ORIGINAL ARTICLES

Peripheral T-lymphocyte subpopulations in different clinical stages of chronic HBV infection correlate with HBV load

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Received: December 26, 2008 Revised: May 24, 2009
Accepted: May 31, 2009
Published online: July 21, 2009

Abstract

AIM: To characterize the peripheral T-cell subpopulation profiles and their correlation with hepatitis B virus (HBV) replication in different clinical stages of chronic HBV infection.

METHODS: A total of 422 patients with chronic HBV infection were enrolled in this study. The patients were divided into three stages: immune-tolerant stage, immune active stage, and immune-inactive carrier stage. Composition of peripheral T-cell subpopulations was determined by flow cytometry. HBV markers were detected by enzyme-linked immunosorbent assay. Serum HBV DNA load was assessed by quantitative real-time polymerase chain reaction.

RESULTS: $CD8^+$ T-cells were significantly higher in patients at the immune-tolerant stage than in patients at the immune-active and -inactive carrier stages (36.87 ± 7.58 *vs* 34.37 ± 9.07, 36.87 ± 7.58 *vs* 28.09 ± 5.64, $P < 0.001$). The peripheral blood in patients at the immune-tolerant and immune active stages contained more $CD8^+$ T-cells than $CD4^+$ T-cells (36.87 ± 7.58 *vs* 30.23 ± 6.35, 34.37 ± 9.07 *vs* 30.92 ± 7.40, $P < 0.01$), whereas the peripheral blood in patients at the immune-inactive carrier stage and in normal controls contained less $CD8^+$ T-cells than $CD4^+$ T-cells (28.09 ± 5.64 *vs* 36.85 ± 6.06, 24.02 ± 4.35 *vs* 38.94 ± 3.39, $P < 0.01$). ANOVA linear trend test showed that $CD8^+$ T-cells were significantly increased in patients with a high viral load (39.41 ± 7.36, 33.83 ± 7.50, 31.81 ± 5.95 and 26.89 ± 5.71, $P < 0.001$), while $CD4^+$ T-cells were significantly increased in patients with a low HBV DNA load (37.45 ± 6.14, 33.33 ± 5.61, 31.58 ± 6.99 and 27.56 ± 5.49, $P < 0.001$). Multiple regression analysis displayed that log copies of HBV DNA still maintained its highly significant coefficients for T-cell subpopulations, and was the strongest predictors for variations in $CD3^+$, $CD4^+$ and $CD8^+$ cells and $CD4^+/CD8^+$ ratio after adjustment for age at HBV-infection, maternal HBV-infection status, presence of hepatitis B e antigen and HBV mutation.

CONCLUSION: Differences in peripheral T-cell subpopulation profiles can be found in different clinical stages of chronic HBV infection. T-cell impairment is significantly associated with HBV load.

You J, Zhuang L, Zhang YF, Chen HY, Sriplung H, Geater A, Chongsuvivatwong V, Piratvisuth T, McNeil E, Yu L, Tang BZ, Huang JH. Peripheral T-lymphocyte subpopulations in different clinical stages of chronic HBV infection correlate with HBV load. *World J Gastroenterol* 2009; 15(27): 3382-3393 Available from:

INTRODUCTION

Hepatitis B virus (HBV) infection is a dynamic process with variable biochemical, virological and histological profiles at different stages of chronic HBV infection depending on host and viral factors. Furthermore, the profiles may change at a variable pace over time[1-5]. Based on the virological and biochemical parameters, chronic HBV infection can be divided into three stages: immune-tolerant stage, immune active stage and immune-inactive carrier stage[5-7]. Patients at the immune-tolerant stage have no symptoms, but their serum hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) are detectable, and HBV DNA levels are high, while their serum aminotransferase level is normal or minimally elevated, and their histological activity is minimal. This stage often lasts decades in perinatal HBV infection patients. The serum HBV DNA level decreases, and the serum aminotransferase level increases, symptoms may appear and flares of aminotransferase may be observed in patients at the immune active stage. The time of this stage ranges months-years in patients with chronic HBV infection. Flares of aminotransferase are followed by HBeAg seroconversion in some patients. The immune-inactive carrier stage is characterized by a low HBV DNA level and a normal aminotransferase concentration. HBV clearance is closely associated with the appearance of a vigorous virus-specific T-cell response[3,4]. In contrast, HBV persistence and chronic hepatitis are associated with a markedly diminished HBV-specific T-cell response[4,8-11]. Interestingly, different profiles of chronic hepatitis B are also associated with different magnitudes of HBV-specific immune response[12]. Episodes of acute flares during chronic HBV infection are associated with the recovery of HBV-specific CD4+ T cell response[13,14]. Increased HBV-specific CD8+ T cell response has only been demonstrated in patients with a low HBV replication, irrespective of the inflammation degree[15-17]. These data have led to the hypothesis that the composition of T cell subpopulations is different in different clinical stages of chronic HBV infection, which may be related to the HBV load. Thus, characterization of T cell profiles is relevant to the improved understanding of chronic HBV infection and the design of antiviral therapy. This study was to characterize the different stages of chronic HBV infection by analyzing the composition of T cell subpopulations in peripheral blood and its correlation with HBV replication.

MATERIALS AND METHODS

Subjects

Four hundred and twenty-two consecutive chronic HBV infection patients were recruited from Department of Infectious Diseases of First Affiliated Hospital of Kunming Medical University, Third Kunming People's Hospital, and Yunnan General Hospital of Chinese People's Armed Police Forces, between January 2006 and May 2007. The patients were diagnosed according to the criteria for viral hepatitis[5-7].

All patients fulfilled the following criteria: positive HBsAg for at least 12 mo, no other concomitant causes of liver disease (hepatitis C and D, HIV infection and alcohol consumption of more than 60 g/d), relatively rare liver diseases (autoimmune hepatitis and metabolic liver disease), on immunosuppressive therapy or antiviral therapy for HBV-infection within the recent 12 mo before entry. None of the patients was a drug user, or exposed to hepatotoxin. All patients gave their informed consent to participate in the study. The study protocol, conforming to the guidelines of Declaration of Helsinki, was approved by the Ethics Committees of the Faculty of Medicine of Prince of Songkla University and the First Affiliated Hospital of Kunming Medical University.

One hundred healthy individuals (61 males, 39 females), with a mean age of 33.24 ± 10.28 years, served as controls.

Serological test of liver function and evaluation of HBVs markers

Serum alanine aminotransferase (ALT), aspartate trans-aminase (AST) and total bilirubin levels were measured with routine automated techniques (upper limit: 40 U/L, 40 U/L and 17.1 μmol/mL, respectively). HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, and anti-HBcAb IgM) were detected by enzyme-linked immunosorbent assay.

Detection of HBV DNA and HBV pre-core mutant

Serum HBV DNA load in individuals was detected by real-time polymerase chain reaction (RT-PCR) using a Lightcycler PCR system (FQD-33A, Bioer) with a lower detection limit of approximately 1000 viral genome copies/mL with a reagent kit package insert (Shenzhen PG Biotech Co. Ltd.) following its manufacturer's instructions. The primer was provided with the kit, the reaction volume was 40 μL. PCR was performed at 37℃ for 5 min and at 94℃ for 1 min, followed by 40 cycles at 95℃ for 5 s and at 60℃ for 30 s. HBV pre-core A1896 mutant was detected by PCR (PE9600, Perkin Elmer Co., Ltd. USA) with the reagent kit package insert (Shanghai Haoyuan Biotech Co. Ltd) following its manufacturer's instructions.

Detection of peripheral blood T lymphocyte subsets

The key compositions of cellular immune are T-lymphocytes and their subpopulations. CD3+, CD4+ and CD8+ cells are the major function subgroups of T cells and play an important role in response to HBV infection, which can reflect the cellular immune function and immunoregulation and are usually regarded as a valuable index of the changes in immunity of patients[3,4]. This index was used in our study to evaluate the cellular immune function of chronic HBV infection patients.

Blood samples were collected into heparinized vacuum tubes. Whole blood samples were analyzed using a Muti-Q-Prep processor (Coulter, USA) and an Epics-XL flow cytometer (Coulter, USA). Lymphocytes were analyzed using a gate set on a forward scatter *vs* a side scatter, and a three-color flow cytometry was used to combine CD3, CD4 and CD8. Anti-human monoclonal antibodies, CD3-PE-CY5/CD4-FITC/CD8-PE, were purchased from Immunotech, Ltd, USA. The detected peripheral blood T lymphocyte subsets in each sample were analyzed using the CellQuest software (Coulter, USA). The results were expressed as percentages of $CD3^+$, $CD3^+/CD4^+$ (short for $CD4^+$ below) and $CD3^+/CD8^+$ (short for $CD8^+$ below) cells which were positive for the marker antigen in the total T cell population. The procedures were performed with the reagent kit package insert following its manufacturer's instructions.

Maternal HBV infection status

All medical records of mothers of the subjects were reviewed for previous HBV infection and those who were infected with HBV were identified. In addition, all of them were invited to undergo HBV-marker tests. For those with a positive result, a second set of tests were conducted three months after the first test to confirm their chronic HBV carrier status. If the mother was dead, the cause of death was investigated based on the medical records of HBV-related liver diseases, such as chronic hepatitis B, liver cirrhosis or hepatocellular carcinoma. If yes, the maternal HBV infection status was classified as positive.

Age at HBV infection

In the recent three decades, all children in China have been obligated to have a test for HBV markers when they first go to kindergarten and elementary school. Subsequent obligatory tests are made when they apply for entrance examination of university or for a job. Based on the results of these tests obtained from medical records and interview, we classified the age at the first positive test as < 8 years, between 8-20 years and > 20 years, respectively.

Statistical analysis

Initial calculation came up with a sample size of 50 patients with positive HBV DNA and 50 patients with negative HBV DNA, which could provide the study with a statistical power of 80% at the 0.025 level of significance to detect the difference in T-cell variation values of 33 and 38. However, to cover the potentially confounded problem due to other variables and to have enough subjects for stratifying the HBV DNA load to examine the dose-response relationship, we recruited 422 chronic HBV infection patients and 100 healthy controls.

Descriptive statistical analysis was used to examine the age, gender, serum HBV load, HBeAg status, age at HBV-infection and maternal HBV infection status.

The number of T-lymphocyte subpopulations in normal individuals (HBsAg-negative), which was expressed as mean ± SD, served as a control reference. Effects of different independent demographic, clinical and serological variables on T-cell profiles were analyzed only in HBsAg-positive patients. Univariate analysis of these profiles broken down by individual independent variables was carried out. Independent *t* test was done for 2-level independent variables and one-way ANOVA for more than 2-level variables. The relationship between HBV replication level and peripheral T-lymphocyte subpopulation was analyzed by correlation analysis and ANOVA linear trend test. Finally, multiple linear regression models were employed in multivariate analysis for the assessment of independent effects of variables on peripheral blood T lymphocytes. Variables yielding a *P* value ≤ 0.2 in the univariate analysis were included in multivariate analysis, and the models were refined by backward elimination guided by the change in log likelihood of successive models. *P* < 0.05 was considered statistically significant. Computation was carried out with the aid of R Software version 2.5.1[18].

RESULTS

Characteristics of patients at different stages of chronic HBV infection

The demographic, virological, serological, and clinical characteristics of patients are summarized in Table 1. The mean age of 422 chronic HBV infection patients (males accounted for 63.5%) was 30.59 ± 10.40 years. Over half of the patients' mothers were HBV positive. Two fifths of the patients were infected with HBV before the age of 8 years. Two thirds of the patients had detectable HBV DNA load and the majority of them (62.8%) had over 10^7 copies of HBV DNA per milliliter, more than half of them were HBeAg positive (51.7%) and nearly one third of them were infected with HBV pre-core region mutant.

Based on the predefined virological and biochemical parameters, all patients were divided into different stages of chronic HBV infection. A total of 112 patients were assigned to the immune-tolerant stage with a mean ALT level of 25.37 ± 12.56 IU/L, a mean viral load of 7.88 ± 1.54 log10 copies of HBV DNA/mL, while 106 patients had more than 10^5 copies of HBV DNA/mL, six patients with positive HBeAg had 10^5 copies of HBV DNA/mL. Of the 112 patients, all with HBeAg positive and 37 had HBV DNA pre-C mutations. The patients at the immune-tolerant stage had a higher maternal HBV infection rate (83.0%) and a younger age (< 8 years) at HBV infection (55.4%). Two hundred and twenty-two patients were assigned to the immune active stage with a mean ALT level of 161.42 ± 139.26 IU/L, a mean HBV load of 5.94 ± 2.21 log10 copies/mL. Of the 222 patients, 96 were HBeAg positive, 71 had HBV DNA pre-C mutations, and 57 had a serum HBV load of less than 10^3 copies/mL, but no other reasons for the elevated aminotransferase level. Eighty-eight patients at the immune-inactive carrier stage had a mean

Table 1 Characteristics of patients with chronic HBV infection (mean ± SD), *n* (%)

| Characteristics | Patients (*n* = 422) | Clinical stage of chronic HBV infection | | | |
		Tolerant (*n* = 112)	Active (*n* = 222)	Inactive carrier (*n* = 88)	*P*
Sex (Male/female)	268/154	67/45	156/66	45/43	< 0.05[1]
Mean age (yr)	30.59 ± 10.40	29.45 ± 11.72	29.69 ± 9.30	34.34 ± 10.53	< 0.001[2]
Patients with MH	233 (55.2)	93 (83.0)	115 (51.8)	25 (28.4)	< 0.001[1]
Age at HBV-infection (yr)					< 0.001[1]
< 8	166 (39.3)	62 (55.4)	92 (41.4)	12 (13.6)	
8-20	127 (30.1)	33 (29.5)	80 (36.0)	14 (15.9)	
> 20	103 (24.4)	9 (8.0)	46 (20.7)	48 (54.5)	
Unknown	26 (6.2)	8 (6.3)	4 (1.8)	14 (15.9)	
Serum ALT (IU/L)	116.52 ± 82.66	25.37 ± 12.56	161.42 ± 139.26	31.15 ± 19.67	< 0.001[2]
Serum AST (IU/L)	82.61 ± 79.89	22.20 ± 14.63	116.63 ± 107.84	25.29 ± 12.52	< 0.001[2]
Serum TBIL (μmol/mL)	15.66 ± 9.22	15.08 ± 2.55	20.78 ± 14.25	13.55 ± 11.24	< 0.001[2]
HBV DNA positive	285 (67.5)	112(100)	165 (74.3)	8 (9.1)	< 0.001[1]
Serum HBV DNA (copies/mL)					< 0.001[1]
$\leqslant 1.0 \times 10^3$	137 (32.5)	0	57 (25.7)	80 (90.9)	
$> 1.0 \times 10^3$-1.0×10^5	46 (10.9)	6 (5.4)	32 (14.4)	8 (9.1)	
$> 1.0 \times 10^5$-1.0×10^7	60 (14.2)	22 (19.6)	38 (17.1)	0	
$> 1.0 \times 10^7$	179 (42.4)	84 (75.0)	95 (42.8)	0	
HBV DNA (Log, copies/mL)	5.87 ± 2.43	7.88 ± 1.54	5.94 ± 2.21	3.11 ± 0.37	< 0.001[2]
HBeAg positive	218 (51.7)	112 (100)	96 (43.2)	10 (11.4)	< 0.001[1]
HBV pre-C region mutation positive	122 (28.9)	37 (33.0)	71 (32.0)	14 (15.9)	< 0.01[1]

[1]χ^2 test; [2]ANOVA. HBV: Hepatitis B virus; MH: Maternal HBV-infection status.

Table 2 Characteristics of chronic HBV infection patients at the immune-tolerant and immune-active stages (mean ± SD), *n* (%)

| Characteristics | Immune-tolerant stage (*n* = 112) | | | Immune-active stage (*n* = 222) | | |
	HBV DNA (< 1.0 × 10⁷ copies/mL) (*n* = 28)	HBV DNA (> 1.0 × 10⁷ copies/mL) (*n* = 84)	*P*	HBV DNA (< 1.0 × 10⁷ copies/mL) (*n* = 127)	HBV DNA (> 1.0 × 10⁷ copies/mL) (*n* = 95)	*P*
Sex (Male/female)	13/15	54/30	> 0.05[1]	87/40	69/26	> 0.05[1]
Mean age (yr)	30.80 ± 10.20	30.61 ± 10.90	> 0.05[2]	31.17 ± 9.82	27.71 ± 8.18	< 0.01[2]
Patients with MH	16 (57.1)	77 (91.7)	< 0.001[1]	48 (37.8)	67 (70.5)	< 0.001[1]
Age at HBV-infection (yr)			> 0.05[1]			< 0.001[1]
< 8	14 (50.0)	48 (54.8)		34 (26.8)	58 (61.0)	
8-20	10 (35.7)	23 (31.2)		54 (42.5)	26 (27.4)	
> 20	2 (7.1)	7 (7.5)		38 (29.9)	8 (8.4)	
Unknown	2 (7.1)	6 (6.5)		1 (0.8)	3 (3.2)	
Serum ALT (IU/L)	25.32 ± 12.6	29.54 ± 11.28	> 0.05[2]	111.36 ± 86.5	211.33 ± 173.20	< 0.001[2]
Serum AST (IU/L)	24.54 ± 16.33	24.59 ± 18.31	> 0.05[2]	79.08 ± 60.87	151.84 ± 136.65	< 0.001[2]
Serum total bilirubin (μmol /mL)	12.55 ± 6.28	11.83 ± 8.59	> 0.05[2]	17.68 ± 8.28	23.80 ± 17.97	< 0.001[2]
HBV DNA positive	28 (100)	84 (100)	> 0.05[1]	70 (55.1)	95 (100)	< 0.001[1]
HBV DNA (Log, copies/mL)	5.62 ± 0.81	8.63 ± 0.82	< 0.001[2]	4.31 ± 1.36	8.13 ± 0.74	< 0.001[2]
HBeAg positive	28 (100.0)	84 (100.0)	> 0.05[1]	30 (23.6)	66 (69.5)	< 0.001[1]
HBV pre-C region mutation positive	1 (3.6)	36 (42.9)	< 0.001[1]	29 (22.8)	42 (44.2)	< 0.01[1]

[1]χ^2 test; [2]*t*-test.

ALT level of 31.15 ± 19.67 IU/L, a mean HBV load of 3.11 ± 0. 37 log₁₀ copies/mL. Eighty patients had undetectable serum HBV DNA, and 8 patients with negative HBeAg had a serum HBV load of less than 10^5 copies/mL. Ten patients had positive HBeAg and undetectable HBV DNA, and 14 patients had HBV pre-core mutant infection. Patients at the immune-inactive carrier stage also had a low maternal HBV infection rate (28.4%) and an older age (> 20 years) at HBV-infection (54.5%). All parameters had a statistically significant difference in patients at the three stages of chronic HBV infection. Profess from immune tolerance to chronic

HBV infection was significantly associated with HBV replication status including a higher HBV load, HBeAg expression, and HBV pre-core mutation (*P* < 0.001).

The characteristics of patients with a high HBV load (HBV DNA > 10^7 copies/mL) and a low HBV load (HBV DNA < 10^7 copies/mL) at the immune-tolerant and -active stages are summarized in Table 2. Patients with a high HBV load had a higher maternal HBV infection rate (91.7%) and a higher positive HBV pre-C mutation rate (42.9%) than those with a lower HBV load at the immune-tolerant stage (*P* < 0.001). A similar pattern was observed in patients at the immune active

Table 3 Peripheral T-cell subsets broken down by various factors in normal controls and chronic HBV infection patients (mean ± SD)

Groups	Patients (n)	CD3+	CD4+	CD8+	CD4+/CD8+ ratio
HBV status[b]					
Negative (normal control)	100	71.07 ± 4.76	38.94 ± 3.39	24.02 ± 4.35	1.67 ± 0.33
Positive	422	56.42 ± 13.16	31.97 ± 7.30	33.73 ± 8.63	1.04 ± 0.45
Clinical stages of HBV infection[c]					
Tolerant	112	50.78 ± 13.26	30.23 ± 6.35	36.87 ± 7.58	0.86 ± 0.29
Active	222	55.51 ± 12.50	30.92 ± 7.40	34.37 ± 9.07	1.00 ± 0.47
Inactive carrier	88	65.89 ± 9.09	36.85 ± 6.06	28.09 ± 5.64	1.37 ± 0.39
HBV DNA loads (copies/mL)[d]					
$\leqslant 1.0 \times 10^3$	137	65.96 ± 8.58	37.45 ± 6.14	26.89 ± 5.71	1.46 ± 0.42
$> 1.0 \times 10^3$-1.0×10^5	46	62.28 ± 7.50	33.33 ± 5.61	31.81 ± 5.95	1.10 ± 0.36
$> 1.0 \times 10^5$-1.0×10^7	60	60.05 ± 12.97	31.58 ± 6.99	33.83 ± 7.50	0.97 ± 0.26
$> 1.0 \times 10^7$	179	46.39 ± 9.93	27.56 ± 5.49	39.41 ± 7.36	0.73 ± 0.23
HBeAg status[b]					
Negative	204	62.62 ± 10.67	34.83 ± 7.16	29.66 ± 7.35	1.27 ± 0.48
Positive	218	50.61 ± 12.62	29.29 ± 6.37	37.53 ± 7.98	0.83 ± 0.29
HBV pre-C region mutation status[b]					
Negative	300	58.40 ± 12.89	33.63 ± 6.94	31.73 ± 8.04	1.15 ± 0.44
Positive	122	51.55 ± 12.57	27.89 ± 6.56	38.63 ± 8.06	0.78 ± 0.37
Maternal HBV-infection status[b]					
Negative	189	62.34 ± 10.89	35.31 ± 6.32	29.64 ± 6.87	1.27 ± 0.43
Positive	233	51.61 ± 12.89	29.27 ± 6.93	37.04 ± 8.49	0.85 ± 0.37
Age at HBV infection[c] (yr)					
< 8	166	51.83 ± 12.86	28.17 ± 6.61	37.31 ± 8.16	0.80 ± 0.29
8-20	127	57.17 ± 12.27	33.05 ± 5.90	33.39 ± 7.91	1.07 ± 0.41
> 20	103	61.42 ± 11.89	36.02 ± 7.27	28.86 ± 7.89	1.35 ± 0.49
Unknown	26	62.25 ± 14.39	34.93 ± 6.34	31.78 ± 7.58	1.19 ± 0.45

Effects of various independent demographic, clinical and serological variables on T-cell profile of HBsAg positive individuals were analyzed. [b]$P < 0.001$ *vs* measurement of each T-cell parameter; [c]$P < 0.01$, [d]$P < 0.001$ *vs* ANOVA linear trend test.

stage. Furthermore, patients with a high HBV load had a younger age at HBV infection and a higher HBeAg expression level than those with a low HBV load at the immune active stage.

Composition of peripheral blood T lymphocyte subpopulations

The composition of T lymphocytes in peripheral blood of all chronic HBV infection patients is summarized in Table 3. Univariate analyses showed that T-cell impairment was significantly associated with both clinical stages of chronic HBV infection and higher HBV loads, serum HBeAg expression, HBV pre-C region mutation, history of maternal HBV infection, and younger age at HBV-infection. The linear dose-response relationship between T-lymphocyte subpopulations and HBV DNA copies was highly significant (linear trend test, $P < 0.001$). The number of CD3+ and CD4+ cells and CD4+/CD8+ ratio were negatively correlated with the serum HBV load in chronic HBV infection patients (r = -0.67, -0.58, -0.69, $P < 0.0001$), while the number of CD8+ cells was positively correlated with the HBV load (r = 0.64, $P < 0.0001$).

The composition of peripheral blood T-lymphocytes in patients at different immune stages of chronic HBV infection is shown in Figure 1. The number of peripheral blood CD8+ T-cells was higher in patients at the immune-tolerant stage than in patients at the immune-active and -inactive carrier stages, whereas the number of CD4+ T cells was lower in patients at the immune-tolerant stage than in patients at the immune-inactive carrier stage ($P < 0.001$), suggesting that patients at the immune-tolerant stage have the highest number of peripheral blood CD8+ T-cells and the lowest number of peripheral blood CD4+ T-cells and ratio between CD4+/CD8+ cells than those at the immune-active and -inactive carrier stages. The peripheral blood contained more CD8+ T-cells than CD4+ T-cells in patients at the immune-tolerant and -active stages, whereas the peripheral blood contained more CD4+ T-cells than CD8+ T-cells in patients at the immune-inactive carrier stage and normal controls ($P < 0.01$).

The proportion of CD3+, CD4+ and CD8+ T-cells in peripheral blood of patients at the immune-tolerant stage is summarized in Figure 2A. The mean percentage of CD3+ and CD4+ T-cells was higher, whereas the mean percentage of CD8+ T-cells was lower in patients with a low HBV load than in patients with a high HBV load ($P < 0.001$). A similar pattern was also observed between patients with a high HBV load and those with a low HBV load at the immune active stage ($P < 0.001$, Figure 2B).

The mean percentage of CD3+ and CD4+ T-cells was higher in patients with negative HBeAg than in those with positive HBeAg at the immune active stage, whereas CD8+ T-cells was lower in patients with negative HBeAg than in those with positive HBeAg ($P < 0.001$, Figure 3B). A similar pattern was observed between patients with negative HBeAg and those with positive HBeAg at the immune-tolerant stage ($P > 0.05$, Figure 3A).

Linear regression predicting peripheral blood T lymphocyte subpopulation

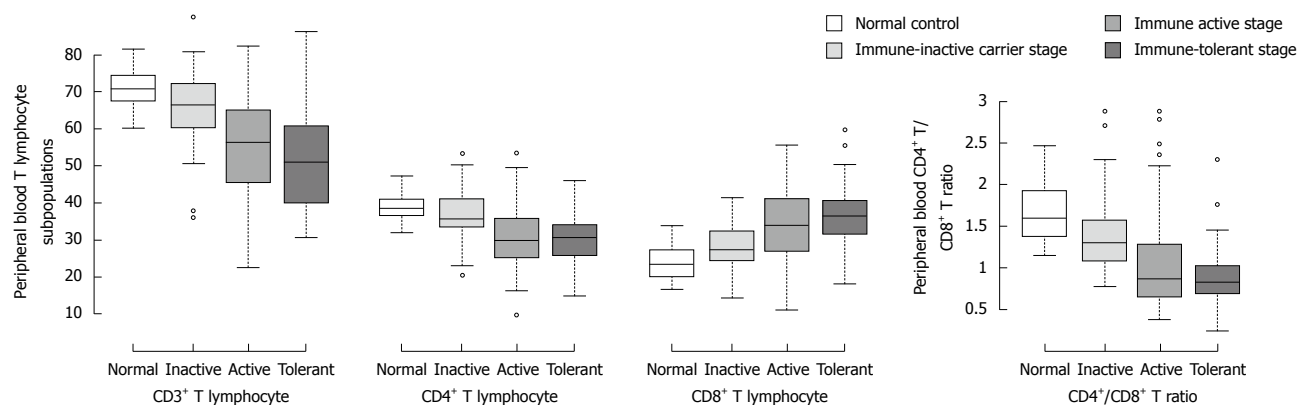Linear regression models of CD3+, CD4+ and CD8+

Figure 1 Peripheral blood T-lymphocyte subpopulations in patients at different clinical stages of chronic HBV infection. The mean percentages of CD3^{+}, CD4^{+} and CD8^{+} T cells and CD4^{+}/CD8^{+} ratio were shown in patients at the different stages and control. Top of the box represents the 75%, the bottom of the box represents the 25%, the solid line in the middle of the box represents the median. Whiskers above and below the box indicate the 90% and 10%, while filled circles represent outliers. A statistically significant difference in T cells but not in CD4^{+} cells was observed between chronic HBV infection patients and normal control ($P < 0.01$). In the peripheral blood of patients at the immune-tolerant and immune-active stages, CD8^{+} T-cells were the dominant lymphocytes compared to CD4^{+} T-cells, whereas in the peripheral blood of patients at the immune-inactive carrier stage and normal controls, CD4^{+} T-cells were the dominant lymphocytes compared to CD8^{+} T-cells.
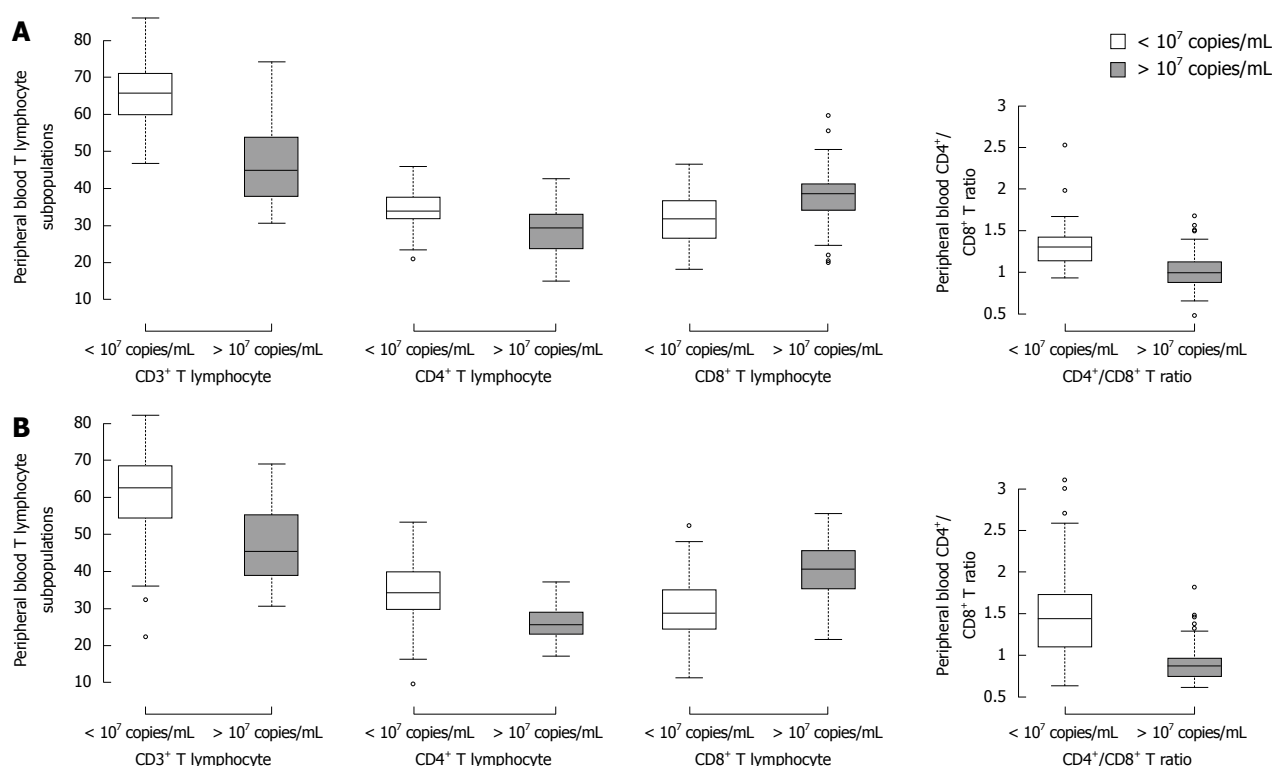

Figure 2 Mean percentages of CD3^{+}, CD4^{+} and CD8^{+} T-cells in patients at immune-tolerant stage (A) and immune active stage (B) of chronic HBV infection. Patients were divided into two groups based upon HBV DNA load. The proportion of CD8^{+} T-cells was significantly higher in patients with a high HBV load than in patients with a low HBV load at the immune-tolerant stage (38.66 ± 7.11 *vs* 31.50 ± 6.40, $P < 0.001$) and at the immune active stage (40.07 ± 7.55 *vs* 30.11 ± 7.69, $P < 0.001$). The percentage of CD4^{+} T-cells was significantly higher in patients with a low HBV load than in patients with a high HBV load at the immune-tolerant stage (33.77 ± 5.81 *vs* 29.05 ± 6.11, $P < 0.001$) and at the immune active stage (34.41 ± 7.23 *vs* 26.25 ± 4.52, $P < 0.001$). Significant differences were found in CD3^{+} T-cells and CD4^{+}/CD8^{+} ratio between patients with a high HBV load and a low HBV load at the immune-tolerant stage (45.90 ± 10.69 *vs* 65.40 ± 8.70, 0.78 ± 0.21 *vs* 1.11 ± 0.32, $P < 0.001$) and at the immune active stage (46.82 ± 9.25 *vs* 62.01 ± 10.52, 0.69 ± 0.23 *vs* 1.24 ± 0.47, $P < 0.001$). CD8^{+} T-cells were predominant compared with CD4^{+} T-cells in patients with a high HBV load, whereas CD4^{+} T-cells were predominant compared with CD8^{+} T-cells in patients with a low HBV load.

cells and CD4^{+}/CD8^{+} ratio as dependent variables are listed in Table 4. After adjustment for all independent variables, serum HBV load was the key predictor for T-cell profiles. The number of CD3^{+} and CD4^{+} T lymphocytes was significantly reduced, the number of CD8^{+} T cells was increased, and the CD4^{+}/CD8^{+} ratio was decreased in patients at the immune-tolerant stage. Those with chronic HBV infection at a younger age

had less CD4^{+} T cells and a lower CD4^{+}/CD8^{+} ratio than those with chronic HBV infection at an older age. HBeAg expression, HBV pre-C mutation, and maternal HBV-infection status also had partial independent effects on T-lymphocyte profiles.

The relationship between T-lymphocyte subpopulations and HBV load stratified by age at HBV infection is shown in Figure 4A. No significant difference of T-cell
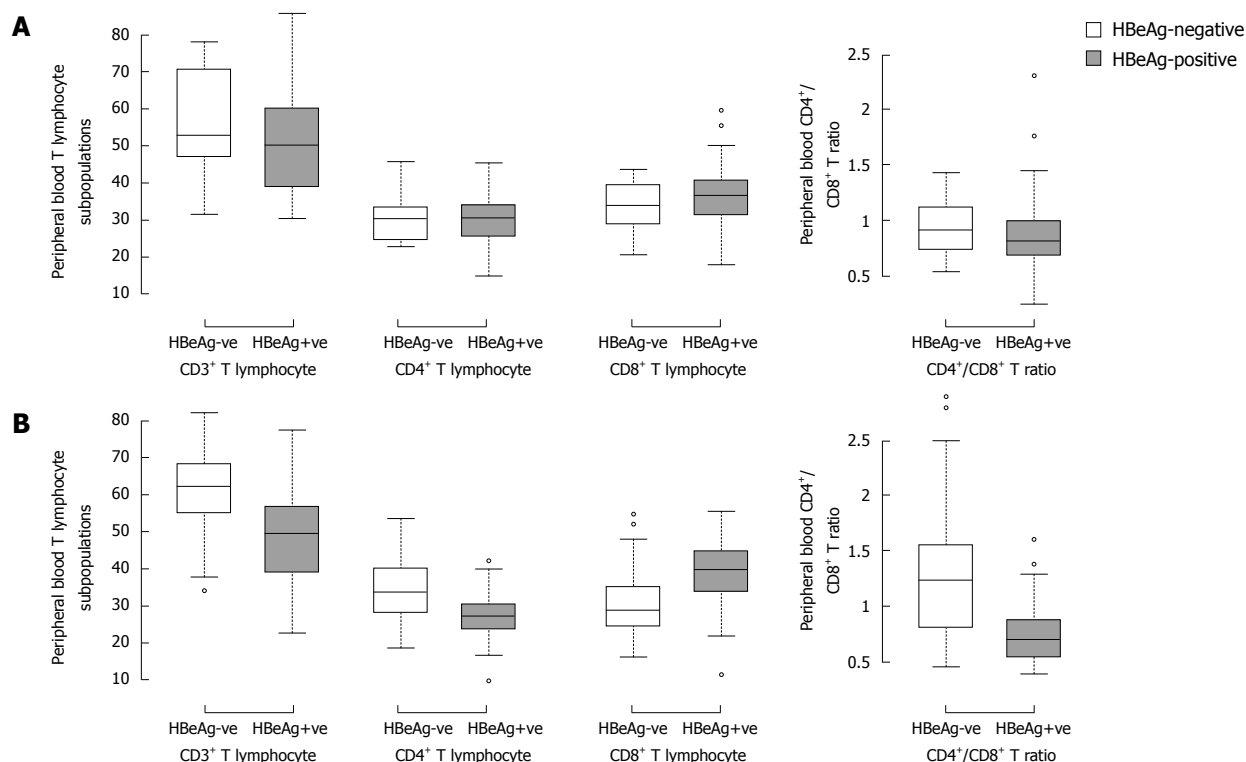
Figure 3 Mean percentages of CD3^{+}, CD4^{+} and CD8^{+} T-cells in peripheral blood of patients at the immune-tolerant stage (A) and immune active stage (B). Patients were divided into two groups based upon the HBeAg status. The proportion of CD8^{+} T-cells was significantly higher in HBeAg positive patients than in HBeAg negative patients at the immune active stage (39.24 ± 8.05 *vs* 30.66 ± 8.01, *P* < 0.001). The percentage of CD4^{+} T-cells was significantly higher in HBeAg negative patients than in HBeAg positive patients at the immune active stage (33.60 ± 7.41 *vs* 27.39 ± 5.75, *P* < 0.001). Significant differences were found in CD3^{+} T-cells and CD4^{+}/CD8^{+} ratio between HBeAg positive and negative patients (48.99 ± 11.06 *vs* 60.48 ± 11.22, 0.74 ± 0.25 *vs* 1.21 ± 0.50, *P* < 0.001). CD8^{+} T-cells were predominant compared with CD4^{+} T-cells in patients with a high HBeAg expression level, whereas CD4^{+} T-cells were predominant compared with CD8^{+} T-cells in patients with a low HBeAg expression level. In immune-tolerant-patients, no significant difference was observed in parameters of T-cell profile between HBeAg negative and positive patients (*P* > 0.05).

Table 4 Multiple linear regression predicting peripheral blood T lymphocyte subpopulations (*n* = 422)

	CD3^{+} T lymphocytes			CD4^{+} T lymphocytes			CD8^{+} T lymphocytes			CD4^{+}/CD8^{+} ratio		
	β	SE	*P*	β	SE	*P*	β	SE	*P*	β	SE	*P*
Intercept	85.35	2.64		43.32	1.53		16.48	1.73		1.89	0.08	
Serum HBV load (Log, copies/mL)[1]	-3.62	0.34	< 0.0001	-1.29	0.19	< 0.0001	1.80	0.22	< 0.0001	-0.1	0.01	< 0.0001
HBeAg[2]	-2.74	1.40	0.06	-1.95	0.81	0.01	3.42	0.92	< 0.001	-0.16	0.04	< 0.01
HBV pre-C region mutation[3]	-0.67	1.13	0.55	-2.79	0.66	< 0.0001	3.38	0.74	< 0.0001	-0.15	0.04	< 0.0001
Clinical stages of HBV infection[4]			0.014			< 0.0001			< 0.001			< 0.0001
Active	-4.19	1.34		-3.64	0.77		3.63	0.88		-0.19	0.04	
Inactive carrier	-5.17	1.98		-3.55	1.15		4.93	1.30		-0.26	0.06	
Age at HBV infection[5] (yr)			0.038			< 0.001			0.37			< 0.001
8-20	-1.07	1.25		2.21	0.73		0.14	0.82		0.07	0.04	
> 20	-3.55	1.49		2.35	0.86		-0.55	0.98		0.15	0.05	
Unknown	2.51	2.17		3.18	1.25		-0.94	1.42		0.16	0.07	
Maternal HBV infection status[6]	-3.23	1.21	< 0.01	-1.35	0.70	0.06	2.14	0.79	< 0.01	-0.08	0.04	0.03

The linear regression model was employed in multivariate analysis to assess the independent effects of variables on peripheral blood T lymphocyte subpopulation. β: Coefficients from the model; SE: Standard error; [1]Continuous variable; [2]Reference group with positive HBeAg; [3]Reference group without pre-C mutation; [4]Reference group at tolerant stage; [5]Reference group with the age < 8 years at HBV infection; [6]Reference group without maternal HBV infection status.

subsets was found in patients at different HBV infection ages after adjustment for serum HBV load. A similar pattern of the relationship between T-lymphocyte subpopulations and HBV load stratified by maternal HBV carrier status was also observed in patients with chronic HBV infection (Figure 4B). No significant difference was found in T-cell subsets between patients in maternal HBV carrier status after adjustment for serum HBV load.

DISCUSSION

This study demonstrated that there were clear differences of peripheral T cell subpopulation profile in patients at different clinical stages of chronic HBV infection. An impaired balance of T-cell subsets was related to an increased proportion of CD8^{+} T lymphocytes, a decreased proportion of CD4^{+} T lymphocytes and a
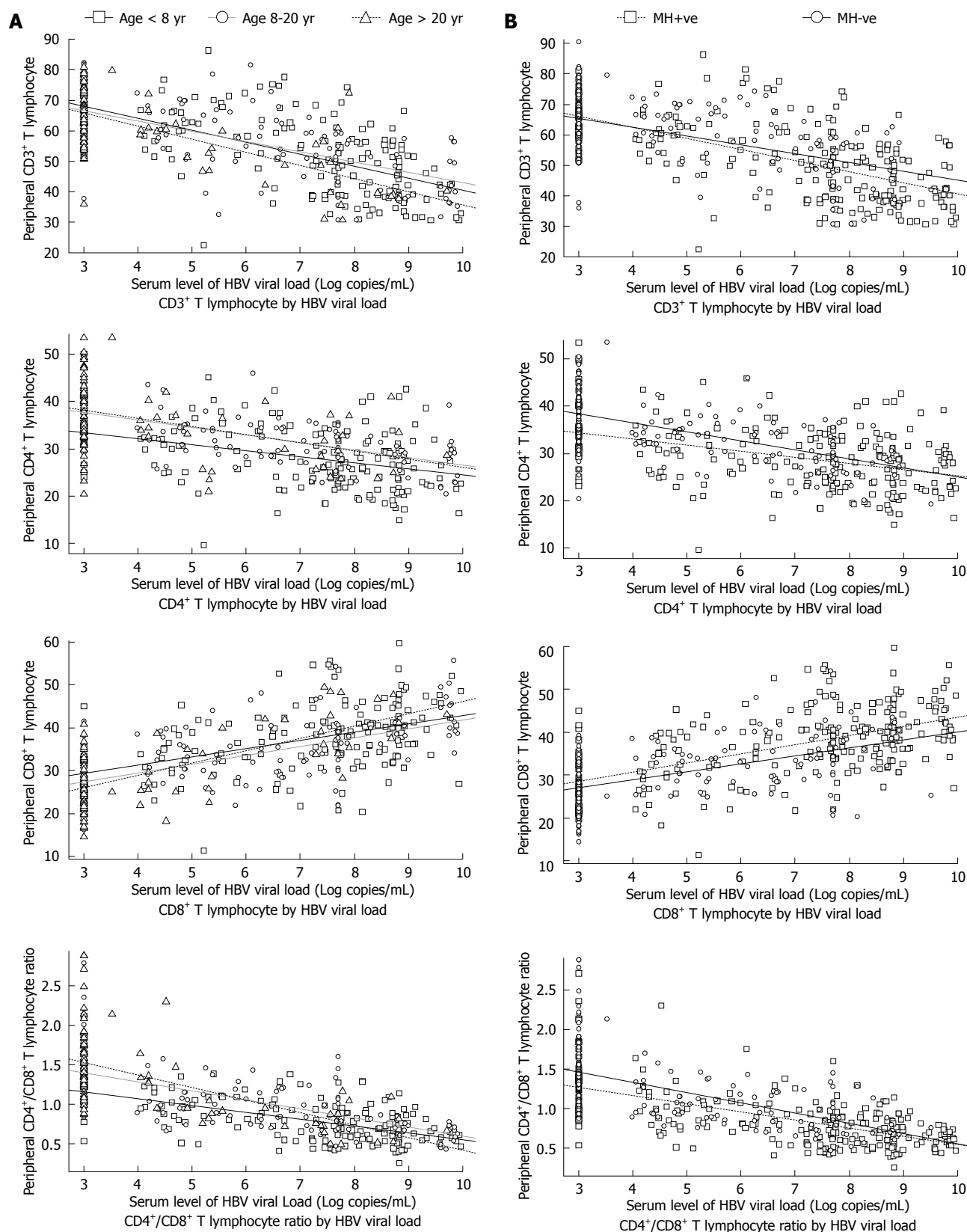
Figure 4 Correlation between T-cell subsets and HBV load stratified by age at HBV infection (A) and maternal HBV infection status (B). Three regression lines (with different slopes) were drawn for patients at different ages of HBV infection. The coefficient of the interaction term "HBVDNA: age-at-HBV-infection" was not statistically significant for each parameter of T lymphocyte subpopulations (*P* > 0.05). A similar pattern was seen when stratified by maternal HBV infection status.

decreased ratio between CD4^{+} and CD8^{+} cells. T-cell impairment had a linear dose-response relationship with the HBV DNA load.

The composition of T-cell subpopulations in

peripheral blood was significantly different in patients at different clinical stages of chronic HBV infection, indicating that patients at the immune-tolerant stage have the highest number of CD8^{+} T-cells, the lowest number

of CD4^{+} T-cells, and lowest ratio between CD4^{+} and CD8^{+} cells than those at the immune-active and inactive carrier stages. It has been reported that the composition of peripheral blood lymphocyte populations in patients at different stages of chronic HBV infection with research subject sample sizes ranging from 29 to 47[19,20], which are different from our study and is, thus, unable to detect any significant difference of T-cell subsets in patients at different clinical stages of chronic HBV infection. Using a larger sample size that covered a wide range of different stages of chronic HBV infection in this study, we showed the discrimination power of the composition of T cell profile in peripheral blood.

An impaired balance of T-cell subsets is related to an increased proportion of CD8^{+} T lymphocytes, a decreased proportion of CD4^{+} T lymphocytes, and decreased ratio between CD4^{+} and CD8^{+} cells during chronic HBV infection[21-27]. However, the results of previous studies are controversial results[28-32]. The fact that chronic hepatitis B represents a dynamic disease state may explain such divergent results. Therefore, any analysis of the composition of peripheral blood lymphocyte populations needs to be viewed within the context of disease progression. In this study, we attempted to correlate the trend in HBV infection, diseases progression, and stages of HBV infection with the T-cell phenotypes based on the virological and biochemical profiles (Figure 1). Our results revealed that T-cell impairment was significantly associated with the HBV replication level, indicating that serum HBV load is a strong predicting factor for T-lymphocyte subpopulations. Interestingly, our results are consistent with previous findings[19]. It has been reported that the proportion of CD8^{+} T-cells and CD4^{+} T-cells in liver is increased, respectively, in patients with a high HBV load and in those with a low HBV load at the immune-clearance stage[19]. Pham et al[33] also reported that the CD4^{+}/CD8^{+} ratio in liver-derived lymphocytes is correlated with HBV replication in patients with chronic hepatitis B. The decreased proportion of CD4^{+} T-cells and CD4^{+}/CD8^{+} ratio in chronic HBV infection patients with a high HBV load and HBeAg expression may be a consequence but not the cause of a high HBV DNA load[3-5,12]. The impact of HBV load on antiviral T-cell responses has been precisely characterized in animal models of virus infections (like LCMV), showing that the sustained presence of viral antigens leads to a progressive functional decline of virus-specific CD8^{+} T-cell responses and ultimately virus-specific T-cell deletion[34-36]. Similarly, the frequency and function of circulating and intrahepatic HBV-specific CD8^{+} T cells are inversely proportional to the HBV DNA load in HBV-infected patients[37-39]. High HBV loads are associated with the ablation of CD8^{+} T cell responses and IL-2 production impaired by virus-specific CD4^{+} T cells[35]. Matloubian et al[40] demonstrated that CD4^{+} T cells are required to sustain the CD8^{+} T cell responses during chronic HBV infection. Furthermore, Lamivudine treatment can transiently restore the efficient antiviral T cell responses in patients

with chronic hepatitis B and alleviate their viremia[41-44], indicating that HBV load plays an important role in the pathogenesis of T cell hypo-response in chronic HBV infection patients.

Although no information on the proportion of T-cell profile in liver was provided in this study, Pernollet et al[45] demonstrated that intrahepatic lymphocyte subsets are correlated with peripheral lymphocyte subsets and there is an exchange between intrahepatic and peripheral compartments. Similarly, two recent works have shown that the same specific T lymphocytes can be isolated from either the liver compartment or the PBL of chronically HCV-infected patients[46,47]. For this reason, the authors of one of these works proposed a characterization of the lymphocyte response to HCV in the peripheral blood instead of in the liver compartment[46]. We can also hypothesize that T lymphocytes leave the liver after encountering antigen there, although it has recently been shown that the majority of activated CD8^{+} T cells entering the liver undergo apoptosis within the liver[48,49]. On the other hand, it has been reported that peripheral blood mononuclear cells can be infected with HBV[50,51]. Nuti et al[52] suggested that intrahepatic lymphocytes do not undergo clonal expansion within the liver but migrate from extrahepatic sites to the chronically infected liver, where they display the function of effector cells and subsequently die, implying that maintenance of the intrahepatic lymphocyte pool depends on continuous immigration of lymphocytes.

Multivariate multiple regression analysis in our study demonstrated a partial independent effect of HBeAg and pre-core mutant infection on T-cell profile, which is supported by previous studies[53-69]. It is well known that factors including HBeAg[53-59] and viral mutations[60-69] may contribute to the outcome and pathogenesis of HBV infection by inducing tolerance to specific T cells, reducing their potential to kill cells, inhibiting antigen processing and presentation, thereby decreasing the visibility of infected hepatocytes in the immune system. In addition, our results indicate that there was no significant difference of the proportion of T-cell subsets in patients at different ages of HBV infection and in patients of maternal HBV carrier status after adjustment for serum HBV load. In our study, patients with a maternal carrier history were usually infected with HBV at a younger age (Table 1) and a higher HBV load was detected in most of them, suggesting that HBV infection from the mother and/or at a younger age predisposes to tolerance to HBV infection and, thus, has a higher BHV load.

The differences of peripheral T cell subpopulation profile in patients at different clinical stages of chronic HBV infection and the strong relationship between peripheral T-lymphocyte subpopulations with a HBV load were observed in this study. Unfortunately, no information on the proportion of virus-specific T-cell subpopulations was provided in this study. Whether the increased CD8^{+} T cells contain an increment of virus-specific CD8^{+} T cells and the decreased CD4^{+} T cells include a decrement of HBV-specific CD4^{+} T cells

remains unclear. Further studies are needed to confirm their relationship and the correlation between intrahepatic and peripheral lymphocyte subsets.

In conclusion, HBV viral load in patients at the immune-tolerant and immune active stages contributes to the variations in peripheral T cell subpopulation profile, which is relevant to the design of individualized new anti-viral strategies. Further study is required to better understand the complex host-virus interaction that determines the persistence and outcome of HBV infection.

ACKNOWLEDGMENTS

COMMENTS

Background

Hepatitis B virus (HBV) infection is a serious public health problem worldwide and a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The pathogenesis of persistent HBV infection and hepatitis B is very complex and has not been clarified until now. HBV infection, a dynamic process with variable biochemical, virological and histological profiles at its different stages, depends on host and viral factors. Furthermore, the profiles may change at a variable pace over time. Based on the virological and biochemical parameters, chronic HBV infection patients can divided into immune-tolerant stage, immune active stage, and immune-inactive carrier stage.

Research frontiers

Outcome of HBV infection and the pathogenesis of liver disease are determined by immune-medicated host-virus interaction, which is difficult to fully elucidate because the host range of HBV is limited to human beings and chimpanzees. The pathogenesis of liver disease and interaction between virus and host remain the hotspots in this field.

Innovations and breakthroughs

The pathogenesis of cellular immune disorder and viral replication remains unknown. In the present study, peripheral T-lymphocyte subpopulations in chronic HBV infection patients were detected by flow cytometry and HBV load was determined by real-time polymerase chain reaction. The results suggest that differences in the peripheral T cell subpopulation profile were observed in patients at different clinical stages of chronic HBV infection. HBV load in patients at the immune-tolerant and immune active stages contributes to the variations in peripheral T cell subpopulation profile. The substantial linear dose-response relationship and strong independent predictive effect of HBV load on T-lymphocyte subpopulations suggested a close proximity of the causal pathway between them, indicating that HBV load plays an important role in the pathogenesis of T-cell impairment in chronic HBV infection patients.

Applications

The results, which suggest that a high HBV load contributes to the impaired balance of T-cells in chronic HBV infection patients, have practical implications because understanding of the immune response to HBV infection is useful in developing appropriate therapeutic strategies for controlling viral hepatitis and disease progression, improving current knowledge about the prognosis of persistent HBV infection. In addition, a high HBV load can predict the variations in peripheral blood T-lymphocyte subpopulations of chronic HBV infection patients.

Terminology

$CD4^+$ T cells, classically referred to helper T cells that are required for the efficient effector cytotoxic/suppressor $CD8^+$ T- and B-cell antibody production, play an important role in HBV infection by secreting Th1 cytokines that down-regulate HBV replication, and by promoting $CD8^+$ T- and B-cell responses. $CD8^+$ T cells go on to clear HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms, thus reducing the circulating virus level, while B-cell antibody production neutralizes free viral particles, thus preventing (re) infection. $CD3^+$, $CD4^+$ and $CD8^+$ cells are the major function subgroups of T cells and play an important role in the response to HBV infection, which can reflect the cellular immune function and immunoregulation and are usually regarded as a valuable index of the changes in immunity of patients.

Peer review

This is a descriptive study of the peripheral T cell sub-populations in chronic hepatitis B based in a large cohort of patients.

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