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Serum Interleukin (IL)-9 and IL-10, but not T-Helper 9 (Th9) Cells, are Associated With Survival of Patients With Acute-on-Chronic Hepatitis B Liver Failure

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Abstract: CD4⁺ T helper (Th) cells are reported to be essential for initiating and maintaining an effective immune response to hepatitis B virus (HBV) infection. Th9 cells are a new subset of CD4⁺ Th cells that produce interleukin (IL)-9 and IL-10. The present study aimed to investigate the percentage of Th9 cells relative to the number of CD4⁺ cells in peripheral blood.

We also measured serum IL-9 and IL-10 levels in different stages of HBV infection and their relationship with progress and prognosis of liver disease. Whole blood samples from 111 patients with HBV infection, including 39 chronic hepatitis B (CHB), 25 HBV-liver cirrhosis (HBV-LC), 21 acute-on-chronic liver failure (ACLF) patients, and 26 healthy controls were collected.

The percentage of Th9 cells and serum IL-9 and IL-10 levels were determined. There was no significant difference in the percentage of Th9 cells and serum IL-9 and IL-10 levels among different groups, nor were these related to hepatitis B e antigen status, complications of cirrhosis, inflammation index, or prognosis indexes. There was no change in the percentage of Th9 cells before and after antiviral treatment in CHB patients. There was no correlation of Th9 cells with survival of ACLF patients. However, IL-9 and IL-10 levels were significantly higher in the nonsurvived ACLF patients compared to survived ACLF patients. Furthermore, baseline IL-9 level predicted the prognosis of ACLF patients with 87.5% sensitivity and 61.5% specificity.

Thus, our data indicate that Th9 cells were unlikely involved in the pathogenesis of HBV infection, but elevation in IL-9 and IL-10 may signal poor prognosis for ACLF.

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Abbreviations: ALB = serum albumin, ALT = alanine aminotransferase, CHB = chronic hepatitis B, ELISA = enzyme-linked immunosorbent assay, HAV = hepatitis A virus, HBV = hepatitis B virus, HBV-ACLF = hepatitis B virus-related acute-on-chronic liver failure, HBV-LC = hepatitis B virus-related liver cirrhosis, HC = health controls, HCC = hepatic carcinoma, HCV = hepatitis C virus, HDV = hepatitis D virus, HIV = human immunodeficiency virus, MELD = model for end-stage liver disease, TBil = total bilirubin.

INTRODUCTION

Hepatitis B virus (HBV) infection is a severe public health burden, and approximately one-third of the world population has serological evidence of resolved or ongoing infection.^{1,2} HBV infection causes a broad-spectrum of liver diseases ranging from acute hepatitis B, chronic infection with no biochemical evidence of liver injury to progressive chronic hepatitis B (CHB), which may advance to liver cirrhosis (LC), liver failure (LF), and hepatocellular carcinoma.^{1,3} HBV is thought to be noncytotoxic during infection, and the host's immune response to HBV infection is responsible for viral pathogenesis and clearance of HBV infection.^{4,5}

Many cytokines involved in the host's innate and adaptive immune responses have been suggested to contribute to effective antiviral immunity and outcomes of HBV infection.⁶ HBV-specific cytotoxic T lymphocytes and CD4⁺ T helper (Th) lymphocytes are 2 major components of the HBV-specific immune response.^{7,8} CD4⁺ Th cells are a group of lymphocytes that produce cytokines regulating strength and duration of immunity and inflammation. CD4⁺ Th cells can be classified into Th1 that secretes interleukin (IL)-2 and interferon (IFN)-gamma, Th2 that produces IL-4, Th17 (IL-17), Th22 (IL-22), and the newly discovered Th9 (IL-9) cells.^{9–11} Several studies have demonstrated that an imbalance in Th1/Th2 and/or T regulatory (Treg)/Th17 was involved in the progression of HBV infection and is attributed to liver injury and fibrosis.^{12–15}

The Th9 cell is a new subset of Th cells. It secretes IL-9 and IL-10 and functions mainly through IL-9.^{10,16} The Th9 was reported to contribute to various diseases, such as allergic disease,^{17–19} autoimmune disease,^{20,21} cancer,²² transplant rejection,²³ and parasitic infection.²⁴ IL-9 has also been suggested to be involved in the pathogenesis of respiratory

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syncytial virus and EB virus infection.^{25,26} A recent study²⁷ showed that the percentage of Th9 cells in peripheral blood mononuclear cells (PBMCs) and serum IL-9 level were significantly elevated in HBV-LC patients compared to healthy individuals. Furthermore, a higher expression of CCL20 and CC chemokine receptor 6 (CCR6) was detected on Th9 cells, suggesting that Th9 cells may contribute to the pathogenesis of HBV-LC through the CCL20/CCR6 axis. Our previous study also showed that the serum IL-9 level was significantly higher in HBV-LC than in healthy individuals.²⁸ These observations led us to hypothesize that Th9 cells, as well as IL-9 and IL-10, are immune components participating in the pathogenesis of HBV infection. This study aimed to understand the involvement of Th9 cells in HBV infection and their relationship with progress and prognosis of liver disease. Specifically, we measured the percentage of Th9 cells and serum IL-9 and IL-10 levels in different stages of HBV infection. We also analyzed the kinetic changes in Th9 cells, IL-9, and IL-10 levels over time in CHB patients.

PATIENTS AND METHODS

Patients

This study retrospectively enrolled 85 chronic HBV infected patients including 39 CHB, 25 HBV-LC and 21 acute on chronic LF (ACLF) patients, who were hospitalized or followed from July 2012 to January 2013 in the Department of Infectious Diseases of the First Hospital of Quanzhou, affiliated with Fujian Medical University. The criteria for diagnoses of CHB,²⁹ HBV-LC³⁰ and ACLF³¹ have been previously described. Patients were excluded if they were (1) treated with antivirals or immunomodulating agents, such as thymosin and glucocorticoid hormones; (2) concomitantly infected with hepatitis A virus, hepatitis C virus (HCV), hepatitis D virus (HDV), or human immunodeficiency virus (HIV); or (3) concomitantly infected with alcohol- or drug-induced liver diseases. Blood samples from 26 healthy individuals were collected as health controls (HC). The study protocol was approved by the Ethics Committee of the First Hospital

of Quanzhou affiliated with Fujian Medical University ([2013]60), and written informed consent was obtained from each participant.

Treatment and Follow-up

Out of the 39 CHB patients, 23 were treated with nucleos(-)ide analogs (NAs, *i.e.*, entecavir, telbivudine, lamivudine, or adefovir dipivoxil) and were followed for 7.2 ± 3.4 months. A total of 21 ACLF patients were followed for >6 months. The patients were divided into survived ($n = 13$) and nonsurvived ($n = 8$) groups based on the final outcomes at the time this study was completed. The detailed characteristics of the 2 groups were described in a previous study.³²

Laboratory Data

Blood samples were collected from all patients the next morning after admission and at the end of follow-up for the 23 CHB patients who were treated with NAs. All biochemical and serological markers were detected in the Department of Laboratory of the First Hospital of Quanzhou affiliated with Fujian Medical University, and the details of them have been described in our previous study.^{33,34} The Child–Pugh³⁵ score and the model for end-stage liver disease (MELD)³⁶ score were calculated using clinical and experimental parameters.

Cell Preparation and Flow Cytometry

PBMCs were isolated from 3 mL of blood by ficoll density gradient centrifugation and adjusted to a density of 2×10^6 /mL in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA). Two milliliters of PBMCs were seeded on to a 6-well plate and maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY), 25 ng/mL phorbol myristate acetate, 1 μ g/mL ionomycin, and 1.7 μ g/mL monensin (all from eBioscience, San Diego, CA), and incubated at 37 °C and 5% CO₂ conditions for 5 hours. Then the cells were collected and incubated with monoclonal antibodies CD3-PC5 and CD8-ECD (Beckman Coulter Immunotech, Brea, CA) for 15 minutes. The cells were permeabilized

TABLE 1. Clinical Characteristics of the Subjects Enrolled in the Study

Characteristic	HC (n = 26)	CHB (n = 39)	HBV-LC (n = 25)	ACLF(n = 21)	P Value
No. (%) male	12 (46.2)	28 (71.8)	20 (80.0)	19 (86.4)	0.01
Age (yr)	25.5 (23.0–49.3)	33.0 (23.0–54.0)	53.0 (41.6–69.4)	36.0 (22.2–55.8)	<0.01
No.(%) HBeAg positive	–	27 (70.7)	7 (28.0)	12 (57.1)	<0.01
HBV DNA (lg copies/mL)	–	5.9 (4.7–6.9)	4.9 (3.0–6.8)	6.0 (4.2–7.1)	0.01
ALB (g/L)	–	41.5 (31.5–46.0)	30.4 (21.2–40.9)	30.0 (22.3–36.5)	<0.01
TBIL (μ mol/L)	–	21.6 (14.5–172.6)	33.9 (14.6–264.5)	279.3 (180.8–534.5)	<0.01
ALT (IU/L)	–	206.6 (68.0–1067.0)	40.0 (16.6–128.4)	347.0 (62.4–1585.4)	<0.01
INR	–	–	1.3 (0.9–1.8)	1.6 (1.1–2.9)	0.06
Cr (μ mol/L)	–	–	79.5 (57.2–123.4)	71.0 (41.6–120.4)	0.06
Child–Pugh stage:					
A, n	–	–	6	1	–
B, n	–	–	12	6	–
C, n	–	–	7	14	–
MELD score	–	–	10.3 (3.6–24.4)	20.4 (13.9–25.3)	<0.01

ALB = serum albumin, ALT = alanine aminotransferase, CHB = chronic hepatitis B, Cr = creatinine, HBeAg = hepatitis B e antigen, HBV-LC = hepatitis B virus related liver cirrhosis, INR = international normalized ratio, MELD = model for end-stage liver disease, NC = normal controls, TBil = total bilirubin.

with FIX and PERM reagent A (Invitrogen, Carlsbad, CA) for 15 minutes, and FIX and PERM reagent B (Invitrogen, Carlsbad, CA) for 10 minutes. Then the cells were incubated with anti-IL-17A-FITC and anti-IL-9A-PE (eBioscience, San Diego, CA) for 20 minutes. After washing with phosphate buffer saline, the phenotypes of the stained cells were analyzed by flow cytometry (COULTER Epics XL, Beckman, Brea, CA). Cells with CD3⁺CD8⁻ markers were recognized as CD4⁺ T cells and cells with IL-9⁺IL-17⁻ markers among the T cells were recognized as Th9 cells. The number of CD3⁺CD8⁻IL-9⁺IL-17⁻ cells divided by the total number of CD3⁺CD8⁻ cells indicated the percentage of Th9 cells in CD4⁺ cells.

Enzyme Linked Immunosorbent Assays (ELISA)

Serum IL-9 and IL-10 levels were determined using commercial ELISA kits according to the manufacturer’s instructions (Market Inc, San Jose, CA). The colorimetric changes in the wells were read at a wavelength of 450 nm in a microplate reader (ELx800, BioTek Instruments, Inc., Winooski, Vermont).

Statistical Analysis

All data were analyzed using SPSS version 13.0 (SPSS Inc., Chicago, IL). Continuous variables are expressed as the median (10th–90th percentile) unless specified. Comparison of differences was performed using the Kruskal–Wallis *H* test, Mann–Whitney nonparametric *U* test and Chi-square test. Paired comparisons were performed using Wilcoxon signed-rank test. The possible relationship between variables was evaluated using Spearman’s rank correlation. The diagnostic value of variables was assessed by the area under the receiver operating characteristic (ROC) curves. A 2-side *P* value < 0.05 was considered significant.

RESULTS

Clinical Characteristics of Enrolled Subjects

Clinical characteristics of the enrolled subjects are summarized in Table 1. Patients with HBV-LC were older than those with CHB, ACLF, and HC (*P* < 0.01). The hepatitis B e (HBe) antigen (HBeAg) positive percentage, HBV DNA, and

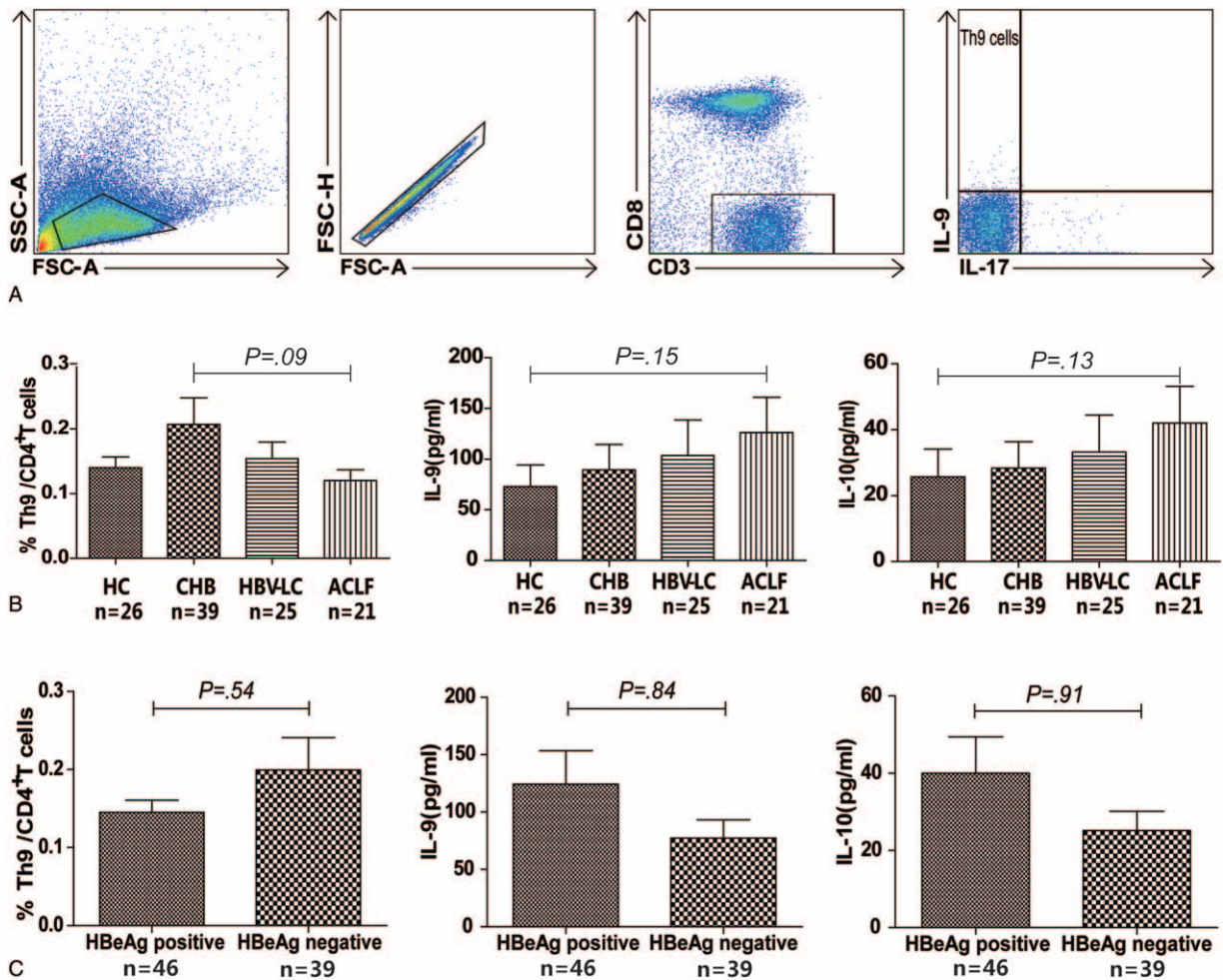


FIGURE 1. Frequencies of Th9 cells and serum levels of IL-9 and IL-10 in different stages of HBV infection. Sequential gating strategy for Th9 identification (CD3⁺CD8⁻IL-9⁺IL-17⁻) using flow cytometry from freshly isolated PBMC (A). The percentage of Th9 cells and the serum level of IL-9 and IL-10 were analyzed in HC, CHB, HBV-LC, and ACLF groups (B), and in HBeAg-positive and HBeAg-negative groups (C) using the Mann–Whitney *U* test. ACLF = acute-on-chronic liver failure; CHB = chronic hepatitis B; HBV-LC = hepatitis B virus-related liver cirrhosis, HC = health controls, IL = interleukin; .

TABLE 2. Correlation Between Th9 Cells, IL-9, and IL-10 and Laboratory Parameters

	Th9 cells		IL-9		IL10	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age	0.09	0.42	-0.18	0.10	-0.19	0.08
ALB	0.14	0.19	-0.17	0.13	-0.16	0.15
TBIL	-0.06	0.61	0.14	0.20	0.15	0.15
ALT	0.08	0.48	0.09	0.44	0.10	0.35
HBV DNA	0.02	0.90	-0.01	0.94	-0.01	0.98

ALB = serum albumin, ALT = alanine aminotransferase, TBil = total bilirubin.

alanine aminotransferase (ALT) levels were significantly lower in HBV-LC patients ($P < 0.01$) compared to CHB and ACLF patients. Serum albumin (ALB) levels were lower in HBV-LC and ACLF patients compared to CHB patients. Total bilirubin (TBil) were significantly higher in ACLF patients compared to CHB and HBV-LC patients ($P < 0.01$). The MELD scores were higher in ACLF patients compared to HBV-LC patients ($P < 0.01$).

Th9 Cells, IL-9, and IL-10 Were not Related to Progression of HBV-Related Liver Injury

Th9 percentages in CD4⁺ T cells and serum IL-9 and IL-10 levels were analyzed in all groups. No significant differences in both the percentages of Th9 cells and the serum IL-9 and IL-10 levels were observed between the HC group and HBV-infected

patients (all $P > 0.05$) (Figure 1B). However, the Th9 percentage was slightly higher in the CHB group compared to the other 3 groups (0.15% [0.04–0.37%] in the CHB group vs 0.12% [0.02–0.26%] in the HC group, 0.11% [0.05–0.45%] in the HBV-LC group, and 0.10% [0.03–0.23%] in the ACLF group; Figure 1B).

The Th9 percentages and the serum IL-9 and IL-10 levels were further analyzed based on the HBeAg status of all patients. The percentage of Th9 cells was slightly lower in HBeAg-positive group compared to the HBeAg-negative group (0.12% [0.05–0.28%] vs 0.13% [0.03–0.45%], $P = 0.54$), whereas the serum IL-9 and IL-10 levels were slightly higher in the HBeAg-positive groups compared to the HBeAg-negative groups (IL-9: 37.7 [4.0–495.1] pg/mL vs 32.5 [7.7–197.7] pg/mL, $P = 0.84$; IL-10: 14.8 [1.8–165.0] pg/mL vs 12.2 [2.9–77.5] pg/mL, $P = 0.91$) (Figure 1C).

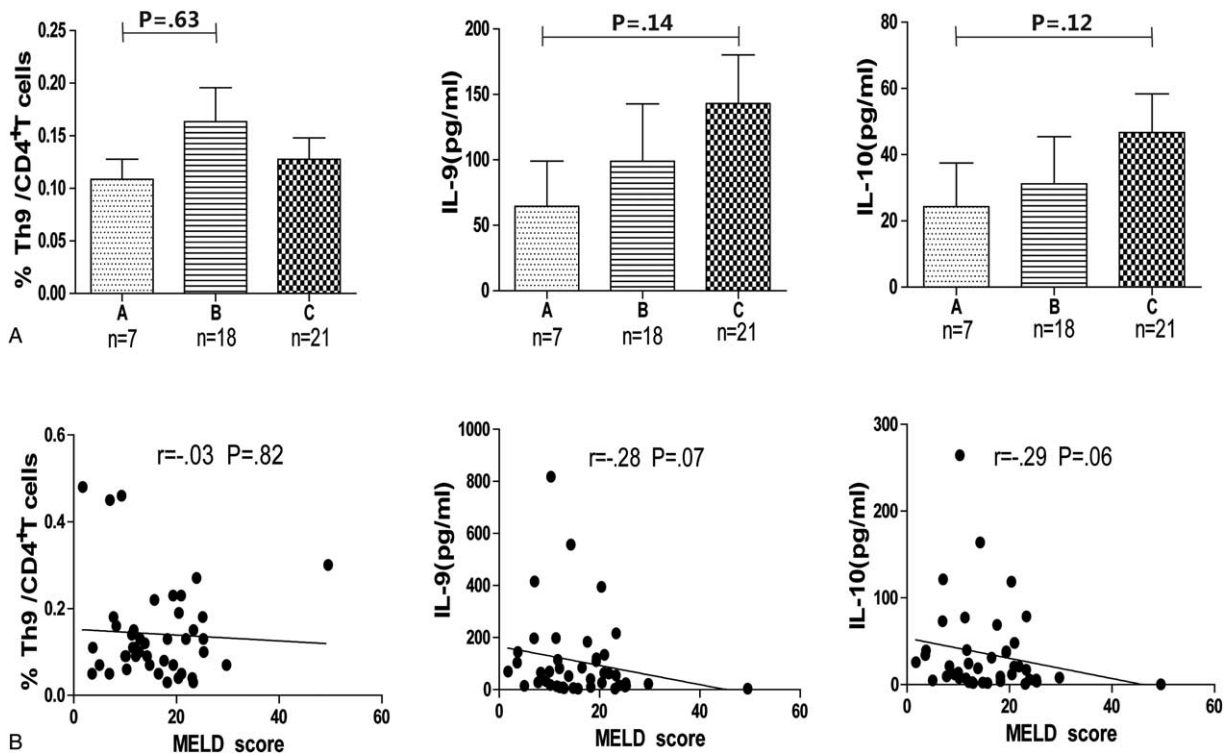


FIGURE 2. Frequencies of Th9 cells and serum levels of IL-9 and IL-10 did not correlate with disease progression in HBV-LC and ACLF patients. Correlations between the frequencies of Th9 cells, levels of IL-9 and IL-10, MELD, and Child–Pugh scores were analyzed using the Spearman’s rank correlation test. ACLF = acute-on-chronic liver failure; HBV-LC = hepatitis B virus-related liver cirrhosis; MELD = model for end-stage liver disease.

Th9 Cells, IL-9, and IL-10 Levels Were not Related to Severity of Liver Injury in Patients With HBV Infection

Previous studies showed that Treg and Th17 cells were related to the extent of liver injury.^{37,38} Extent of liver damage and the impact on liver functions can be reflected by elevated

ALT, TBil, and reduced ALB levels. The relationship of Th9 cells, as well as IL-9 and IL-10 levels, with these biochemical parameters were analyzed. There was no obvious correlation between the percentage of Th9 cells, levels of IL-9, and IL-10 with age, TBil, ALB, ALT, and HBV DNA levels ($P > 0.05$) (Table 2). Also, there was no clear association of the Th9

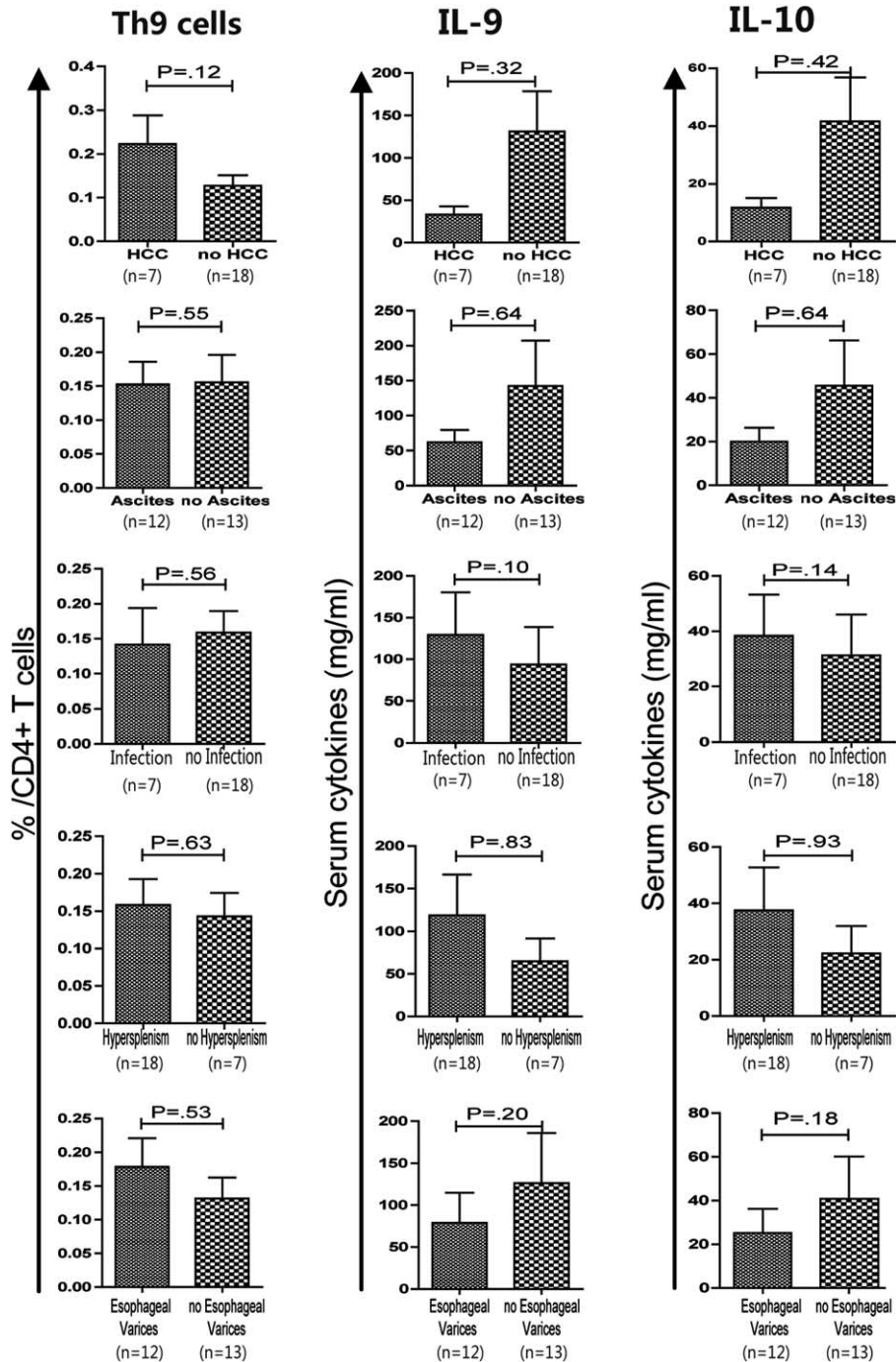


FIGURE 3. No association was found between the frequencies of Th9 cells and serum levels of IL-9 and IL-10 with HBV-LC patients with and without complications. Correlations of Th9 cell frequency, and IL-9 and IL-10 levels in patients with or without HCC, ascites, infection, hypersplenism, and esophageal varices were analyzed using the Mann-Whitney *U* test. HBV-LC = hepatitis B virus-related liver cirrhosis; HCC = hepatocellular carcinoma.

percentages and the serum levels of IL-9 and IL-10 with the Child–Pugh and MELD scores (Figure 2A and B).

Th9 Cells, IL-9, and IL-10 Were not Related to the Progression of HBV-LC

HBV-LC patients were divided into 2 groups to determine if there was a possible relationship between Th9 cells and serum IL-9 and 10 levels, based on the presence or absence of complications. As shown in Figure 3, there was no significant difference in Th9 cell frequencies and IL-9 and IL-10 levels between the HBV-LC patients with and without complications ($P > 0.05$). No significant difference was noted in Th9 cell frequencies, IL-9, and IL-10 levels between patients with and without HCC ($P = 0.12, 0.32, \text{ and } 0.42$, respectively) (Figure 3).

IL-9 and IL-10, but not Th9 Cells, Increased After Treatment in CHB Patients

A total of 23 CHB patients with an average age of 35.9 ± 9.7 (23–59) years were treated with NAs for 7.2 ± 3.4 months, and 17 of them were males. At the end of follow-up, ALT level and HBV DNA load were markedly decreased compared to baseline levels (all $P < 0.01$). IL-9 and IL-10 levels were significantly increased ($P < 0.01$ and 0.02 , respectively), whereas the Th9 proportion was slightly reduced after treatment ($P = 0.34$) (Figure 4).

Serum IL-9 and IL-10 Levels, but not the Percentage of Th9 Cells, Predicted the Prognosis of ACLF Patients

Our previous study indicated that the ratio of Treg to Th17 cells was associated with survival of ACLF patients.¹³ We wondered if the percentage of Th9 cells was also related to survival of ACLF patients. In this study, the percentage of Th9 cells was slightly lower in the nonsurvived group compared to

the survived group ($0.09 [0.03–0.19]$ pg/mL vs $0.12 [0.04–0.25]$ pg/mL, $P = 0.29$), but the serum levels of IL-9 and IL-10 were significantly higher in the nonsurvived group compared to the survived group (IL-9: $200.2 [26.6–556.7]$ pg/mL vs $40.3 [4.6–126.1]$ pg/mL, $P = 0.01$; IL-10: $73.8 [11.9–171.5]$ pg/mL vs $9.3 [2.1–44.8]$ pg/mL, $P = 0.01$) (Figure 5A). The possible predictive values of IL-9 and IL-10 were further explored by ROC curve analysis. Although the same area under the curve (AUC) value ($0.84, P = 0.01$) was reached for both IL-9 and IL-10, only IL-9 showed a high sensitivity and specificity for predicting the survival of ACLF patients (87.5% and 61.54% at the cutoff value of 53.19 pg/mL, respectively) (Figure 5B).

DISCUSSION

In this study, the percentage of Th9 cells in the total CD4⁺ T cells and the serum levels of IL-9 and IL-10 were analyzed in different stages of HBV infection. We found that Th9 cells, and IL-9 and IL-10 levels were not related to the severity of liver injury, progression, and prognosis of HBV-related chronic liver diseases. No significant change in Th9 cell frequency was observed before and after NAs treatment in CHB patients.

Our findings were different from a published study²⁷ that detected the frequency of Th9, serum levels of IL-9 and CCL-20, and CCL20 and CCR6 expression in liver tissues in 18 HBV-LC patients and 6 healthy individuals. This study found that Th9 cell percentages and IL-9 level were significantly higher in HBV-LC patients compared to healthy individuals. The study also suggested that these factors were positively related to the severity of liver fibrosis, indicating that Th9 cells may be involved in the pathogenesis of HBV-LC through the CCL20/CCR6 axis. The difference between these 2 studies may be attributed to different sample size and severity of liver cirrhosis.

HBV-related ACLF is the most severe complication triggered by flare-ups of liver injury and is accompanied by high mortality. Cytokine storm is considered one of factors in the

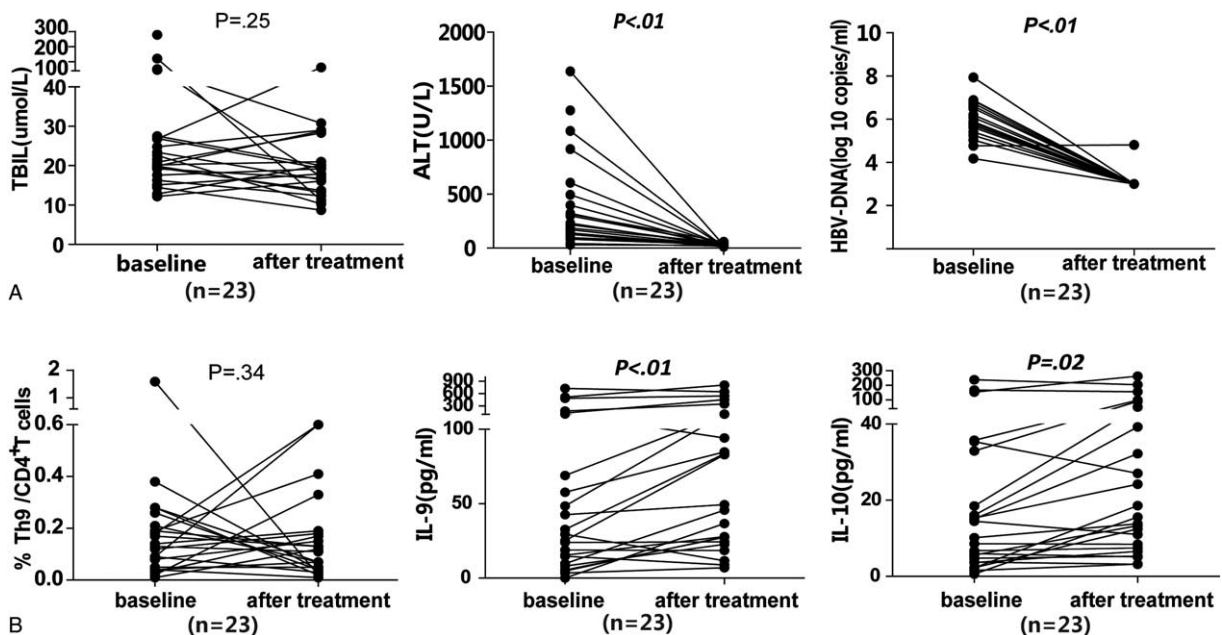


FIGURE 4. Changes in the Th9 cell frequencies and the serum levels of cytokines and laboratory parameters before and after nucleos(t)ide analog antiviral treatment in 23 patients with CHB. Data were analyzed with the Wilcoxon signed-rank test. ALT = alanine amino-transferase; CHB = chronic hepatitis B; Tbil = total bilirubin.

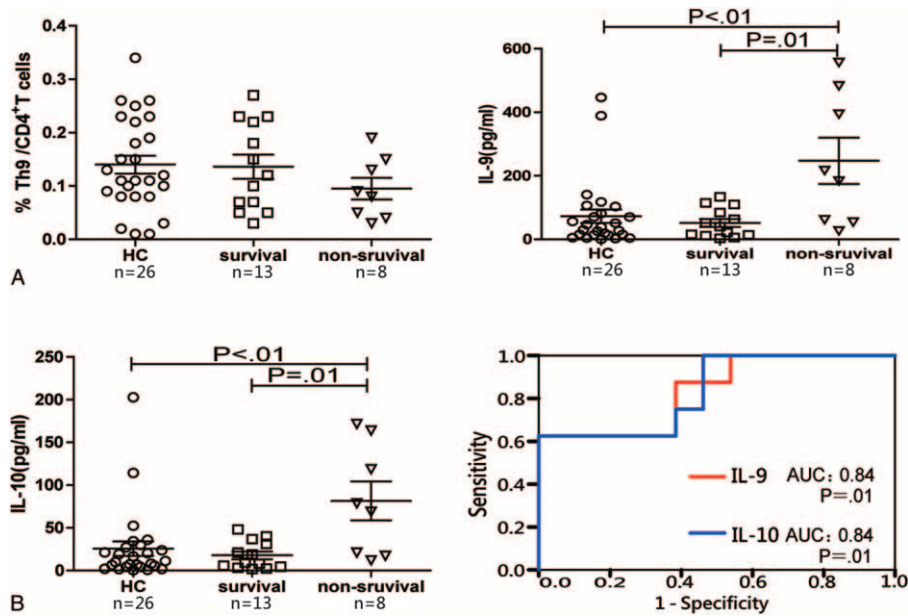


FIGURE 5. Correlations of frequencies of Th9 cells and serum levels of cytokines with survival of ACLF patients. (A) Frequencies of Th9 cells, serum levels of IL-9 and IL-10 in the survived and nonsurvived ACLF patients were analyzed using the Mann–Whitney *U* test. (B) Receiver operating characteristic curve of IL-9 and IL-10 for the prognosis prediction in ACLF patients. ACLF = acute-on-chronic liver failure; HC = health controls.

pathologic process of ACLF.³⁹ Several studies have shown the association of Treg and Th17 cells and the cytokines they secrete with development of ACLF.^{13,40} However, it is unclear whether Th9 cells are also involved in the pathogenesis of ACLF. The present study showed that the percentage of Th9 cells was lower in ACLF compared to HC, and it was lower in the nonsurvived ACLF than the survived ACLF, although the difference was not significant. The data indicated that Th9 cells were unlikely involved in the pathogenesis of ACLF, or the Th9 cells migrated to the livers in response to severe liver injury and inflammation.⁴¹

However, serum levels of IL-9 and IL-10 were significantly higher in the nonsurvived ACLF patients compared to the survived ACLF patients and HC. In fact, the IL-9 level was predictive of ACLF prognosis, suggesting a possible involvement of IL-9 and IL-10 in the progression of ACLF. The increased IL-9 and IL-10 levels in the nonsurvived ACLF patients could be caused by other immune cells that also secrete IL-9 and IL-10 as part of the cytokine storm, in addition to Th9 cells. Our results suggest that a significant elevation in cytokine production is a sign for high mortality in ACLF patients.

A previous study reported that the IL-9 level was higher in CHB patients, but decreased after 6 months of telbivudine treatment.⁴² In this study, the IL-9 and IL-10 levels were significantly increased after treatment. This discrepancy may be explained by the number of T cells that produce IL-9 and IL-10. Other Th cells, such as Th2 and Th17, were activated to secrete more IL-9 and IL-10 once HBV replication was profoundly inhibited by NAs therapy.¹⁰ This hypothesis was supported by our previous study that Th17 cell frequencies and IL-17 level were increased in CHB patients who mounted a complete immune response.³³

We found no clear correlation of Th9 cells with HBV infection and related liver injury. This may not only reflect the fact that there was a very low percentage of Th9 cells in the

peripheral blood,^{39,20} which was as low as 0.01% (range 0.01–1.59%) in the present study, but also IL-9 and IL-10 can be secreted by other CD4⁺ T cells. Thus, the changes in the serum levels of IL-9 and IL-10 in HBV infection may not necessarily reflect the function of the Th9 cells.

There are several limitations in the present study. First, we did not measure Th9 cells in the livers because liver samples are not available. Second, Th9 cell-related transcription activity was not analyzed to determine the IL-9 and IL-10 mRNA levels. Third, other CD4⁺ T cells, such as Th2, Th17, and Treg that can also secrete IL-9, were not measured, and we were unable to determine if the elevated IL-9 and IL-10 levels were secreted by the Th9 cells alone or by other Th cells. We hope to address these limitations in future studies.

In conclusion, we found that Th9 cells and IL-9 and IL-10 were unlikely related to HBV infection, degree of liver injury, and progression of HBV infection-related cirrhosis. However, serum levels of IL-9 and IL-10 appeared to be predictive of ACLF prognosis in patients.

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