological variables on T-cell profile were analyzed only among HBsAg-positive individuals. In univariate analysis, breakdown of these profiles by individual independent variables was carried out. An independent t test was done for two-level independent variables and one-way ANOVA for more than two-level variables. The relationship between HBV replication and peripheral T-lymphocyte subpopulation was analyzed by correlation analysis and ANOVA linear trend test. Finally, a multiple linear regression model was employed for multivariate analysis to assess the independent effects of variables on peripheral blood T lymphocytes. Variables yielding P ≤ 0.2 in univariate analysis were included in the multivariate analysis, and the models were refined by backward elimination, guided by a change in log likelihood of successive models. A final P < 0.05 was considered statistically significant. Computations were carried out with the aid of R software version 2.5.1[18].

**RESULTS**

**Demographic characteristics and clinical features of CHB patients**

The demographic, virological, serological and clinical characteristics of the patients are summarized in Table 1. Over two-fifths of the CHB patients acquired the infection before the age of 8 year. Almost three-quarters had detectable levels of HBV DNA. Among these, the majority (57.7%) had > 10^5 copies/mL. Around half of the patients’ mothers were HBV-positive. A little less than half were HBeAg-positive (46.6%). Among various courses of CHB, all severe-CHB patients had detectable levels of HBV DNA; the majority of severe-CHB patients...
Table 2  Peripheral T-cell subsets in normal control and CHB patients broken down by various factors (mean ± SD)

| Groups                        | n   | CD3⁺ | CD4⁺ | CD8⁺ | CD4⁺/CD8⁺ ratio |
|-------------------------------|-----|------|------|------|-----------------|
| HBV status                   |     |      |      |      |                 |
| Negative (normal control)     | 100 | 71.07 ± 4.76 | 38.94 ± 3.39 | 24.02 ± 4.35 | 1.67 ± 0.33     |
| Positive                      | 206 | 55.44 ± 12.39 | 30.92 ± 7.48 | 34.39 ± 9.22 | 1.01 ± 0.49     |
| Maternal HBV-infection status |     |      |      |      |                 |
| Negative                      | 102 | 60.36 ± 10.68 | 34.92 ± 6.52 | 30.43 ± 7.76 | 1.25 ± 0.49     |
| Positive                      | 104 | 50.60 ± 12.09 | 26.99 ± 6.18 | 38.29 ± 8.91 | 0.77 ± 0.34     |
| Age at HBV-infection (yr)     |     |      |      |      |                 |
| < 8                           | 86  | 49.83 ± 11.64 | 25.74 ± 5.16 | 39.11 ± 8.68 | 0.70 ± 0.24     |
| 8-20                          | 71  | 59.28 ± 11.47 | 34.15 ± 6.31 | 31.49 ± 7.52 | 1.18 ± 0.47     |
| > 20                          | 45  | 60.29 ± 11.13 | 35.58 ± 7.27 | 29.68 ± 8.80 | 1.33 ± 0.54     |
| Unknown                       | 4   | 53.20 ± 12.45 | 32.63 ± 4.43 | 37.78 ± 5.54 | 0.88 ± 0.19     |
| Liver diseases status         |     |      |      |      |                 |
| Mild-CHB                      | 101 | 60.38 ± 11.51 | 33.57 ± 8.36 | 30.29 ± 8.91 | 1.23 ± 0.54     |
| Moderate-CHB                  | 54  | 56.69 ± 10.27 | 30.53 ± 4.79 | 35.26 ± 7.72 | 0.93 ± 0.31     |
| Severe-CHB                    | 51  | 44.32 ± 8.71  | 26.08 ± 5.19 | 41.63 ± 6.18 | 0.65 ± 0.19     |
| HBV DNA load (copies/mL)      |     |      |      |      |                 |
| ≤ 1.0 × 10⁵                   | 57  | 66.13 ± 7.38  | 37.92 ± 5.94 | 25.18 ± 5.39 | 1.58 ± 0.43     |
| > 1.0 × 10⁵-1.0 × 10⁶         | 32  | 60.93 ± 8.02  | 32.73 ± 6.42 | 33.18 ± 5.36 | 1.03 ± 0.33     |
| > 1.0 × 10⁶-1.0 × 10⁷         | 31  | 54.28 ± 12.91 | 29.63 ± 7.07 | 35.17 ± 8.18 | 0.88 ± 0.25     |
| > 1.0 × 10⁷                   | 86  | 46.72 ± 9.31  | 26.07 ± 4.52 | 40.69 ± 7.35 | 0.67 ± 0.22     |
| HBeAg status                 |     |      |      |      |                 |
| Negative                      | 110 | 61.09 ± 10.67 | 33.99 ± 7.48 | 30.17 ± 8.04 | 1.24 ± 0.52     |
| Positive                      | 96  | 48.99 ± 11.06 | 27.39 ± 5.75 | 39.24 ± 8.05 | 0.73 ± 0.25     |

⁺P < 0.001 for all comparisons of +ve vs -ve for each measure and each T-cell parameter; ᵇP < 0.01, ᵇᵇP < 0.001 for ANOVA Linear trend test.

had higher viral load (82.4%), higher titer of HBV DNA in serum, higher serum positivity of HBeAg (78.4%), higher maternal HBV infection status (70.6%) and lower age at HBV-infection than mild-CHB and moderate-CHB patients (all P < 0.01). The severity of liver diseases was significantly associated with HBV replication status, including higher viral load and HBeAg expression, all with P < 0.001 (Table 1).

Over two-thirds of the CHB patients with MH acquired the infection before the age of 8 year (79.8%), whereas age of HBV infection of > 20 years was more common in non-MH patients. Higher positivity of serum HBV DNA and of HBeAg were observed in MH patients than in non-MH individuals (92.3% vs 52.0%, OR = 10.96, 95% CI 4.7-28.85; 56.7% vs 36.3%, OR = 2.29, 95% CI 1.27-4.19; respectively). The percentage of cases with higher-viral-load viremia (> 10⁷ copies/mL) was higher in MH patients than that in the non-MH group (57.7% vs 25.5%, OR = 3.96, 95% CI 2.12-7.54). A greater proportion of non-MH individuals had an undetectable-viral-load viremia (< 10³ copies/mL) compared with MH individuals (48.0% vs 7.7%, OR = 10.96, 95% CI 4.7-28.85).

A higher double-positivity of serum HBV DNA and HBeAg existed in MH patients significantly higher than in non-MH patients (56.7% vs 36.3%, OR = 2.29, 95% CI 1.27-4.19).

**Peripheral T-lymphocyte subpopulation composition in CHB patients**

CHB patients had significantly decreased total CD3⁺ and CD4⁺ subpopulations and CD4⁺/CD8⁺ ratio, and increased CD8⁺ subsets compared with uninfected controls, all with P < 0.001. Univariate analysis showed that T-cell failure was significantly associated with higher viral load, serum HBeAg expression, severity of liver disease, history of maternal HBV infection, and lower age at HBV infection (Table 2). Linear dose-response relationship between the level of T-lymphocyte subpopulation and copies of HBV DNA was also highly significant (linear trend test P < 0.001). A negative correlation existed between the levels of CD3⁺ and CD4⁺ cells and CD4⁺/CD8⁺ ratio and serum HBV viral load, whereas a positive correlation existed between the level of CD8⁺ cells and viral load, all with P < 0.0001. Correlation between T lymphocytes and viral load is shown in Figures 1 and 2.
Furthermore, there was a significant decreasing trend of CD3⁺ and CD4⁺ cells and CD4⁺/CD8⁺ ratio with increasing hepatocytic damage; this was inverse for CD8⁺ cells. A similar pattern was also seen among age at HBV infection, all with a linear trend test P value < 0.01.

Table 3  Multiple linear regression predicting peripheral blood T lymphocyte subpopulations (n = 206)

|                | CD3⁺ T lymphocyte | CD4⁺ T lymphocyte | CD8⁺ T lymphocyte | CD4⁺/CD8⁺ ratio |
|----------------|-------------------|-------------------|-------------------|-----------------|
| Intercept      | 69.39             | 3.51              | 35.6              | 23.21           | 1.55 | 0.12 |
| Serum HBV load (Log, copies/mL) | -2.58 | 0.45 | < 0.0001 | -1.31 | 0.26 | < 0.0001 | 2.05 | 0.33 | < 0.0001 | -0.11 | 0.02 | < 0.0001 |
| HBeAg¹       | 2.37              | 0.15              | 0.71              | 0.47            | -1.16 | 1.21 | 0.34 | 0.06 | 0.06 | 0.32 |
| Liver diseases status³ | < 0.01 | 0.63 | 0.04 | 0.04 |
| Moderate-CHB  | -0.36             | 1.58              | -1.15             | 0.93            | 2.34 | 1.15 | -0.15 | 0.05 |
| Severe-CHB    | -6.14             | 1.88              | -1.65             | 1.11            | 3.52 | 1.37 | -0.14 | 0.06 |
| Age at HBV-infection (yr)³ | 0.31 | < 0.001 | 0.06 | < 0.001 |
| 8-20          | -0.06             | 2.09              | 4.1               | 1.24            | -0.72 | 1.52 | 0.13 | 0.07 |
| > 20          | -1.72             | 2.55              | 4.03              | 1.51            | -0.16 | 1.86 | 0.15 | 0.09 |
| Unknown       | 1.93              | 4.94              | 6.17              | 2.92            | -0.52 | 3.61 | 0.14 | 0.17 |
| Maternal HBV-infection status³ | 3.61 | 2.05 | 1.78 | 1.21 | 0.14 | -2.76 | 1.49 | 0.07 | 0.14 | 0.07 | 0.05 |

β: Coefficients from the model; 1Continuous variable; 2Reference group: HBeAg-negative; 3Reference group: mild-CHB; 4Reference group: < 8 yr old age at HBV infection; 5Reference group: With maternal HBV-infection status.

Furthermore, there was a significant decreasing trend of CD3⁺ and CD4⁺ cells and CD4⁺/CD8⁺ ratio with increasing hepatocytic damage; this was inverse for CD8⁺ cells. A similar pattern was also seen among age at HBV infection, all with a linear trend test P value < 0.01.

**Linear regression predicting peripheral blood T-lymphocyte subpopulation from relevant parameters**

In Table 3, linear regression models are separately summarized for CD3⁺, CD4⁺ and CD8⁺ cells and CD4⁺/CD8⁺ ratio, which are the dependent variables. After adjustment for all independent variables listed in the table, serum HBV viral load was the key predictor for T-cell profile. The severity of liver disease reduced the number of CD3⁺ T lymphocytes, increased the number of CD8⁺ T cells, and decreased the CD4⁺/CD8⁺ ratio. Those who had infection at a young age had a lower CD4⁺ T cell count and CD4⁺/CD8⁺ ratio than those who acquired infection later in life. Maternal infection history and serum HBeAg expression had no independent effect on T-lymphocyte profile.

**DISCUSSION**

This study demonstrated disorder of cellular immune function in CHB patients. The level of T-cell dysfunction had a linear dose-response relationship with the load of HBV DNA. Furthermore, the study also illustrated that the strong independent effect of HBV viral load seemed to eliminate and/or weaken the effects of liver disease severity, maternal carrier status, early age of infection and HBeAg positivity on the impairment of T-cell function.
Our findings indicate that CHB patients have T-cell failure. The same finding has also been demonstrated previously, namely, that the chronicity of HBV infection is caused by a deficiency in cellular immune function[16-20], and hepatocyte damage is mainly caused by immunological injury[21-29]. However, the mechanism has not been defined[3]. Apart from direct biological effects of viral variants, there is a growing consensus that the host immune response, especially the virus-specific T-cell response, is the key determinant influencing the course of disease and the onset of liver disease[30,31]. The significant decrease in total T lymphocyte (CD3+ T) revealed that there is a lack of immunologically competent cells involved in cellular immunoreactivity against HBV infection. A lack of CD4+ T cells can impair CD8+ T-cell activity and antibody production[32], while the inability to mount a virus-specific CD8+ T-cell response results in a level of circulating virus that cannot be cleared by antibodies alone[33-35]. Activation-induced cell death (AICD) is related to a decrease in lymphocytes and functional defects. This phenomenon can cause decreased immune clearance. This may be an important reason for persistent infection with HBV. AICD in peripheral blood T lymphocytes in CHB has been demonstrated previously[36,37]. Thus, AICD is considered an important modulator in down-regulating the "burst" of responding T cells in patients with CHB[38,39].

Our results revealed T-cell failure was significantly associated with viral replication level. The substantial linear dose-response relationship and strong independent predictive effect of HBV DNA, but not other variables, on T-lymphocyte subpopulations suggests the possibility of a causal relationship between them. However, the cross-sectional nature of our data did not allow us to identify the temporal direction of the causal relationship between these two variables. Mizukoshi et al[38] have suggested antiviral therapy of persistently infected patients appears to increase the frequency of HBV-specific CD4+ T-cell responses during the first year of treatment. Boni et al[39,40] have reported antiviral treatment can overcome CD8+ T-cell hyporesponsiveness in subjects with CHB, which suggests the T cells are present but suppressed. It has been reported by Pham et al[41] in 21 CHB patients that the ratio of CD4+/CD8+ liver-derived lymphocytes, but not of peripheral blood lymphocytes, appears to be related to the level of HBV replication, which reveals a positive correlation with viral load. The evidence that an efficient antiviral T-cell response can be restored by antiviral monotherapy in CHB, concurrently with reduction of viremia, indicates the importance of viral load in the pathogenesis of T-cell hyporesponsiveness.

The strong independent effect of viral load on T-cell impairment and viral factors (viral variants) might explain the disappearance of the effect of other variables in multivariate analysis. Among our patients, the majority were characterized by young age at first HBV infection, maternal carrier status, and high serum viral load, especially in severe CHB patients. In addition to HBV DNA, HBeAg is also a serological marker for viral replication, which plays a crucial role in chronicity of HBV infection and high viral load, by inducing immunological tolerance to HBV in the fetus. The tolerance-inducing effect of HBeAg has been well characterized in mice[42-44] and likely contributes to the low level of core-specific T-cell responses present in HBeAg-positive CHB patients[45]. Clinical evidence supports the tolerogenic effect of HBeAg[45,46]. Also, viral mutations that abrogate or antagonize antigen recognition by virus-specific T cells have been reported in patients with CHB[45,46]. Although the results from univariate analysis in our study showed T-cell dysfunction was significantly related to HBeAg, the association disappeared in multivariate analysis. One possible reason is that some of the subjects were infected with pre-C stop codon mutation virus (pre-C/C mutant), which resulted in a loss of HBeAg. In these patients, therefore, viral replication may have persisted, despite elimination of HBeAg and seroconversion to anti-HBe. While the loss of HBeAg appears irrelevant to the biology of the virus, it may play an important role in the interaction of the virus with the immune system. This may weaken the independent association between HBeAg and T-cell failure, so that the sample size in our study could not detect this magnitude of association. Moreover, those who had a history of maternal carriage usually acquired infection at a younger age, and a higher HBV viral load was detected in the majority of those who had infection at a younger age. In the same way, those who had severe liver damage were usually positive for maternal HBV carrier status and acquired infection in early life, thus a high viral load was measured in these patients. This phenomenon suggests that infection from the mother and/or at younger age predisposes to tolerance to HBV infection and thus, higher viral load.

Our study clearly showed the severity of the liver disease was significantly associated with functional disorder of T-lymphocytes, and the effect was independent of viral load. Hepatocyte damage may also be correlated directly with T-cell failure, rather than through the load copies of viral replication. Previous studies have suggested that hepatocyte damage is mainly caused by immunological injury[9,21-30]. HBV is a typical non-cytopathic virus that can induce tissue damage of variable severity by stimulating a protective immune response that can simultaneously cause damage and protection, by killing an intracellular virus through the destruction of virus-infected cells[5]. Therefore, immune elimination of infected cells can lead to the termination of infection when it is efficient, or to a persistent necroinflammatory disease when it is not[37]. Destruction of infected cells, however, is not the only mechanism implicated in the elimination of intracellular virus, as demonstrated by studies carried out in animal models of HBV infection and in human hepatitis B, which demonstrate the importance of cytokine-mediated, non-cytolytic mechanisms of anti-viral protection[30-32].

The strength of this study lies in the large sample size and the measurements of T-lymphocyte subpopulations using modern advanced FCM technology and viral load with quantitative real-time PCR. A limitation of this study is that the specificity of T-lymphocyte subpopulations and liver-derived T-lymphocytes were not explored concurrently. Although a strong relationship between T-lymphocyte subpopulations and viral load was illustrated, further studies are needed to confirm the causal relationship between them.
Our results, which suggest high viral load contributes to functional impairment of T cells in CHB patients, have practical implications for understanding the pathogenesis and control of persistent viral infection and diseases progression and prognosis. This is because patients with CHB are at risk of persistent viral infection that leads to liver failure, cirrhosis and even hepatocellular carcinoma. We should take into account effective intervention strategies such as anti-viral and/or immunotherapy to prevent progression and long-term consequences. Inhibition of viral replication with agents such as lamivudine may enhance the likelihood that therapeutic stimulation of the T-cell response will induce HBV antigen seroconversion, ultimately leading to recovery from disease. Further clinical studies are needed to explore this possibility in persistent HBV-infected patients.

In conclusion, we found a strong, independent predictive effect of viral load on T-lymphocyte subpopulations, which suggests a causal relationship between viral load and T-cell failure. T-cell dysfunction might contribute to viral persistence. HBV establishes persistent infection mainly by vertical transmission from HBV-infected mothers to neonates, and the immunomodulatory effects of HBsAg might play an important role in this setting. High viral load may be one important factor that contributes to T-lymphocyte failure, and is more important than HBsAg in this regard. Clearly, additional studies are required to better understand the complex host-virus interactions that determine the persistence and outcome of HBV infection.

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