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ORIGINAL ARTICLE

Increased $CD4^+CD25^+$ regulatory T cells correlate with poor short-term outcomes in hepatitis B virus-related acute-on-chronic liver failure patients

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Background: The roles of $CD4^+CD25^+$ regulatory T cells (Treg) in chronicity of hepatitis B virus (HBV) infection have been confirmed. We aimed to explore alteration of Treg in patients with HBV-related acute-on-chronic liver failure (ACLF).
Methods: Thirty-two HBV-related ACLF patients, 44 chronic hepatitis B patients, and 41 healthy controls were recruited. We detected frequencies of peripheral Treg and intrahepatic forkhead winged helix transcription factor $(Foxp3)^+$ cells. Inhibitory activity of Treg was assessed by functional suppression assays. Serum interferon-γ and interleukin-10 were also determined.
Results: Peripheral Treg and intrahepatic $Foxp3^+$ cells were more markedly increased in ACLF than chronic hepatitis B and controls (all $p < 0.001$), and the $Foxp3^+$ cells located predominantly in the portal areas. The Treg frequency was positively correlated with HBV DNA load,

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international normalized ratio, model of end stage liver disease score, and serum interleukin-10 level in ACLF patients. Functional assays *in vitro* demonstrated that ACLF patients exhibited higher suppressive effects of Treg on proliferations of autologous $CD4^+CD25^-$ T cells than controls. On logistic regression, prolonged international normalized ratio and higher peripheral Treg frequency predicted 30-day survival of ACLF.

Conclusion: The patients with HBV-related ACLF exhibit increased amounts of Treg, of which redistribution from periphery to liver seems to modulate liver inflammation. Higher Treg amounts are associated with more severe liver disease in ACLF, and its level in combination with international normalized ratio may assist prediction of short-term outcomes of HBV-related ACLF.

Introduction

Hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) refers to an acute deterioration of liver function due to a precipitating event such as microbial infection, reactivation of viral hepatitis, hepatotoxic drugs, surgical procedures, or variceal bleeding occurring in patients with any form of underlying chronic HBV infection.[1,2] In China, HBV infection constitutes >80% of all etiologies of ACLF owing to a high carriage rate of HBV.[3,4] HBV-related ACLF exhibits relatively high mortalities and liver transplantation is the most promising treatment.[5,6]

Recent studies have shed light on $CD4^+CD25^+$ regulatory T cells (Treg) that are actively engaged in the maintenance of immune tolerance to both self and nonself antigens by suppressing aggressive T-cell response.[7,8] This specialized subpopulation of $CD4^+$ T cells constitutively expresses interleukin (IL)-2 receptor α-chain (CD25), and represents approximately 2—4% of peripheral $CD4^+$ T cells.[9] The forkhead-winged helix transcription factor (Foxp3) has been demonstrated to be a unique molecule for the development and functions of $CD4^+CD25^+$ Treg.[10,11]

Accumulating evidence indicates that increased Treg numbers are associated with persistence of HBV infection by downregulating HBV-specific effector T cell responses.[12—15] Depletion of this proportion of cells *in vitro* can enhance proliferation of effector cells and secretion of interferon-γ (IFN-γ) against HBV antigens in a dose-dependent manner.[12,16] By contrast, the suppressive role of Treg may prevent excessive immunopathological damages induced by sustained immune activation and inflammation.[17,18] ACLF often represents a complicated state of host immune dysregulation, in which exacerbated innate immune responses and aberrant adaptive immune responses may mediate hepatic inflammation.[19—23] However, it is still controversial whether Treg is increased or decreased in HBV-related ACLF due to discrepant results.[16,24]

In this study, we examined the frequency of peripheral $CD4^+CD25^+$ Treg and the distribution of liver-infiltrating $Foxp3^+$ cells in patients with HBV-related ACLF. We also investigated inhibitory functions of Treg, as well as its association with short-term prognosis in HBV-related ACLF.

Patients and methods

Patients

A total of 32 patients with HBV-related ACLF and 44 with chronic hepatitis B (CHB) were recruited at the Third Affiliated Hospital of Hebei Medical University, Shijiazhuang, China from September 2009 to December 2011. Forty-one healthy individuals were selected as controls matched for sex and age. The study protocol was approved by the hospital Ethics Committee. Written informed consent was obtained from the patients or their families prior to enrollment. According to the *Diagnostic and Treatment Guidelines for Liver Failure* issued by Chinese Society of Hepatology and Chinese Society of Infectious Disease,[25] the inclusion criteria for HBV-related ACLF included: (1) carriage of hepatitis B surface antigen ≥6 months; (2) detectable serum HBV DNA; (3) acute deterioration in liver functions based on total bilirubin (TBIL) ≥ 171μM and prothrombin activity ≤40%; and/or (4) recent development of complications such as hepatic encephalopathy (HE), ascites, spontaneous bacterial peritonitis, or hepatorenal syndrome. The definitions of CHB patients have been described in detail elsewhere.[16] Patients who had coinfection of hepatitis A, C, E, or D viruses or human immunodeficiency virus, autoimmune liver diseases, biliary obstruction, hemolytic diseases, pregnancy, or malignancy were excluded. No patient had received anti-HBV or immunosuppressive agents 6 months prior to enrollment.

Samples

A blood sample was obtained from each participant at enrollment. Tissue was collected from six explanted livers of HBV-related ACLF, 23 biopsied livers from CHB, and six healthy donors, respectively. One third of each specimen was stored in RNA fixer (GENEray Biotechnology, Shanghai, China) at −80 °C until RNA extraction was conducted. The remainder was fixed in 10% formalin for immunohistochemistry.

Laboratory tests

Serum albumin, alanine transaminase (ALT), aspartate transaminase (AST), TBIL, and creatinine (CREA) were

determined by Fully Automatic Biochemical Analyzer (AU2700, Olympus, Tokyo, Japan). Prothrombin time was measured by Fully Automatic Coagulometer (Ac.T 5diff AL; Beckman-Coulter Inc., Brea, CA, USA) for calculating its derived parameters PTA and international normalized ratio (INR). HBV serological markers including hepatitis B surface antigen, anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe, and anti-hepatitis B core were determined by commercially enzyme immunoassay kits (Yingkexinchuang Sci-Tech Co., Ltd., Xiamen, China). Serum HBV DNA load was quantified by a real-time polymerase chain reaction (PCR) diagnostic kit D.A. Gene, Zhongshan University, Guangzhou, China with the lower limit threshold of 500 copies/mL. The model for end-stage liver disease (MELD) score was calculated as follows: $3.78 \times \text{Ln[TBIL (mg/dL)]} + 11.2 \times \text{LnINR} + 9.57 \times \text{Ln[CREA (mg/dL)]} + 6.43 \times$ (constant for etiology: 0 = if cholestatic or alcoholic, 1 = otherwise).

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from 2 mL fresh heparinized blood by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) density gradient centrifugation. Then the isolated PBMCs were incubated with fluorescein isothiocyanate-conjugated anti- CD4 and phycoerythrin-conjugated anti-CD25 antibodies (eBioscience, San Diego, CA, USA) at room temperature for 20 minutes. After staining, the cells were washed twice with phosphate-buffered saline (PBS), and then fixed in 1% paraformaldehyde. The stained cells were subsequently processed on an EPICS-XL FCM (Beckman-Coulter) for analysis, and $CD4^+CD25^+$ Treg cells were gated within the lymphocyte population. Isotype-matched control antibodies were used to separate positive and negative cells on FITC and PE fluorescence channels. All data were analyzed by (Beckman-Coulter).

Quantitative real-time PCR

Total RNA was extracted from the PBMCs and liver tissues using Redzol reagent (Saibaisheng-SBS, Beijing, China) according to the manufacturer's protocol. The extracted RNA was reverse transcribed to first strand cDNA using the PrimeScript RT reagent Kit (Fermentas, Burlington, ON, Canada). Quantification of gene expressions were carried out in a thermal cycler (ABI 7500; Applied Biosystems, Foster City, CA, USA) using a SYBR-green RealMaster Mix kit (TianGen Biotech, Beijing, China). The amplification programs were an initial 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 1 minute. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The sequences of primers were: Foxp3: forward: 5′-CCTCCCCCATCATATCCTTT-3′ and reverse: 5′-TTGGGGTTTGTGTTGAGTGA-3′ (size: 236 bp); and GAPDH: forward: 5′-ACCACAGTCCATGCCATCACT-3′ and reverse: 5′-TCCACCACCCTGTTGCTGTA-3′ (size: 452 bp). For each sample, PCR was performed twice in triplicates, and all data were analyzed by the thermal cycler's software to calculate the ΔCt value (ΔCt = Ct value of the target gene minus Ct value of the internal control gene). Relative expression of Foxp3 to the internal control was calculated using the $2^{-\Delta\Delta Ct}$ method.[26]

Immunohistochemistry

Paraffin-embedded, 10% formalin fixed liver tissues were sectioned in 5 μm. Antigen retrieval was achieved via pressure cooking for 8 minutes in citrate buffer (PH 6.0). After blocking the endogenous peroxidase activity with 3% H_2O_2, all sections were incubated with monoclonal antibody of mouse-anti-human Foxp3 (1:80 dilution; Abcam, Cambridge, UK) overnight at 4°C. Two-Step immunohistochemistry Detection Reagent (Zhongshan Golden Bridge Biotech, Beijing, China) containing secondary rabbit anti-mouse antibody, and 3,3′-diaminobenzidine tetrahydrochloride Substrate Kit (Zhongshan Golden Bridge Biotech) were further applied for detection according to manufacturer's protocols. The $Foxp3^+$ cells were enumerated in three high-powered (400×) fields (hpf) by two independent observers. The average percentage of positive cells was taken for each specimen.

Enzyme-linked immunosorbent assay

Serum IFN-γ and IL-10 were measured using commercially available enzyme-linked immunosorbent assay kits (Rapidbio, West Hills, CA, USA) according to the manufacturer's instructions. All samples were analyzed in duplicate.

$CD4^+CD25^+$ Treg isolation

$CD4^+CD25^+$ Treg were isolated from fresh PBMCs by CD4-negative selection, followed by CD25-positive selection, using MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The reminder population was $CD4^+CD25^-$ T cells, which were regarded as responder cells. The purity of two-cell subpopulations, determined by flow cytometry, exceeded 90% for $CD4^+CD25^+$ Treg and 85% for $CD4^+CD25^-$ T cells.

Inhibitory function assay

The isolated $CD4^+CD25^-$ T cells (2.0×10^5/well) were seeded into 96-well plates, and then stimulated with 1 μg/mL of plate-bound anti-CD3 antibody (eBioscience) and 1 μg/mL of anti-CD28 antibody (eBioscience) in RPMI 1640 medium containing 10% FCS. Autologous $CD4^+CD25^+$ Treg were added to $CD4^+CD25^-$ T cells at ratios of 1:1, 1:2, and 1:5. Control cultures were performed using $CD4^+CD25^-$ T cells instead of $CD4^+CD25^+$ Treg under identical conditions. The cells were co-cultured in triplicate and incubated for 72 hours at 37°C with 5% CO_2. Eighteen hours prior to the end of culture, 0.5 μCi/well of ^{3}H-thymidine was added. The amount of incorporated ^{3}H-thymidine was determined by liquid scintillation spectroscopy. Percentage inhibition was calculated as 1 − (counts/minute in the presence of $CD4^+CD25^+$ Treg/counts/minute in the absence of $CD4^+CD25^+$ Treg) × 100%.

Statistical analysis

All data were analyzed by SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Continuous variables are expressed as mean ± standard deviation. Normally distributed data were analyzed using one-way ANOVA, followed by

Table 1 Clinical and biochemical characteristics of all participants

	HBV-related ACLF ($n = 32$)	Chronic hepatitis B ($n = 44$)	Controls ($n = 44$)
Age	45 ± 12	37 ± 13	39 ± 16
Sex (male/female)	25/7	29/15	26/15
Albumin (g/L)	31.1 ± 4.4*,**	41.4 ± 6.3***	43.9 ± 3.2
ALT (U/L)	309 ± 331**	171 ± 134***	19 ± 11
AST (U/L)	325 ± 348*,**	108 ± 78***	20 ± 7
TBIL (μM)	407.0 ± 145.4*,**	28.6 ± 55.3***	11.3 ± 4.0
DBIL (μM)	218.3 ± 70.5*,**	12.1 ± 32.4***	2.2 ± 1.0
CREA (μM)	102.8 ± 42.4*,**	69.6 ± 10.6	68.6 ± 12.8
PTA (%)	27.6 ± 7.7*,**	102.2 ± 17.4	107.7 ± 12.4
INR	2.6 ± 0.8*,**	1.1 ± 0.1***	1.0 ± 0.1
HBeAg (+)/HBeAg (−)	15/17	25/19	0/0
HBV DNA level ($\log_{10}$ copies/mL)	5.3 ± 1.4	5.8 ± 1.2	ND
Severe complications	19 (59.4)*,**	0 (0)	0 (0)
HE	9 (28.1)*,**	0 (0)	0 (0)
SBP	11 (34.4)*,**	0 (0)	0 (0)
HRS	5 (15.6)*,**	0 (0)	0 (0)
VB	2 (6.3)*,**	0 (0)	0 (0)
30-day mortality	17 (53.1)*,**,[a]	0 (0)	0 (0)

[a] One patient who received liver transplantation within the 30-day observation was arbitrarily assigned to the nonsurvivor group.
Data are presented as *n* (%) or mean ± SD, unless otherwise indicated.
*$p < 0.01$ versus CHB.
**$p < 0.01$ versus healthy controls.
***$p < 0.01$ versus healthy controls.
ACLF = acute-on-chronic liver failure; HBeAg = hepatitis B e antigen; ALT = alanine transaminase; AST = aspartate transaminase; TBIL = total bilirubin; DBIL = direct bilirubin; CREA = creatinine; PTA = prothrombin activity; INR = international normalized ratio; HBV = hepatitis B virus; HE = hepatic encephalopathy; HRS = hepatorenal syndrome; ND = not done; SBP = spontaneous bacterial peritonitis; VB = variceal bleeding.

Student–Newman–Keuls test or Tamhane's T2 test according to the tests of homogeneity of variances. For non-normally distributed data, differences between groups were analyzed using nonparametric Kruskal–Wallis *H* test, followed by Mann–Whitney *U* test for multiple comparisons. Pearson Chi-square test or Fisher's exact test was used to analyze categorical variables as appropriate. Bivariate correlation analysis was assessed by Spearman correlation test. All statistical tests were two-tailed, and $p < 0.05$ was considered to be statistically significant.

Logistic regression analysis with forward stepwise variables selection was used to demonstrate the independent predictors for 30-day mortality of patients with HBV-related ACLF. The covariates included age, sex, HBeAg status, HBV DNA loads, ALT, AST, TBIL, CREA, INR, peripheral Treg frequency, and serum IL-10 level.

Results

Demographic and clinical characteristics

The demographic and clinical characteristics of patients with HBV-related ACLF, patients with CHB, and healthy controls are summarized in Table 1. There were no significant differences in HBV DNA loads and ratios of HBeAg positive/HBeAg negative between ACLF patients and CHB patients. Of all ACLF patients, 19 (59.4%) exhibited severe complications, including nine with HE, 11 with spontaneous bacterial peritonitis, five with hepatorenal syndrome, and two with variceal bleeding.

Peripheral $CD4^+CD25^+$ Treg frequency

As shown in Fig. 1, ACLF patients had a significantly higher frequency of peripheral Treg (6.33% ± 1.49%; median 6.21%) than CHB patients (4.16% ± 1.31%; 3.85%; $p < 0.001$) and healthy controls (2.20% ± 0.69%; 2.33%; $p < 0.001$). There is also a significant difference between CHB patients and healthy controls ($p < 0.001$). In addition, ACLF patients with severe complications (6.51% ± 1.50%; 6.74%) displayed higher levels of circulating Treg than patients without any complications (5.94% ± 1.42%; 5.91%), but there was no significance ($p = 0.552$).

Hepatic $Foxp3^+$ cells frequency

The highest mean frequency of $Foxp3^+$ cells was detected in livers from ACLF patients (14.7 ± 3.6/hpf; 14/hpf) compared with CHB patients (4.6 ± 1.4/hpf; 5.0/hpf; $p < 0.001$) and healthy controls (0.4 ± 0.2/hpf; 1/hpf; $p < 0.001$). More $Foxp3^+$ cells were also observed in CHB patients than healthy controls ($p < 0.001$). The distribution of $Foxp3^+$ cells were predominantly in portal areas, and occasionally scattered in hepatic sinusoids (Fig. 2).

Foxp3 mRNA expression

The level of Foxp3 mRNA in healthy controls was assigned as a reference value of 1. The Foxp3 mRNA levels in both PBMCs and livers were significantly upregulated in ACLF patients (PBMCs: $n = 13$; 8.09 ± 1.34 and livers: $n = 6$;

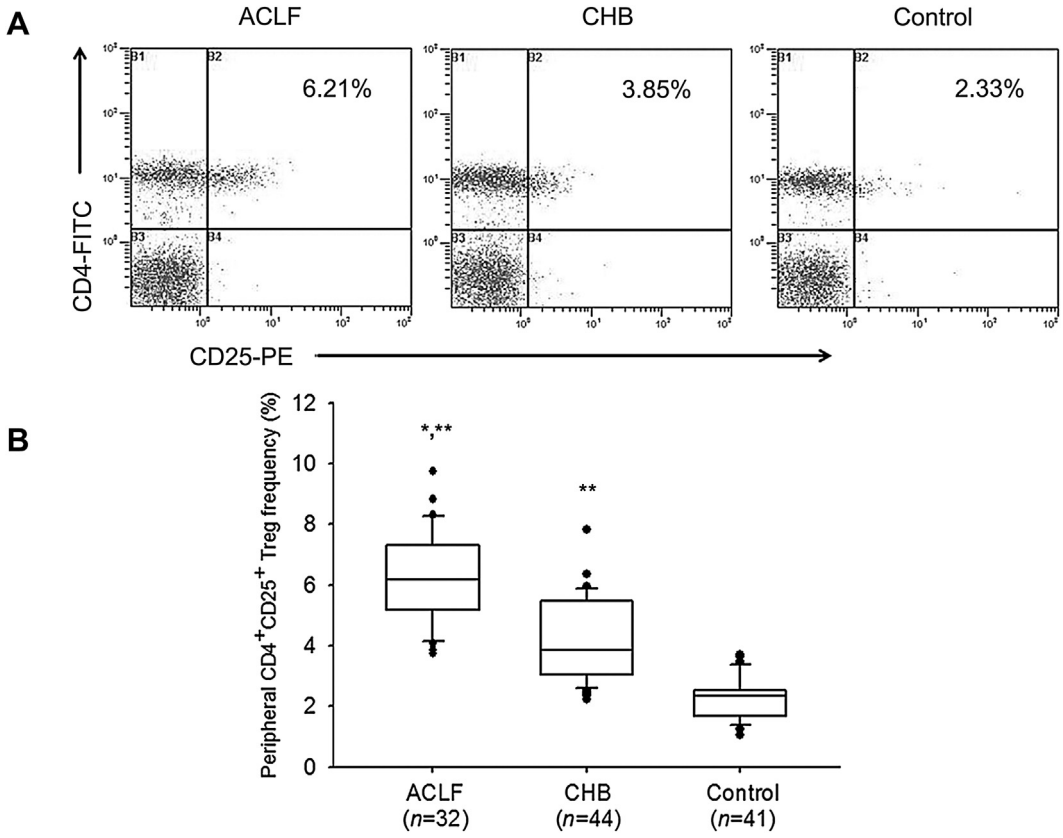

Figure 1. Comparison of peripheral percentages of $CD4^+CD25^+$ regulatory T cells (Treg) within total $CD4^+$ T cells among patients with acute-on-chronic liver failure (ACLF), patients with chronic hepatitis B (CHB), and healthy controls. (A) Representative dot plots obtained by flow cytometry using antibodies against both CD4 and CD25 molecules. (B) Frequencies of $CD4^+CD25^+$ Treg in the above three groups. Data are expressed as box plots, in which the horizontal lines illustrate the 25th percentile, 50th percentile, and 75th percentile, whereas the vertical lines represent the 10th percentile and 90th percentiles. *$p < 0.001$ versus CHB, **$p < 0.001$ versus healthy controls.

5.89 ± 2.06) than CHB patients (PBMCs: $n = 15$; 3.33 ± 1.06 and livers: $n = 13$; 2.12 ± 0.25; all $p < 0.001$) and healthy controls (PBMCs: $n = 10$ and livers: $n = 6$; all $p < 0.001$; Fig. 3).

Inhibitory activity of $CD4^+CD25^+$ Treg

The suppressive ability of $CD4^+CD25^+$ Treg on proliferation of the responder cells ($CD4^+CD25^-$ T cells) was investigated in six ACLF patients, 10 CHB patients, and eight healthy controls. The $CD4^+CD25^-$ T cells exhibited strong proliferation upon anti-CD3/anti-CD28 stimulations in the absence of Treg. Reconstitution of Treg could significantly inhibit proliferation of autologous responder cells in a dose-dependent manner. The inhibitory degrees according to different ratios of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ Treg (1:1, 1:2, and 1:5) were $61.27\% \pm 9.72\%$, $49.51\% \pm 8.18\%$, and $25.52\% \pm 5.34\%$ for ACLF, $53.62\% \pm 7.74\%$, $46.33\% \pm 7.37\%$, and $23.34\% \pm 5.50\%$ for CHB, $35.71\% \pm 5.40\%$, $27.30\% \pm 6.35\%$, and $18.10\% \pm 3.59\%$ for healthy controls, respectively (all $p < 0.05$ when ACLF vs. controls and CHB vs. controls). Although Treg isolated from both ACLF and CHB patients exhibited increased suppressive activity toward responder cells than healthy controls, no significant difference of inhibitory percentage was observed between ACLF and CHB patients (Fig. 4).

Peripheral Treg and disease severity

In ACLF patients, there was a positive correlation between circulating Treg and serum HBV DNA loads ($r = 0.36$, $p = 0.046$; Fig. 5A). However, no correlation was found between circulating Treg and HBeAg status, even though four HBeAg negative patients who showed precore/core promotor mutations had been excluded. There were no correlations between Treg frequency and serum ALT, AST, TBIL, and CREA; whereas it was positively correlated with INR ($r = 0.64$, $p < 0.001$, Fig. 5B) and the MELD score ($r = 0.37$, $p = 0.042$, Fig. 5C).

Th1/Th2 shift and peripheral Treg

Serum IFN-γ level in ACLF patients (0.96 ± 0.37 pg/mL) was significantly elevated than CHB patients (0.40 ± 0.17 pg/mL, $p < 0.001$) and healthy controls (0.37 ± 0.17 pg/mL, $p < 0.001$). Our study also revealed that serum IL-10 was significantly increased in ACLF patients (0.77 ± 0.33 pg/mL) compared with CHB patients (0.48 ± 0.27 pg/mL,

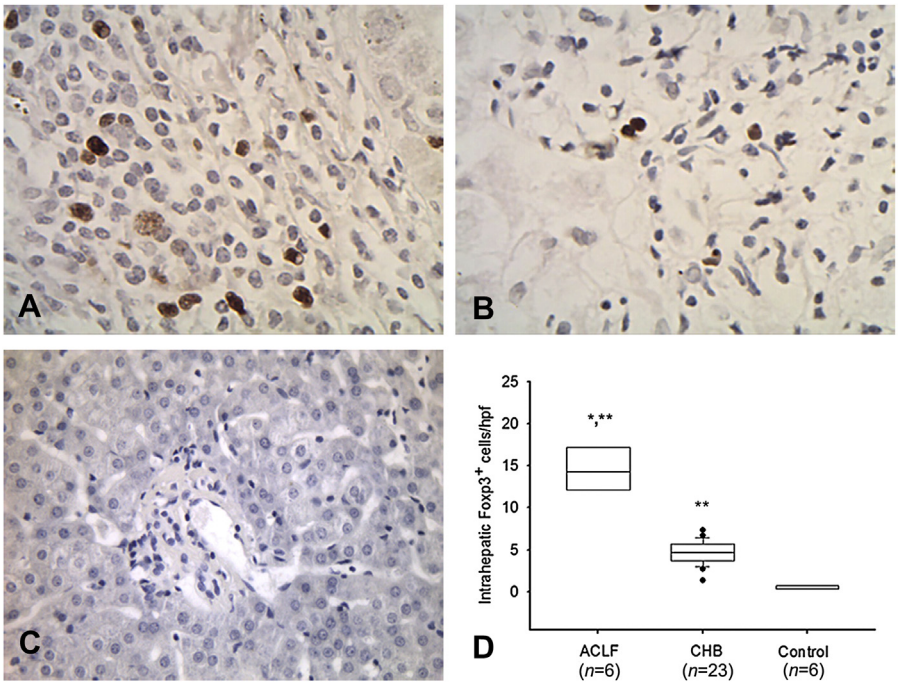

Figure 2. Immunohistochemistry of forkhead winged helix transcription factor (Foxp3) in livers. Foxp3$^+$ cells localized mainly in the inflammatory areas or occasionally scattered in hepatic sinusoids. (A—C) Representative graphs of hepatic expression and distribution of Foxp3 in patients with acute-on-chronic liver failure (ACLF), patients with chronic hepatitis B (CHB), and healthy controls. (D) Comparison of the amounts of Foxp3$^+$ cells among above three groups. Data are expressed as median, 25th percentile, and 75th percentile. * $p < 0.001$ versus chronic hepatitis B, ** $p < 0.001$ versus healthy controls (original magnification: 400×).

$p < 0.001$) and healthy controls (0.54 ± 0.38 pg/mL, $p < 0.001$), and its level in ACLF patients was correlated with peripheral Treg frequency ($r = 0.41$, $p = 0.025$, Fig. 5D). Moreover, there was a higher ratio of IFN-γ/IL-10 in ACLF patients (1.49 ± 0.83) as compared with CHB patients (1.25 ± 1.08; $p = 0.037$) and healthy controls (0.98 ± 0.63; $p = 0.004$).

Peripheral Treg correlates with poor short-term prognosis

We investigated the kinetics of peripheral Treg in 26 ACLF patients who had received only standard medical treatment except liver transplantation by determination of their

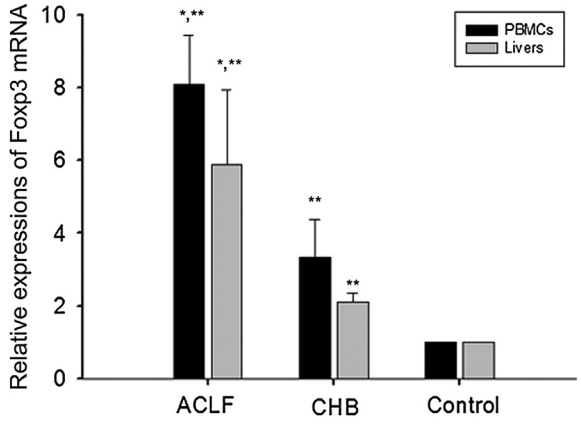

Figure 3. Comparisons of relative forkhead winged helix transcription factor (Foxp3) mRNA expressions in both peripheral blood mononuclear cells (PBMCs) and livers among patients with acute-on-chronic liver failure (ACLF), patients with chronic hepatitis B (CHB), and healthy controls. * $p < 0.01$ versus CHB, ** $p < 0.01$ versus healthy controls.

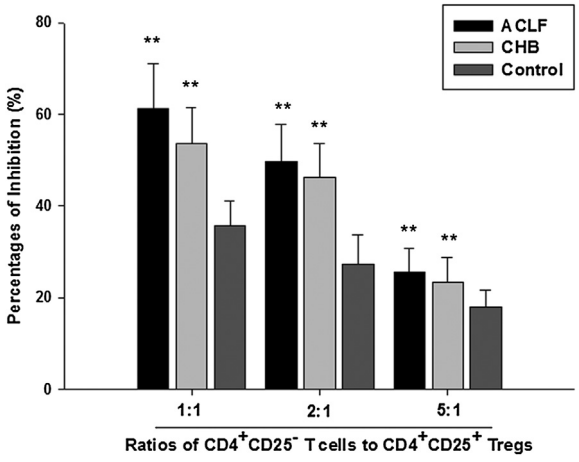

Figure 4. Comparisons of suppressive activity of CD4$^+$CD25$^+$ Treg on proliferation of autologous CD4$^+$CD25$^-$ T cells among acute-on-chronic liver failure (ACLF) patients, chronic hepatitis B (CHB) patients and healthy controls when CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^+$ Treg cocultured at various ratios of 1:1, 2:1, and 5:1. ** $p < 0.05$ versus healthy controls at the same ratio of CD4$^+$CD25$^+$ Treg to CD4$^+$CD25$^-$ T cells.

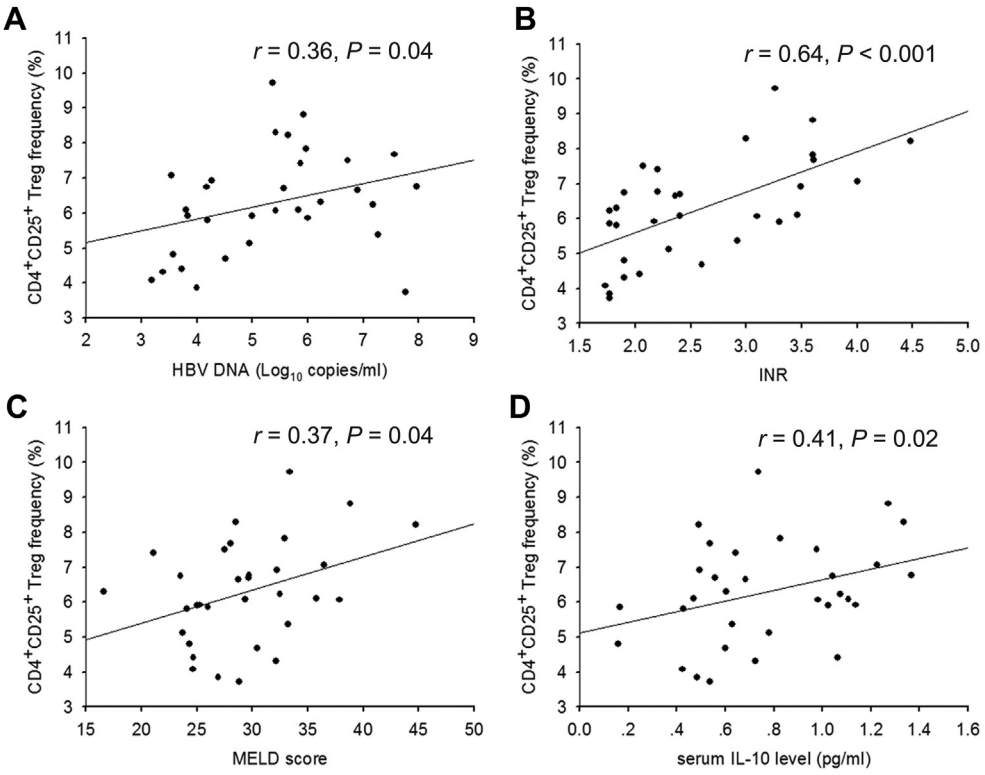

Figure 5. Significant correlations were found between circulating frequencies of $CD4^+CD25^+$ Treg and Log_{10} (A) hepatitis B virus (HBV) DNA, (B) international normalized ratio, (C) model for end-stage liver disease (MELD) score, and (D) serum level of interleukin-10 international normalized ratio (INR), model for end-stage liver disease (MELD) score, and serum level of interleukin-10 in patients with HBV-related acute-on-chronic liver failure.

frequencies every 7 days. According to the outcomes after 30-day observation, these patients were divided arbitrarily into the survival group ($n = 10$) and the nonsurvival group ($n = 16$). Table 2 describes the baseline characteristics of the above two groups. As shown in Fig. 6, the nonsurvivors (6.68% ± 1.32%) had a higher baseline Treg frequency compared with the survivors (5.44% ± 1.18%, $p = 0.024$), and showed an increasing trend with mean levels of 7.23% ± 1.34%, 7.79% ± 1.20%, 8.82% ± 0.91%, and 8.91% ± 0.18% on Day 7, Day 14, Day 21, and Day 28, respectively. By contrast, the survival group presented as a decline with levels of peripheral Treg of 5.53% ± 1.28%, 5.28% ± 1.03%, 4.45% ± 0.87%, and 3.47% ± 0.61% at each time point (all $p < 0.001$ nonsurvivors vs. survivors). Furthermore, logistic regression analysis demonstrated that higher INR (odds ratio 5.9; 95% confidence interval 1.6—24.0; $p = 0.026$) and increased peripheral Treg frequency (odds ratio 3.9; 95% confidence interval 1.4—10.9; $p = 0.042$) can be used to predict 30-day survival of HBV-related ACLF.

Table 2 The baseline data of survivors and nonsurvivors with hepatitis B virus-related acute-on-chronic liver failure receiving standard medical treatment

	Survivors ($n = 10$)	Nonsurvivors ($n = 16$)
Age	44 ± 15	43 ± 10
Sex (male/female)	10/0	12/4
Albumin (g/L)	32.1 ± 4.8	30.9 ± 4.5
ALT (U/L)	346 ± 455	297 ± 277
AST (U/L)	212 ± 155	501 ± 526
TBIL (μM	336.8 ± 153.4	430.8 ± 119.0
DBIL (μM)	187.5 ± 72.8	234.2 ± 65.3
CREA (μM)	87.9 ± 39.7	99.5 ± 38.3
INR	1.9 ± 0.2	2.8 ± 0.7*
HBeAg (+)/HBeAg (−)	3/7	7/9
HBV DNA level (log_{10} copies/mL)	4.6 ± 1.1	5.4 ± 1.5
IFN-γ	0.939 ± 0.439	0.995 ± 0.361
IL-10	0.579 ± 0.316	0.900 ± 0.292**

Data are presented as mean ± SD.
*$p < 0.01$.
**$p < 0.05$.
ALT = alanine transaminase; AST = aspartate transaminase; TBIL = total bilirubin; DBIL = direct bilirubin; CREA = creatinine; INR = international normalized ratio; IFN-γ = interferon-γ; IL-10 = interleukin 10; HBeAg = hepatitis B e antigen; HBV = hepatitis B virus.

Discussion

HBV infection remains a serious public health problem, worldwide affecting 350—400 million people.[27] A small proportion of patients with chronic HBV infection may develop ACLF which exhibits high mortalities and poor prognosis. Although pathogenesis of HBV-related ACLF is extremely complex and still remains unclear, dysregulation

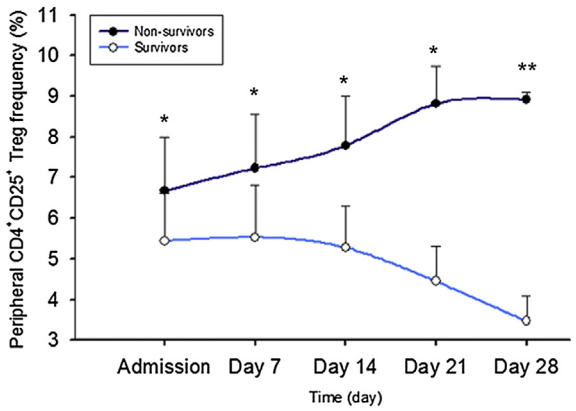

Figure 6. Kinetics of peripheral $CD4^+CD25^+$ Treg in patients with hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) receiving standard medical treatment for 30 days. Peripheral $CD4^+CD25^+$ Treg remain at high levels in the nonsurvival group, whereas the survival group displays a gradual decline. The circles and the error bars represent means and standard deviations. * $p < 0.01$ versus the survival group at each time-point; ** no comparison between two groups due to insufficient sample sizes.

of host immune responses induced by host-HBV interactions is proposed to be the most contributing factor.[28,29] It has been suggested that Treg may play a crucial role in controlling immunopathological damage, but it may also contribute to hyporesponsiveness against infection.

The relationship between Treg and HBV-related ACLF is poorly understood. We observed that ACLF patients had a markedly higher peripheral $CD4^+CD25^+$ Treg, as well as increased inhibitory activity against $CD4^+CD25^-$ responder cells, than healthy controls. Our data are identical to those observed by Xu et al,[16] who showed that patients with chronic severe hepatitis B (i.e., HBV-related ACLF) had the highest circulating $CD4^+CD25^{high}$ Treg among patients with different phases of HBV infections. These results support the notion that upregulation of Treg in ACLF may suppress immune responses, thereby limiting liver injuries and inflammation caused by necrosis or apoptosis of hepatocytes. However, Wang et al[24] demonstrated the opposite finding that decreased peripheral $CD4^+CD25^+CD127^{low}$ Treg could aggravate liver injuries by enhancing immunological responsiveness to HBV in HBV-related ACLF. The discrepancies between these studies may be largely attributed to differences in the molecular markers selected for identifying Treg. Previous studies have confirmed that early-stage ACLF are usually characterized by immune activation, but "sepsis-like" immune paralysis usually accompanies late-stage ACLF.[19,30] Thus, we propose that host immune status in different phases of ACLF may also be responsible for numbers and functions of Treg. Although higher Treg were observed in patients with severe complications with no statistical significance, a larger sample size may lead to a statistical significance.

The Treg cells, much like effector T cells, can accumulate and migrate into the sites of infection or inflammation, where they deal directly with the activated T cells and exert their effects against immune pathology.[31–34] Liver can rapidly mobilize and/or recruit $Foxp3^+CD4^+$ Treg with suppressive activity in response to intrahepatic appearance of activated $CD8^+$ cells,[35] which have been shown to be increased in livers of patients with HBV-related ACLF.[16,20] Because liver is the principle organ affected by HBV infection, we investigated the profile of hepatic $Foxp3^+$ cells in HBV-related ACLF. Our immunohistochemistry study showed profound accumulations of $Foxp3^+$ cells in livers of HBV-related ACLF patients, and this cell population localized predominantly in lymphocyte-rich or inflamed areas, whereas normal liver did not comprise such striking cell population, indicating their direct role in the regulation of local immunity and injuries.

Interestingly, we did not find any correlation between circulating Treg and serum ALT, which reflects the degree of liver inflammation. However, a positive correlation was observed between peripheral Treg and INR. We firstly demonstrated that increased Treg had a correlation with the MELD score, which is recommended as a prognostic score for ACLF.[2] The above results suggest that increased Treg may be a predictive value for the severity of ACLF.

The shift of Th1/Th2 response presenting as profoundly imbalanced ratio of IFN-γ/IL-10 has been observed in our study. IFN-γ is postulated to be a principle proinflammatory cytokine involved in stimulating hepatic inflammation and aggravating liver damage.[36] High level of intrahepatic IFN-γ was associated with severe liver damage in human ACLF.[20] Thus, substantially increased Treg may be a protective factor for immune homeostasis by antagonizing enhanced production and effects of IFN-γ. We have also observed a compensatory increase in serum anti-inflammatory cytokine—IL-10 in ACLF patients, and the IL-10 level was positively correlated with peripheral Treg. It has been reported that one potential mechanism that $CD4^+CD25^+$ Treg control immune responses is due to IL-10, by which the peripheral conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ Treg is also promoted.

Our study also explored the kinetics of peripheral Treg and their relationship with short-term prognosis in HBV-related ACLF. The results demonstrate that early lower level and a decline trend of Treg seem to be followed by a likelihood of better short-term prognosis. A possible mechanism may be that when a patient's condition has been improved, activated Treg may undergo death through an activation-induced cell death pathway.[37] Moreover, migration of peripheral Treg into the inflamed liver may also account for the restoration of peripheral Treg to normal range. By contrast, strikingly high levels of peripheral Treg were persistent in nonsurvivors, suggesting that liver inflammation may not be regulated by intrahepatic—peripheral redistribution of Treg due to severe immune dysmodulation. In addition, some molecules increased in ACLF such as lipopolysaccharide, pathogen associated molecular patterns, and inflammatory tissue factors may be capable of favoring production and function of Treg.[7] Because both ACLF patients and CHB patients had similar inhibitory functions of peripheral Treg, it is also likely that partial proportion of increased Treg in ACLF may have substantially functional deficiencies that could not exert their physiological properties.

In conclusion, patients with HBV-related ACLF exhibit increased circulating $CD4^+CD25^+$ Treg and intrahepatic $Foxp3^+$ cells, of which redistribution from periphery to liver seems to modulate local inflammation. Persistent high levels

of peripheral Treg are not only associated with a more severe degree of liver disease, but are also likely to assist in the prediction of short-term outcomes of HBV-related ACLF. With potentially anti-inflammatory functions, immune-modulation treatment targeting Treg may provide potential strategies for the interventions of HBV-related ACLF.

Conflicts of interest

The authors declare that they have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the manuscript.

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