

Changes in circulating Foxp3⁺ regulatory T cells and interleukin-17-producing T helper cells during HBV-related acute-on-chronic liver failure

Xue-Song Liang, Cheng-Zhong Li, Yin Zhou, Wei Yin, Ya-Yun Liu, Wen-Han Fan

Xue-Song Liang, Cheng-Zhong Li, Yin Zhou, Wei Yin, Ya-Yun Liu, Wen-Han Fan, Department of Infectious Diseases, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

Yin Zhou, Department of Biochemistry and Molecular Biology, College of Basic Medical Sciences, Second Military Medical University, Shanghai 200433, China

Author contributions: Liang XS conceived the study, participated in the design of the study, performed the statistical analysis and drafted the manuscript; Li CZ participated in the design of the study and enrolled the subjects; Zhou Y performed most of the experiments; Yin W collected all of the human material; Liu YY and Fan WH were responsible for patient follow up; all authors read and approved the final manuscript.

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Correspondence to: Xue-Song Liang, Associate Professor, Department of Infectious Diseases, Changhai Hospital, Second Military Medical University, No. 168 Changhai Road, Yangpu District, Shanghai 200433, China. liangxuesong2000@163.com
Telephone: +86-21-31161902 Fax: +86-21-31161910

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Abstract

AIM: To longitudinally investigate cytokine gene expression and protein levels in Th17 and Treg cells, to observe T-cell phenotypes during hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACHBLF) and to analyze changes in Th17 and Treg phenotypes during disease progression.

METHODS: We measured the expression of seven Th17/Treg differentiation-related genes and serum concentrations of the corresponding cytokines in 18 ACHBLF, 18 chronic hepatitis B (CHB) disease controls and 10 healthy controls (HCs) by real-time quantitative

PCR and enzyme linked immunosorbent assay. Peripheral Th17 and Treg cell frequencies were analyzed by flow cytometry.

RESULTS: From the onset of ACHBLF, patients presented with a conducive Th17 differentiation cytokine environment accompanied by high Th17 frequency and high serum IL-17 levels, which were sustained throughout the disease course. The Treg-related cytokine IL-2 and Foxp3 were also up-regulated from disease onset, and Foxp3 gene expression showed a gradually increasing trend during ACHBLF. The circular phenotype of Treg and Th17 cells showed changes from the onset of ACHGLF. At disease onset, Th17 frequency increased significantly compared with both CHB and HCs, but Treg cell frequency decreased significantly compared with CHB. During the ACHBLF event, Th17 frequency remained higher compared with HCs, but decreased sharply from the peak point to the recovery point; Treg cell frequency increased gradually during the ACHBLF event. Treg and Th17 cell counts correlated with ACHBLF development; in all patients, serum IL-17 levels significantly correlated with patient serum ALT levels. In survivors, Th17 frequency at the onset point and the Treg to Th17 ratio at the peak point correlated with the patient's model for end stage liver disease (MELD) plus sodium (MELD-Na) score. The Treg to Th17 ratio and the Th17 frequency at onset were significant predictors of patient survival. Low Treg/Th17 cell ratios at the onset predicted poor survival. Survivors exhibited an initial decrease in the circulating Treg/Th17 ratio from the onset to the peak time, and subsequently displayed a continuous increase.

CONCLUSION: Treg and Th17 cells showed changes in genes, protein levels and T cell phenotypes during ACHBLF events. An increased Treg/Th17 ratio was associated with the survival of ACHBLF patients.

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Key words: Hepatitis B virus; Treg; Th17; Immune; Hepatitis B virus-related acute-on-chronic liver failure

Core tip: In this study, we longitudinally investigated Foxp3⁺Treg cells and IL-17⁺Th cells by measuring gene levels, protein levels and T-cell phenotypes during HBV-related acute-on-chronic liver failure (ACHBLF) progression. From the onset of ACHBLF, there were changes in Foxp3⁺Treg and Th17 cell frequencies. An increased Treg/Th17 ratio was associated with the survival of ACHBLF patients.

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INTRODUCTION

Hepatitis B virus (HBV) is a major human pathogen, infecting 350 million people worldwide. Adults infected with HBV can either exhibit acute self-limited HBV infection or progress to chronic infection, while viral infection *in utero* or early in life generally results in chronic hepatitis B (CHB)^[1]. Some CHB patients may rapidly progress towards liver failure, a condition referred to as acute-on-chronic liver failure (ACLF)^[2]. HBV-related acute-on-chronic liver failure (ACHBLF) is one of the most severe consequences of HBV infection. ACHBLF refers to liver failure occurring in patients with CHB, CHB-related liver cirrhosis, or chronic asymptomatic HBV carriers^[3]. In China, as a result of the high prevalence of HBV infection, ACHBLF cases account for more than 80% of ACLF cases^[4,5]. The precise mechanisms underlying the deterioration of liver function occurring in ACLF remain unclear; however, the impairment of cellular immunity is believed to be a contributing factor^[6-9].

Recently, a novel and unique pro-inflammatory T cell subset, interleukin-17 (IL-17)-producing CD4⁺T helper cells (Th17), was identified. Several key cytokines, including IL-1 β , IL-6, tumor necrosis factor alpha (TNF- α) and IL-23, create a cytokine milieu that regulates the differentiation and expansion of human Th17 cells^[10-13]. Th17 and its related cytokines may play an important role in the pathogenesis of HBV infection^[6-9,14-16].

Conversely, another CD4⁺T cell subset, Foxp3⁺ regulatory T cells (Treg), which are characterized by their constitutive expression of CD25 and Foxp3 and by immunological suppression^[17-21], can restrain the immune response, thus limiting liver damage^[9,22-24]. In chronic HBV infection, an imbalance between Tregs and effector T cells has recently been described by several groups. These studies suggest that either the number or functional imbalance of Tregs in the blood or liver may be a reason for

persistent HBV infection and disease progression^[9,24-26].

Although increased Th17 and Treg frequencies in patients with CHB have been reported, little is known about the relationship between circulating Th17 and Tregs and their role in ACHBLF development. Recently, we studied the changes in Treg and Th17 cell balance in the development of acute and chronic hepatitis B virus infection and found that in ACHBLF patients, peripheral blood Th17 cell frequency increased significantly compared with that seen in healthy controls (HCs), but the frequency of Treg cells did not increase synchronously, creating an imbalance between Treg and Th17 cells in ACHBLF^[9]. Zhai *et al.*^[27] also found that at the onset of ACHBLF, the frequency of Th17 cells increased in ACHBLF patients and that the ratio of Th17 to Treg cells correlated with patient survival. However, Th17 and Treg cell dynamics in ACHBLF patients have not been previously reported.

Our study longitudinally analyzed the frequency of Treg and Th17 cells and related cytokine protein and gene expression in a cohort of 18 patients with ACHBLF. Our data document changes in Treg and Th17 cell populations during ACHBLF.

MATERIALS AND METHODS

Subjects

A total of 18 patients with ACHBLF have been described in our recent study^[9]. In brief, inclusion criteria included (1) HBV-related liver cirrhosis or chronic HBV infection based on a histopathologic diagnosis or compatible laboratory data and sonographic findings; (2) recent development of jaundice, ascites, hemodynamic instability and/or encephalopathy grade III-IV, compatible with the definition of hepatic decompensation and necessitating further treatment in the ICU; (3) no evidence of hepatocellular carcinoma or other metastatic liver tumors that could affect liver function; and (4) no immunosuppressive medication within the last 3 mo prior to study entry^[9,28]. All of the patients were longitudinally followed. Table 1 summarizes the clinical data acquired from the investigations of these patients at the onset point (1-2 wk after clinical onset), peak point (the time of peak total bilirubin level, 2-3 wk after clinical onset), and recovery point (total bilirubin levels decreased by more than 30%, typically 7-8 wk after clinical onset)^[29]. The disease control group was composed of 18 age-, gender- and Child-Pugh stage-matched patients with CHB. The diagnostic criteria for CHB were as follows: positive for hepatitis B surface antigen (HBsAg) and anti-hepatitis B core (anti-HBc) for more than 6 mo; negative for antibodies to hepatitis C virus, hepatitis D virus, hepatitis G virus, and HIV-1 and -2; no other causes of chronic liver damage; and persistently elevated serum ALT levels and positive serum HBV DNA for at least 6 mo. Ten healthy adults (age range: 24-42 years, 7 females and 3 males) were included as HCs. All patients and controls were Chinese. The study was approved by the local medical ethics committee of Shanghai Changhai Hospital, and informed

Table 1 Clinical characteristics of patients with hepatitis B virus-related acute-on-chronic liver failure

No.	Age (yr)	Sex	Onset				Peak				Recovery			
			Week after clinical onset	ALT (U/L)	TBIL (μmol/L)	HBV DNA (Log ¹⁰ copies/mL)	PTA (%)	MELD-Na	Week after clinical onset	ALT (U/L)	TBIL (μmol/L)	HBV DNA (Log ¹⁰ copies/mL)	PTA (%)	MELD-Na
1	58	M	1	112	497.3	5.69	48.01	24.70	2	72	239.5	4.58	51.36	22.56
2	48	M	1	65	309.5	5.21	38.30	18.38	2	64	267.5	4.25	45.56	20.45
3	45	M	1	1002	400.5	3.67	21.65	26.86	3	316	576.8	2.08	16.06	58.53
4	50	F	1	302	520.1	6.36	16.35	52.47	2	Died				
5	59	M	1	435	170.6	5.22	40.36	16.84	3	62	60.2	4.15	60.13	11.99
6	50	M	2	145	620.0	3.29	46.40	31.14	3	165	613.7	3.29	46.81	27.99
7	38	M	1	92	449.5	4.57	24.24	26.48	2	89	481.9	3.29	22.78	25.77
8	30	M	1	504	293.6	7.14	42.65	21.78	3	148	403.4	3.12	42.65	25.07
9	42	M	1	282	439.1	5.88	42.31	18.93	2	151	449.6	3.29	37.50	21.70
10	61	M	1	81	465.6	4.37	27.53	29.51	2	61	405.0	3.11	27.53	32.25
11	44	M	2	53	599.8	2.67	28.12	25.36	3	58	381.5	2.23	27.53	46.89
12	55	F	1	35	182.8	4.30	25.78	20.31	2	46	208.5	ND	27.24	20.78
13	52	M	1	213	381.1	5.89	10.96	39.84	2	584	540.2	ND	4.70	62.03
14	48	M	1	355	364.7	6.07	32.43	25.63	2	63	546.2	2.12	29.20	29.60
15	45	F	1	21	508.8	3.35	40.99	33.77	2	31	481.2	2.98	44.07	38.17
16	30	M	1	93	395.0	3.19	18.67	29.42	2	86	353.8	3.19	19.14	36.92
17	53	M	2	138	541.5	2.67	30.73	28.79	3	107	639.6	2.32	27.53	50.07
18	33	M	1	138	216.0	5.68	47.21	22.43	2	63	259.2	4.11	74.58	16.17

consent was acquired from each individual.

Peripheral blood mononuclear cell isolation, total RNA extraction and complementary DNA synthesis

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized peripheral blood of the studied subjects by standard Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density centrifugation. Total RNA was extracted from PBMCs using the RNeasy kit (Qiagen, Valencia, CA, and United States). All samples were treated with DNaseI to eliminate potential genomic DNA contamination. The quality and quantity of the RNA were determined using an ultraviolet spectrophotometer (NANODROP1000, Thermo). Target RNA was reverse-transcribed using the Omniscript RT Kit (Qiagen, Valencia, CA, United States). All samples were treated according to identical protocols and in parallel. RNA and cDNA were stored at -80 °C until further processing.

Quantification of gene expression

The panels of genes of interest (GOIs) are listed in Table 2. The *GAPDH* gene was selected as an endogenous reference. Candidate primer sets were first designed by Primer Express Software (Applied Biosystems, Foster City, CA, United States) and then validated manually to satisfy the following criteria: (1) amplicon length between 50 and 150 base pairs; (2) amplification efficiencies, as determined by a template linear dilution method as described previously^[30], were approximately equal for the GOIs and 18S rRNA; and (3) primers were verified to generate a single product specific to target genes by both blast algorithm (<http://www.ncbi.nlm.nih.gov/blast/>) and melting curve analysis. The expression of GOIs was determined by RQ-PCR using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 50 μL with the following thermal conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Samples were assayed in triplicate. The expression ratio of each GOI between different groups was analyzed using the delta comparative cycle threshold method^[31].

Table 2 T helper type 17 and T regulatory differentiation-related cytokines

Genes	GenBank accession number	Primers (5' to 3')	Reported function
<i>IL-1β</i>	NM_000576	Forward: GCTGATGGCCCTAAACAGATGAA Reverse: TGAAGCCCTTGTCTGTAGTGGTG	Induce human Th17 polarization ^[17]
<i>IL-6</i>	NM_000600	Forward: AAGCCAGAGCTGTGCAGATGAGTA Reverse: TGTCTGCAGCCACTGGTTTC	Induce human Th17 polarization ^[17]
<i>IL-23/p19</i>	NM_016584	Forward: GCAGCCTGAGGGTCACCACT Reverse: GGCGGCTACAGCCACAAA	Unique subunit of IL-23
<i>IL-17A</i>	NM_002190	Forward: TGTCCACCATGTGGCCTAAGAG Reverse: GTCCGAAATGAGGCTGTCTTTGA	Main effective cytokine of Th17 cells ^[7]
<i>TGF-β1</i>	NM_000660	Forward: AGCGACTCGCCAGAGTGGTTA Reverse: GCAGTGTGTATCCCTGCTGTCA	Induce mouse Th17 and Treg polarization, suppress human Th17 polarization ^[9,10,17]
<i>IL-2</i>	NM_000586	Forward: CAACTCTGTCTTGCATTGCACTAA Reverse: AATGTGAGCATCTGGTGAGTTTG	Induce human and mouse Treg polarization ^[10]
<i>FoxP3</i>	NM_014009	Forward: GTTCACACGCATGTTTGCCTTC Reverse: CACAAAGCACTTGTGCAGACTCAG	Master regulatory transcription factors of Treg lineage ^[21]

Flow cytometric analysis

For Th17 cell examination, the PBMCs were isolated from peripheral blood. PBMCs (2×10^6) were stimulated further for 5 h with 50 ng/mL phorbol myristate acetate, 1 mmol/L ionomycin (both from Sigma, St Louis, MO, United States) and 10 mg/mL brefeldin A (TocrisCookson, Bristol, United Kingdom) in complete RPMI-1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, United States). Upon harvest, cells were first surface-stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 antibodies for 20 min, fixed and permeabilized with Perm/Fix solution, and then stained intracellularly with phycoerythrin (PE)-conjugated anti-human IL-17A.

For Treg cell examination, peripheral blood (100 μ L) was first surface-stained with FITC-conjugated anti-human CD4 antibodies and allophycocyanin (APC)-conjugated anti-human CD25 antibodies for 30 min, then lysed with FACSTM lysing solution (BD PharMingen) and treated with eBioscience fix/perm mixture (eBiosciences) according to the manufacturer's instructions. Finally, the cells were incubated with PE-conjugated anti-human Foxp3 antibodies overnight. Isotope controls were used to ensure antibody specificity. Flow cytometry was performed using a FACSCalibur (Becton Dickinson, San Jose, CA). FACS data were analyzed using CellQuest software (Becton Dickinson Rutherford, NJ)^[28]. All antibodies were purchased from BD Biosciences (San Jose, CA, United States).

Enzyme linked immunosorbent assay

Serum concentrations of IL-1 β , IL-6, IL-23, IL-17A, TGF- β 1, and IL-2 were measured by commercially available enzyme linked immunosorbent assay kits (R and D Systems, Minneapolis, MN, United States) according to the protocols provided by the manufacturer. All samples were assessed in triplicate.

Virological and biochemical assessments

The levels of HBsAg, HBeAg, anti-HBs, anti-HBc, anti-HBe, anti-HCV, anti-HDV, anti-HGV, anti-HIV-1, and

anti-HIV-2 were measured using commercially available kits (Abbot Laboratories, North Chicago, IL) in our clinical lab. Serum HBV-DNA levels were measured by fluorescent quantitative PCR with commercially available kits (PE/B/MJ/L, Shenzhen, China) according to the manufacturer's instructions. The threshold of the HBV DNA detection limit was 500 copies/mL.

Statistical analysis

All data were analyzed using IBM SPSS statistic version 19 (IBM, Com). The Kruskal-Wallis test was used to evaluate the differences among more than 2 groups. The Mann-Whitney *U* test was used to evaluate the difference between two groups. Spearman's correlation test was used to assess the correlation of immune factors and clinical characters. The area under the receiver operating characteristic (ROC) curve was used to compute predictive values of different factors on survival rates. For all tests, two-sided *P* < 0.05 was considered significant.

RESULTS**Clinical characteristics and treatment of patients with ACHBLF**

The clinical and biochemical details of the studied patients are listed in Table 1. We measured the expression of 7 genes and the serum concentrations of six corresponding cytokines, which have been reported previously to contribute to the differentiation of Th17 and Treg cells^[32,33]. The name, GenBank accession number, primer sequences and reported function of each gene are presented in Table 2.

Patients with ACHBLF received conservative management, including nutritional support, hepatoprotective drugs, antiviral therapy (lamivudine 100 mg/d) and prevention and control of complications, but did not receive any immune-modulating therapy. Patients were divided subsequently into non-survivor (NS) patients (who died or received a liver graft) and survivors (who survived or whose total bilirubin level decreased by more than 30% by the end of study) according to an earlier report^[29]. Four patients died during the study, and one patient received a liver graft.

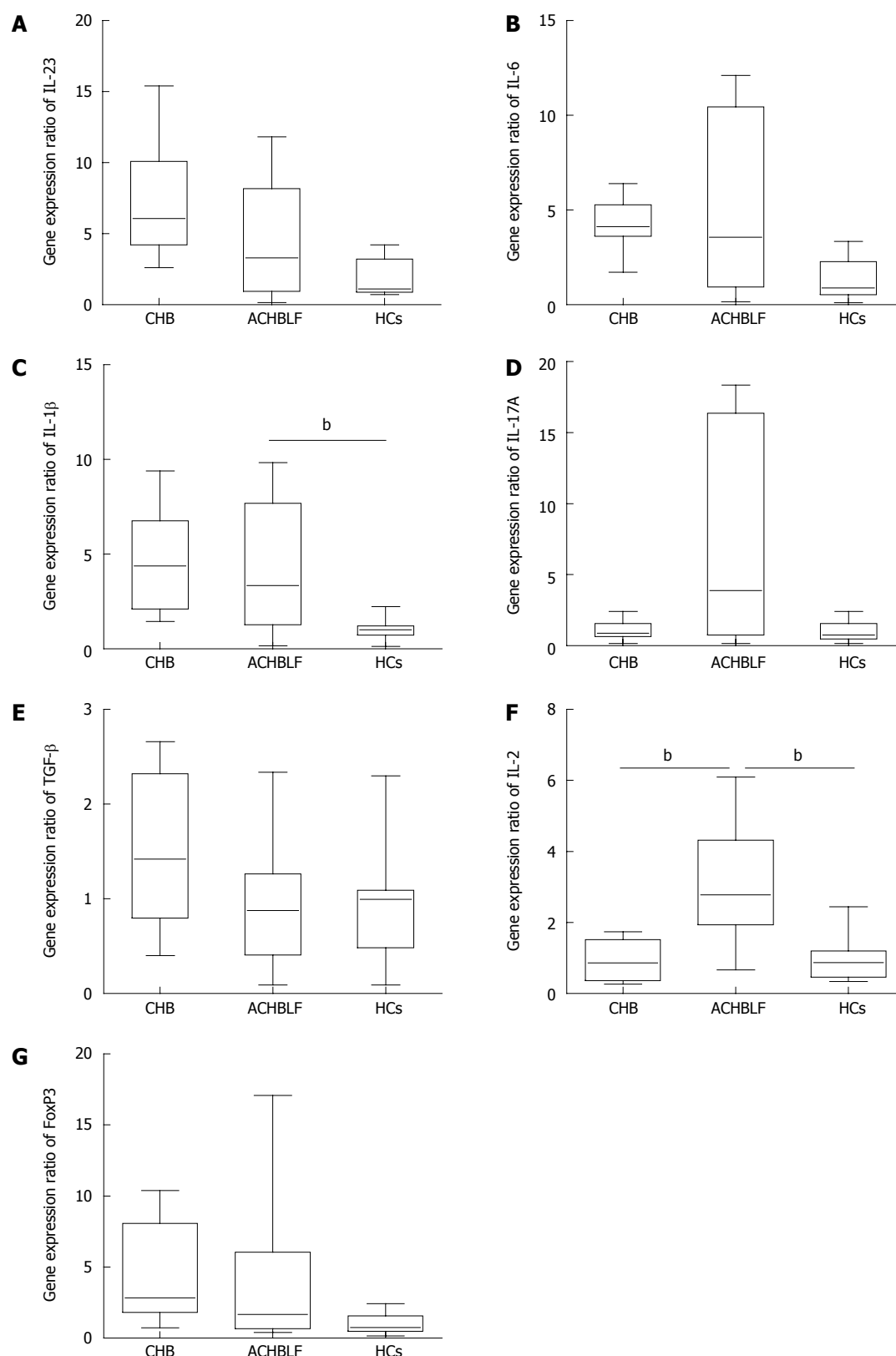


Figure 1 Expression of interleukin-17-producing T helper cell and Foxp3⁺ regulatory T cell-related genes in patients with hepatitis B virus-related acute-on-chronic liver failure at the onset point. Compared with healthy controls (HCs), all interleukin-17-producing T helper cell (Th17)-related cytokines were up-regulated, but only IL-1 β was up-regulated significantly. Compared with general chronic hepatitis B (CHB), there were no significant differences in Th17-related cytokine gene expression at the onset of hepatitis B virus-related acute-on-chronic liver failure (ACHBLF). For Foxp3⁺ regulatory T cell (Treg)-related liver gene expression, IL-2 expression was significantly up-regulated compared with both CHB patients and HCs. Data are presented as whisker-box plots, and the difference between ACHBLF and the HC or CHB groups was analyzed by a Mann-Whitney test. Boxes represent the interquartile range, with the median represented by the line inside the box. Upper whisker, the highest value less than or equal to the 75 percentile plus 1.5 times interquartile range; lower whisker, the lowest value greater than or equal to the 25 percentile minus 1.5 times interquartile range. Outliers were excluded. ^b*P* < 0.01 vs ACHBLF group.

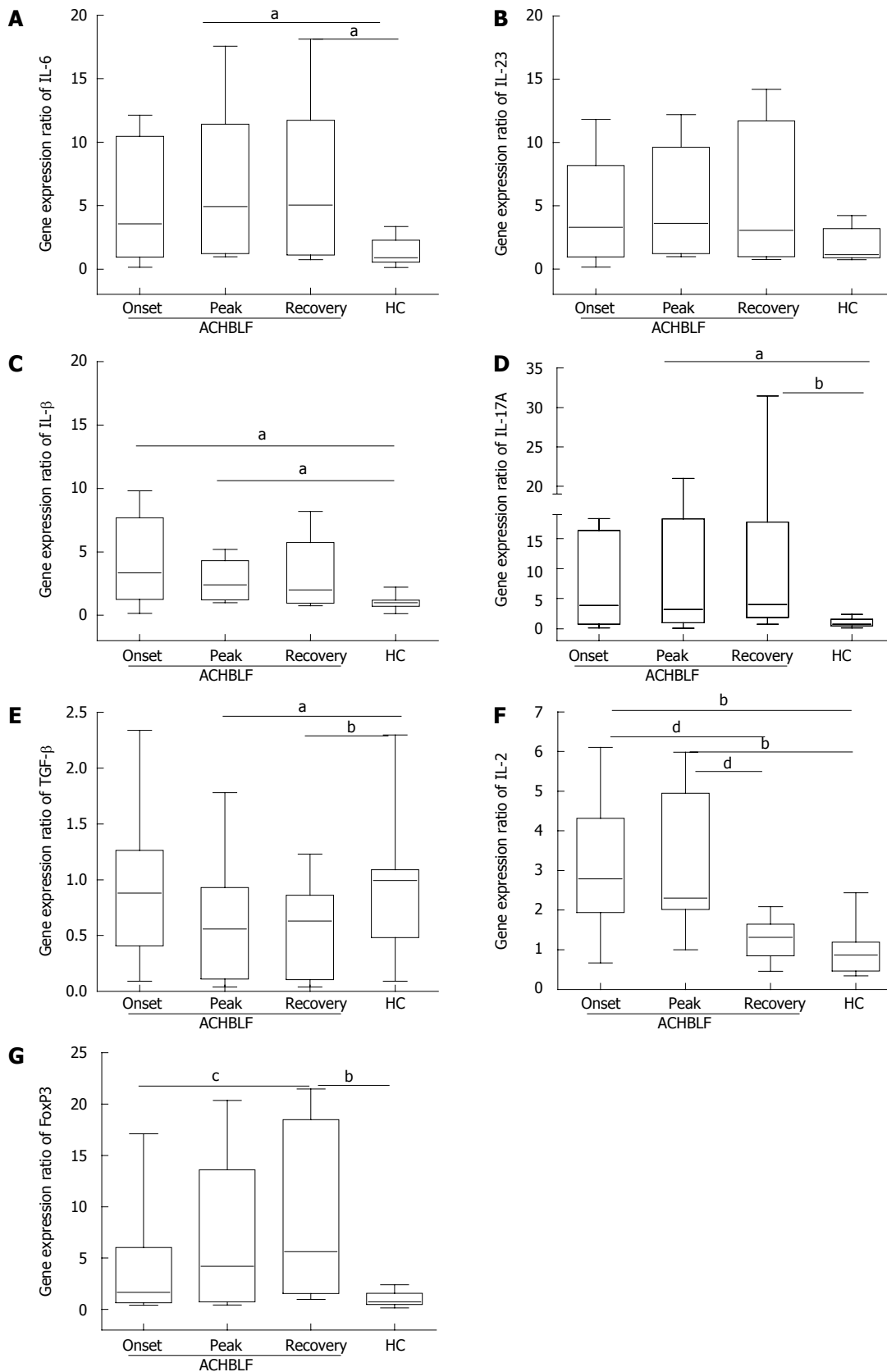


Figure 2 Dynamic cytokine milieu for interleukin-17-producing T helper cells and Foxp3⁺ regulatory T cells in patients with hepatitis B virus-related acute-on-chronic liver failure. The gene expression of pro-th17 cytokines showed higher levels throughout the hepatitis B virus-related acute-on-chronic liver failure (ACHBLF) event compared with HCs, especially for IL-6 and IL-1 β (A and C). FoxP3 gene expression was up-regulated gradually during ACHBLF events (G). Data are presented as whisker-box plots. Boxes represent the interquartile range, with the median represented by the line inside the box. Upper whisker, the highest value less than or equal to the 75 percentile plus 1.5 times interquartile range; lower whisker, the lowest value greater than or equal to the 25 percentile minus 1.5 times interquartile range. Outliers were excluded. Differences between different time points were analyzed by the Kruskal-Wallis test and with HCs by the Mann-Whitney test. ^a*P* < 0.05, ^b*P* < 0.01 vs HC; ^c*P* < 0.05, ^d*P* < 0.01 vs recovery group.

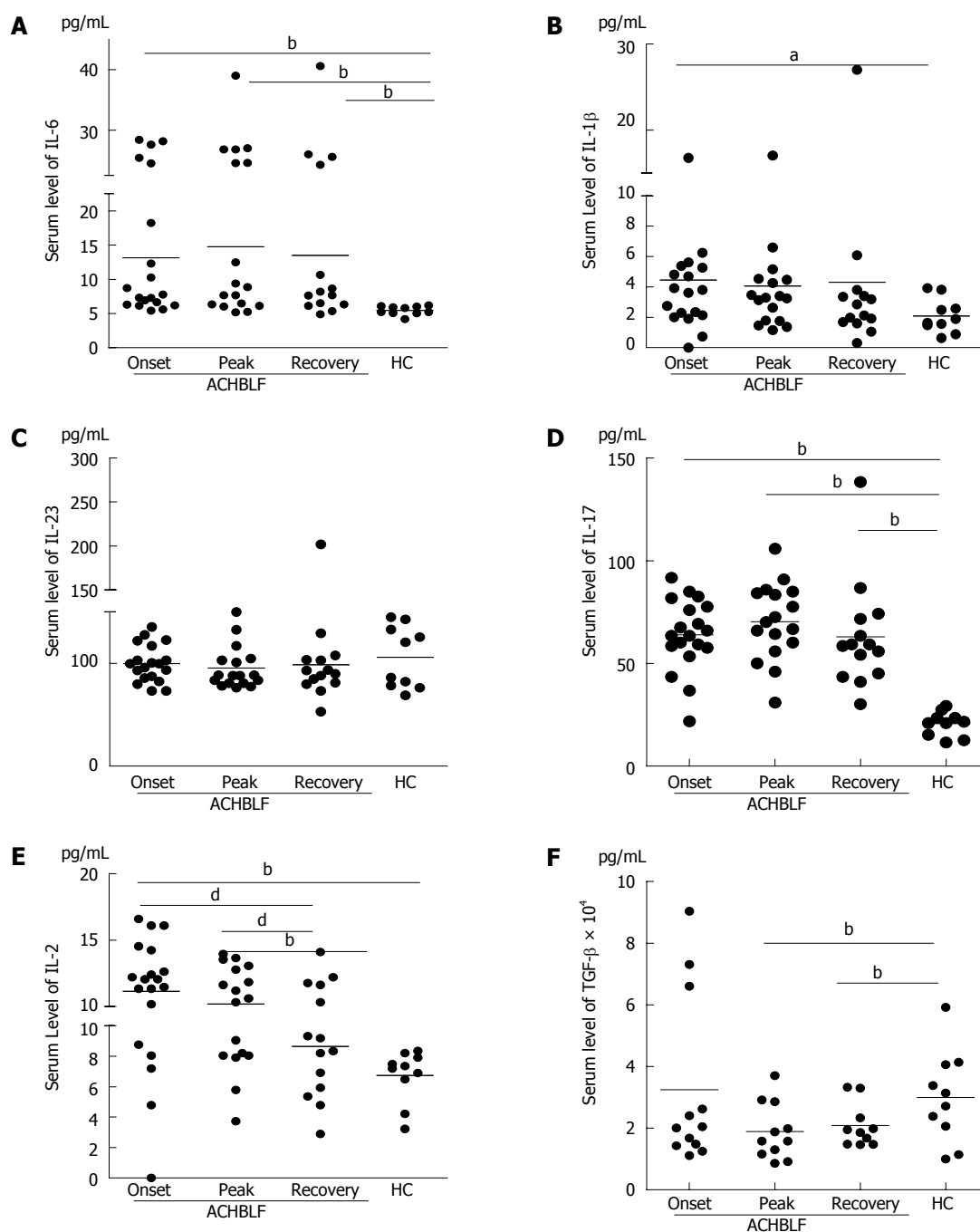


Figure 3 Serum levels of interleukin-17-producing T helper cell and Foxp3⁺ regulatory T cell-related cytokines in patients with hepatitis B virus-related acute-on-chronic liver failure. A: Interleukin (IL)-6; B: IL-1 β ; C: IL-23; D: IL-17; E: IL-2; F: Transforming growth factor (TGF)- β 1 were tested. Data are presented as the mean \pm standard deviation. Differences between different time points were analyzed by the Kruskal-Wallis test and with HCs by the Mann-Whitney test. ^a $P < 0.05$; ^b $P < 0.01$ vs HC; ^c $P < 0.01$ vs recovery group.

Both Th17 and Treg cell-related cytokines were up-regulated at the onset of ACHBLF

Compared with HCs, the gene expression of the pro-Th17 cytokines IL-1 β , IL-6 and IL-23/p29 was up-regulated, especially IL-1 β , at the onset of ACHBLF (Figure 1A-C). We also observed higher expression of IL-17A (Figure 1D). However, there were no significant differences between the ACHBLF and CHB patients.

Although the expression of TGF- β 1 in the patients with ACHBLF did not differ from that of the HCs and

CHB patients (Figure 1E), IL-2 gene expression was up-regulated significantly compared with the HCs and CHB patients (Figure 1F). We also observed higher levels of *Foxp3* gene expression (Figure 1G).

Dynamic Treg and Th17 cell differentiation environment at both gene and protein levels during ACHBLF

During ACHBLF, the differentiation environment was conducive to Th17 cell differentiation (Figure 2A-C and Figure 3A-C), which was demonstrated by significantly

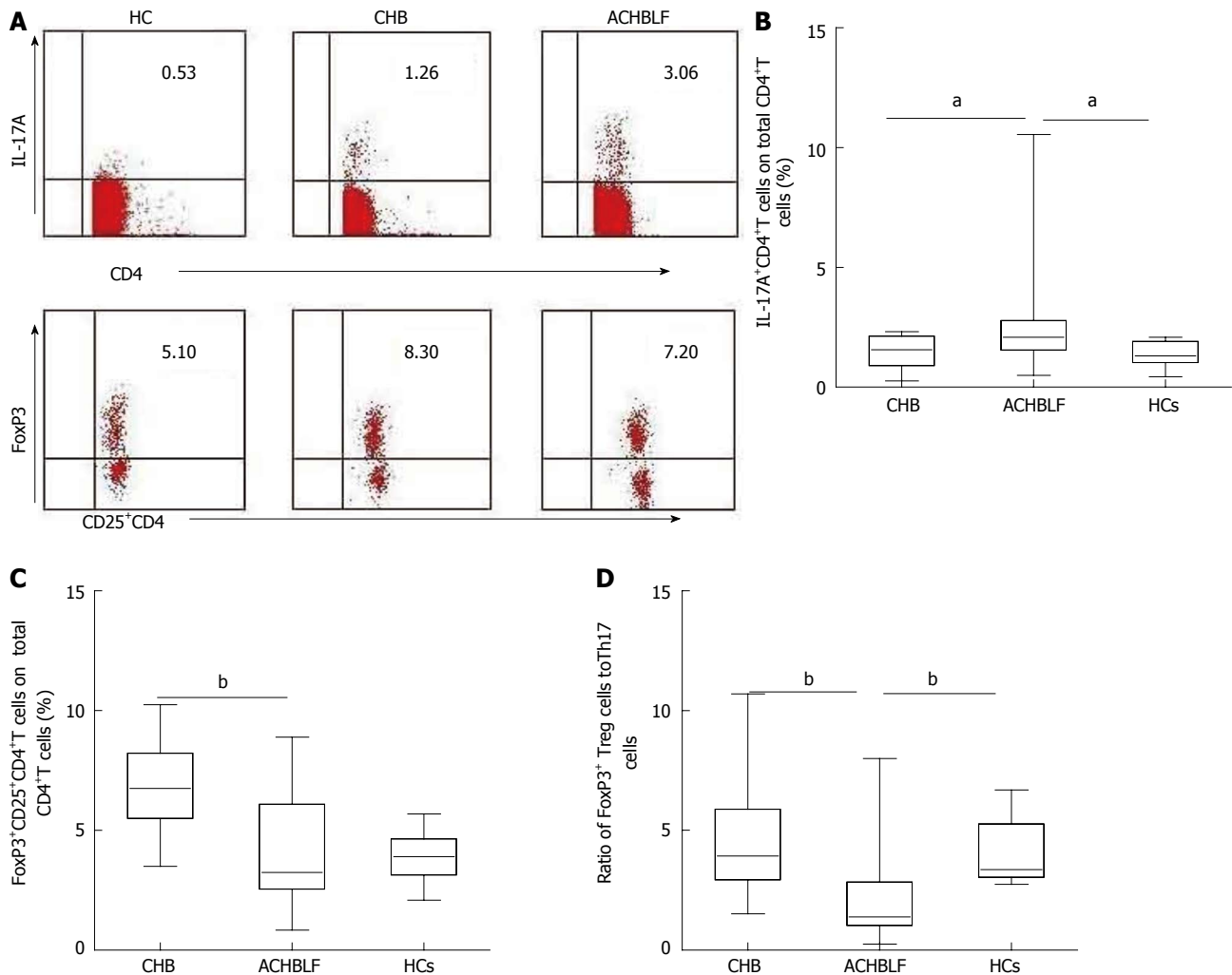


Figure 4 Population of interleukin-17-producing T helper cells as a percentage of total CD4⁺ cells in the peripheral blood mononuclear cells was evaluated by flow cytometry. A: Representative dot plots of intracellular IL-17 and Foxp3 staining in patients with HBV-related acute-on-chronic liver failure (ACHBLF); B: Percentage of IL-17A⁺CD4⁺T cells in total CD4⁺T cells; C: Percentage of FoxP3⁺CD25⁺CD4⁺T cells in total CD4⁺T cells; D: Ratio of Treg cells to Th17 cells. CHB: Chronic hepatitis B. Differences between ACHBLF and HCs or CHB group were analyzed by the Mann-Whitney test. ^a*P* < 0.05, ^b*P* < 0.01 vs ACHBLF group.

elevated IL-6 and IL-1 β levels both at the gene and protein expression level (Figures 2A, C and 3A, B). However, Treg cell-related cytokine expression levels gradually decreased during ACHBLF (Figures 2E, F and 3E, F). Corresponding to this Th17 differentiation environment, patients possessed higher IL-17A levels at both the gene and protein levels during the ACHBLF event (Figure 2D and Figure 3D). Interestingly, *Foxp3* gene expression was up-regulated gradually during the ACHBLF event. *Foxp3* gene levels at the recovery point were significantly higher than HCs and at onset time point (Figure 2G).

Treg and Th17 cell numbers during ACHBLF

Based on our knowledge of the cytokine milieu in patients with ACHBLF at the onset point, we further evaluated the Treg and Th17 cell subsets in PBMCs, defined as the percentage of Th17 cells and Treg cells in the total CD4⁺ T cell population using flow cytometry (Figure 4A). The Th17 cell population was increased in patients with ACHBLF ($2.83\% \pm 2.61\%$) compared with patients

with CHB ($1.48\% \pm 0.71\%$, *P* = 0.04) and HCs ($1.37\% \pm 0.51\%$, *P* = 0.01; Figure 4B), whereas Treg decreased in ACHBLF patients ($4.08\% \pm 2.28\%$) compared with CHB patients ($6.71\% \pm 1.76\%$, *P* = 0.001) (Figure 4C).

Given the nonsynchronous changes of Treg and Th17 cells at the onset of ACHBLF, to better understand the relationship between these two types of immune cells, we used the Treg/Th17 ratio. The Treg/Th17 ratio of ACHBLF at the onset point was decreased significantly ($2.83\% \pm 2.60\%$) compared with HCs ($4.09\% \pm 1.39\%$, *P* = 0.003) and the CHB patients ($4.67\% \pm 2.30\%$, *P* = 0.007) (Figure 4D).

These data indicate that a significant imbalance in the numbers of circulating CD4⁺T cells occurs in patients with ACHBLF.

Increased ratio of Treg to Th17 cells in patients with ACHBLF

To characterize the changes in the circulating CD4⁺T subset, all 18 patients with ACHBLF were longitudinally

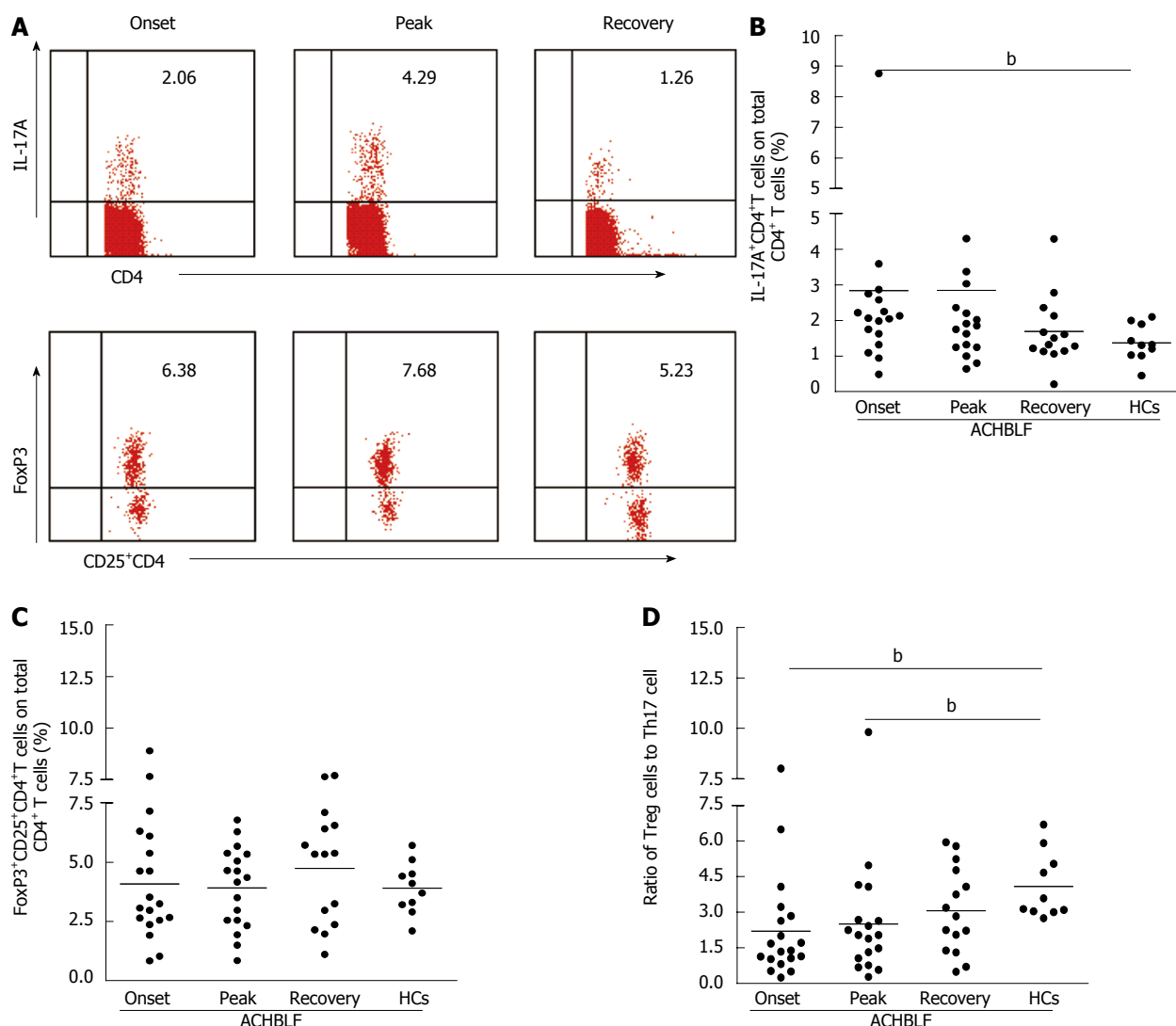


Figure 5 Ratio of Foxp3⁺ regulatory T cells to interleukin-17-producing T helper cells was dynamically increased during hepatitis B virus-related acute-on-chronic liver failure. A: Representative dot plots of intracellular IL-17 and FoxP3 staining in different stages of a hepatitis B virus-related acute-on-chronic liver failure (ACHBLF) individual. Values in the upper right quadrant indicate the circulating Th17 cells percentage and the FoxP3⁺ percentage of total CD4⁺ T cells; B: The dynamics of Th17 cell frequency at the onset, peak and recovery phases of ACHBLF; C: The dynamics of Treg cells at the onset, peak and recovery phases of ACHBLF; D: The dynamics of the ratio of Treg to Th17 cell frequency at the onset, peak and recovery phases of acute-on-chronic HBV-related liver failure. The data were analyzed by one-way ANOVA Kruskal-Wallis test, $P < 0.05$ indicates significance. ^b $P < 0.01$ vs HCs.

followed. Intracellular IL-17 and FoxP3 staining in different stages of ACHBLF were tested using flow cytometry (Figure 5A). As shown in Figure 5B, during ACHBLF events, patients had a sustained higher frequency of Th17 cells in the peripheral blood compared with HCs. The frequency of Th17 cells at the peak point was increased slightly compared with at the onset point, but there was no significant difference between the two clinical phases. However, at the recovery time point, the frequency of Th17 cells ($1.69\% \pm 0.97\%$) was decreased sharply from the peak time point ($2.84\% \pm 2.92\%$). The frequency of Treg cells was not changed significantly during the entire clinical phase (Figure 5C).

We further analyzed the changes in the ratio of Tregs to Th17 cells during ACHBLF events. The ratio of Treg to Th17 cells was increased gradually from the onset point to the recovery point, and at the recovery point, the ratio of Treg to Th17 ($3.07\% \pm 1.80\%$) was similar to

that of the HCs ($4.09\% \pm 1.39\%$, $P = 0.17$) (Figure 5D).

Relationship between disease development and the Th17-Treg dynamic in patients with ACHBLF

To identify the relationship between host immune changes and disease development, we used total bilirubin (TBIL), alanine aminotransferase (ALT) and a model for end stage liver disease (MELD) plus sodium (MELD-Na) score to evaluate disease development^[34,35]. We performed correlation analyses between these main biochemical measures and the MELD-Na score and Th17 frequency, Treg frequency and the ratio of Treg to Th17 cells at the onset, peak, and recovery phases of the disease among our 18 ACHBLF patients. The data revealed that all of these host immune characteristics positively or negatively correlated with disease development (Table 3). However, in all patients, only serum IL-17 levels at peak point markedly correlated with the ALT level ($r = -0.56$, $P = 0.02$). We

Table 3 Correlations between clinical characteristics and host immune data during the onset, peak, and recovery phases of hepatitis B virus-related acute-on-chronic liver failure

	Onset						Peak						Recovery																	
	Th17			Treg			IL-17			Th17			Treg			IL-17			Th17			Treg			Th17			IL-17		
	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value		
Total patients	ALT	0.17	0.49	-0.02	0.92	-0.004	0.98	-0.17	0.48	-0.20	0.44	0.11	0.68	0.42	0.10	-0.56	0.02	-0.17	0.56	-0.33	0.24	0.00	1.00	-0.24	0.41					
	TBIL	0.06	0.81	-0.21	0.38	-0.20	0.41	-0.28	0.25	0.04	0.88	-0.11	0.68	-0.04	0.88	-0.30	0.23	-0.30	0.29	-0.15	0.59	-0.09	0.76	-0.36	0.19					
	MELD-Na	0.09	0.71	-0.02	0.91	-0.18	0.46	-0.01	0.96	0.02	0.95	-0.09	0.74	0.07	0.79	-0.06	0.82	-0.50	0.06	-0.01	0.98	0.15	0.60	-0.27	0.34					
Survivors	ALT	-0.11	0.71	0.17	0.56	0.25	0.40	-0.12	0.68	-0.42	0.15	0.12	0.73	0.58	0.04	-0.53	0.06	-0.19	0.52	-0.17	0.57	0.19	0.53	-0.29	0.32					
	TBIL	-0.17	0.57	0.09	0.75	0.14	0.63	-0.33	0.26	-0.29	0.34	-0.34	0.25	0.01	0.99	-0.14	0.65	-0.35	0.23	0.05	0.85	0.06	0.83	-0.42	0.14					
	MELD-Na	-0.54	0.05	0.008	0.98	0.30	0.32	0.01	0.96	-0.08	0.80	-0.33	0.27	-0.10	0.75	0.24	0.44	-0.55	0.05	0.18	0.54	0.31	0.29	-0.32	0.27					

ALT: Alanine aminotransferase; TBIL: Total bilirubin; PTA: Prothrombin activity; MELD-Na: Model for end-stage liver diseases and sodium; Th17: Interleukin-17-producing T helper cells; Treg: Foxp3⁺ regulatory T cell; Treg/Th17: Ratio of Treg to Th17 cells. Data were analyzed by Spearman correlation test.

further analyzed patients in the survival group and found that the ratio of Treg to Th17 at the peak point positively correlated with ALT levels ($r = 0.58, P = 0.04$). Furthermore, we also found that in the survival group, changes in the Treg to Th17 ratio from the onset to the peak point markedly correlated with TBIL levels ($r = -0.57, P = 0.04$).

Predictive value of Treg, Th17 and the Treg to Th17 ratio on 28-d survival in ACHBLF

Because Treg, Th17, and IL-17 levels and the ratio of Treg to Th17 were shown to be associated with ACHBLF development, we further evaluated the early predictive value of these factors on ACHBLF outcome. We evaluated the value of these factors at onset and at peak point in predicting ACHBLF survival by the end of a 28-d follow-up period. ROC curve analysis showed that Th17 frequency and the ratio of Treg to Th17 at the onset point were significant in predicting 28-d ACHBLF survival. The Th17 frequency was a negative predictor and the Treg to Th17 ratio was a positive predictor [the area under the ROC was 0.1 (95%CI: 0.000-0.248, $P = 0.01$) for Th17 frequency at onset and 0.846 (95%CI: 0.65-1.00, $P = 0.02$) for the ratio of Treg to Th17 at onset]. There was no significant predictive value of Treg frequency during the event, IL-17 levels during the event, and changes in the ratio of Treg to Th17 from the onset to peak point (Table 4).

To further investigate the predictive value of the Treg to Th17 frequency ratio during ACHBLF events on the survival of ACHBLF patients, we divided the ACHBLF patients into survivors and non-survivors (NS)^[29]. As shown in Figure 6A, we found that the ratio of Treg to Th17 frequency at the onset point in NS patients ($0.91\% \pm 0.24\%$) was significantly lower than that in survivors ($2.64\% \pm 2.19\%$, $P = 0.02$). Furthermore, we also found that survivors exhibited an initial decrease in the circulating ratio of Treg to Th17 frequency from the onset point to the peak point. Subsequently, survivors displayed a continuous increase of the Treg to Th17 ratio, accompanied by a total bilirubin level decrease of more than 30% (Figure 6B). However, dead/transplanted patients lacked these sequential responses compared with survivors. The Treg to Th17 ratio remained persistently low in these patients (Figure 6C). These data suggest that the restoration of the balance of Treg and Th17 cells may represent a prognostic marker for a favorable clinical result in ACHBLF patients.

DISCUSSION

In the present study, we demonstrated that a Treg and Th17 immune imbalance exists in ACHBLF patients by measuring gene, protein and T cell phenotypes. We also longitudinally identified the pattern of dynamic change in Treg and Th17 cells during ACHBLF. We found that compared with CHB, ACHBLF patients possessed a favorable Th17 differentiation environment, accompanied by a sustained higher Th17 frequency and IL-17 levels. The results suggest that Th17 and related pro-inflammatory cytokine up-regulation played a vital role in disease progression from CHB to ACHBLF and also played a critical role in ACHBLF development. Zhang *et al*^[7] previously found that circulating Th17 cells increased with disease progression from CHB to ACHBLF. Another CD4⁺T cell subset, Tregs, plays a critical role in HBV persistence during CHB events.

Table 4 Predictive value of Foxp3⁺ regulatory T cell and interleukin-17-producing T helper cells in hepatitis B virus-related acute-on-chronic liver failure patient survival

Variables	Area	Standard error	Asymptotic sign	95%CI	
				Low boundary	Upper boundary
Treg frequency at onset	0.577	0.163	0.622	0.258	0.896
Th17 frequency at onset	0.108	0.081	0.012	0.000	0.266
Ratio of Treg to Th17 at onset	0.846	0.100	0.027	0.650	1.000
Serum IL-17 level at onset	0.554	0.136	0.730	0.288	0.820
Treg frequency at peak	0.523	0.166	0.882	0.197	0.849
Th17 frequency at peak	0.462	0.168	0.805	0.132	0.791
Ratio of Treg to Th17 at peak	0.585	0.160	0.588	0.270	0.899
Ratio of Treg to Th17 change from onset to peak	0.308	0.132	0.218	0.049	0.566
Serum IL-17 level at peak	0.785	0.108	0.068	0.573	0.997

We found that when disease progressed from CHB to ACHBLF, although the Treg differentiation cytokine IL-2 was up-regulated, circulating Treg frequency began to decrease and showed lower levels during ACHBLF events compared with CHB events. These data suggest that Treg down-regulation may promote liver inflammation and accelerate disease progression from CHB to ACHBLF and ACHBLF development.

The development of ACHBLF involves dysregulation of both the innate and adaptive immune systems. Many different cell types, including T lymphocytes, monocytes and dendritic cells, are primed in ACHBLF. These cells are believed to play a pivotal role in the pathogenesis of ACHBLF^[4,6,8,12,26]. Treg and Th17 cells are both involved in the pathogenesis of HBV infection. Tregs and Th17 cells are closely associated with each other; the two types of T cells not only share the same origin but are also mutually antagonistic in function. Thus, the balance between the two types of T cells could impact inflammation control and autoimmune inflammation^[36,37]. Many studies have found that an imbalance between Th17 and Treg cells is closely related to the development of a number of diseases, including HBV infection^[9,27,36-39]. Recently, two different groups have demonstrated imbalances between Treg and Th17 cells in ACHBLF patients and that this imbalance plays an important role in the progression of ACHBLF^[27,40]. Furthermore, several studies have demonstrated that antiviral therapy induces a viral load reduction that partly restores antiviral immunity in patients with CHB^[41-44]. We also previously reported that for acute and chronic HBV infected patients, the balance between Treg and Th17 cell frequency was different. Treg and Th17 immune imbalance only exists in CHB and ACHBLF^[9]. In this study, we further demonstrated that immune imbalance of Treg and Th17 cells exists in ACHBLF measured by both gene and protein levels from the clinical onset. Furthermore, the imbalance of Treg to Th17 changed along the progression of the disease and correlated with the outcome of the disease. Furthermore, to avoid a quick viral load reduction that induces immune restoration in the early part of the ACHBLF event, in this study, all patients accepted lamivudine therapy to improve the long-term prognosis of ACHBLF.

Although it is now recognized that an imbalance be-

tween Th17 and Tregs exists in ACHBLF patients, our knowledge about the dynamic interplay between Tregs and Th-17 cells during ACHBLF events is limited. In this study, we longitudinally followed ACHBLF patients and demonstrated that the immune imbalance between Treg and Th17 dynamically changed along ACHBLF progression, but mechanistic data were absent in our study. At onset, the ratio of Treg to Th17 cells, with a significant Th17 increase, was decreased significantly compared with CHB patients and HCs. Then, the Treg to Th17 ratio dynamically increased from the onset to the recovery point, and finally, the ratio of Treg to Th17 was increased to similar levels as HCs at the recovery point. These data suggest that the Treg and Th17 balance modulates liver damage and the outcome of ACHBLF.

Based on the role of Th17 and Treg in ACHBLF and the relationship between the two types of T cells, we studied the relationship between the two types of T cells and ACHBLF progression. We used the MELD-Na score to evaluate ACHBLF disease development, ALT and TBIL to evaluate liver inflammation, and the ratio of Treg to Th17 to represent the interplay of the two types of T cells. We found that in all patients, the frequency of the two types of T cells and their ratio during ACHBLF events correlated with the ALT, TBIL and MELD-Na score positively or negatively, but only serum IL-17 levels at peak point significantly correlated with ALT levels (Table 4). However, in survivors, we found that Th17 frequency at onset and recovery showed a relationship with MELD-Na (Table 4), and the ratio of Treg to Th17 at the peak point significantly correlated with ALT levels. These data suggested that MELD-Na is an improved model for the prediction of mortality in patients with end-stage liver disease awaiting liver transplantation but cannot exactly represent the stage of ACHBLF, especially in patients whose acute liver damage developed quickly^[45].

The mortality of ACHBLF is high and there are few early predictors for survival in ACHBLF. Zhai *et al.*^[27] found that the ratio of Th17 to Treg cells was inversely associated with survival in HBV-related ACLF patients. In this study, we not only found that a lower ratio of circulating Treg to Th17 frequency at the onset predicts a poor survival rate but also found that survivors exhibited an initial decrease in the circulating ratio of Treg to Th17

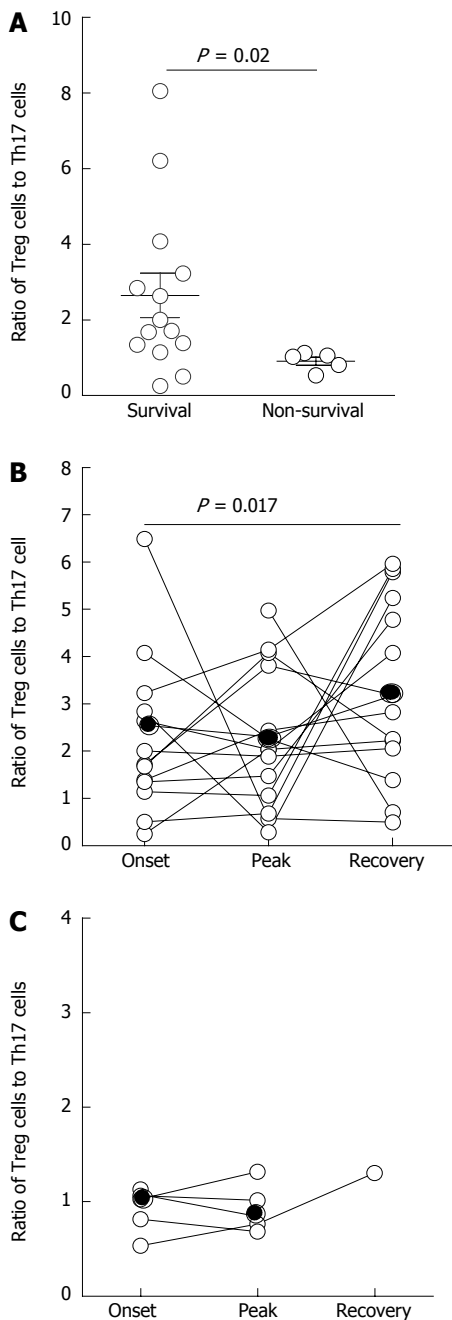


Figure 6 Predictive value of the Foxp3⁺ regulatory T cells to interleukin-17-producing T helper cells cell ratio during disease development on the survival of hepatitis B virus-related acute-on-chronic liver failure patients. A: A lower ratio of Treg cells to Th17 frequency in non-survival ACHBLF patients at clinical onset; B: The distribution of the ratio of Treg cells to Th17 cells in surviving ACHBLF patients; C: The distribution of the ratio of Treg cells to Th17 cells in non-surviving ACHBLF patients. Each symbol represents one individual, and each line represents the changes in an individual patient's ratio of Treg to Th17 frequency. The solid line depicts the mean ratio of all patients.

cells from onset to peak time and subsequently displayed a continuous increase in the ratio of Treg to Th17 cells, accompanied by a total bilirubin level decrease of more than 30%. According to these data, we speculate that the interplay between Tregs and Th-17 is important for maintaining the balance between a limited immune response and pathological damage.

In summary, our findings suggest that an immune imbalance between Treg and Th17 cells exists in ACHBLF patients, and the immune imbalance dynamically changes with disease progression. The ratio of Treg to Th17 cell frequency is dynamically associated with survival in ACHBLF patients. A low Treg to Th17 cell ratio at onset predicts poor survival. Survivors exhibit an initial decrease in the circulating ratio of Treg to Th17 cells from onset to peak time and subsequently display a continuous increase in the ratio of Treg to Th17 cells.

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COMMENTS

Background

FoxP3⁺Treg cells and IL-17⁺Th cells are both involved in the progression of hepatitis B virus (HBV) infection. During chronic HBV infection there is an imbalance between the two related types of immune T cells. Little is known about the change in the pattern of the two types of T cells during HBV-related acute-on-chronic liver failure (ACHBLF) progression.

Research frontiers

Studies have previously demonstrated that there is an imbalance between FoxP3⁺Treg and IL-17⁺Th cells during ACHBLF. The ratio of Th17 to Treg cells is inversely associated with survival in HBV-related ACLF patients.

Innovations and breakthroughs

In this study, the authors longitudinally investigated FoxP3⁺Treg cells and IL-17⁺Th cells using gene expression and protein levels and also T-cell phenotypes during ACHBLF. At the onset of ACHBLF, patients presented with an environment conducive for Th17 differentiation, but Treg cell-related cytokines showed a gradually increasing trend over time. FoxP3⁺Treg and IL-17⁺T cells showed changes in gene, protein level and T cell phenotypes during ACHBLF events. An increased Treg/Th17 ratio was associated with survival in ACHBLF patients.

Applications

ACHBLF is one of the most severe consequences of HBV infection. The mortality of ACHBLF is high, and there are few early predictors for survival in ACHBLF. The ratio of Treg to Th17 cell frequency is dynamically associated with survival in ACHBLF patients. A low Treg to Th17 cell ratio at disease onset predicts poor survival, and survivors exhibit an initial decrease in the circulating ratio of Treg to Th17 cells from onset to peak time; therefore, monitoring the changes in the Treg to Th17 cell ratio could predict survival in ACHBLF.

Terminology

ACHBLF refers to acute liver failure occurring in patients with chronic HBV infection (CHB), CHB-related liver cirrhosis, or chronic asymptomatic HBV carriers. ACHBLF manifests as jaundice and coagulopathy, complicated within 4 wk by ascites and/or encephalopathy.

Peer review

The manuscript aimed to longitudinally investigate the expression levels of Treg cells in patients with ACHBLF and to analyze changes in Th17 and Treg cells during the follow-up period. Overall, the data are interesting. Although the numbers of patients are small, they are well characterized. However, an absence of mechanistic data was observed in this manuscript.

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