



Original Article

PD-1 Involvement in Peripheral Blood CD8⁺ T Lymphocyte Dysfunction in Patients with Acute-on-chronic Liver Failure

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Abstract

Background and Aims: Programmed cell death-1 (PD-1) plays an important role in downregulating T lymphocytes but the mechanisms are still poorly understood. This study aimed to explore the role of PD-1 in CD8⁺ T lymphocyte dysfunction in hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF). **Methods:** Thirty patients with HBV-ACLF and 30 healthy controls (HCs) were recruited. The differences in the numbers and functions of CD8⁺ T lymphocytes, PD-1 and glucose transporter-1 (Glut1) expression from the peripheral blood of patients with HBV-ACLF and HCs were analyzed. *In vitro*, the CD8⁺ T lymphocytes from HCs were cultured (HC group) and the CD8⁺ T lymphocytes from ACLF patients were cultured with PD-L1-IgG (ACLF+PD-1 group) or IgG (ACLF group). The numbers and functions of CD8⁺ T lymphocytes, PD-1 expression, glycogen uptake capacity, and Glut1, hexokinase-2 (HK2), and pyruvate kinase (PKM2) expression were analyzed among the HC group, ACLF group and ACLF+PD-1 group. **Results:** The absolute numbers of CD8⁺ T lymphocytes in the peripheral blood from patients with HBV-ACLF were lower than in the HCs ($p < 0.001$). The expression of PD-1 in peripheral blood CD8⁺ T lymphocytes was lower in HCs than in patients with HBV-ACLF ($p = 0.021$). Compared with HCs, PD-1 expression was increased ($p = 0.021$) and Glut1 expression was decreased ($p = 0.016$) in CD8⁺ T lymphocytes from the HBV-ACLF group. *In vitro*, glycogen uptake and functions of ACLF CD8⁺ T lymphocytes were significantly lower than that in HCs ($p = 0.017$; all $p < 0.001$). When PD-1/PD-L1 was activated, the glycogen uptake rate and expression levels of Glut1, HK2, and PKM2 showed a decreasing trend (ACLF+PD-1 group compared to ACLF group

, all $p < 0.05$). The functions of CD8⁺ T lymphocytes in the ACLF+PD-1 group [using biomarkers of Ki67, CD69, IL-2, interferon-gamma, and tumor necrosis factor-alpha- were lower than in the ACLF group (all $p < 0.05$). **Conclusions:** CD8⁺ T lymphocyte dysfunction is observed in patients with HBV-ACLF. PD-1-induced T lymphocyte dysfunction might involve glycolysis inhibition.

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Introduction

Hepatitis B virus (HBV) infection-induced acute-on-chronic liver failure (ACLF) (i.e. HBV-ACLF) is a common clinical condition of critical liver diseases, with rapid progression and 28- and 90-day transplantation-free mortality rates of 32.8% and 51.2%, respectively.¹ The pathophysiology of ACLF is related to an initial widespread immune activation, systematic inflammatory response syndrome, and secondary sepsis due to immune dysfunction.² ACLF increases the risk of secondary infection and infection-related death.^{3–6} ACLF is an important cause of hepatic encephalopathy, hepatorenal syndrome, ascites, hyponatremia, and infectious shock.⁷ The exact mechanisms involved in the immune dysfunction of patients with HBV-ACLF are poorly understood.

Wasmuth *et al.*⁸ proposed that the pathogenesis of ACLF is similar to that of sepsis-like immune paralysis, manifesting by reduced expression of human leukocyte antigen-DR molecules on the surface of monocytes in peripheral blood, inactivation of immune function, and reduced production of tumor necrosis factor-alpha (TNF- α). T lymphocyte dysfunction (manifested by increased apoptosis, weakened proliferative ability, and decreased reactivity or non-reactivity of T lymphocytes in ACLF)⁹ is observed in ACLF. Similar to sepsis, ACLF leads to decreased numbers of CD4⁺ and CD8⁺ T lymphocytes in peripheral blood,¹⁰ as well as low activation of CD8⁺ T lymphocytes in patients with ACLF.¹¹ The production of T lymphocyte-related cytokines in peripheral blood is also decreased.¹² Therefore, T lymphocyte dysfunction plays an important role in the immune suppression of patients with ACLF, but the specific mechanisms are still unknown.¹

Keywords: Acute and chronic liver failure; Programmed cell death 1; Immune function; Glycolysis; Glut1.

Abbreviations: ACLF, acute-on-chronic liver failure; Glut1, glucose transporter 1; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HC, healthy control; HK2, hexokinase-2; IFN- γ , interferon-gamma; MFI, mean fluorescence intensity; PBMC, prepared peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PD-1, programmed cell death-1; PD-L1/2, programmed cell death 1-ligand 1/2; PKM2, pyruvate kinase; TNF- α , tumor necrosis factor-alpha.

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Table 1. Characteristics of the ACLF patients and HCs at the time of hospital admission

Indicator	ACLF patients, n=30	HCs, n=30	p ^a
Age in years	53.2±9.2	50.5±11.2	0.595
Female, n (%)	12 (40.0)	13 (43.3)	0.962
BMI in kg/m ²	23.6±3.6	25.6±2.9	0.496
ALT in U/L	121.4±90.8	33.3±11.1	<0.001*
AST in U/L	129.6±41.7	24.3±6.3	<0.001*
TBIL in µmol/L	410.4±143.7	17.7±5.8	<0.001*
PTA, %	28.5±4.2	120.0±5.1	<0.001*
INR	2.1±0.3	0.9±0.2	<0.001*
Albumin in g/L	29.1±3.5	39.5±1.6	<0.001*
Urea in mmol/L	5.2±1.4	5.4±1.1	0.474
Creatinine in µmol/L	70.3±6.3	67.1±3.1	0.133
MELD score	23.5±5.5	–	–

*p<0.05. ^ap-values were acquired by chi-square test or t-test. ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HCs, healthy controls; INR, international normalized ratio; MELD, model for end-stage liver disease; PTA, prothrombin activity; TBIL, total bilirubin.

ATP provided by glycolysis and the electron transport chain is the fuel for the activity and function of any cell.^{13,14} The activation of T lymphocytes is accompanied by changes in glucose metabolism. Naïve T lymphocytes are dependent on oxidative phosphorylation for energy, but once stimulated, they differentiate into effector T lymphocytes, with the reprogramming of metabolic patterns, increasing the expression of glucose transporter 1 (Glut1) on the cell membrane, promoting glycogen uptake and using it as the main energy supply, and reducing oxidative phosphorylation.¹⁵ Glycolysis levels affect T lymphocyte proliferation, activation, and immune function.^{16,17}

Programmed death-1 (PD-1) is mainly expressed in activated CD8⁺ T lymphocytes, and its ligands are programmed cell death 1-ligand 1 and 2 (PD-L1 and PD-L2).¹⁸ The PD-1/PD-L1/2 co-stimulatory signaling pathway plays an important negative regulatory role in T lymphocyte activation, proliferation, and cytokine secretion.¹⁹ In chronic lymphocytic leukemia, PD-1 inhibits key glycolytic enzymes [hexokinase-2 (HK2) and pyruvate kinase (PKM2)] in monocytes, and blocking PD-1 can restore their glycolytic levels.²⁰ *In vitro*, PD-1 inhibits the uptake and use of glycogen in T lymphocytes and affects the differentiation of T lymphocytes.²¹ Whether PD-1 mediates its negative function by regulating the glycolysis of T lymphocytes is currently unknown. Therefore, this study explored how PD-1 mediates T lymphocyte dysfunction in ACLF by regulating the glycolytic pathway. This study provides insights into the immune dysfunction observed in ACLF.

Methods

Subjects

Thirty patients with HBV-ACLF from the Gastroenterology Department of Shanxi Baijiu Hospital were screened from June 2018 to June 2019, and 30 healthy controls (HCs) were recruited during the same period from among healthy volunteers. The study protocol was approved by the Ethics Committee of Shanxi Baijiu Hospital, Taiyuan (No. 2017LL039), China. The study was conducted in accordance with the Declaration of Helsinki. All patients and healthy volunteers pro-

vided written informed consent prior to study inclusion.

For patients with HBV-ACLF, the inclusion criteria were: 1) ACLF with positivity for hepatitis B surface antigen (HBsAg) or positivity for HBV DNA; and 2) meeting the diagnostic criteria for ACLF by the Asian Pacific Association for the Study of the Liver:²² total bilirubin ≥85 µmol/L, international normalized ratio ≥1.5, or prothrombin activity ≤40%. For the HCs, the inclusion criteria were: 1) normal liver function; and 2) negative HBsAg.

The exclusion criteria for all the subjects were: 1) alcoholic, drug-induced, or other viral hepatitis; 2) cancer; or 3) other diseases involving the immune system.

The clinical data of the subjects are shown in Table 1.

Detection of the expression levels of CD8⁺ T lymphocyte subsets

EDTA-K₂ anticoagulation tubes were used to collect the peripheral blood samples to detect the absolute number of CD8⁺ T lymphocytes. Whole blood (100 µL) was added to a test tube with standard microspheres and 10 µL of CD3-PC5- and CD8-PE-labeled monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ, USA). The suspension was mixed thoroughly and incubated at room temperature for 20–30 m. Then, 2 mL of erythrocyte solution was added, mixed thoroughly, and incubated at room temperature for 10 m. The tubes were centrifuged at 1,500 rpm for 5 m, and the supernatant was discarded. Normal saline (2 mL) was added, the sample centrifuged at 1,500 rpm for 5 m, and the supernatant was discarded. Then, normal saline (1 mL) was added to resuspend the cells, and the sample subjected to flow cytometric analysis using a FACS Calibur system (BD Diagnostics, Sparks, MD, USA). Data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Detection of CD8⁺ T lymphocytes

The peripheral blood mononuclear cells (PBMCs) were sorted using Miltenyi cell sorting magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Positive selection was applied. CD8⁺ T lymphocytes magnetic bead antibody (20 µL; Miltenyi Biotec GmbH) was added, incubated for 15

m at 4°C, and 500 µL of magnetic bead buffer was added. After being washed three times, the cell suspension was passed through a magnetic column. The CD8⁺ T lymphocytes were retained in the column and subsequently eluted. The whole process was performed under sterile conditions. The cells were washed twice to remove the magnetic beads. After cells were added to the CD8-FITC-labeled antibodies (BD Biosciences), the purity of the sorted CD8⁺ T lymphocytes was detected by flow cytometry. Purity >99% was required for the subsequent experiments.

Evaluation of PD-1 and Glut1 expression levels

The sorted CD8⁺ T lymphocytes were resuspended in 2% phosphate-buffered saline (PBS) and stained with FITC-conjugated anti-PD-1 antibody (R&D Systems, Minneapolis, MN, USA) and FITC-conjugated anti-Glut1 antibody (Biolegend, San Diego, CA, USA). Mouse IgG2b (BD Biosciences) was used as an isotype control. The cells were incubated on ice for 30 m, washed twice with 2% PBS, and analyzed for PD-1 and Glut1 by flow cytometry.

Cell culture

According to a previous study,²³ the cells were purified using a CD8⁺ T lymphocyte purification column and cultured for 48 h. The CD8⁺ T lymphocytes sorted from HCs were immunized with anti-human CD28 (0.5 µg/mL) and anti-human CD3 (20 U/mL) antibodies (HC group). For the CD8⁺ T lymphocytes sorted from patients with HBV-ACLF, two groups were divided out. One group was cultured with anti-human CD28 (0.5 µg/mL) and anti-human CD3 (20 U/mL)+PD-L1-IgG fusion protein (Cat. No. 16-9989-82, at 10 µg/mL; eBioscience, San Diego, CA, USA) (ACLF+PD-1 group); the other group was added with anti-human CD28 (0.5 µg/mL) and anti-human CD3 (20 U/mL) + IgG (Cat. No. 16-4714-82; eBioscience) (ACLF group).

Detection of CD8⁺ T lymphocyte proliferative ability (Ki67), cell viability (CD69), and cytokine production [IL-2, interferon-gamma (IFN-γ), and TNF-α]

The CD8⁺ T lymphocytes were treated with CD3/CD28 and IL-12 stimulation *in vitro* for the detection of proliferative ability (Ki67) and cell viability (CD69). First, the cells were resuspended with 2% PBS, added with Ki67 (Cat. No. 558616; BD Biosciences) and CD69 (Cat. No. 310904; Biolegend) antibodies, incubated on ice for 30 m, washed twice with 2% PBS, and detected by flow cytometry. For the detection of IL-2 (Cat. No. 500310; Biolegend), IFN-γ (Cat. No. 502515; Biolegend), and TNF-α (Cat. No. 559321; BD Biosciences), phorbol myristate acetate (Enzo Life Sciences, Inc., Farmingdale, NY, USA) and ionomycin (Enzo Life Sciences, Inc.) were added, and the cells were cultured for 5 h. In the third hour of cultivation, the protein transport inhibitor Monensin 3 µM (BD Biosciences) was added. After centrifugation and washing, the cells were treated with fixation and membrane-breaking agents. The fluorescence-labeled antibodies were added and incubated for 30 m and detected by flow cytometry. The results were expressed as mean fluorescence intensity (MFI).

Detection of glucose uptake

At total of 1×10⁶ cells of CD8⁺ T lymphocytes were taken and

cultured at room temperature for 2 h in PBS, washed with PBS, added with 1 µCi/ml of 2-deoxy-D-[³H]-labeled glucose, and incubated for 20 m. The cells were rinsed three times with pre-chilled PBS to stop the reaction. All operations were performed according to the instructions of the glucose uptake assay kit (Cat. No. ab136955; Abcam, Cambridge, UK). The glucose levels were quantified using the FLUOstar Omega plate reader (BMG LABTECH GmbH, Ortenberg, Germany). The results were expressed as the average of three tests.

Western blotting

The total cell proteins were extracted from the HC, ACLF, and ACLF+PD-1 groups, respectively. The protein concentration was determined by the bicinchoninic acid method, and the proteins (35 µg per sample) were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrotransferring the proteins to polyvinylidene fluoride membranes, the membranes were cut into strips and incubated with the corresponding antibody solution (anti-β-actin mouse monoclonal antibody, anti-HK2 mouse monoclonal antibody, and anti-PKM2 mouse monoclonal antibody) (Millipore Corp., Billerica, MA, USA) in 5% nonfat-dried milk at 4°C, with gentle agitation, overnight. After washing with Tris-buffered saline three times (30 m each time), the membranes were incubated with the secondary antibody, with gentle agitation, at room temperature, for 1 h. Finally, the membranes were washed three times with Tris-buffered saline, developed with a chemiluminescence solution, and photographed.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). According to the Kolmogorov-Smirnov test, if the data fit the normal distribution pattern, continuous data were presented as mean ± standard deviation, and if the data did not fit the normal distribution pattern, median was presented. The clinical characteristic variables were analyzed using the independent-samples *t*-test and Pearson's chi-square test between ACLF patients and HCs. The numbers and functions of CD8⁺ T lymphocytes, PD-1 expression, glycogen uptake capacity, and Glut1, HK2 and PKM2 expression were analyzed using independent-samples *t*-test between the two groups. Two-sided (except for the chi-square test) *p*-values <0.05 were considered statistically significant.

Results

Absolute numbers of CD8⁺ T lymphocytes and the expression of PD-1 in the peripheral blood from HCs and patients with HBV-ACLF

The absolute numbers of CD8⁺ T lymphocytes in the peripheral blood from patients with HBV-ACLF were lower than in the HCs (333.88±147.74 vs. 872.50±206.64, *p*<0.001) (Fig. 1). The expression of PD-1 in peripheral blood CD8⁺ T lymphocytes was lower in HCs (7.02±2.12%) than in patients with HBV-ACLF (13.33±2.52%) (*p*=0.021) (Fig. 2).

Glycolysis and immune function analysis of CD8⁺ T lymphocytes in peripheral blood from HCs and patients with HBV-ACLF

The expression of Glut1 in CD8⁺ T lymphocytes in the pe-

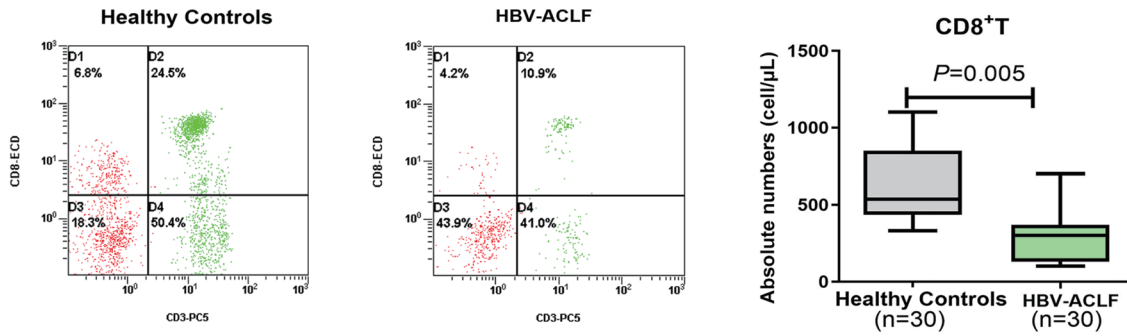


Fig. 1. Proportion and absolute numbers of peripheral CD8⁺ T cells in patients with HBV-ACLF and HCs. HBV-ACLF, hepatitis B virus infection-induced acute-on-chronic liver failure; HCs, healthy controls.

peripheral blood of patients with ACLF was lower than that in HCs ($13.33 \pm 1.40\%$ vs. $19.27 \pm 2.05\%$, $p=0.016$) (Fig. 3). *In vitro*, the glycogen uptake capacity of CD8⁺ T lymphocytes from patients with HBV-ACLF was significantly lower than that from HCs (2.8 ± 0.11 vs. 3.6 ± 0.14 pmol/cell, $p=0.017$) (Fig. 4). The expressions of Glut1, HK2, and PKM2 in CD8⁺ T lymphocytes in patients with HBV-ACLF were significantly lower than those in HCs (all $p < 0.001$) (Fig. 5).

Compared with HCs, the peripheral blood CD8⁺ T lymphocytes from patients with HBV-ACLF were in an immune

paralysis state. The cell viability (CD69) of ACLF CD8⁺ T lymphocytes was weaker than that of HCs (MFI: $1,722.9 \pm 142.5$ vs. $3,017.4 \pm 132.1$, $p < 0.001$). The proliferative ability (Ki67) of ACLF CD8⁺ T lymphocytes was weaker than that of HCs (MFI: $1,737.2 \pm 139.3$ vs. $2,603.4 \pm 172.8$, $p < 0.001$). The productive levels of cytokines of ACLF CD8⁺ T lymphocytes were lower than in HCs [IL-2 (MFI: $330,067.2 \pm 10,033.3$ vs. $150,586.9 \pm 9,157.2$, $p < 0.001$), IFN- γ (MFI: $2,423.2 \pm 115.6$ vs. $1,737.4 \pm 161.2$, $p < 0.001$), and TNF- α (MFI: $10,947.5 \pm 819.3$ vs. $4,049.6 \pm 241.5$, $p < 0.001$)] (Fig. 6).

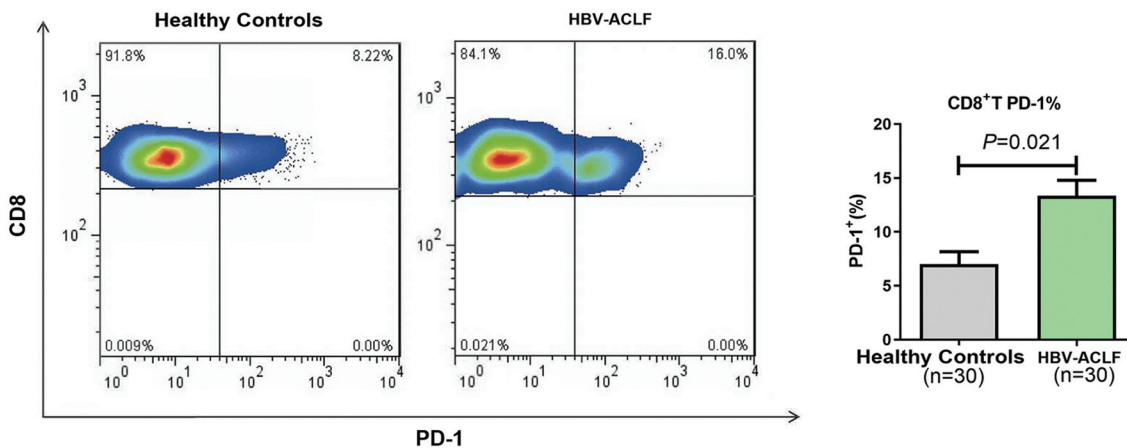


Fig. 2. Expression of PD-1 in peripheral CD8⁺ T cells of patients with HBV-ACLF and HCs. HBV-ACLF, hepatitis B virus infection-induced acute-on-chronic liver failure; HCs, healthy controls; PD-1, programmed cell death-1.

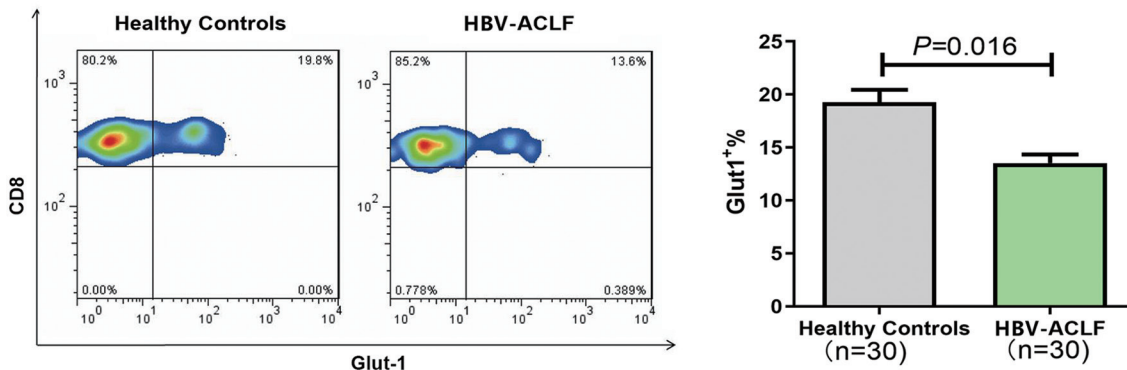


Fig. 3. Expression of Glut1 in peripheral CD8⁺ T cells of patients with HBV-ACLF and HCs. HBV-ACLF, hepatitis B virus infection-induced acute-on-chronic liver failure; HCs, healthy controls.

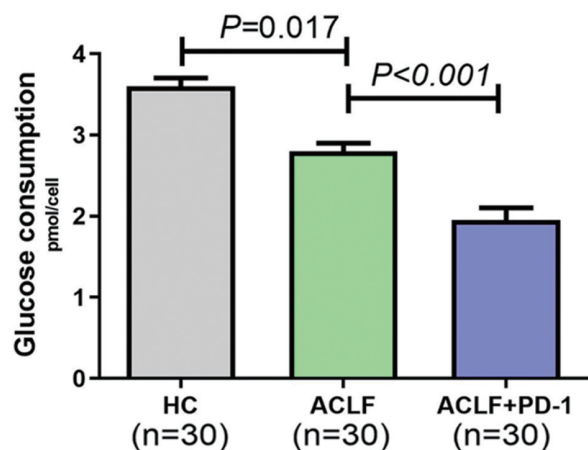


Fig. 4. Glycogen uptake capacity of CD8⁺ T lymphocytes from the Healthy controls (HCs), Acute-on-chronic liver failure (ACLF), and ACLF+PD-1 groups. ACLF, acute-on-chronic liver failure; HCs, healthy controls; PD-1, programmed cell death-1.

Effects of PD-1 on glycolysis and immune functions of CD8⁺ T lymphocytes in vitro

When PD-1/PD-L1 was activated, the expressions of Glut1, HK2, and PKM2 in CD8⁺ T lymphocytes in the ACLF+PD-1 group were lower than in the ACLF group (all $p < 0.001$) (Fig. 5). At the same time, the glycogen uptake in patients in the ACLF+PD-1 group was lower than in the ACLF group (2.0 ± 0.21 vs. 2.8 ± 0.11 pmol/cell, $p < 0.001$) (Fig. 4).

With regard to the immune function of CD8⁺ T lymphocytes in the PD-1/PD-L1 activated state, the cell viability (CD69) (MFI: 916.7 ± 43.3 vs. $1,722.9 \pm 142.5$, $p < 0.001$) and proliferative ability (Ki67) (MFI: 940.3 ± 71.3 vs. $1,737.2 \pm 139.3$, $p < 0.001$) of CD8⁺ T lymphocytes in the ACLF+PD-1 group were lower than in the ACLF group. The levels of secreted IL-2 (MFI: $64,267.1 \pm 3,643.7$ vs. $150,586.9 \pm 9,157.2$, $p < 0.001$), IFN- γ (MFI: $1,307.1 \pm 95.6$ vs. $1,737.4 \pm 161.2$, $p = 0.031$), and TNF- α (MFI: $2,099.5 \pm 119.3$ vs. $4,049.6 \pm 241.5$, $p < 0.001$) were lower than in the ACLF group (Fig. 7).

Discussion

Patients with HBV-ACLF often show immune dysfunction

and are prone to secondary infection, related complications, and mortality.³⁻⁶ T lymphocyte dysfunction is an important mechanism of ACLF immune suppression.^{10,11} The results of this study showed that PD-1 regulates CD8⁺ T lymphocyte dysfunction in patients with ACLF and that the immune dysfunction possibly involves the glycolytic pathway.

The pathogenesis of immune depletion in ACLF is similar to that observed in sepsis. The dysfunction or depletion of CD8⁺ T lymphocytes manifests as decreases in cell proliferation and secretion of effector cytokines (IL-2, IFN- γ , and TNF- α).²⁴ This study showed that the absolute number, viability, proliferative ability, and cytokine secretion of CD8⁺ T lymphocytes in the peripheral blood of patients with ACLF were lower than that of HCs. In addition, PD-1 expression was increased in CD8⁺ T lymphocytes in the peripheral blood of patients with HBV-ACLF. In order to explore the role of PD-1/PD-L1 signaling in the function of CD8⁺ T lymphocytes, PD-L1 was added to the culture medium to activate PD-1/PD-L1 signaling, and the viability, proliferation, and cytokines secretion abilities of the CD8⁺ T lymphocytes were weakened. The upregulation of PD-1 expression is an important mechanism involved in T lymphocyte immune dysfunction in cancer²⁵ and other conditions.²⁶ In the early stage of acute HBV infection, the upregulation of PD-1 expression on CD8⁺ T lymphocytes in peripheral blood helps reduce the damage to the liver by CD8⁺ T lymphocytes,²⁷ but this immune suppression participates in secondary infections. HBV-ACLF is often associated with immune depletion. The number of immune cells with upregulated PD-1 expression in liver tissues of patients with HBV-ACLF is higher than that of patients with chronic hepatitis B and HCs.²⁸ Similarly, Liu *et al.*²⁹ found that PD-1 expression is upregulated in CD8⁺ T lymphocytes in the peripheral blood of patients with HBV-ACLF and is directly proportional to the severity of the disease. Therefore, those results suggest that the PD-1/PD-L1 signaling pathway is involved in CD8⁺ T lymphocyte dysfunction or depletion in patients with HBV-ACLF.

The expression levels of Glut1 and the key glycolytic enzymes HK2 and PKM2 in the CD8⁺ T lymphocytes in the peripheral blood of patients with HBV-ACLF were decreased. The glycogen uptake rate of CD8⁺ T lymphocytes in peripheral blood of patients with HBV-ACLF was lower than that of HCs, and the glycogen metabolism provides ATP to the immune cells for their activities and functions.¹³ Once exposed to external stimuli, naïve T lymphocytes differentiate into effector T lymphocytes, a process accompanied by reprogramming of the metabolic patterns that involves increased Glut1 expression on the cell membrane and increased glycogen uptake.³⁰⁻³² In contrast, when Glut1 expression is decreased, the glycogen uptake and aerobic glycolysis of T

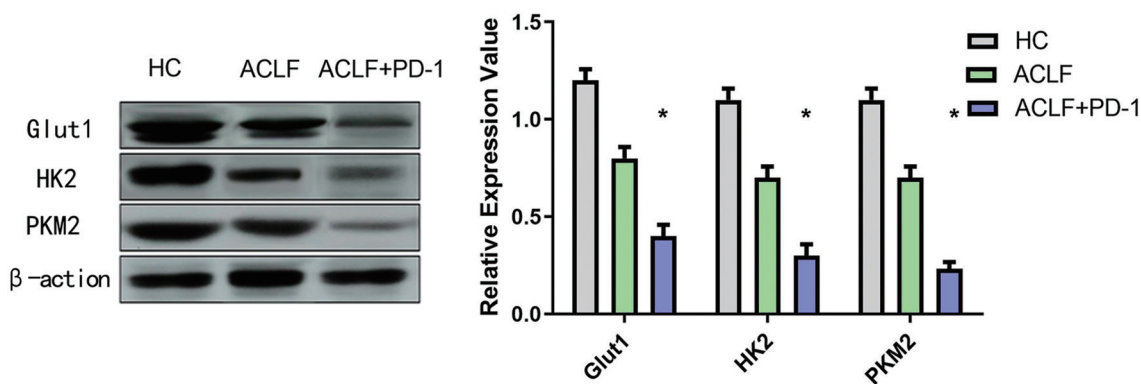


Fig. 5. Expression of Glut1 and key glycolytic enzymes (HK2 and PKM2) in the HCs, ACLF, and ACLF+PD-1 groups. *ACLF vs. ACLF+PD-1, $p < 0.05$, $n = 3$ experiments. ACLF, acute-on-chronic liver failure; Glut1, glucose transporter 1; HCs, healthy controls; HK2, hexokinase-2; PD-1, programmed cell death-1; PKM2, pyruvate kinase.

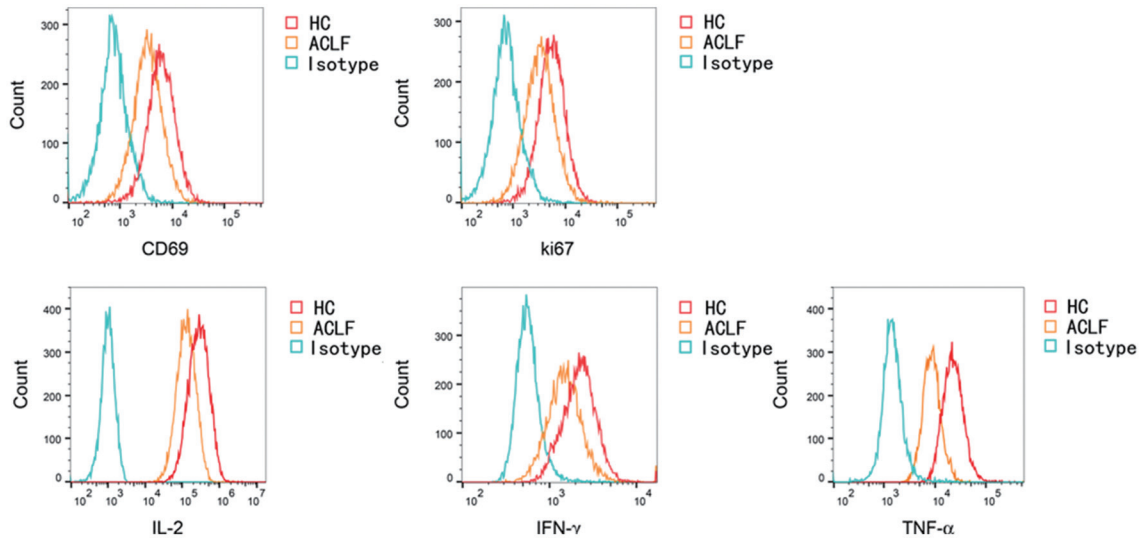


Fig. 6. Compared with HCs, the CD8+ T lymphocytes in peripheral blood from patients with HBV- ACLF were in an immune paralysis state. The cell viability (CD69) of ACLF patients (MFI: 1,723±143) was weaker than that of HCs (MFI: 3,017±132, $p < 0.001$). The proliferative ability (Ki67) of ACLF (MFI: 1,737±139) was weakened compared with that of HCs (MFI: 2,603±173, $p < 0.001$). Compared with HCs, the levels of secreted IL-2 (MFI: 330,067±10,033 vs. 150,587±9,157, $p < 0.001$), IFN-γ (MFI: 2,423±116 vs. 1,737±161, $p < 0.001$) and TNF-α (MFI: 10,948 ±819 vs. 4,050±242, $p < 0.001$) were decreased in the ACLF group. ACLF, acute-on-chronic liver failure; HBV- ACLF, hepatitis B virus infection-induced acute-on-chronic liver failure; HCs, healthy controls; IFN-γ, interferon-gamma; MFI, mean fluorescence intensity; TNF-α, tumor necrosis factor-alpha.

lymphocytes are reduced, thereby reducing the proliferation and differentiation capacity of T lymphocytes, especially of the effector T lymphocytes.¹⁶ In the present study, PD-L1 was added to the culture medium of CD8⁺ T lymphocytes from patients with HBV-ACLF to activate PD-1/PD-L1 signaling, and the expression levels of Glut1, HK2, and PKM2 were found to be significantly decreased, as was the glycolysis uptake capacity. Similarly, in chronic lymphocytic leukemia, PD-1 inhibits the glycolytic pathway of monocytes and blocking PD-1 can restore their glycolytic level, indicating that PD-1 can affect the regulation of T lymphocyte glycolysis.²⁰ The expression of Glut1 is under control of the

PI3K pathway, which can be interfered by PD-1.²³ The restoration of PI3K when blocking PD-1 is probably involved in Glut1 expression and the restored glycolysis.³³

A previous study involving non-small lung cancer indicated that blocking the PD-1/PD-L1 axis produces more effects on the function of CD8⁺ T cells than blocking the PD-1/PD-L2 axis.²⁵ This differential effect of PD-L1 and PD-L2 should be explored in ACLF CD8⁺ T cells in future studies. In addition, in cancer-associated CD8⁺ T cell dysfunction, blocking PD-1 only results in a partial restoration of the CD8⁺ T cell functions, indicating that other pathways are involved.^{25,34} Therefore, pathways like that regulated by lymphocyte-ac-

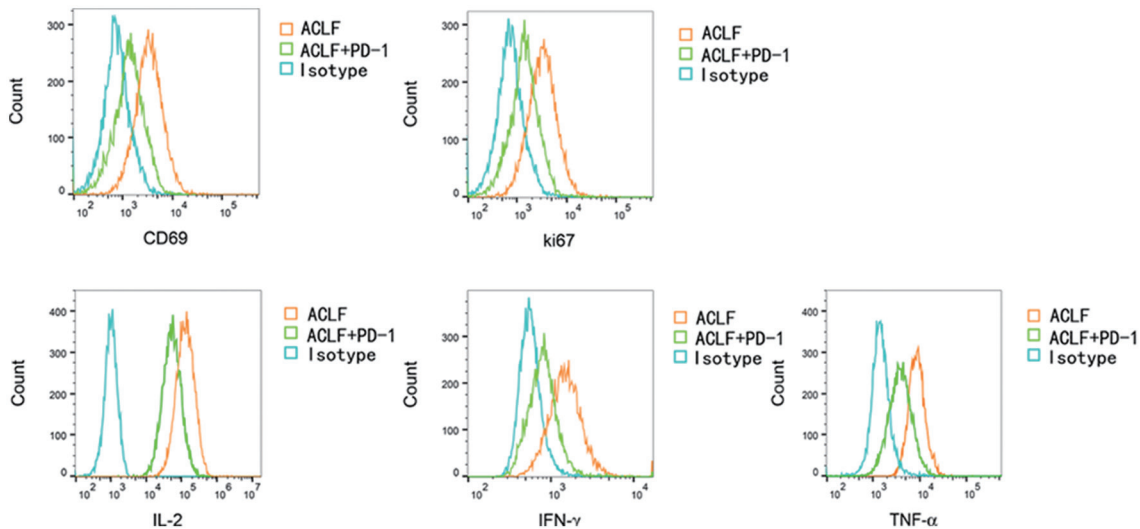


Fig. 7. When PD-1/PD-L1 was activated, the cell viability (CD69) (MFI: 917±43 vs. 1,723±143, $p < 0.001$) and proliferative ability (Ki67) (MFI: 940±71 vs. 1,737±139, $p < 0.001$) of CD8+ T lymphocytes in patients in the ACLF+PD-1 group were lower than those in the ACLF group. The levels of IL-2 (MFI: 64,267±3,644 vs. 150,587±9,157, $p < 0.001$), IFN-γ (MFI: 1,307±96 vs. 1,737±161, $p = 0.031$), TNF-α (MFI: 2,100±119 vs. 4,050±242, $p < 0.001$) were lower than those in the ACLF group. ACLF, acute-on-chronic liver failure; IFN-γ, interferon-gamma; MFI, mean fluorescence intensity; PD-1, programmed cell death-1; PD-L1/2, programmed cell death 1-ligand 1/2; TNF-α, tumor necrosis factor-alpha.

tivation gene-3 could also be involved in CD8⁺ T cell dysfunction,³⁵ and the related impact on energy metabolism of immune cells should be explored in ACLF. Dysfunctional T cells and T cells with activated PD-1 pathway are prone to apoptosis,³⁶⁻³⁸ and whether the dysfunctional energy metabolism contributes to apoptosis in CD8⁺ T cells in ACLF should be explored in the future.

In cancer, blocking the PD-1 axis shows benefits through the abrogation of immune tolerance toward the tumor.³⁹ In the present study, activating the PD-1 axis resulted in lower expression of IL-2, IFN- γ , and TNF- α . Decreased IL-2 expression will contribute to CD8⁺ T cell dysfunction,⁴⁰ and a decreased IFN- γ and TNF- α production will contribute to the onset of secondary infection.^{41,42} Whether blocking the PD-1 axis could be beneficial in patients hospitalized for ACLF remains to be examined, but the findings from the present study indirectly suggest that blocking PD-1 in such patients might have therapeutic value by restoring the activity of CD8⁺ T cells, which could decrease the occurrence of secondary infections. This is of importance since another study showed that the expression of PD-L1 and PD-L2 in the liver of patients with ACLF is higher than in patients with chronic hepatitis B without ACLF.²⁸ A recent review summarized the theoretical basis for the use of immune checkpoint inhibitors in patients with cirrhosis and ACLF,⁴³ but this theoretical topic will still have to be examined in future trials.

This study has limitations. It only brushed on the surface of the relationship between PD-1 and glycolysis, and additional studies are necessary to determine the exact relationships between the two. In addition, molecular studies are necessary to determine the exact genes and proteins that are regulated in this process.

In summary, the findings from this study suggest that PD-1 induces CD8⁺ T lymphocyte dysfunction in patients with HBV-ACLF, possibly by regulating the glycolytic pathway. The results of this study, thus, help clarify the role of PD-1 in the occurrence of immune suppression in ACLF, providing a new potential effective target molecule for the prevention and treatment of immune dysfunction in patients with ACLF as well as a new theoretical basis for disease prevention and treatment.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conception and design (JY), administrative support (JH),

provision of study materials or patients (YL), collection and assembly of data (NZ, TL), data analysis and interpretation (XZ), manuscript writing and final approval of the manuscript (all authors).

Data sharing statement

No additional data are available.

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