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Expression of Tim-3/Galectin-9 pathway and CD8+T cells and related factors in patients with cystic echinococcosis

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ABSTRACT

Objective: One of the primary reasons for the successful patriotization of Echinococcus multilocularis in patients is its ability to induce host immune tolerance. This study examined the expression of the immunosuppressive Tim-3/Galectin-9 pathway, CD8+T cells, and related factors in AE patients. The aim was to analyze the relationship between the Tim-3/Galectin-9 pathway and CD8+T cells in this disease and further understand the mechanism of immune tolerance induced by cystic echinococcosis.

Methods: Using flow cytometry, we evaluated the expression of CTL, $CD8^+CD28$-T cells, $CD8^+CD28$ + IFN-γ + T cells, $CD8^+CD28$+perforin + T cells, $CD8^+CD28$+granzyme B + T cells, $CD8^+CD28$-IL-10 + T cells, $CD8^+CD28$-TGF-β+T cells, and Tim-3 expression on CD8+T cells in the peripheral blood of control (n = 30) and AE patients (n = 33). qRT-PCR was used to measure CD107a and Tim-3/Galectin-9 mRNA levels in PBMCs from the control and AE groups. Immunohistochemistry was employed to detect IL-10, TGF-β, and Tim-3/Galectin-9 expressions in the infected livers of AE patients.

Results: AE patients exhibited a significant decrease in peripheral blood CTL ratio ($P < 0.001$) and an increase in $CD8^+CD28$+IFN-γ+T cell ratio ($P < 0.001$). No significant changes were observed in the ratios of $CD8^+CD28$+perforin + T cells ($P = 0.720$) and $CD8^+CD28$+granzyme B + T cells ($P = 0.051$). The proportions of $CD8^+CD28$-T cells ($P < 0.001$), $CD8^+CD28$-IL-10 + T cells ($P < 0.001$), and $CD8^+CD28$-TGF-β+T cells ($P < 0.001$) were notably higher than in the control group. The expression of Tim-3 on CTL and $CD8^+CD28$-T cells in AE patients was significantly upregulated ($P < 0.001$, $P < 0.001$). AE patients displayed a substantial decrease in peripheral blood PBMC CD107a mRNA levels ($P < 0.001$) and significant elevations in Tim-3/Galectin-9 mRNA levels ($P < 0.001$, $P < 0.001$). A negative correlation was observed between CD107a mRNA levels and both Tim-3 ($r\hat{}2 = 0.411$, $P < 0.001$) and Galectin-9 ($r^2 = 0.180$, $P = 0.019$) mRNA levels. Expressions of IL-10 ($P < 0.001$), TGF-β ($P < 0.001$), and Tim-3/Galectin-9 ($P < 0.001$, $P < 0.001$) in AE patient-infected livers were significantly higher than in uninfected regions. IL-10 and TGF-β expressions showed a positive correlation with Tim-3/Galectin-9.

Conclusion: This study suggests that the high expression of Tim-3 on CD8+T cell surfaces in AE patients might promote an increase in $CD8^+CD28$-T cells and related factors, while suppressing CTL and related factor expressions. This potentially induces the onset of immune tolerance, which is unfavorable for the clearance of Echinococcus multilocularis in patients, leading to the exacerbation of persistent infections.

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1. Introduction

Alveolar echinococcosis (AE) is a disease caused by Echinococcosis multilocularis (E.m), the larvae of Echinococcus multilocularis (E. m), parasitizing the human body (温浩. 包虫病学, 2015). An important reason in patients successfully parasitized by Echinococcus multilocularis is that infection can induce immune tolerance or evasion in the body (Gottstein, 1991; 景涛. 一种可能的泡球蚴免疫逃避行为, 1999; Wen et al., 1995; Wen and Craig, 1994). In this study, it was previously observed that a major cause of persistent infection with Echinococcus multilocularis was due to immune dysfunction of T cells, which showed immune imbalance in Th1/Th2, Treg/Th17 and other cells, so that the body could not effectively remove pathogens, and developed in a direction conducive to Echinococcus multilocularis infection (庞楠楠 et al., 2011; 赵慧 et al., 2012). Other studies have found that PD-1 is highly expressed on the surface of T cells around the lesion in AE patients mediating T cell immunodepleting in hepatic alveolar echinococcosis, resulting in immune tolerance in the liver region, and is involved in the process of fibrogenesis (智德, 2022). However, recent studies on parasitic infections have found that CD8 + T cell activation and failure are closely associated with disease progression. Leishmania donovani has been found to evade the response of CD8 + T cells by limiting CD8 + T cells, inducing their functional exhaustion, and promoting cell death (Barbosa Santos et al., 2017). CD8 + T cells are one of the main effector cells of anti-infective immune response, and their differentiation status is closely related to the intensity of immune response and the degree of infection. In chronic infection, CD8 + T cells tend to present a state of exhaustion, mainly characterized by reduced effector function and decreased proliferative capacity. CD8 + T cells are subdivided into naive T cells, effector T cells, and memory T cell subsets according to their differentiation status. Niave CD8 + T cells can differentiate into a variety of effector cells with different effects (Mayer et al., 2019). Among them, cytotoxic T lymphocytes (CTL cells) can differentiate to monitor the body and clear infections and play an important role in the immune system against pathogens (such as viruses, bacteria, and tumors). CTLs can kill target cells through a granulose-dependent perforin/granzyme pathway (Trapani and Smyth, 2002), and the magnitude of killing ability can be reflected by the amount of CDl07a they express (Wattrang et al., 2015). CTLs belong to Tc1 cells. Other CD8 + T-αβ cell subsets generated by different cytokine environments include Tc2, Tc9, Tc17, and CD8 + regulatory T cells (Casalegno Garduño and Däbritz, 2021). Dysfunction of CD8 + T can also trigger excessive immune responses, which lead to immune-mediated body damage or pathological responses. Like various T helper cell subsets, these cells express specific transcription factors and generate characteristic cytokines and effector molecules. Among them, CD8 + CD28-T cells belong to immunosuppressive CD8 + T cells and can secrete IL-10 and TGF-β to play a role (Filaci et al., 2007). Several studies have shown that exhausted CD8 + T cells express receptors such as PD-1, CTLA-4, TIM3, and LAG3 on their surface, which perform immunosuppressive functions and mediate immune tolerance or evasion (Hashimoto et al., 2022; Fairfax et al., 2020; Yang et al., 2021; Ma et al., 2019). T celll immunoglobulin and mucin domain containing molecule-3 (Tim-3) is a member of the TIM family. Tim-3 was demonstrated to be a molecule with inhibitory regulatory effects on immune responses, regulating apoptosis and immune tolerance in T cells (Rodriguez-Manzanet et al., 2009). Galectin-9 is a ligand for Tim-3 (Tang et al., 2013). Tim-3/Galectin-9 plays an important role in a variety of infectious diseases. Both Tim-3 + CD8 + T cells and Tim-3 + CD4 + T cells are significantly increased in patients infected with HIV (Gonçalves Silva et al., 2017; Prévost et al., 2020). Tim-3 + CD4 + T cells and Tim-3 + CD8 + T cell function exhibited a state of attrition compared to Tim-3 negative cells. In addition, blocking Tim-3 with neutralizing antibodies restored T-cell antiviral immune responses to some extent (Jones et al., 2008). Galectin-9, a ligand of Tim-3, is significantly up-regulated on NK cells in HIV-infected individuals, and Galectin-9 expression is associated with impaired expression of the cytotoxic effector molecules granzyme B, perforin (Motamedi et al., 2019; Rahmati et al., 2023). Studies on both HBV and chronic hepatitis C virus (HCV) infection have found that Tim-3 is involved in the regulation of T cell, especially CD8 + T cell damage during chronic infection (Dong et al., 2017). In addition, the number of Tim-3 and Galectin-9 positive cells in the lung and mediastinal lymph node tissues of Plasmodium infected mice was found to be significantly increased compared with normal controls in parasitic infections; the Tim-3/Galectin-9 pathway acts on Kupffer cells and may be involved in the immunopathological response of the liver of Plasmodium berghei infected mice by regulating the secretion of type I interferons (Zhang et al., 2019). During schistosome infection, Th1 cell apoptosis mediated by Tim-3/Galectin-9 pathway may be an important mechanism of Th2 immune shift induced by schistosomes, and after inhibiting Tim-3/Galectin-9 pathway, Th1 cells proliferate significantly, making Th2 immune polarization affected to some extent (Tang et al., 2015). In addition, studies on alveolar echinococcosis have also found Tim-3/Galectin-9 signaling pathway may be involved in the development of persistent infection of Em by regulating the production of Th1 and Th2 cytokines (Li et al., 2022).

Effective CD8 + T cell responses are the result of reciprocal balance between positive and negative costimulatory signals. At present, there are few reports on the role of negative costimulatory signal Tim-3 in regulating CD8 + T cell immune response in hydatid cyst infection. Therefore, in this study, we detected the expression of Tim-3/Galectin-9 pathway and CD8 + T cells and related factors in AE patients and analyzed the relationship between Tim-3/Galectin-9 pathway and CD8 + T cells in alveolar echinococcosis to provide new ideas and theoretical basis for further understanding the causes of host immune tolerance induced by alveolar echinococcosis and future treatment.

2. Material and methods

2.1. Material

2.1.1. Study subjects

The subjects of the study were divided into a control group (n = 30) and an infection group (n = 33).

Control Group: This group consisted of 30 healthy individuals, with 18 males and 12 females. Their age ranged from 20 to 65 years, with an average age of 43.25 ± 14.09 years.

Infection Group: This group included 33 patients with AE (Alveolar Echinococcosis) undergoing their first surgery. There were 18 males and 15 females, with ages ranging from 12 to 68 years and an average age of 40.39 ± 14.05 years (as shown in Table 1).

This study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (Ethics number:

Tables 1–1
Clinical characteristics of enrolled subjects.

Clinical features	All kinds of patients	
	HC(n = 30)	AE (n = 33)
Age	43.25 ± 14.09	40.39 ± 14.05
Sex ratio		
Female: Male	12:18	15:18
Ethnic group		
Han ethnic group	10	7
Uygur	6	1
Tibetan ethnic group	0	15
Kazak	5	5
The Mongol nationality	1	3
Other	8	2
Eosinophil (%)	1 ± 0.5	2.8 ± 2.4
Liver function		
AST (U/L)	30.42 ± 7.05	369.51 ± 235.81
ALT (U/L)	28.67 ± 10.78	350.42 ± 215.19
GGT (U/L)	38.94 ± 13.28	106.73 ± 85.26

20160218-14). All patients and healthy volunteers willingly signed an informed consent form.

2.1.1.1. Inclusion criteria. Infection Group: The AE patients were selected from those diagnosed with AE at the Hepatobiliary Echinococcosis Department of the Digestive Vascular Surgery Center of the First Affiliated Hospital of Xinjiang Medical University between January 2019 and October 2021. All patients were diagnosed using the criteria set by the unofficial organization for echinococcosis of the World Health Organization. Patients were included based on their medical history, clinical signs, symptoms, laboratory, and radiological examinations, with the diagnosis of AE further confirmed by intraoperative pathology.

Control Group: Healthy individuals were selected from those who underwent health check-ups at the Health Management Examination Center of Xinjiang Medical University between January 2019 and October 2021. Those with a history of exposure to endemic areas, chronic infections (viral, bacterial, parasitic, etc.), other significant diseases, hepatobiliary diseases, or a history of excessive alcohol consumption were excluded.

2.1.1.2. Exclusion criteria. Infection Group: AE patients with chronic infectious diseases, other hepatobiliary diseases, autoimmune diseases, malignant tumors such as liver cancer, or those who have been on long-term immunosuppressive drugs like corticosteroids.

Control Group: Individuals with a history of exposure to endemic areas, chronic infections (viral, bacterial, parasitic, etc.), autoimmune diseases, other significant diseases like malignant tumors such as liver cancer, or those on long-term immunosuppressive drugs like corticosteroids.

2.1.1.3. Sample collection. For AE patients, 5 mL of venous blood with EDTA-2K anticoagulant was collected on the second day after admission. Part of the anticoagulated blood was used for flow cytometry detection; the rest was reserved for DNA extraction for qRT-PCR testing. Paraffin sections of the liver tissues of AE patients were obtained from the Department of Pathology of the First Affiliated Hospital of Xinjiang Medical University. These liver tissue paraffin sections were used for immunohistochemical testing. The collection and processing of samples from the control group were done in the same manner.

2.2. Methods

2.2.1. Flow cytometry detection

Upon collecting venous blood anticoagulated with EDTA-2K from both the infection and control groups, red blood cells were lysed within 4 h using red cell lysis solution. For every 100 μL of whole blood, 2 mL of 1 × red cell lysis solution was added. After thorough shaking and mixing, the mixture was left at room temperature for 8–12 min for lysis, until the cell suspension became clear and transparent. The cells were then washed, resuspended, and the concentration adjusted to 2×10^6/mL. The sample was divided into two:

For surface staining: 5 μL of fluorescent monoclonal antibodies conjugated with different fluorochromes were added, including anti-human APC-cy7-CD3, anti-human PE-cy7-CD8, anti-human FITC-CD28, and anti-human PE-Tim-3. Next, 5 μL of isotype control was added and incubated in the dark at 4 °C for 30 min. After washing, cells were resuspended in 500 μL PBS, transferred to a flow cytometry tube, and analyzed using software on the machine.

For intracellular staining: 4 μL of BD Leukocyte Activation Cocktail with BD GolgiPlug (a stimulant and protein transport inhibitor) was added and mixed well. The cells were then incubated in a 37 °C, 5% CO2 incubator or a 37 °C water bath for 4–6 h. Anti-human APC-cy7-CD3, anti-human PE-cy7-CD8, and anti-human FITC-CD28 (all from BD Company, USA) were then added and incubated in the dark at room temperature for 15–30 min. Post fixation and permeabilization of cells, intracellular staining was conducted. Cells were distributed into four tubes, with each tube receiving 80–100 μL of 1 × Perm/Wash Working Solution. Tube one had anti-human APC–IFN–γ added, tube two had anti-human APC-Perforin and anti-human PE-Granzyme B, tube three had anti-human PE-IL-10, and tube four had anti-human PE-TGF-β1. Each antibody was added at a volume of 5 μL, mixed thoroughly, and incubated in the dark at 2–8 °C for 40–50 min. After washing, cells were resuspended in 500 μL PBS and analyzed using a flow cytometer (Beckman Company, USA, DXflex model), with data subsequently analyzed using the appropriate software.

2.2.2. Immunohistochemistry detection

Paraffin blocks (n = 33) were sectioned into 3–4 μm thick pathological tissue slices using a microtome, which were then placed in a 60 °C oven for overnight baking. The tissue sections underwent a series of procedures including: Deparaffinization 、Antigen retrieval、Blocking endogenous peroxidase activity、Serum sealing; Primary antibody incubation with the following antibodies: Rabbit anti-human Tim-3 at a working concentration of 1:300 (Beijing Bioss Biotechnology Co., Ltd.), Rabbit anti-human Galectin-9 at a working concentration of 1:200 (Beijing Bioss Biotechnology Co., Ltd.), Rabbit anti-human IL-10 at a working concentration of 1:200 (Wuhan Servicebio), Rabbit anti-human TGF-β1 at a working concentration of 1:500 (Wuhan Servicebio)Secondary antibody incubation with:HRP-labeled goat anti-rabbit at a working concentration of 1:200 (Wuhan Servicebio), Chromogenic reaction with DAB (Wuhan Servicebio)、Counterstaining of cell nuclei、Dehydration and sealing of the slide, Finally, the sections were examined under a microscope, and images were captured and analyzed.

2.2.3. qRT-PCR detection

Primer sequences for human CD107a, Tim-3, and Galectin-9 were retrieved from GeneBank. The target gene cDNA sequences for CD107a, Tim-3, Galectin-9, and the reference gene β-actin were searched online in Genbank. Primers were designed using the DNA MAN software and synthesized by Shanghai Sangon Biotech Company (as shown in Table 2).

Total RNA from peripheral blood was extracted from both the control group (n = 30) and AE patients (n = 33). The purity and concentration of total RNA were determined. A spectrophotometer (Thermo SCIENTFIC, USA) was used for quantification. The nucleic acid analyzer measured the OD260 and OD280 values, and the OD260/OD280 ratio was calculated. If this ratio fell between 1.8 and 2.0, it indicated that the extracted RNA was relatively pure, free from residual proteins and DNA. The RNA was then reverse transcribed into cDNA using a reverse transcription kit (Takara, Japan).

The qRT-PCR assay was performed using fluorescent dye methodology (BIO-RAD, USA). The reaction parameters were: For Foxp3: 95 °C for 30 s (pre-denaturation), 95 °C for 15 s, and 60 °C for 30 s. For Tim-3: 95 °C for 30 s (pre-denaturation), 95 °C for 15 s, and 60 °C for 30 s.

For Galectin-9: 95 °C for 30 s (pre-denaturation), 95 °C for 15 s, and 60 °C for 30 s. For β-actin: 95 °C for 30 s (pre-denaturation), 95 °C for 15 s, and 58 °C for 30 s. Fluorescence was detected over 40 cycles, with a melting curve plotted. The number of cycles taken for the fluorescence signal in each reaction tube to reach the set threshold (Ct value) was recorded. Each sample was tested in triplicate.

For data analysis, qRT-PCR was employed to evaluate the differential gene expression across different tissues. The relative quantification method, often referred to as the (RQ) = $2^{-\Delta\Delta Ct}$ method, was frequently used to quantify the target genes by determining the Ct value corresponding to a specific fluorescence threshold.

2.2.4. Statistical analysis methods

All experimental data in this study were statistically analyzed using GraphPad Prism 5 software. Data are presented as mean ± standard deviation (SD). Grouped t-tests and Pearson linear correlation analyses were employed for comparative analyses between groups. A P-value of

Table 2
Gene primers for QRT PCR (human).

Gene name	Upstream primer (5'→3')	Downstream primer (5'→3')	PCR fragment size (bp)
Foxp3	CTCTTCTTCCTTGAACCCCAT	CTGGAGGAGTGCCTGTAAG	121
Tim-3	GAGTTACGGGACTCTAGATTGG	TGTTTTCTTCTGAGCGAATTCC	247
Galectin9	CTGGACAGATGTTCTCTACTCC	ACCACAGCATTCTCATCAAAAC	223
β-actin	CTCCATCCTGGCCTCGCTGT	GCTGTCAcCTTCACCGTTCC	269

less than 0.05 was considered statistically significant.

3. Results

3.1. A significant decrease in $CD8^+CD28^+$ T cell (CLT) ratio and a significant increase in $CD8^+CD28^-$ T cell and associated intracellular factor ratios in the peripheral blood of AE patients

Firstly, using flow cytometry, the proportions of CTL cells in the peripheral blood from the control group (n = 30) and AE patients (n = 33) were determined (as shown in Fig. 1). The results indicated that the percentage of CTLs in the peripheral blood of the control group was (19.44 ± 1.12) %. In contrast, the AE patients exhibited a percentage of (12.01 ± 3.57) %. Compared with the control group, the level of CTLs in the peripheral blood of AE patients significantly decreased ($P < 0.001$).

Through the staining of the surface and the inside of $CD8^+$ T cells in the peripheral blood, and utilizing flow cytometry, the ratios of $CD8^+CD28$+IFN-γ+ T cells, $CD8^+CD28^+$ Perforin + T cells, and $CD8^+CD28^+$ Granzyme B + T cells in both the control group and AE patients were measured (as depicted in Fig. 2). The results showed: in the control group:CD8+CD28+IFN-γ+ T cells accounted for (2.16 ± 0.27)%, $CD8^+CD28^+$ Perforin + T cells were (15.60 ± 4.39)%, $CD8^+CD28^+$ Granzyme B + T cells made up (2.42 ± 0.42)%, In AE patients:CD8+CD28+IFN-γ+ T cells comprised (6.94 ± 3.16)%, $CD8^+CD28^+$ Perforin + T cells were (15.23 ± 4.70)%, $CD8^+CD28^+$ Granzyme B + T cells made up (2.73 ± 0.80)%, Compared to the control group, the proportion of $CD8^+CD28$+IFN-γ+ T cells in AE patients significantly increased ($P < 0.001$). However, there was no significant difference in the ratios of $CD8^+CD28^+$ Perforin + T cells ($P = 0.720$) and $CD8^+CD28^+$ Granzyme B + T cells ($P = 0.051$). This suggests that the ability of CTLs in the peripheral blood of AE patients to secrete perforin and granzyme B, which are associated intracellular factors, is inhibited.

As indicated in Fig. 1, the percentage of $CD8^+CD28^-$ T cells in the peripheral blood of the control group were (13.06 ± 1.97) %. In comparison, AE patients exhibited a percentage of (35.51 ± 3.32) %. Relative to the control group, the level of $CD8^+CD28^-$ T cells in the peripheral blood of AE patients significantly increased ($P < 0.001$). This suggests that alterations in the $CD8^+$ T cell subpopulation ratios might be associated with alveolar echinococcosis (AE).

Analysis of Intracellular Factor Expression Levels in $CD8^+CD28^-$ T Cells of AE Patients Using Flow Cytometry, by staining the surface and inside of $CD8^+$ T cells and employing flow cytometry, the ratios of $CD8^+$CD28-IL-10+ T cells and $CD8^+$CD28-TGF-β+ T cells in the peripheral blood from both the control group (n = 30) and AE patients (n = 33) were determined. As depicted in Fig. 3:In the control group: CD8+CD28-IL-10+ T cells accounted for (1.68 ± 0.31)%, $CD8^+$CD28-

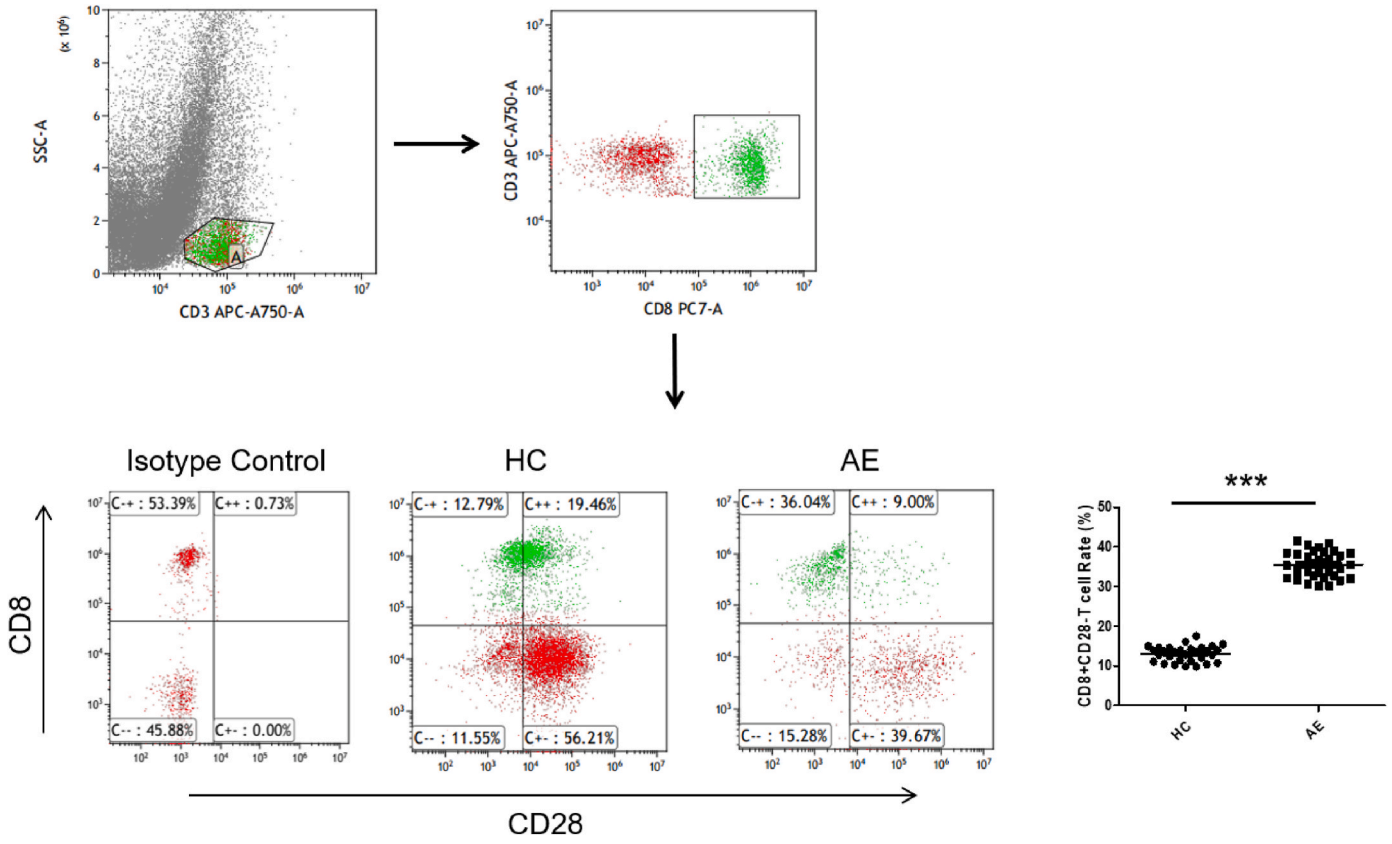

Fig. 1. Flow cytometry of CD8+Tcells in peripheral blood.

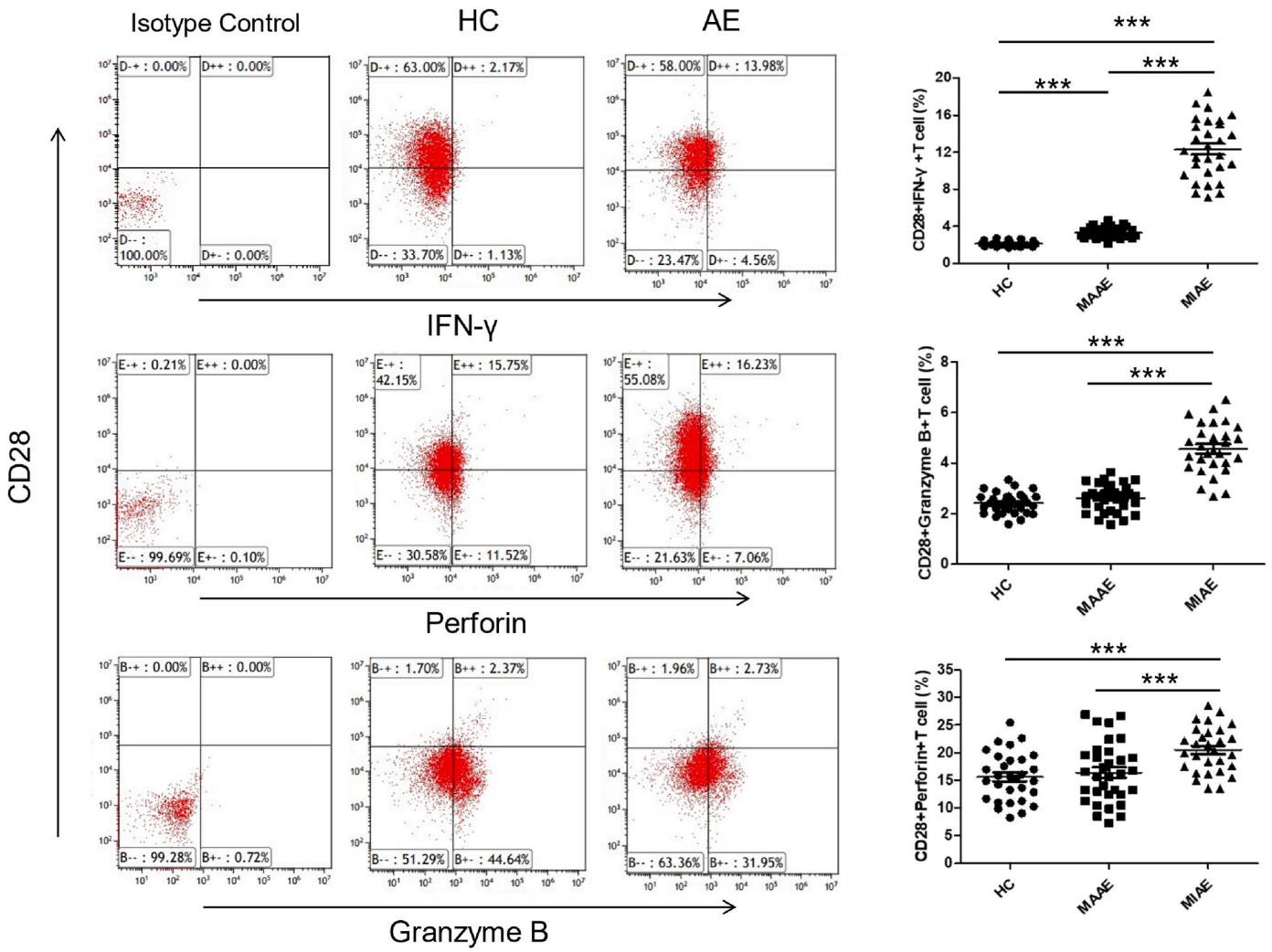

Fig. 2. Flow cytometric expression of intracellular factors in peripheral blood CTL of patients with AE and control group.

TGF-β+ T cells comprised (7.48 ± 1.34)%; In AE patients:CD8+CD28-IL-10+ T cells made up (5.37 ± 1.34)%, $CD8^+$CD28-TGF-β+ T cells were (18.12 ± 2.48)%; When compared to the control group, the ratios of both $CD8^+$CD28-IL-10+ T cells ($P < 0.001$) and $CD8^+$CD28-TGF-β+ T cells ($P < 0.001$) in AE patients were significantly elevated.

The findings above indicate a shift in the expression of $CD8^+$ T cells in AE patients, characterized by a diminished cytotoxic ability and an enhanced inhibitory function, possibly associated with the active progression of the infection.

3.2. Upregulation of Tim-3 expression on $CD8^+$ T cells in the peripheral blood of AE patients

To understand the relationship between Tim-3 and $CD8^+$ T cells in alveolar echinococcosis (AE), flow cytometry was utilized to measure the expression of Tim-3 on CTLs and $CD8^+CD28^-$ T cells in the peripheral blood of both the control group (n = 30) and AE patients (n = 33). As presented in Fig. 4:

The percentage of $CD8^+$CD28+Tim-3+ T cells in the peripheral blood of the control group was (1.46 ± 0.33)%, In contrast, AE patients had a percentage of (6.31 ± 2.13)% for $CD8^+$CD28+Tim-3+ T cells, Relative to the control group, the percentage of $CD8^+$CD28+Tim-3+ T cells in AE patients' peripheral blood notably increased ($P < 0.001$).

Additionally, flow cytometry was employed to evaluate the expression of Tim-3 on $CD8^+CD28^-$ T cells in the peripheral blood of both groups. As depicted in Fig. 4:

The control group had a percentage of $CD8^+$CD28-Tim-3+ T cells at (5.13 ± 1.87) %

For AE patients, this percentage was (13.48 ± 3.97) %

Compared with the control group, there was a significant increase in the percentage of $CD8^+$CD28-Tim-3+ T cells in the peripheral blood of AE patients, and the difference was statistically significant ($P < 0.001$). These results suggest that increased Tim-3 expression on CD8 + T cells may be associated with disease in vesicular maintenance disease.

3.3. There is a negative correlation between CD107a and Tim-3 and Galectin-9 mRNA levels in peripheral blood PBMC of AE patients

To understand the expressions of CD107a and Tim-3/Galectin-9 genes, the mRNA expressions of CD107a and Tim-3/Galectin-9 were measured in peripheral blood PBMC of controls (n = 30) and AE patients (n = 33) using QRT-PCR. The results showed that the expression of CD107a mRNA ($P < 0.001$) was significantly increased in peripheral blood PBMC from AE patients compared to controls (Fig. 5). Expression of both Tim-3 ($P < 0.001$, $P < 0.001$) and galectin-9 ($P < 0.001$) mRNA was significantly elevated in peripheral blood PBMCs from AE patients (Fig. 6, Fig. 7).

To investigate the relationship between CTL function related factor CD107a and Tim-3 mRNA levels in multilocular echinococcus infection, Pearson linear correlation analysis was used for statistical analysis in this study. The results showed that there was a negative correlation between CD107a and Tim-3 ($P < 0.001$) and Galectin-9 ($P = 0.019$)

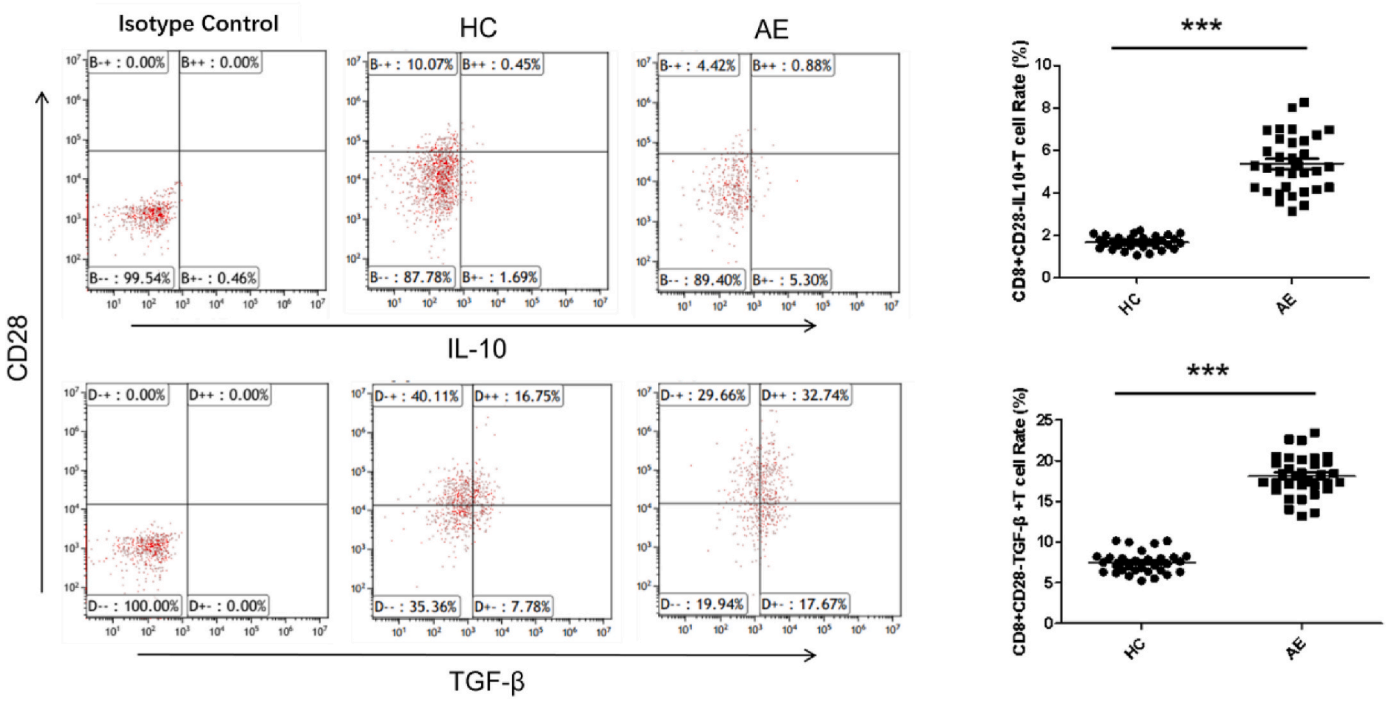

Fig. 3. Flow cytometry of intracellular factor expression in CD8$^+$CD28-T cells in peripheral blood.

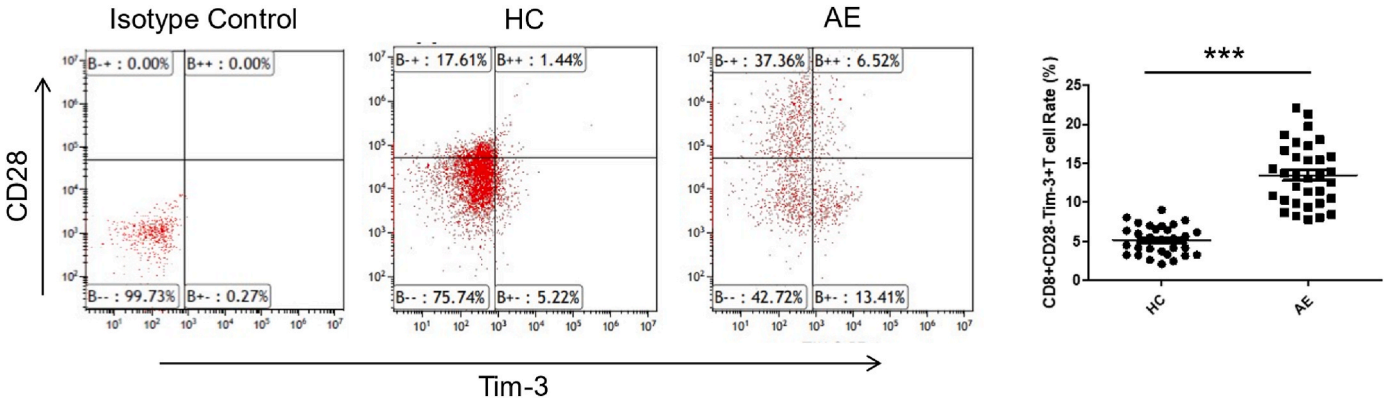

Fig. 4. Flow cytometry of Tim-3 expression on CD8+T cells in peripheral blood.

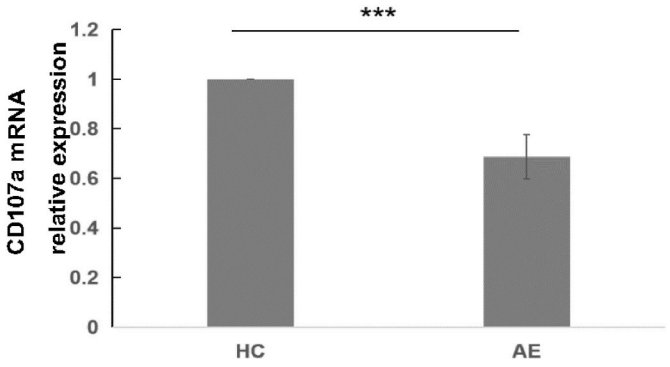

Fig. 5. The level of CD107a mRNA in PBMC.

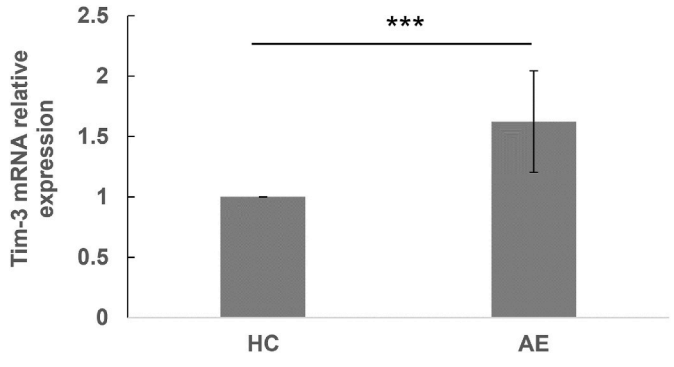

Fig. 6. The level of Tim-3 mRNA in PBMC.

mRNA levels in PBMC of AE patients, and the difference was statistically significant. (Fig. 8).

3.4. Expression of IL-10 and TGF-β1 was higher in liver tissue from AE patients proximal to infection than at levels distal to infection

Increased Expression of Tim-3 on CD8$^+$ T Cells in Alveolar

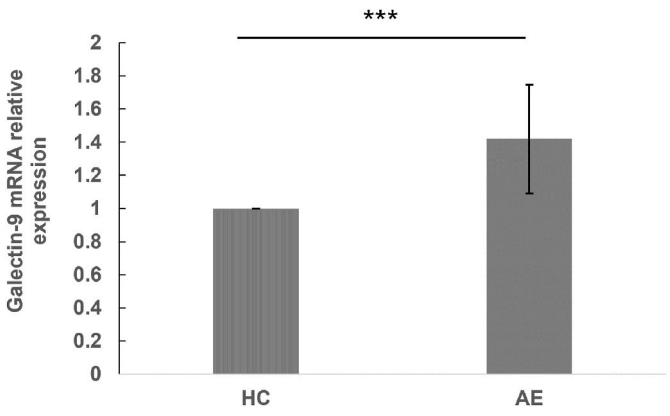

Fig. 7. The level of Galectin-9 mRNA in PBMC.

Echinococcosis Suggests Potential Association with the Disease Elevated Expression of IL-10 and TGF-β1 Near Infection Sites in the Liver Tissue of AE Patients.

To understand the expression of factors related to $CD8^{+}CD28^{-}$ T cells in the infected liver tissues of AE patients, the immunohistochemical method was used to examine the expression of IL-10 and TGF-β1 in areas near and distant from the infection. The results revealed that liver tissues in AE patients near the infection sites were eroded and damaged by multilocular echinococci. Surrounding these eroded areas, a significant infiltration of lymphocytes was observed, with a positive expression rate for IL-10 at (28.310 ± 7.865) % and for TGF-β1 at (29.567 ± 8.378) %. In contrast, in areas of the liver distant from the infection, the tissues remained uneroded by the multilocular echinococci, maintaining a relatively intact hepatic cell structure without significant lymphocyte infiltration. Here, the positive expression rates of IL-10 and TGF-β1 were notably lower, recorded at (2.477 ± 1.150) % and (3.073 ± 1.852) % respectively. Compared to areas distant from the infection in AE patient's liver tissue, the expression of IL-10 and TGF-β1 in areas proximate to the infection significantly increased, and the differences were statistically significant ($P < 0.001$ for both IL-10 and TGF-β1), as illustrated in Fig. 9.

3.5. Elevated expression of Tim-3/Galectin-9 near infection sites in liver tissue of AE patients

To investigate the expression of Tim-3/Galectin-9 in the infected liver tissues of AE patients, an immunohistochemical approach was utilized to assess the expression of Tim-3 and Galectin-9 both near and distant from infection sites. The results indicated that within the abundant lymphocytes observed near the infection sites in AE patient's liver tissues, the positive expression rate of Tim-3 was (23.353 ± 5.703) %, and for Galectin-9, it was (43.747 ± 6.629) %. Conversely, in areas of the liver distant from the infection, the positive expression rates of Tim-3 and Galectin-9 were significantly lower, recorded at (1.503 ± 0.576) % and (1.367 ± 0.589) % respectively. When compared to areas distant from the infection, the expression levels of Tim-3 and Galectin-9 near infection sites in AE patient's liver tissues were significantly elevated, with the differences being statistically significant ($P < 0.001$ for both Tim-3 and Galectin-9), as depicted in Fig. 10.

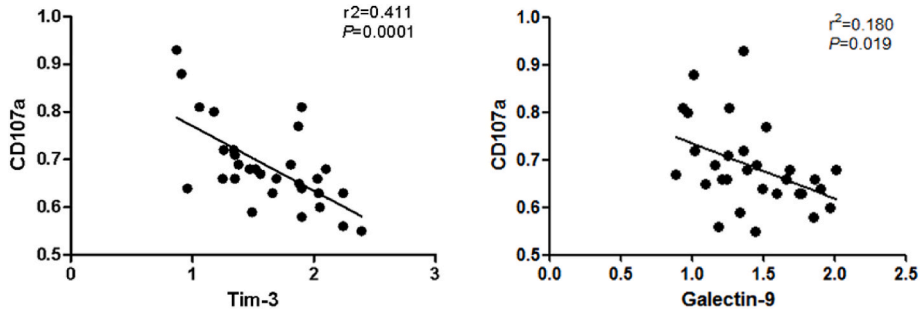

Fig. 8. Correlation analysis between CD107a and Tim-3/Galectin-9 mRNA levels.

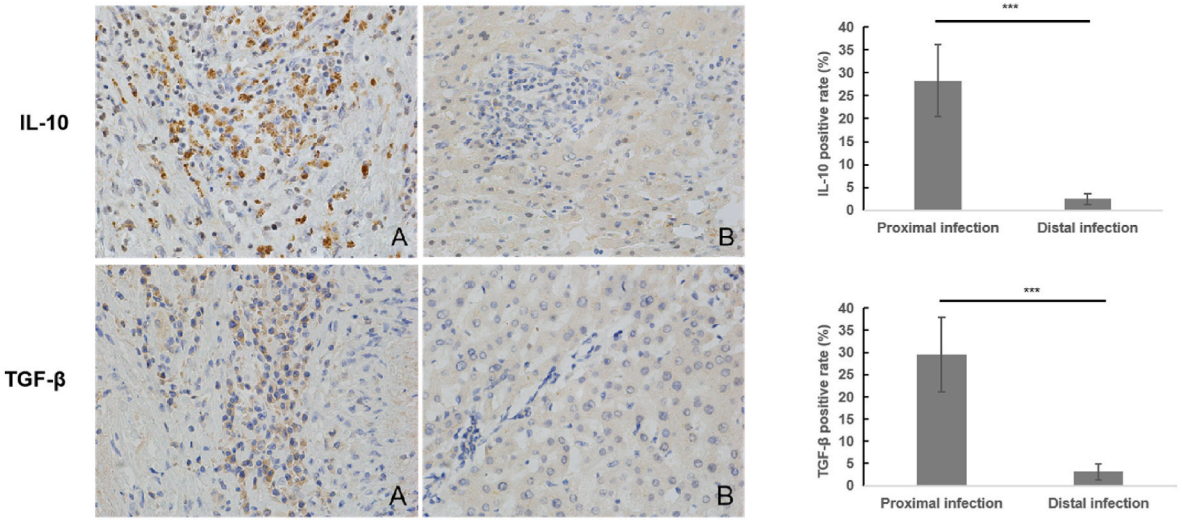

Fig. 9. Expression of IL-10 and TGF-β1 in liver tissue of AE patients.

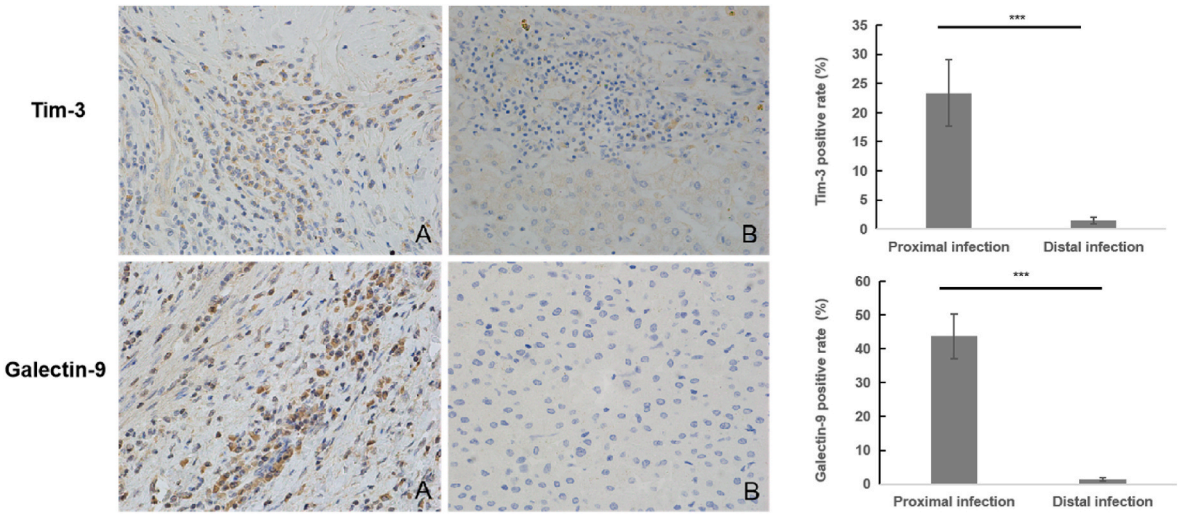

Fig. 10. Expression of Tim-3 and Galectin-9 in liver tissue of AE patients.

3.6. Positive correlation between CD8$^+$CD28-T cell-related factors IL-10, TGF-β1, and Tim-3/Galectin-9 expression in AE patient livers

To understand the relationship between the CD8$^+$CD28-T cell functional factors IL-10, TGF-β1, and Tim-3/Galectin-9 in the infected livers of AE patients, Pearson linear correlation analysis was employed. The results revealed a significant positive correlation between IL-10 ($P < 0.001$) and TGF-β1 ($P < 0.001$) with Tim-3/Galectin-9 expression in the liver tissues of AE patients, as illustrated in Fig. 11.

4. Discussion

Echinococcus multilocularis infection is currently a worldwide public health disease that urgently needs to be solved (伍卫平 and 王谦, 2018). Earlier studies have found that the disease can cause functional changes in CD4 + T cell subsets and related factors, resulting in Echinococcus multilocularis can induce host immune tolerance or immune evasion phenomenon. However, recent studies on chronic infection and tumors have found that CD8 + T cells are closely associated with inflammatory disease progression (Vieyra-Lobato et al., 2018a; Shimokawa et al., 2020; Agle et al., 2018). CD8 + T-cell failure is also a major cause of immune tolerance. Therefore, in this study, we found that the proportion of CTL and the expression of related factors in the peripheral blood and damaged liver of AE patients were significantly changed compared with the control group after collecting samples from clinical AE patients for detection. Among them, the proportion of CTL in peripheral blood was significantly reduced, and the expression of factors CD107a, IFN-γ, perforin and granzyme B, which reflect their related functional levels, was also significantly decreased. Therefore, it is considered that alveolar echinococcosis is closely related to the changes in the number of CTLs and the levels of related factors, and functional failure may occur, and the persistent infection and lesion metastasis of

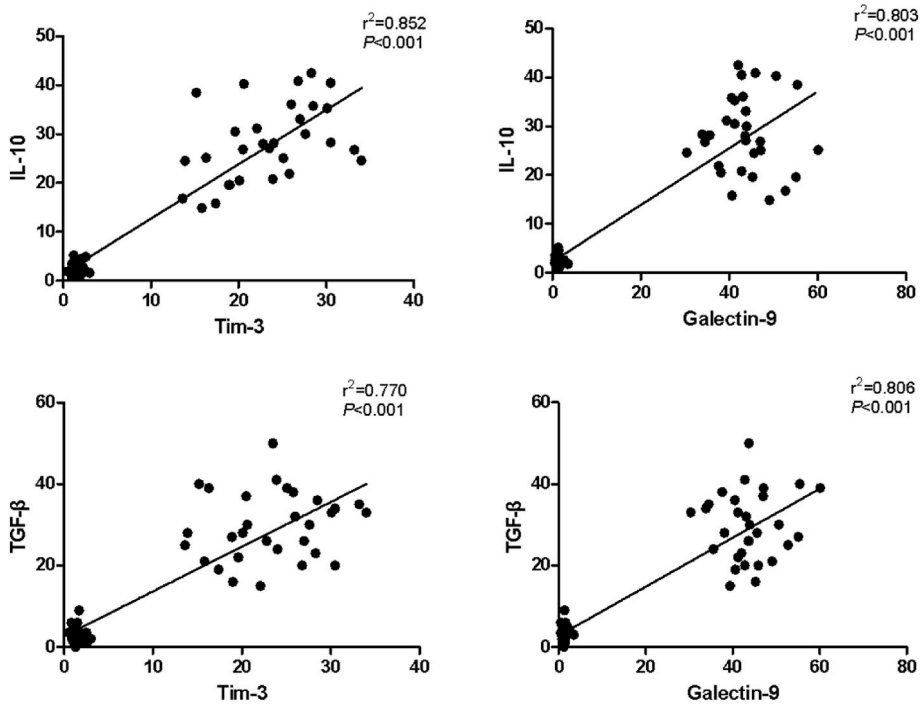

Fig. 11. Association of IL-10 and TGF-β1 with Tim-3/Galectin-9 in the liver of AE patients.

this disease may be related to it. In addition, in addition to CTL with killing effect, there are a group of CD8 + CD28-T cells with immunosuppressive function, which mainly play an inhibitory role through soluble cytokines TGF-β and IL-10 (Vieyra-Lobato et al., 2018b; Wu et al., 2016) and can "actively" effectively inhibit the activity of effector cells and play a negative role in immune regulation. In this study, we found that the proportion of CD8 + CD28-T cells in peripheral blood of AE patients was significantly increased compared with the control group, and the proportions of intracellular factors IL-10 and TGF-β were significantly increased compared with the control group. In the liver tissue of AE patients, a large band of lymphocytes appeared around the eroded liver tissue proximal to the infection compared with the distal end of the liver tissue not infected with Echinococcus multilocularis, in which CD8 expression was significantly increased, and the expression of the related cytokines IL-10 and TGF-β1 protein levels was also significantly increased, considering that there were more CD8 + T cells in the lymphocytes present in the infected liver, which may be mainly CD8 + CD28-T cells. The above results suggest that the imbalance of CTL and CD8 + CD28-T cells in CD8 + T cells may occur in AE patients, which is manifested as the enhancement of the number and function of CD8 + CD28-T cells, and the decrease of the number and related function of CTL. Persistent infection of Echinococcus multilocularis, erosion and destruction of the liver, and metastasis of the lesion may all be associated with this imbalance. CTL is the main immune effector cells of anti-infection, and its quantity and quality play a very important role in the outcome of infection. Therefore, in this study, we consider that CTLs in AE patients do not exert normal immune surveillance or killing function and are in a state of immunosuppression or depletion, thereby weakening the ability to kill target cells. At the same time, CD8 + CD28-T cells with immunosuppressive function dominate in vivo, on the one hand, they inhibit the killing effect of CTLs on target cells; on the other hand, they directly promote immune tolerance to E. multilocularis infection in patients by directly secreting excessive inhibitory cytokines IL-10 and TGF-β, which leads to E. multilocularis being able to present an active state in patients and continuously grow and metastasize. Next, in this study, we found that Tim-3, which has inhibitory function, was significantly up-regulated on CD8 + T cells in the peripheral blood of AE patients by further observation and analysis, which may be related to the imbalance of CD8 + T cells in patients with this disease. Although, Tim-3 is highly expressed on both CTL and CD8 + CD28-T cells, this study suggests that Tim-3 may have different effects on the surface of different CD8 + T cells. The differential sensitivity regarding suppression of HIV-specific CD8 + T cells by Treg cells is closely related to Tim-3 (Elahi et al., 2011). As a newly discovered immune checkpoint molecule, different studies have found that Tim-3/Galectin-9 expression is significantly increased in a variety of chronic infections (Shahbaz et al., 2020; Carroll et al., 2020; Yu et al., 2018; Okoye et al., 2020; Osuch et al., 2020), and T cells with positive Tim-3 expression are unable to proliferate or produce cytokines, which can induce T cell "depletion" and dysfunction (Caraballo Cortés et al., 2019; Hoffmann et al., 2016), which in turn leads to immune tolerance and immune escape in infection. Many studies have demonstrated its role in mediating CTL hyperresponsiveness. Studies on chronic infections such as HIV and HCV have found that Tim-3 promotes the development of infection by inhibiting CTL function (de Armas et al., 2019; Sumida et al., 2013). Tim-3 + CD8 + T cells were found to be significantly increased in infected individuals in HIV and chronic HCV infection. Both HIV and HCV-specific CD8 + T cells showed functional depletion due to overexpression of Tim-3. Tim-3 expression was found to be significantly increased on hepatic T cells, especially CD8 + T cells, in HBV infection (Fisicaro et al., 2017). These results suggest that Tim-3 plays a regulatory role in antiviral T cell responses in HBV infection. In addition, Tim-3 expression was up-regulated in virus-specific CD8 + cells after herpes simplex virus (HSV) infection. Exogenous Galectin-9 decreases CD8 + T cell-mediated immune responses to HSV and impedes viral clearance (Coulon et al., 2020). In the tumor setting, most tumor-infiltrating Treg cells highly express Tim-3 and appear to represent a specialized tissue-resident Treg subset with enhanced suppressive activity. Available data have implicated Tim-3/Galectin-9 interaction in Treg function (Gautron et al., 2014; Banerjee et al., 2021). Compared with Tim-3 positive Treg cells, Tim-3 negative Treg cells have superior suppressive function, as shown by higher expression of known Treg cell effector molecules such as IL-10 and higher Foxp3 content (Liu et al., 2018a; 徐子琴 et al., 2019). Blocking the Tim-3/Galectin-9 pathway resulted in a marked decrease in suppressive activity of Tregs cultured in vitro (Kim et al., 2019; Liu et al., 2018b). Another study showed that Tim-3 + Treg cells would be functionally superior to Tim-3 − Treg cells in an in vitro suppression assay, and they would secrete higher levels of IL-10 and TGF-β, showing a stronger suppressive ability (Gupta et al., 2012).

This study found a correlation between CD8+T cell expression and Tim-3/Galectin-9 in cases of alveolar echinococcosis (AE). In peripheral blood, the expression levels of CTL-related factor CD107a mRNA showed a negative correlation with Tim-3/Galectin-9. Meanwhile, in the livers of AE patients, a positive correlation was observed between $CD8^{+}$CD28-T cell-related factors IL-10, TGF-β, and Tim-3/Galectin-9 expression. Therefore, we speculate that the observed imbalances in CD8+T cell subgroups within AE patients might be associated with heightened expression of Tim-3 on the surface of CD8+T cells. Considering the results in conjunction with other studies, high expression of Tim-3 on CTL surfaces might directly influence their quantity and functionality, potentially leading to CTL functional exhaustion. Additionally, Tim-3 might promote the proliferation of $CD8^{+}$CD28-T cells and cytokine secretion, thereby enhancing the suppressive functionality of cells such as $CD8^{+}$CD28-T. This phenomenon could further inhibit CTL functionality, ultimately promoting the development and progression of multi-chambered Echinococcus granuloses within patients.

However, these conclusions are speculative and are based on initial observations from this study. The exact mechanisms require further elucidation through in vitro experiments and animal studies. Such research will help clarify the impact of Tim-3 on CD8+T cell function in AE, offering new perspectives and theoretical foundations for the effective diagnosis and treatment of the disease in the future.

Author contributions

We thank the healthy subjects and patients for their participation. Hui Zhao and Yuyu Ma were responsible for flow cytometry detection and paper writing, madinaimu·aibibula was responsible for qRT-PCR detection, Xiaojin Mo, Ning Xiao and Fengming Tian were responsible for sample collection and collation of patient information, Bin Li was responsible for flow cytometry detection, Hongyue Min was responsible for immunohistochemical detection, Xuanlin Cai was responsible for data statistical analysis, Ting Zhang associate researchers and Professor Xiumin Ma were responsible for experimental and paper guidance.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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