

Original Article

Regulatory T cells and IL-17⁺ T helper cells enhanced in patients with chronic hepatitis B virus infection

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Abstract: Hepatitis B is a worldwide infectious disease caused by hepatitis B virus (HBV), it led to millions of deaths every year, HBV mainly through immune response to damage liver cells. The purpose of this study was to judge the value of Regulatory T cells (Treg) and IL-17⁺ T helper cells (Th17) in different chronic HBV infection stages. 96 patients with chronic HBV infection were enrolled and selected 33 healthy adults as control. Detected the expression of Treg and Th17 cells in peripheral blood by flow cytometry and assayed liver function simultaneously. Compared to the control group, the expression of Treg (6.80 ± 1.92 vs. 4.42 ± 0.97 ; $P < 0.0001$) and Th17 (6.15 ± 4.20 vs. 2.66 ± 1.79 ; $P < 0.0001$) cells were both increased and the ratio of Treg/Th17 was significantly decreased (1.48 ± 0.89 vs. 2.29 ± 1.31 ; $P = 0.0001$) in patients with HBV infection. Spearman correlation analysis demonstrated that the level of Treg and Th17 cells were associated with liver function. ROC curve analysis found that Treg and Th17 cells were suitable for as a screening test for early detection of the disease. In conclusion, the expression of Treg and Th17 cells were increased in chronic hepatitis B patients and these indicators were independent risk factors to hepatitis.

Keywords: Treg, Th17, hepatitis B, cirrhosis, hepatocellular carcinoma

Introduction

Hepatitis B is a worldwide infectious diseases caused by hepatitis B virus (HBV), it can develop to cirrhosis and hepatocellular carcinoma (HCC). Generally, the chronic HBV infection course can be divided into chronic hepatitis B (CHB), hepatitis B-associated liver cirrhosis (HBV-LC) and hepatitis B-associated hepatocellular carcinoma (HBV-HCC), thus led to millions of deaths every year. In 2006, the hepatitis B surface antigen (HBsAg) carrier rate was 0.96% among children under 5 years of age and estimated 84 million HBV carriers in china [1]. Previous studies showed that HBV replication in liver cells mainly through immune response to damage liver cells [2, 3]. HBV infection could induce cellular and humoral immune response, cause autoimmune response and immune dysfunction, unbalance the ratio of T helper cells [4-6] and produce autoantibodies [7-10]. In recent years, regulatory T (Treg) cells and IL-17⁺ T helper (Th17) cells played an important role in autoimmune diseases and many

studies proved that the expression of Treg and Th17 cells were changed in the patients with CHB, HBV-LC or HBV-HCC [11-13], but the systematic study of the change in different stages of chronic HBV infection were lacked.

Therefore, we studied the expression of Treg and Th17 cells at different stages of chronic HBV infection, the purpose of this study was to determine whether the expression of Treg and Th17 cells or the balance of Treg/Th17 were changed in different chronic HBV infection stages and to judge the value of these indicators.

Materials and methods

Subjects

Ninety-six patients of chronic HBV infection (HBV group), median age was 48.27 years (rang: 23-72 y) with 74 males (77.1%) and 22 females (22.9%), including 43 patients of CHB (CHB group), 29 patients of HBV-LC (HBV-LC group), 24 patients of HBV-HCC (HBV-HCC

Table 1. Summary of the characteristics of study participants

	HBV group	Control group	P value
Gender, No.	96	33	
Male	74	23	
Female	22	10	
Age, y (mean±SD)	48.3±15	43.5±13.1	0.11
Chronic hepatitis B virus infection (HBV).			

group) were enrolled in this study. The diagnosis was based on the guidelines for chronic hepatitis B diagnosis of the American Association for the Study of Liver Diseases (AASLD) [14]. Thirty-three healthy individuals were enrolled as normal controls (Control group) shown as **Table 1**. Additional inclusion criteria included no treatment with nucleic acid analogues or α -IFN within 3 years prior to study initiation. Subjects with hepatitis C virus, hepatitis D virus or human immunodeficiency virus were excluded from the study. The study protocol was approved and monitored by the ethics committee of Nanjing Jiangbei Peoples' Hospital, and written informed consent was obtained from the patients.

Flow cytometric analysis

HU FOXP3 STANG KIT and HU TH1/17 PHNOTYP KIT were purchased from BD PharMingen (San Diego, CA). Whole blood from each patient at admission was collected in heparin anticoagulated vacutainer tube. Samples were stained and fixed within the day of collection. For test Treg cells, whole blood samples (200 μ L) were first stained with CD4 FITC, CD25 APC in dark for 30 min, Then FOXP3 PE was added for 30 min. For detect Th-17 cells, whole blood samples (200 μ L) were activated with phorbol-12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (1 mg/ml) for 4 h. After using BD cytofix and perm in dark for 30 min, then mixed in Th1/Th17 Phenotyping Cocktail for 30 min. All specimens were tested on the Flow Cytometry within 3 h. Three-color flow cytometric analyses were performed using FACSCalibur and CellQuest software (Becton Dickinson, San Jose, CA).

Statistical analysis

Results were reported as means±SD for percentages. Statistical comparisons between two groups used independent samples T-test. The one-way ANOVA and Scheff method were used

for multiple comparisons. Spearman correlation analysis was used to evaluate relationships between two variables. Data analysis was done by using SPSS version 13.0 for Windows and MedCal statistical software (ver. 12. 4. 0. 0), Confidence interval was 95%.

Results

Expression of Treg cells significantly increased in HBV group

We defined the Treg cells as the population of CD4⁺CD25⁺FoxP3⁺T cells taken the percentage of total CD4⁺T cells (**Figure 1A**). Compared to control group, the expression of Treg cells were significantly increased in HBV group (96 vs. 33; 6.80±1.92 vs. 4.42±0.97; P<0.0001; **Figure 2A**). Then we analyzed the data in different HBV infection stages, found there were significant differences between control and each HBV infection subgroups (P<0.0001, **Figure 2B**), but there was no difference between CHB, HBV-LC and HBV-HCC groups.

The frequency of Th17 cells significantly increased in HBV group

We next defined the Th17 cells as the population of CD4⁺IL-17A⁺T cells taken the percentage of total CD4⁺T cells (**Figure 1B**). Similar to Treg cells, the frequency of Th17 cells in HBV group was more higher than in control group (96 vs. 33; 6.15±4.20 vs. 2.66±1.79; P<0.0001; **Figure 2C**). Subgroup analysis showed that with the development of HBV infection, there were significant differences between the groups (P<0.0001, **Figure 2D**). But compared to healthy individuals, the expression of Th17 only significant increased in CHB and HBV-LC groups.

Treg/Th17 decreased in HBV group

To reveal whether there was an imbalance between Treg and Th17 cells, we analyzed the ratio of Treg/Th17 and confirmed that compared to control group, Treg/Th17 was decreased in HBV group (96 vs. 33; 1.48±0.89 vs. 2.29±1.31; P=0.0001; **Figure 2E**), and showed significant differences between the different HBV infection stages (P<0.0001; **Figure 2F**), similar to expression of Th17 cell, Treg/Th17 in CHB and HBV-LC groups expressed more lower than in control group.

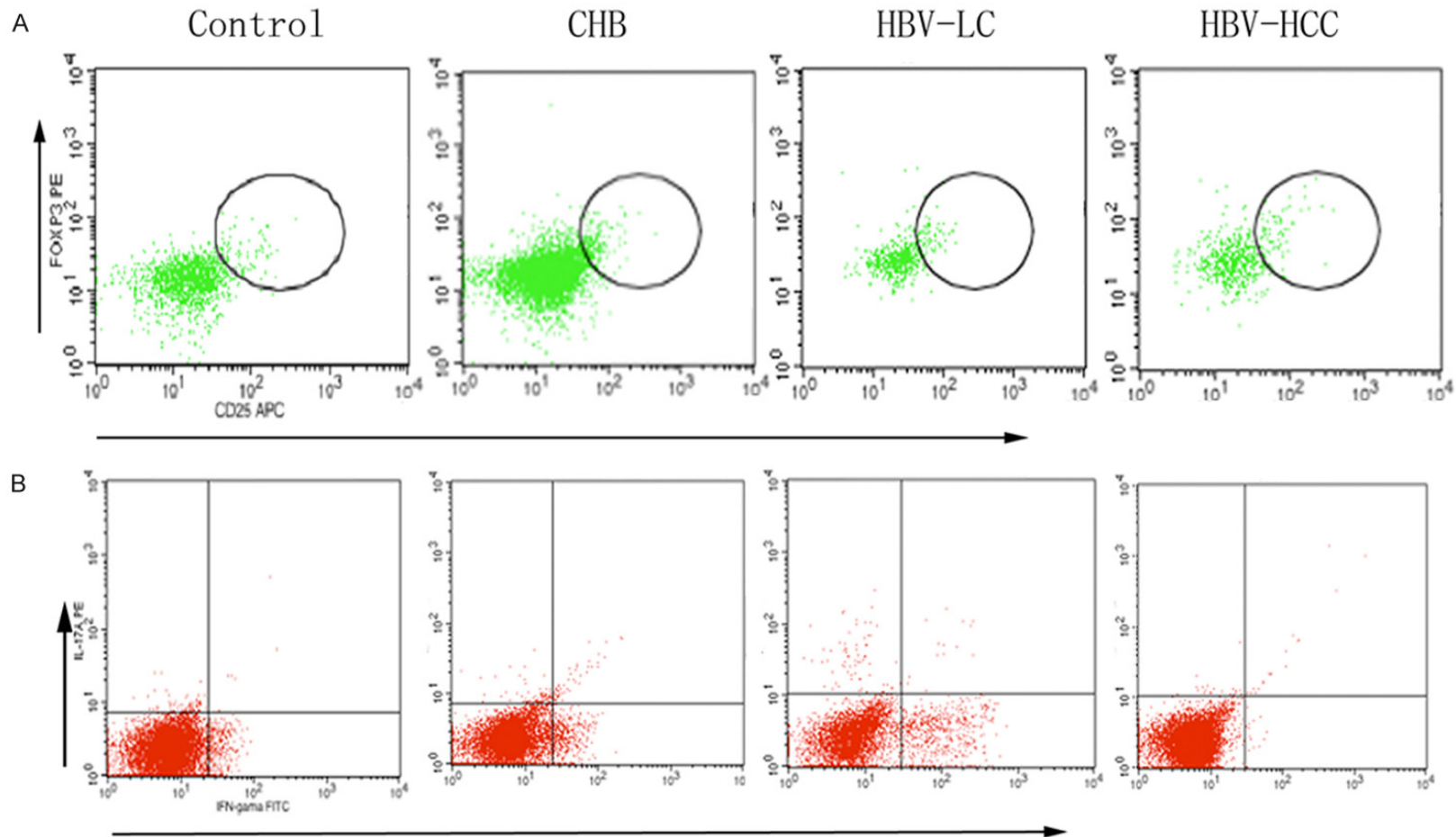


Figure 1. Representative dot plots of FoxP3 and IL-17A. A. Representative dot plots of FoxP3 expression in peripheral CD4⁺T cells of Controls, CHB, HBV-LV, HBV-HCC. B. Representative dot plots of IL-17A a expression in peripheral CD4⁺T cells of Controls, CHB, HBV-LV, HBV-HCC. Chronic hepatitis B virus infection (HBV), chronic hepatitis B (CHB), hepatitis B-associated liver cirrhosis (HBV-LC), hepatitis B-associated hepatocellular carcinoma (HBV-HCC), regulatory T cells (Treg), IL-17⁺ T helper cells (Th17).

Treg, Th17 and chronic HBV infection

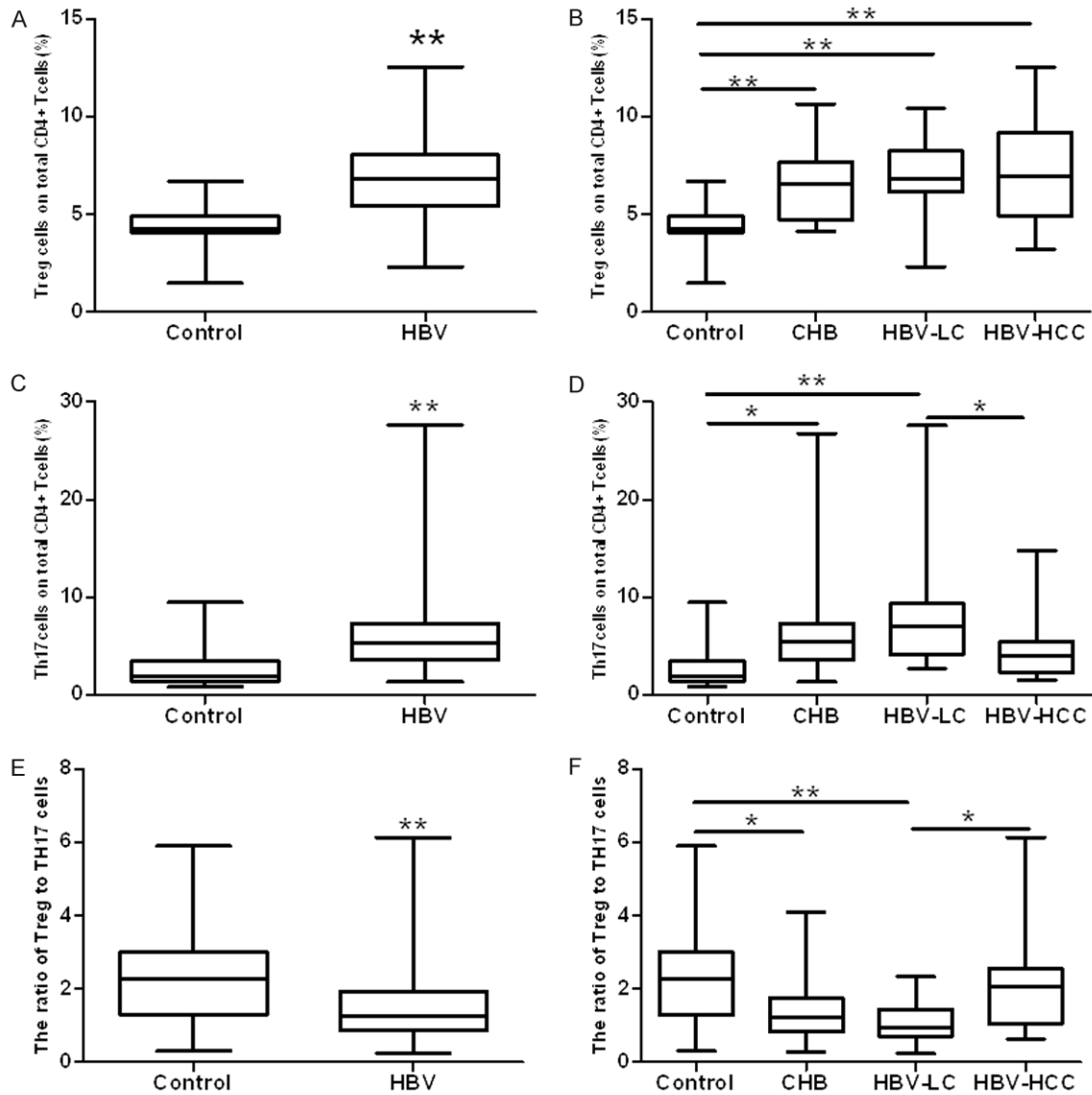


Figure 2. Results with Treg, Th17 and Treg/Th17 ratio between chronic HBV infection patients and controls. Compared with Control, Treg, Th17 and Treg/Th17 were all increased significantly (A, C, E). When to subgroups, Treg was increased in all chronic HBV infection groups (B), Th17 and Treg/Th17 were changed significantly in CHB and HBV-LC groups (D, F) (*P<0.005, ** P<0.001).

Table 2. Results of spearman correlation analysis for Treg to Th17

Group	r	p-value
Control	-0.07	0.7005
HBV	0.33	0.0012**
CHB	0.39	0.0102*
HBV-LC	0.26	0.1795

chronic hepatitis B (CHB), hepatitis B-associated liver cirrhosis (HBV-LC), hepatitis B-associated hepatocellular carcinoma (HBV-HCC), regulatory T cells (Treg), IL-17⁺ T helper cells (Th17). *P<0.005, **P<0.001.

Treg and Th17 cells had positive linear correlation in HBV group

We used spearman correlation analysis to evaluate the relationship between Treg and Th17 cells (Table 2) and found there was positive linear correlation in HBV (R=0.33; P=0.0012; Figure 3A) and CHB (R=0.39; P=0.0102; Figure 3B) groups, and there were no linear correlation in HBV-LC (R=0.27; P=0.1795; Figure 3C) and HBV-HCC (R=0.35; P=0.0985; Figure 3D) groups.

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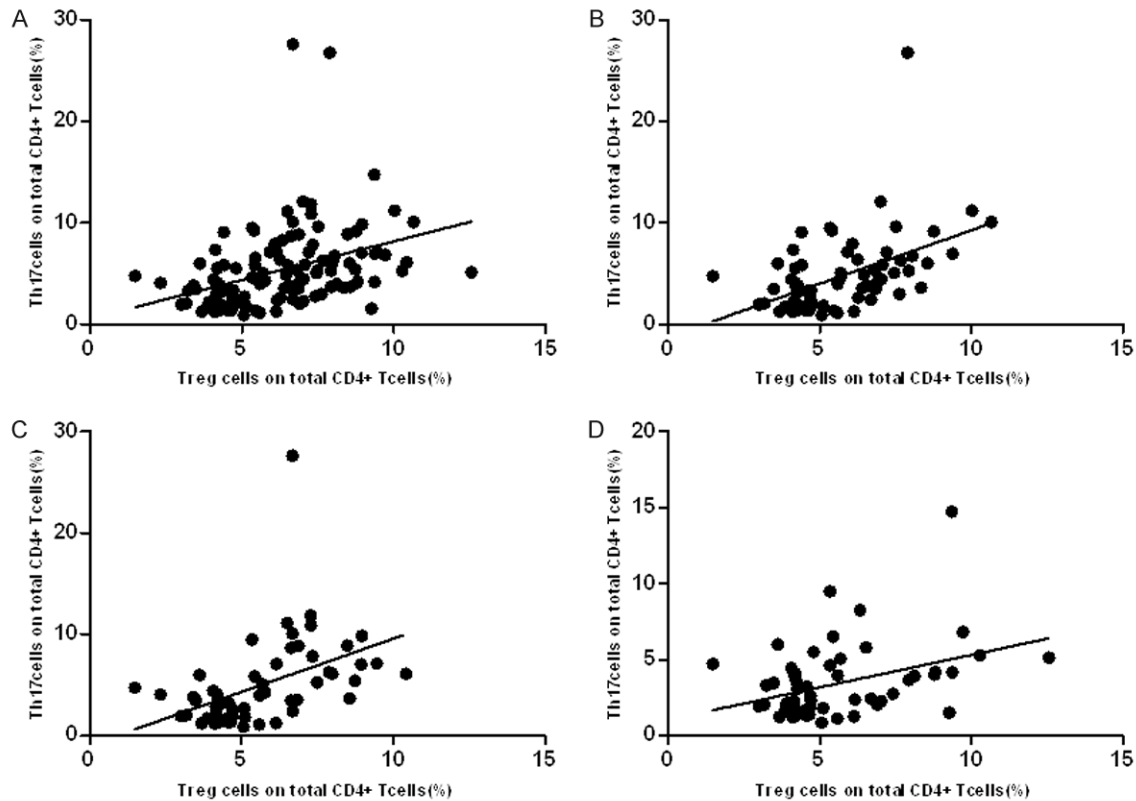


Figure 3. Relationship between Treg, Th17 and Treg/Th17 in chronic HBV infection patients. Spearman correlation analysis showed there were significant positive linear correlations between Treg and Th17 cells in HBV group (A) and CHB group (B), HBV-LC and HBV-HCC groups (C, D) also showed positive linear correlations but without significant difference.

The relationship between Treg cells, Th17 cells, Treg/Th17 and liver inflammation indicators

We selected ALT (alanine aminotransferase), AST (aspartate aminotransferase), PT (prothrombin time), TBIL (total bilirubin), DBIL (direct bilirubin), ALB (albumin), WBC (white blood cell) and HBV-DNA as the biochemical indicators of liver inflammation, and used Spearman correlation analysis to evaluate relationships between liver inflammation and Treg or Th17 cells (**Table 3**). The results revealed that there were significant correlations between Treg cells and PT ($R=0.23$; $P=0.0217$; **Figure 4A**), WBC ($R=-0.27$; $P=0.0088$; **Figure 4B**) in HBV group, Treg cells and ALT ($R=0.32$; $P=0.0347$; **Figure 4C**), AST ($R=0.32$; $P=0.0376$; **Figure 4D**), PT ($R=0.34$; $P=0.0265$; **Figure 4E**) and TBIL ($R=0.32$; $P=0.0341$; **Figure 4F**) in CHB group, Treg cells and TBIL ($R=-0.43$; $P=0.0194$; **Figure 4G**), DBIL ($R=-0.41$; $P=0.0276$; **Figure 4H**), WBC ($R=-0.43$; $P=0.0197$; **Figure 4I**) in HBV-LC group.

Analysis of Treg cells, Th17 cells and Treg/Th17 as chronic HBV infection-specific markers

To examine the selectivity of the blood markers Treg cells, Th17 cells and Treg/Th17 for chronic HBV infection, the data were subjected to ROC curve analysis, and the results were shown in **Table 4**. As is understood from the data of **Figure 5** and **Table 4**, the AUC (area under curve) value of serum Treg and Th17 cells levels were all above 0.75 in the HBV, CHB, HBV-LC and HBV-HCC groups with the Th17 cells sensitivity detected at most more than 80% and specificity more than 75% respectively, Treg cells sensitivity detected at most less than 75% and specificity more than 90% respectively. In addition, when the serum Treg/Th17 level was measured, the AUC value most less than 0.75 in all groups, with the specificity detected at most less than 55% respectively, in all cases, indicated that Treg and Th17 cells were more

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Table 3. Results of spearman correlation analysis for Treg, Th17 and Treg/Th17 to liver inflammation indicators

Group		Mean±SD	Th17		Treg		Treg/Th17	
			r	p-value	r	p-value	r	p-value
HBV	ALT (U/L)	131.2±167.64	0.08	0.4630	0.02	0.8297	-0.06	0.5903
	AST (U/L)	99.58±133.02	0.00	0.9729	0.15	0.1501	0.08	0.4481
	PT (s)	13.06±2.24	0.09	0.3622	0.23	0.0217*	-0.01	0.8908
	TB (μmol/L)	48.02±71.36	0.15	0.1586	0.17	0.1018	-0.08	0.4562
	DB (μmol/L)	23.34±43.26	0.11	0.2677	0.17	0.1034	-0.05	0.5955
	WBC (*10 ⁹ /L)	4.40±1.79	-0.05	0.6246	-0.27	0.0088**	-0.07	0.5236
	ALB (g/L)	37.74±7.3	0.02	0.8406	-0.10	0.3485	-0.05	0.6347
	HBV-DNA (Log10copies /ml)	3.74±3.07	0.09	0.3964	0.01	0.8903	-0.06	0.5679
CHB	ALT (U/L)	230.49±207.5	0.23	0.1417	0.32	0.0347*	0.23	0.3998
	AST (U/L)	120.70±119.4	0.06	0.6907	0.32	0.0376*	0.06	0.6551
	PT (s)	12.21±1.9	0.25	0.1129	0.34	0.0265*	0.25	0.5627
	TB (μmol/L)	35.52±33.85	0.18	0.2549	0.32	0.0341*	0.18	0.8626
	DB (μmol/L)	18.48±22.06	0.20	0.1941	0.28	0.0730	-0.10	0.5351
	WBC (*10 ⁹ /L)	4.73±1.21	-0.02	0.8967	-0.13	0.3905	-0.02	0.9551
	ALB (g/L)	42.23±5.8	0.01	0.9272	-0.15	0.3296	0.01	0.5282
	HBV-DNA (Log10 copies/ml)	5.09±2.78	-0.03	0.8655	-0.01	0.9515	0.04	0.8065
HBV-LC	ALT (U/L)	43.03±35.65	0.09	0.6352	-0.08	0.6841	-0.20	0.2954
	AST (U/L)	52.24±35.21	-0.04	0.8527	-0.16	0.3989	-0.10	0.5589
	PT (s)	14.16±2.35	-0.08	0.6690	-0.13	0.5145	0.02	0.9332
	TB (μmol/L)	46.15±30.34	-0.15	0.4258	-0.43	0.0194*	-0.02	0.9312
	DB (μmol/L)	22.56±18.68	-0.17	0.3765	-0.41	0.0276*	0.00	0.9899
	WBC (*10 ⁹ /L)	3.90±1.87	-0.11	0.5531	-0.43	0.0197*	-0.15	0.4288
	ALB (g/L)	33.04±6.9	0.03	0.8719	0.00	0.9980	-0.07	0.7364
	HBV-DNA (Log10 copies/ml)	2.88±2.73	0.16	0.4497	0.28	0.1625	-0.13	0.5353
HBV-HCC	ALT (U/L)	60.00±48.75	0.17	0.4407	0.13	0.5577	-0.02	0.9195
	AST (U/L)	118.9±203.69	0.10	0.6420	0.29	0.1633	0.12	0.5737
	PT (s)	13.24±2.13	0.01	0.9742	0.17	0.4237	0.08	0.7036
	TB (μmol/L)	72.67±129.96	0.33	0.1118	0.25	0.2481	-0.28	0.1865
	DB (μmol/L)	32.97±79.22	0.13	0.5572	0.27	0.2073	-0.05	0.8338
	WBC (*10 ⁹ /L)	4.43±2.42	0.05	0.8150	-0.17	0.4355	-0.05	0.8181
	ALB (g/L)	35.36±5.44	0.15	0.4714	0.14	0.5114	-0.04	0.8670
	HBV-DNA (Log10 copies/ml)	1.95±2.87	-0.19	0.4113	-0.05	0.8358	0.30	0.2034

alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time (PT), total bilirubin (TBIL), direct bilirubin (DBIL), albumin (ALB), white blood cell (WBC). *P<0.005, **P<0.001.

suitable than Treg/Th17 as chronic HBV infection-specific markers.

Discussion

Chronic HBV infection could cause autoimmune reactions to injury the liver cells directly, or through mediate antibody-dependent cytotoxicity (ADCC) cause liver damage [15-19]. Increasing evidences have shown that immune-mediated liver damage plays an important role

in chronic HBV infection. Bo Yang confirmed Th17 cells and serum IL-17 concentration were significantly higher in CHB and HBV related acute-on-chronic liver failure (ACLF) patients than in asymptomatic chronic HBV carriers (AsC) and control groups, and increased with immune inflammation aggravation from AsC, CHB to ACLF [20]. Zhu S found that levels of Treg and Th17 cells were positively correlated with the degree of inflammatory activity and liver pathology HAI score [21], all these results

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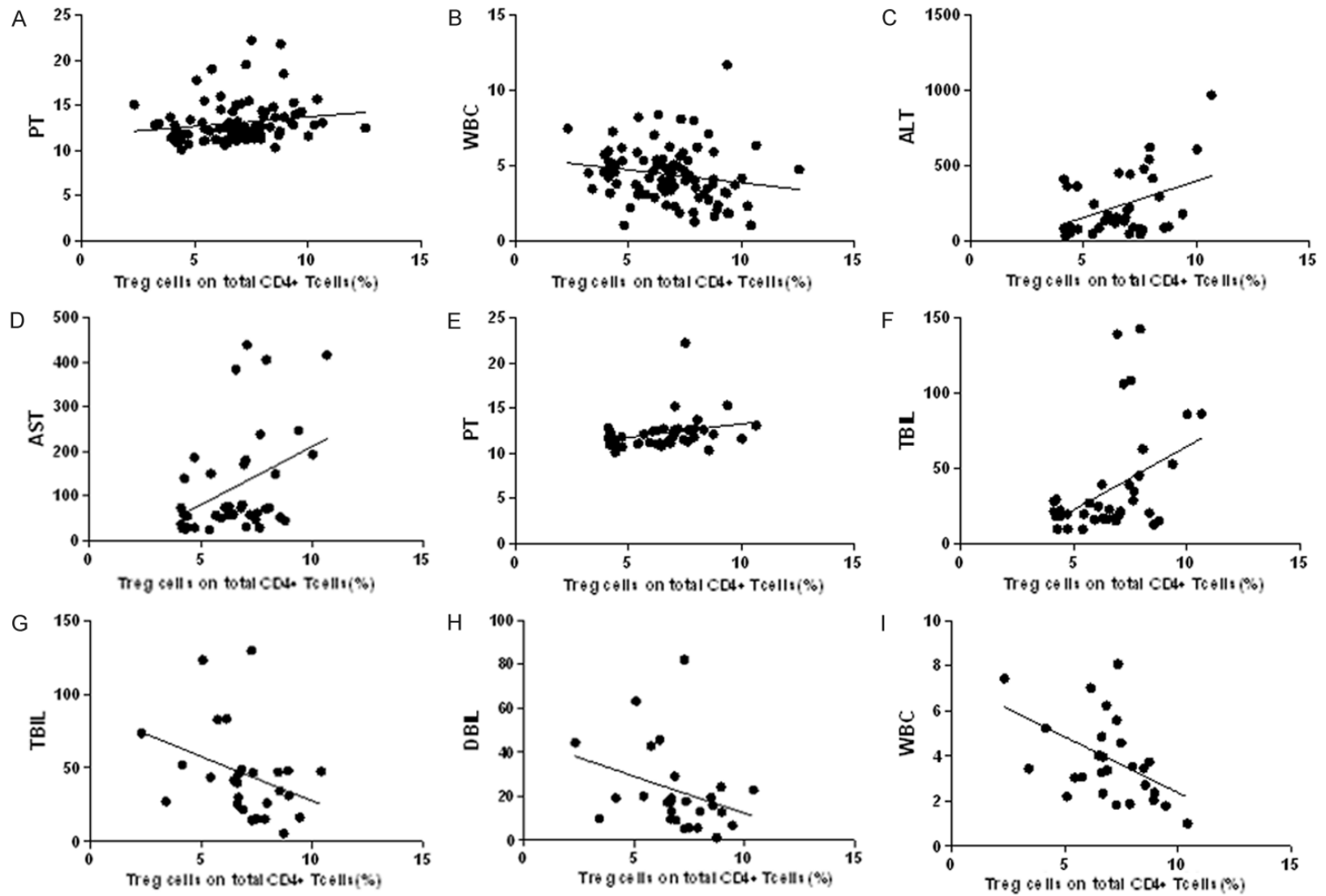


Figure 4. Relationship between Treg and liver inflammation indicators in chronic HBV infection patients. Result of spearman correlation analysis showed there were significant linear correlations between Treg and PT, WBC in HBV group (A, B), ALT, AST, PT and TBIL in CHB group (C-F), TBIL, DBIL and WBC in HBV-LC group (G-I) alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time (PT), total bilirubin (TBIL), direct bilirubin (DBIL), white blood cell (WBC).

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Table 4. Result of ROC analysis of Treg, Th17 and Treg/Th17

Group	Th17				Treg				Treg/Th17			
	AUC	Sensitivity (%)	Specificity (%)	Criterion (%)	AUC	Sensitivity (%)	Specificity (%)	Criterion (%)	AUC	Sensitivity (%)	Specificity (%)	Criterion (%)
HBV	0.84±0.035	80.2	75.8	>3.45	0.857±0.033	71.9	93.9	>5.7	0.703±0.056	88.5	45.5	≤2.45
CHB	0.839±0.045	81.4	75.8	>3.45	0.854±0.043	67.4	93.9	>5.7	0.72±0.06	76.7	60.6	≤1.75
HBV-LC	0.916±0.038	93.1	75.4	>3.45	0.89±0.044	82.8	93.9	>5.7	0.795±0.057	96.6	54.4	≤1.93
HBV-HCC	0.751±0.068	62.5	78.8	>3.56	0.824±0.059	66.7	97	>6.14	0.562±0.077	75	45.5	≤2.45

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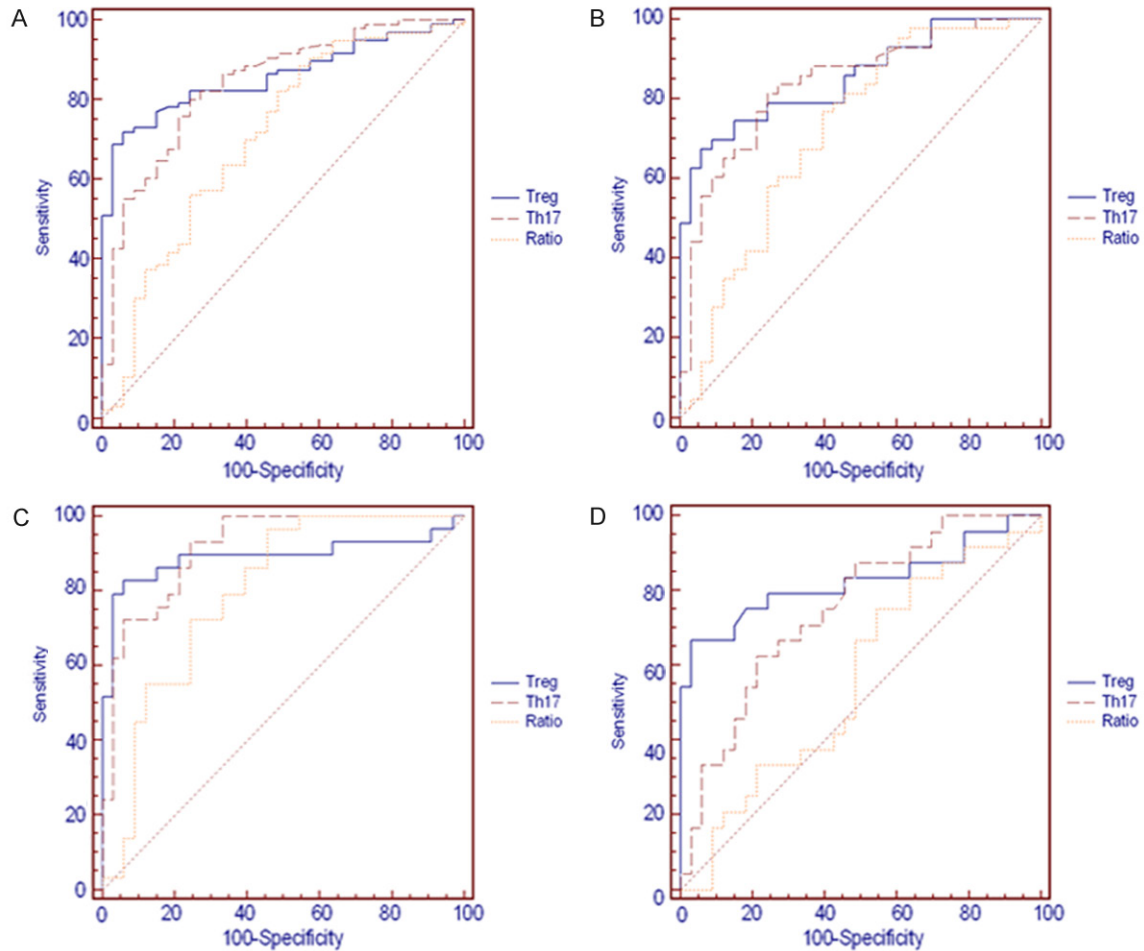


Figure 5. ROC curve analysis of Treg, Th17 and Treg/Th17 in chronic HBV infection patients. ROC curve analysis showed Treg and Th17 cells were significantly higher in all groups, but the AUC (area under curve) value of Treg/Th17 was less than 0.70 in HBV-HCC group.

suggested that Treg and Th17 cells involved in the inflammation and liver cell immune clearance after HBV infection. In this study, we were detected the expression of Treg and Th17 cells in the peripheral blood of patients with chronic HBV infection, and found that compared to the control group, both Treg and Th17 cells increased significantly in the HBV group, it was similar to past studies. Further subgroup analysis revealed that Treg cells increased with disease progression. Similarly, level of Th17 cells also elevated in CHB and HBV-LC groups. We used spearman correlation analysis to evaluate relationships between Treg and Th17 cells and found there was positive linear correlation.

Since Treg and Th17 cells derived from the same original T cells, and has same cell differentiation signaling pathway with TGF- β essen-

tial for their differentiation [22, 23], Th17 cells can increase the immune response by release inflammatory cytokines, and Treg cells can secrete immunosuppressive cytokines to suppress it, the imbalance between Treg/Th17 related to the occurrence of many diseases [24-29]. Xueping Yu found Treg and Th17 cells showed changes in genes, protein levels and T cell phenotypes during ACHBLF events. And the survival of ACHBLF patients was associated with the increased Treg/Th17 ratio [30]. Zhang JY discovered HBC patients taking entecavir antiviral therapy could significantly increase Treg/Th17 ratios [31]. In this study, we found that the imbalance of Treg to Th17 cells correlated with the progression of the disease. The ratio of Treg/Th17 in patients with chronic HBV infection was significantly decreased, which suggested that the Treg/Th17 balance modu-

lated liver damage in chronic HBV infection. Or, on the other hand, Treg/Th17 imbalance may cause HBV infection to chronic. Subgroup analysis showed there also was significant difference between the groups. These data suggested that the interaction between Treg and Th17 cells was important to maintain the balance between the limited immune response and pathological damage. So, these indicators may be independent risk factors to hepatitis.

We also analyzed the relationship between the two types of T cells and chronic HBV infection progression. We found that in all patients, only Treg cells had correlated with PT and WBC levels significantly. In subgroups, we found that Treg cells had correlated with ALT, AST, TBIL and PT levels significantly in CHB group and had correlated with TBIL, DBIL and WBC levels significantly in HBV-LC group, but there were no relationships between Treg cells and ALB, HBV-DNA. Th17 cells and ratio of Treg/Th17 showed no significant relationship with liver inflammation markers either. These data suggested Treg and Th17 cells were independent with HBV replication. Spearman correlation analysis demonstrated that the level of Treg cells was shown to be significantly associated with the biochemical indicators of liver function, it could reflect the changes in liver function more effectively and had more worthy to be used as an independent evaluation.

The results of ROC curve analysis proved that Treg and Th17 cells were suitable for as screening tests for early detection of the disease owing to both high sensitivity and specificity, but the ratio of Treg/Th17 was not reliable especially due to the lack of specificity (45.5% for all stages, 45.5% for the HBV-HCC group).

In conclusion, this study showed that the Treg and Th17 cells were increased in chronic HBV infection patients and changed with the course of disease. While with the course of disease developed, expression of Treg and Th17 cells were increased, and appeared the imbalance between the Treg and Th17 cells. The level of changes may be served for determine the degree of liver damage.

The limited sample population and duration of follow-up in study precluded us from further refinement, as CHB including mild, moderate and severe CHB, HBV-LC including compensat-

ed and decompensate LC. Large-scale multi-center studies are needed to prove the further association of Treg and Th17 cells and chronic HBV infection.

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Disclosure of conflict of interest

None.

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