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Skewed ratios between CD3$^+$ T cells and monocytes are associated with poor prognosis in patients with HBV-related acute-on-chronic liver failure

Feng Shi [a,b,1], Ji-Yuan Zhang [b,1], Zhen Zeng [b], Po Tien [a,*], Fu-Sheng Wang [b,*]

[a] Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[b] Research Center for Biological Therapy, Beijing 302 Hospital, Beijing 100039, China

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ABSTRACT

Tempering of the innate immune response by T lymphocytes has been demonstrated to play a critical role in protecting animals from inflammation-induced death; however, its role in humans remains unknown. Patients with HBV-related acute-on-chronic liver failure (ACLF) share a striking similarity to the inflammatory response in septic shock where a hyperactive innate response is observed. The present study attempted to characterize the features of CD3$^+$ T cells and monocytes and evaluate their clinical implications in 55 patients with HBV-related ACLF, 30 patients with chronic hepatitis B (CHB) and 30 healthy controls (HC). We found that the ratio between circulating CD3$^+$ T cells and monocytes (T/M) was decreased in ACLF patients, due to decreased CD3 counts and increased monocyte counts compared with CHB and HC subjects. We also found that the T/M ratios were decreased from the early to the intermediate stage and reached the lowest value at the late stage in ACLF patients. Analyses with clinical parameters revealed that T/M ratios were negatively correlated with the Model for End-Stage Liver Disease Score and direct bilirubin, and positively correlated with prothrombin activity. Moreover, increased T/M ratios were observed in patients with good prognosis, but not in patients with a poor outcome; and ACLF patients who received liver transplantation exhibited an increased T/M ratio. Importantly, we found that programmed death-1 receptor (PD-1) was drastically upregulated on both CD4$^+$ T and CD8$^+$ T cells in ACLF, which at least in part contributed to the T-cell loss in these patients. Mechanically, the *in vitro* co-culture assay revealed that both CD4$^+$ T and CD8$^+$ T cells, as well as regulatory T cells, could inhibit TNF-α secretion by monocytes. In addition, the TNF-α levels in ACLF serum were negatively correlated with T/M ratios. In conclusion, our study identified the novel potential role of T/M ratio in predicting disease progression and provided novel evidences for further studies of the immunopathogenesis in ACLF.

1. Introduction

Acute-on-chronic liver failure (ACLF) is characterized by jaundice, coagulopathy, hepatic encephalopathy and a high mortality rate [1]. In particular, HBV-related ACLF in China accounts for more than 80% of all ACLF cases due to a high prevalence of chronic HBV infection [2–4]. However, there are still no effective therapeutic options for ACLF patients. Although liver transplantation could cure approximate 90% of patients with liver failure, the shortage of donor livers and considerable cost make this approach impracticable in most patients at present [5,6]. Therefore, the identification of a marker associated with disease severity will be critical for the improved clinical management and the development of new therapeutic options.

There is a large volume of evidence to show that immune dysregulation occurs in HBV-related ACLF [7–11]. These include an exacerbated innate immune responses and an aberrant adaptive immune response, in which the hyperactivated innate responses are considered to play an important role in mediating hepatic inflammation and the apoptosis and necrosis of hepatocytes [7,8,12,13]. Clinical findings and experimental rodent models suggested that ACLF patients were complicated by endotoxemia [14], allowing bacterial components like lipopolysaccharide (LPS) to flow into the liver and activate monocytes/macrophages via the toll-like receptor 4 (TLR4) pathway. Although monocytes contribute directly to immune defense against pathogens, monocyte-produced mediators, such as TNF-α, were believed to be crucial in the

Abbreviations: ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; CHB, chronic hepatitis B; DBil, direct bilirubin; HBV, hepatitis B virus; HC, healthy control; LIL, liver-infiltrating lymphocytes; LPS, lipopolysaccharide; MELD, model for end-stage liver disease; PBMC, peripheral blood mononuclear cells; PD-1, programmed death-1 receptor; PTA, prothrombin activity; TLR, toll-like receptor; T/M, ratios between CD3$^+$ T cells and monocytes.

* Corresponding authors. Address: Research Center for Biological Therapy, Beijing Institute of Infectious Diseases, Beijing 302 Hospital, 100 Western 4th Ring Middle Road, Beijing 100039, China (Fu-Sheng Wang). Fax: +86 010 6387 9735.

E-mail addresses: Tienpo@sun.im.ac.cn (P. Tien), fswang@public.bta.net.cn (F.-S. Wang).

[1] The authors contributed equally to this work.

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pathology of liver disease by targeting mitochondria to initiate death signals in hepatocytes [15–19]. Recent investigations showing the ability of adaptive immune cells, including $CD3^+$ T cells in particular, to temper the innate immune responses triggered by TLRs have gained considerable attention [20,21]. Zhao et al. found that nude and neonatal mice were much more vulnerable to TLR agonists because of their excessive production of proinflammatory cytokines, which indicates that a lack of T cells in the system might result in an uncontrolled innate immune response [22]. As our former study also found a decreased CD3 counts and increased monocyte counts in ACLF patients, we conducted this study to clarify the potential clinical significance of ratios between $CD3^+$ T cells and monocytes (T/M), as well as the mechanism underlying liver damages due to skewed T/M ratios.

2. Materials and methods

2.1. Subjects

Fifty-five HBV-related ACLF, 30 chronic hepatitis B (CHB) patients and 30 healthy controls (HC) were enrolled in this study; and the clinical parameters for different subjects were summarized and compared in Table 1. All ACLF patients had a history of chronic hepatitis with serum $HBsAg^+ > 6$ months and sampled at the very beginning of diagnosis; moreover, these patients were grouped into three stages according to the Chinese Diagnostic and Treatment Guidelines for Liver Failure in 2006 (early, $n = 28$; intermediate, $n = 20$; late, $n = 7$; Table 2) [23]. Within these patients, twenty were successfully enrolled in a follow-up study for three weeks. At the end of follow-up, patients whose total bilirubin (TBil) level decreased by more than 30% were considered to have improved. Ten ACLF patients who had received liver transplantations were also sampled for peripheral blood just before surgeries and two weeks later. Liver tissues from five ACLF patients, seven CHB patients and five healthy liver transplant donors were collected for analysis. The study protocol was approved by the Beijing 302 hospital Ethical Committee, and written informed consent was obtained from each subject before blood and tissue sampling.

2.2. Cell isolation and counts

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation from heparinized blood. Liver-infiltrating lymphocytes (LIL) were isolated based on the following method: in brief, liver tissues were disaggregated by Medimachine (BD Biosciences, San Jose, CA) for 15 s according to the technical instructions. After filtering with Filcons (50 μm, BD Biosciences), LIL were further isolated by density gradient centrifugation. Peripheral lymphocytes and monocyte counts of these patients were determined by an automated differential blood count in our hospital.

$CD3^+$ T, $CD4^+$ T, $CD8^+$ T cells and monocytes were purified from PBMC with the use of CD3, CD4, CD8 and CD14 Microbeads (Miltenyi Biotec, Germany) accordingly. The regulatory T cells were isolated by using $CD4^+$ $CD25^+$ $CD127^{dim}$ regulatory T cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions.

2.3. Flow cytometry analysis

Antibodies conjugated with different types of fluorescein, including phycoerythrin (PE), peridin chlorophyll protein (PerCP) and allophycocyanin (APC), were adopted for flow cytometric analysis. All antibodies were purchased from BD Biosciences (San Jose, CA), except for PE-conjugated anti-programmed death-1 receptor (anti-PD-1) (eBiosciences, San Diego, CA). For superficial staining, PBMC or LIL (1×10^5) were incubated with PE-conjugated anti-PD-1, PerCP-conjugated anti-CD3, and APC-conjugated anti-CD8 for 20 min, before they were subjected to fluorescence activated cell sorting (FACS) analysis (FACS Calibur, BD Biosciences).

2.4. Apoptosis evaluation

Firstly, freshly isolated PBMC (1×10^5) were incubated with PE-conjugated anti-PD-1, Per CP-conjugated anti-CD3 and APC-conjugated anti-CD8 for 20 min. After washing with phosphate buffered solution (PBS), cells were resuspended with binding buffer that contained 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$ and incubated with Annexin-V-FITC (eBiosciences) for 5 min before FACS analysis.

2.5. TNF-α inhibition assay

$CD3^+$ T, $CD4^+$ T, $CD8^+$ T, Treg cells were co-cultured with autologous monocytes (2×10^5) in a 96-well U-bottom plate at different ratios (0:1, 0.1:1, 1:1, or 2:1). The mixed cells were then stimulated with LPS (500 ng/ml, Sigma–Aldrich) for 24 h and monensin (2 μM, Sigma–Aldrich) was added to inhibit cytokine secretion after 4 h of stimulation. Then cells were harvest and subjected to superficial staining with APC-conjugated anti-CD14. After permeabilized with BD Cytofix/Cytoperm Fix/Permeabilization Kit (BD Biosciences), PE-conjugated anti-TNF-α was added for 30 min before FACS analysis.

2.6. Statistical analysis

All data were summarized and presented as means ± standard deviation. The data were analyzed using SPSS software 17.0 (SPSS Inc, Chicago, IL). Comparisons between different groups were

Table 1
Clinical parameters of enrolled subjects.

Group	HC	CHB	ACLF
Case	30	30	55
Age[a]	45 (23–61)	43 (25–57)	48 (18–60)
Sex (male/female)	20/10	23/7	46/9
ALT (U/L)	21 (5–39)	193 (45–328)*	243 (25–2198)*,Δ
AST (U/L)	25 (8–40)	212 (49–335)	308 (39–4303)*,Δ
TBil (umol/L)	9 (5–16)	63 (23–107)*	250 (77–531)*,Δ
DBil (umol/L)	11 (5–20)	45 (17–85)*	190 (56–372)*,Δ
PTA (%)	5 (1.7–10)	97 (75.3–120)*	29 (9.8–39 9)*,Δ
HBV Viral Load (copies/ml)		2 3 × 10^7 (4.5 × 10^4–6 8 × 10^8)*	3.9 × 10^5 (1 × 10^2–8.9 × 10^9)*,Δ
HBeAg (+/−)	0/30	21/9*	35/20*,Δ

Abbreviations: HC, healthy control; ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil, total bilirubin; DBil, direct bilirubin; PTA, prothrombin activity; HBV, hepatitis B virus; HBeAg, e antigen of hepatitis B virus; * $P < 0$ 05, compared with HC; Δ$P < 0$ 05, compared with CHB.
[a] Results were arranged into means and ranges.

Table 2
Criteria for diagonosis and staging acute-on-chronic failure[a].

Parmeters	Early	Intermediate	Late
DBil (μmol/L)		⩾171 μmol/L or increase ⩾ 17.1 μmol/L per day	
PTA	30%–40%	20%–30%	⩽20%
Encephlalopathy	No	GradeI/II	Grade III/IV
Ascites	No	Obvious	Obvious
Complications	No	No	Upper gastrointestinal bleeding, ischemia, severe infections, etc.

[a] According to chinese diagonstic and treatment guidelines for liver failure in 2006.

performed using the Mann–Whitney *U* test. The Wilcoxon matched-pairs *t*-test was used to compare data from the same individuals. A correlation analysis was performed using the Spearman rank correlation test. For all two-tailed tests, $P < 0.05$ was considered to be significant.

3. Results

3.1. T/M ratios were decreased in ACLF, compared with CHB and HC

Firstly, we investigated the absolute numbers of circulating T lymphocytes and monocytes from HBV-related ACLF patients, CHB patients and healthy controls. Results showed that CD3, CD4 and CD8 counts were significantly decreased in ACLF compared with CHB and HC; however, the monocyte counts exhibited a significant increase in ACLF compared with HC, but not with CHB (Fig. 1A). Using the ratios between $CD3^+$ T, $CD4^+$ T, $CD8^+$ T cells and monocytes as indexes, we found that all the ratios shared a similar pattern to decrease from HC to CHB and reached the lowest levels in ACLF (Fig. 1B). Therefore, the ratio between $CD3^+$ T cells and monocytes (T/M) was adopted in the following investigations. To investigate the *in situ* expressions of T/M ratios, we collected liver tissues and found that T/M ratios in LIL were greatly decreased in ACLF compared with their counterparts from HC and CHB (Fig. 1C). Interestingly, the intrahepatic T/M ratios were greatly upregulated in CHB compared with HC, showing a totally opposite status with the peripheral outcomes. The intrahepatic results were further verified by immunohistochemical staining for both CD3 and CD68 (intrahepatic monocyte/macrophage, also named kuffer cells) in liver tissues. Compared with HC and CHB, the expressions of CD3-positive cells were greatly enhanced in ACLF (Supplementary Fig. 1A). However, the expression of CD68-positive cells seemed more dominant in ACLF and the ratios between CD3/CD68 were significantly decreased, in comparison with HC and CHB (Supplementary Fig. 1B).

3.2. Decreased T/M ratios were associated with poor prognosis in ACLF patients

Next, we analyzed the correlation between T/M ratios and disease progression in ACLF patients. As showed in Fig. 2A, we found the T/M ratios decreased from the early to intermediate stage; in particular, patients in the late stage of disease displayed the lowest levels of T/M ratios. We performed a follow-up analysis in twenty ACLF patients, who received standard medical treatment in our unit. We found that, in the patients with improved prognosis, T/M ratios increased from week to week and reached the highest level at the end of the observation (Fig. 2B). However, in the patients with a poor prognosis, the T/M ratios were sustained at low levels or even decreased further (Fig. 2C). To address whether liver transplantation influenced T/M ratios, peripheral blood was collected from ten HBV-related ACLF patients just before surgery and two weeks later. Intriguingly, all patients showed significantly upregulated T/M ratio after liver transplantation (Fig. 2D). Next, we evaluated the correlations between T/M ratios and clinical parameters, such as the Model of End-Stage Liver Disease (MELD) score, direct bilirubin (DBil), prothrombin activity (PTA), total bilirubin (TBil), alanine transaminase (ALT) and hepatitis B virus (HBV) DNA load, which are generally used to evaluate disease severity in HBV-related ACLF patients. Interestingly, we found significant negative correlations in MELD score/DBil in these patients (Supplementary Fig. 2A). Furthermore, the T/M ratios were positively correlated with PTA value. All the correlations maintained within every stage, except the MELD score in late stage (Supplementary Fig. 2A). Nevertheless, no correlation was observed between the T/M ratios and TBil / ALT / HBV DNA (Supplementary Fig. 2B). Thus, all the results above indicated that T/M ratios could serve as a prognostic marker for disease progression in patients with ACLF.

3.3. PD-1 upregulation contributed to T-cell apoptosis in ACLF patients

PD-1 was originally cloned from apoptotic cell lines and could increase the apoptotic sensitivity of T cells in HBV infections [24]. Subsequently, we examined whether PD-1 upregulation affects the numbers of T lymphocytes in HBV-related ACLF patients. We analyzed the frequency of PD-1-expressing T lymphocytes in all subjects, and found that ACLF patients expressed higher levels of PD-1 on the total $CD4^+$ T and $CD8^+$ T cells than CHB and HC did (Fig. 3A). Moreover, PD-L1, one ligand for PD-1, was also greatly enhanced on monocytes and hepatocytes in ACLF (unpublished results). We also found that both PD-1-positive $CD4^+$ T and $CD8^+$ T cells had higher levels of Annexin-V staining than the PD-1-negative counterparts (Fig. 3B and C), which suggested that higher levels of PD-1 expression was associated with greater apoptosis sensitivity of T lymphocytes. Thus, the upregulated PD-1 molecule constrained and reduced the T-cell number in patients with ACLF.

3.4. T lymphocytes could inhibit TNF-α-section by monocytes

To clarify the mechanism underlying immunopathogenesis for ACLF related to the decreased T/M ratios, we designed the *in-vitro* co-culture assay to evaluate the suppressive effect of T lymphocytes on monocytes. The results revealed that T lymphocytes, including $CD3^+$ T, $CD4^+$ T, $CD8^+$ T and Treg cells, did not produce TNF-α under the stimulation with LPS (Fig. 4A). Oppositely, more than one third monocytes would produce TNF-α under the same situation. When co-cultured with $CD3^+$ T, $CD4^+$ T, $CD8^+$ T cells, fewer monocytes would produce TNF-α in a ratio-dependent manner (Fig. 4B and C). We also examined the Treg cells and found that the inhibition efficacies between Treg cells and other T lymphocytes were comparable (Fig. 4B and C). Furthermore, we found that T/M ratios in ACLF patients were negatively correlated with TNF-α levels in the serum, indicating a suppressive role of T lymphocytes in controlling monocytes-derived TNF-α (Fig. 4D).

4. Discussion

The development of ACLF involves dysregulation of both the innate and adaptive immune systems. Many different cell types, including T lymphocytes, monocytes and dendritic cells, are

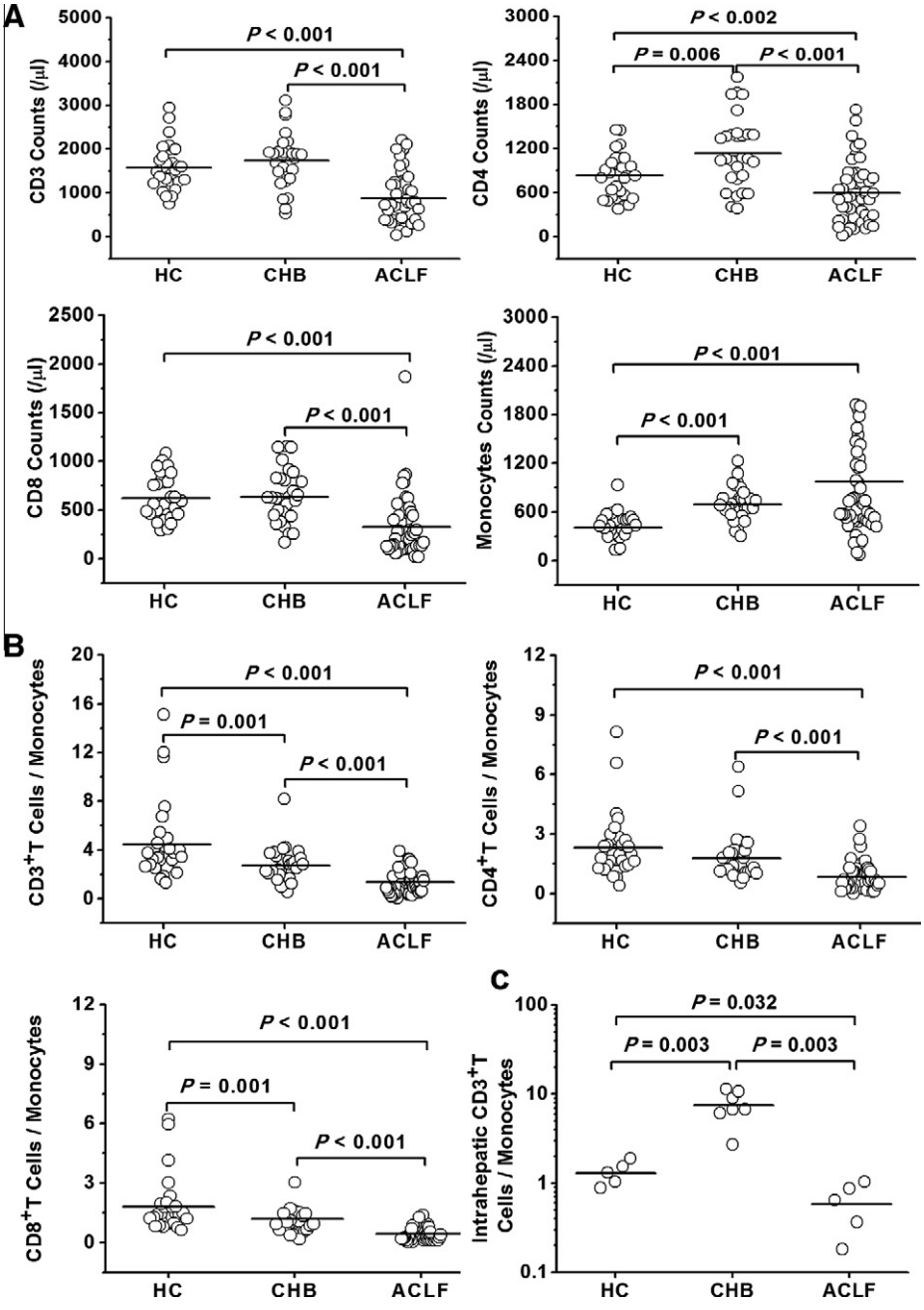

Fig. 1. T/M ratios decreased in ACLF, compared with CHB and HC. (A) The counts of $CD3^+$ T, $CD4^+$ T and $CD8^+$T cells decreased whereas monocytes increased in ACLF ($n = 55$), compared with CHB ($n = 30$) and HC ($n = 30$). (B) The ratios between different T lymphocytes and monocytes decreased in ACLF compared with CHB and HC. (C) The intrahepatic T/M ratios drastically decreased in liver-infiltrating lymphocytes from ACLF patients ($n = 5$), compared with CHB ($n = 7$) and HC ($n = 5$). The black bars indicate the mean values.

primed in ACLF, and these are believed to play a pivotal role in the pathogenesis of ACLF [8,10,13,14]. The clinical findings suggested that chronic liver diseases were usually accompanied with portal hypertension and increased gut permeability, both of which contribute to the increased accumulation of endotoxin (LPS) in the liver and peripheral blood. This activates intrahepatic monocytes/macrophages, kupffer cells, and further leads to a burst of cytokines which are dangerous to the liver. Given the deadly outcomes of ACLF, finding a prognostic marker of this disease to give patients an appropriate indication of time-to-transplantation is urgently needed. In the present study, we found that the decreased T/M ratio was associated with disease progression in ACLF patients, which would provide timely knowledge for clinical management.

Monocytes/macrophages are generally involved in liver injury; however, very little information is known about the suppressive crosstalk between monocytes and T lymphocytes. Two recent investigations demonstrated that $CD3^+$ T cells could temper the innate immune response by inhibiting proinflammatory cytokine secretion [20,22]. In these studies, the authors treated mice with TLR agonists and found that T cell-deficient and neonatal mice were prone to death when compared with wild cohorts. Furthermore, these T cell-deficient and neonatal mice could be saved by the transfer of T lymphocytes, regardless of regulatory T cells. In the present study, we found an increased expression profile in both circulating and intrahepatic monocytes in ACLF patients. Together with the decreased CD3, CD4 and CD8 counts in peripheral, ACLF

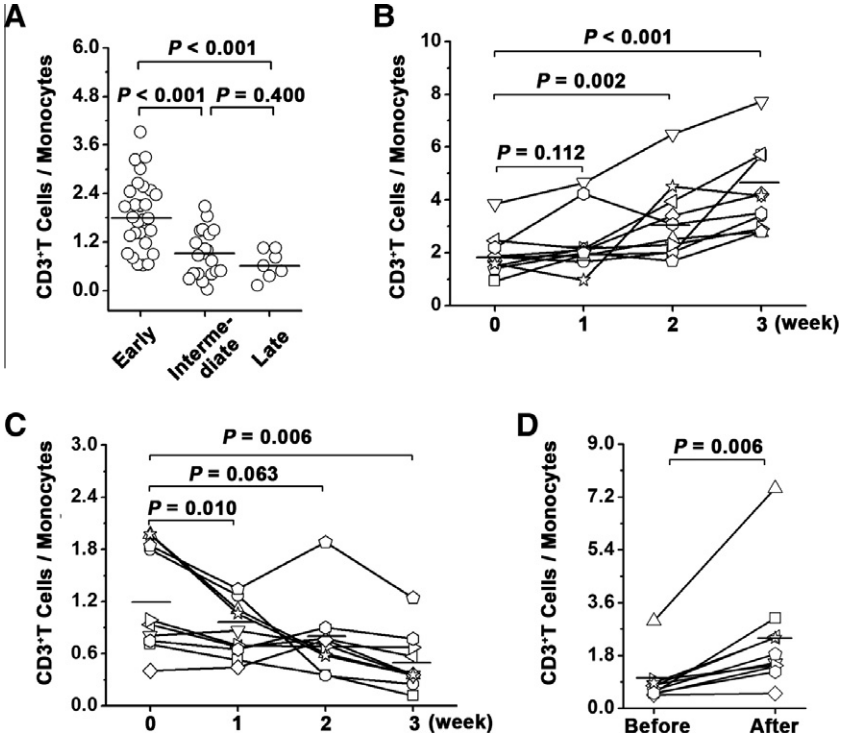

Fig. 2. Decreased T/M ratios predicted poor prognosis in ACLF patients. (A) The T/M ratios decreased from patients in the early stage (n = 28) to those in the intermediate (n = 20) stage and reached its lowest level in the late stage (n = 7) of ACLF. A follow-up observation for T/M ratios was carried out in two cohorts of patients with improved (B) (n = 10) or poor prognosis (C) (n = 10). (D) The peripheral T/M ratios significantly increased in ACLF patients (n = 10) after liver transplantation. The black bars indicate the mean values.

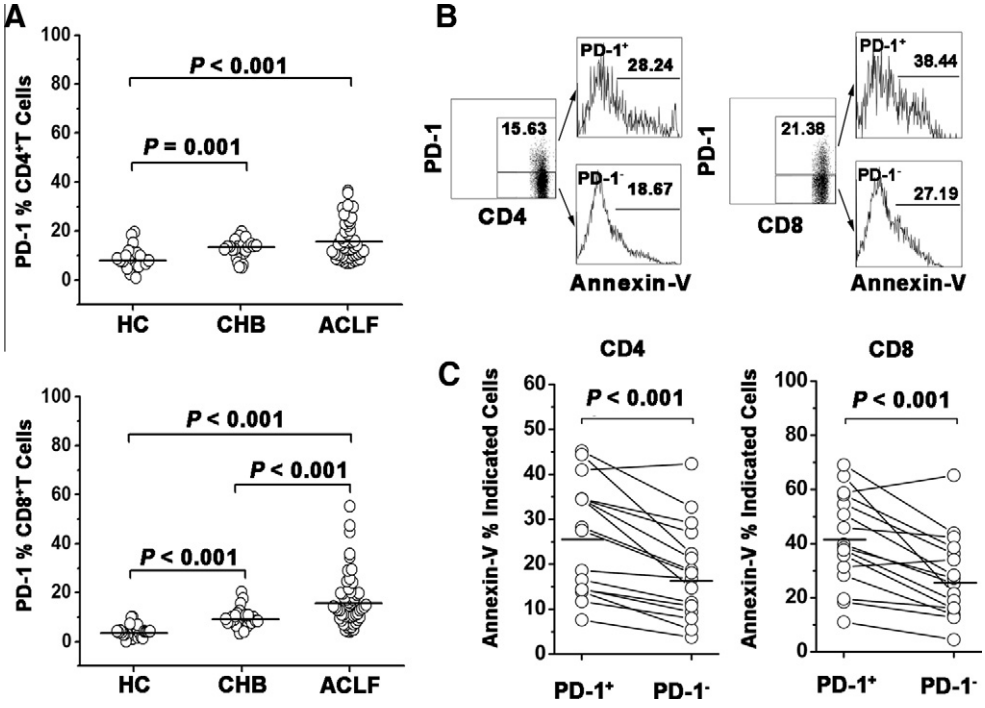

Fig. 3. PD-1 contributed to T-lymphocyte loss in ACLF patients. (A) Combined statistical results showed that the levels of PD-1 on $CD4^+$ T and $CD8^+$ T lymphocytes were upregulated in patients with ACLF (n = 55), compared with CHB (n = 30) and HC (n = 30). Black bars indicate the mean values. The representative (B) and combined statistical (C) results showed that PD-1-positive $CD4^+$ T and $CD8^+$ T lymphocytes were more prone to apoptosis (n = 16). Numbers shown in the representative dot and histogram plots indicate the frequencies of gated cells.

patients possessed a decreased T/M ratio compared with HC and CHB. Similar with former investigations, we also found numerous CD3-positive lymphocytes infiltrating in the ACLF liver. However, the expression of macrophages/monocytes seemed much more predominant and the CD3/CD68 ratios were even lower than HC and CHB. All the data indicated that the skewed T/M ratios were

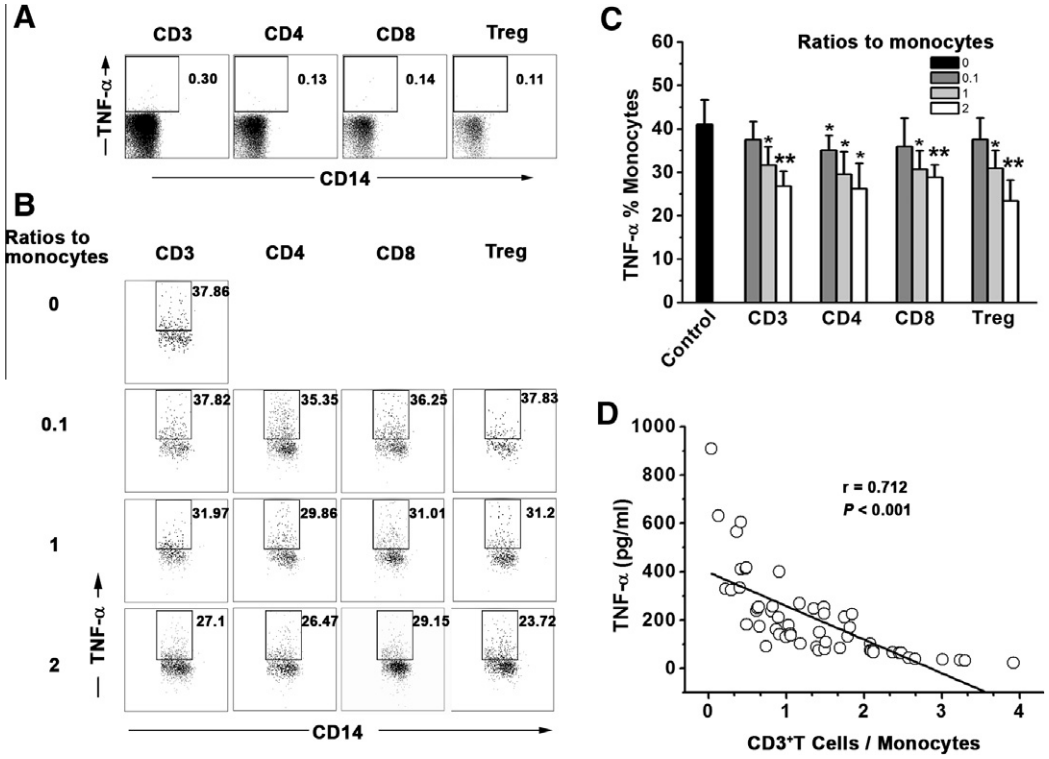

Fig. 4. T lymphocytes could inhibit TNF-α-secretion by monocytes. $CD3^+$ T, $CD4^+$ T, $CD8^+$ T and Treg cells were co-cultured with monocytes at different ratios for 24 h before intracellular TNF-α detection. (A) $CD3^+$ T, $CD4^+$ T, $CD8^+$ T and Treg cells produced a paucity of TNF-α upon stimulation with LPS. Numbers shown in the representative plots indicate the frequencies of gated cells. The representative (B) and combined statistical results (C) showed that $CD3^+$ T, $CD4^+$ T, $CD8^+$ T, as well as Treg cells, could inhibit TNF-α-section by monocytes. $^*P < 0.05$; $^{**}P < 0.01$, compared with control. At least three trials were employed in each experiment. (D) The T/M ratios negatively correlated with TNF-α levels in the serum of ACLF patients ($n = 55$).

correlated disease progression in HBV-infected patients and this result was further verified within ACLF patients for different clinical stages, standard or transplant treatments. To clarify the mechanism underlying immunopathogenesis for ACLF related to the decreased T/M ratios, the *in-vitro* co-culture assay were employed to evaluate the suppressive effect of T lymphocytes on the secretion of TNF-α, the major proinflammatory cytokine for hepatocyte apoptosis and necrosis, by monocytes. Together with the findings in animal model [20,22], human T lymphocytes, including $CD3^+$ T, $CD4^+$ T and $CD8^+$ T cells could constrain the TNF-α-secretion. Moreover, the inhibition potentials between common T lymphocytes and Treg were comparable, indicating a universal and evolutionary characteristic for T lymphocytes to constrain innate immune cells. As nature killer T and gamma–delta T cells were also included in $CD3^+$ T lymphocyte at low frequencies, the following experiments should be employed to identify the similar characteristics for both of the CD3-positive subsets. Still, the mechanism and manner that govern the suppressive crosstalk between T lymphocytes and monocytes should be further investigated.

Although the mechanisms for decreased circulating T lymphocytes remained unclear, our data showed that PD-1, a co-inhibitory receptor that indicates the apoptotic sensitivity of T cells, was highly expressed on both $CD4^+$ T and $CD8^+$ T cells in these HBV-related ACLF patients. Further analyses showed that PD-1-positive cells were prone to apoptosis when compared with their negative counterparts. We also found that the expression of PD-L1, the ligand for PD-1, was enhanced on both monocytes and hepatocytes (unpublished data). Together with our former investigations with regards to PD-1-induced T cell attrition during acute HBV infection [24], our data suggests that PD-1 was upregulated on both $CD4^+$ and $CD8^+$T cells, which at least in part contributed to the T-cell loss in these HBV-related ACLF patients. An alternative explanation for decreased T lymphocytes in peripheral blood is that T lymphocytes are largely recruited and accumulated into the liver through a chemokine-dependent pathway; and previous investigation showed that the intrahepatic $CD8^+$T cell number increased by approximately 50-fold in ACLF patients compared to normal subjects [3]. Although we also observed increased T lymphocytes infiltrated livers that were inflamed, we found that intrahepatic $CD3^+$ T cells did not manifest a relative enhancement compared with monocytes. Moreover, the CHB, as an early stage for ACLF, manifested lower PD-1-expression and higher T-lymphocyte counts in the peripheral blood and especially higher T/M ratios in the liver compared with both HC and ACLF, indicating a tolerant characteristic of the liver and the crucial role of $CD3^+$ T lymphocytes in the maintenance of liver tolerance.

In conclusion, our study identified for the first time the potential role of T/M ratios in predicting disease progression in patients with HBV-related ACLF. We also provide novel explanations for the loss of circulating T lymphocytes, as well as the deleterious effects on acute inflammatory exacerbations in patients with ACLF. Therefore, these findings would extend our understanding of the underlying mechanisms of ACLF from a new perspective.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.096.

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