



Tim-3/Galectin-9 signaling pathway is involved in the cytokine changes in mice with alveolar echinococcosis

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Abstract

Background Tim-3/Galectin-9 is involved in the immune escape of many pathogens. However, the role of Tim-3/Galectin-9 in persistent infection of *Echinococcus multilocularis* (Em), which is related to immune escape, is still unclear.

Objective To investigate the role of Tim-3/Galectin-9 and related cytokines in mice with persistent infection of Em.

Methods Em infection model was established by injecting the protoscoleces. Serum was collected at days 2, 8, 30, 60, 90, 180 and 270 after infection. Lymphocytes were isolated from liver tissue samples with Ficoll. Tim-3 + CD4 + T percentage was analyzed by flow cytometry. CD4 + T cells were isolated from liver tissues of Em infected mice and cultured *in vitro*. The mRNA levels of *Tim-3*, *Galectin-9*, *IFN-γ* and *IL-4* were detected by qRT-PCR. Cytokine levels in serum and culture supernatant (IFN-γ and IL-4) were analyzed by cytometric bead array.

Results The expression of *Tim-3* and *Galectin-9* mRNA significantly increased after 30 days of infection, reached peak on day 90, and then decreased slightly on days 180–270. The expression of *IFN-γ* mRNA, increased on day 2 and 8 after infection, slightly decreased on days 30–60, and obvious decreased on days 90–270, but were still higher than those of the control group. The expression of *IL-4* mRNA gradually increased along with the time of infection. In serum of Em infected mice, level of IFN-γ peaked at day 30 and then gradually decreased; whereas IL-4 level peaked at day 90 and then gradually decreased. *In vitro* experiment found that Tim-3/Galectin-9 directly caused the changes in the levels of IFN-γ and IL-4.

Conclusions 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.

Keywords *Echinococcus* infection · Tim-3/Galectin-9 pathway · Cytokines

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Introduction

Echinococcosis is a zoonosis caused by cestodes of the genus *Echinococcus*. The incidence of echinococcosis is high in Xinjiang, Gansu, Tibet and the Ganzi Tibetan Autonomous Prefecture of Sichuan Province of China [1]. The two main types of this disease are cystic echinococcosis (CE) and alveolar echinococcosis (AE). AE caused by *Echinococcus multilocularis* (*E. multilocularis*) is extremely harmful and untreated patients may develop chronic liver erosions with prolonged asymptomatic phase, during which an invasive tumor-like multi-vesicular and exogenously budding mass-like lesion is developed [2, 3]. Symptoms are often manifested in a late infection stage that is too late to be treated by surgery [4]. More than 90% of patients die in 10–15 years without treatment [5]. Thus, AE is considered as one of the most deadly worm infections, which is also called parasite cancer [6].

The development, reproduction and long-term survival of *E. multilocularis* in the intermediate host all depend on effective immune escape mechanisms. Current studies found that the complex escaping mechanisms of *E. multilocularis* mainly include histological isolation, molecular simulation and immunosuppression [7–10]. T helper type 1 (Th1) / T helper type 2 (Th2) cytokines, interferon- γ (IFN- γ) and interleukine-4 (IL-4) can activate multiple immune-related signaling pathways, induce and regulate the activation, proliferation and differentiation of macrophages, B lymphocytes, and other lymphocytes [11]. These cytokines can mutually interact and inhibit each other and play an important role in regulating the normal functions and balances between Th1 and Th2 cells. These are the important basis for maintaining the normal immune function [12, 13]. In the preliminary study, the process of *E. multilocularis* infection in mice was divided into three stages: (1) early infection (2–30 days after infection), (2) mid-term infection (30–90 days after infection), and (3) late infection (90–270 days after infection). It was found that the immune status of *E. multilocularis*-infected mice changed significantly in these different stages of infection [14].

T cell immunoglobulin domain and mucin domain family 3 (Tim-3) is mainly an inhibitory regulator of immune response molecules and are expressed on the surface of T cells and a variety of immune cells. Galectin-9 (Gal-9), its ligand, has multiple functions. It can induce apoptosis of T cells, while can also activate resting T-cells in the absence of typical T-cell activating signals [15]. Especially, it can activate and expand human Th1 cells [15]. Gal-9 can specifically bind to Tim-3 and has important biological functions, such as induction of cell aggregation and adhesion, chemotaxis, cell activation, and apoptosis [16–19].

The interaction of Tim-3 and Gal-9 can result in the loss of Th1 cell function and reduced expression of IFN- γ (Th1-related cytokine). The established Tim-3/Gal-9 signaling pathway has negative regulation on Th1 type immune response and induction of immune tolerance [20–23]. However, the role of Tim-3/Galectin-9 signaling pathway in the pathogenesis of *E. multilocularis* infection is not yet clear.

In this study, an animal model of *E. multilocularis* infection was established. The effect of Tim-3/Gal-9 signaling pathway in *E. multilocularis* infection was analyzed and discussed.

Materials and methods

Animals

A total of 112 SPF BALB/c mice were selected to establish the animal model of *E. multilocularis* infection. All mice

were purchased from the animal center of Xinjiang Medical University (Urumqi, China), and they were all 6-week-old female mice weighing 20 ± 2 g. The experimental mice were randomly divided into model group ($n=56$) and control group ($n=56$). The *E. multilocularis* suspension was prepared and adjusted to 10,000 protoscoleces/mL as previously described [24]. Then, 100 μ L suspension was injected into the anterior liver lobe of mice in the model group as previously described [25], and the mice in the control group were injected with equal volume of saline at the same position. The mice were normally cultured after inoculation. The two groups were divided into seven subgroups according to the days of infection: 2d, 8d, 30d, 60d, 90d, 180d and 270d groups.

Ethics

The animal model establishment and feeding were carried out in the Research Laboratory of Animal Science (ALLAC certification) of the First Affiliated Hospital of Xinjiang Medical University. All animal experiments were conducted according to the ethical guidelines of the Animal Care and Use Committee and were approved by the Ethical Committee of First Affiliated Hospital of Xinjiang Medical University (License number: A-20130216-155).

Sampling

At each time point, retro-orbital blood sample was collected, and serum was separated and stored at -80 °C for CBA assay. The mice were sacrificed by cervical dislocation immediately after the blood sampling. Liver tissues were collected to isolate lymphocytes.

Isolation and purification of mononuclear cells and CD4⁺T cells from liver

The liver tissues were grinded, digested and centrifuged. The supernatant was collected and centrifuged again (1500 r/min at 4 °C for 10 min) to collect the cell precipitates. The cell precipitates were re-suspended and subjected to Ficoll density gradient centrifugation to isolate liver lymphocytes or Percoll density gradient centrifugation to isolate liver mononuclear cells. After isolation, the cells were stained with 1% trypan blue and counted. CD4⁺T cells were sorted from liver lymphocytes with magnetic cell sorting (MACS) (Miltenyi Biotech, Bergisch Gladbach, Germany).

Flow cytometry

Liver lymphocytes were adjusted to a cell concentration of 5×10^6 cells/mL with PBS and incubated with

anti-CD3-PE-Cy7, anti-human-CD4-PerCP and anti-Tim-3-PE at 4 °C for 20 min. After washing and re-suspension, the percentage of Tim-3 + CD4 + T cells was detected by flow cytometry. The gating strategies were as follows: monocytes were gated based on FSC and SSC. Then, CD3 + T cells were gated on CD3 positive monocytes. Finally, the Tim-3 + CD4 + T cells were gated on Tim-3 positive and CD4 positive cells.

Quantitative RT-PCR

CD4 + T cells were isolated from the livers of mice and the total RNA was extracted with Trizol. The PCR kit and Trizol reagent were bought from Invitrogen Co. (Carlsbad, CA, US). The RNA was reverse-transcribed into cDNA using a reverse transcription kit (Revert Aid™). First strand cDNA synthesis Kit was purchased from Fermentas Co. (Thermo Fisher Scientific, CA, US), and the target gene was amplified. The primer sequences of *Tim-3*, *Galectin-9*, *IFN-γ*, *IL-4* and *GAPDH* genes were searched from GeneBank and designed by DNAMAN software (Version, 5.0, LynnonBiosoft, USA). The primers are listed in Table 1. All the primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

The cDNAs of Tim-3, Galectin-9, IL-4, IFN-γ and GAPDH were used as templates of gene amplification and the PCR products were collected and stored at -20 °C for further use. For each sample, both the housekeeping gene (GAPDH) and the target genes were amplified in triplicate using the following procedure: initial denaturation at 95 °C for 1 min, 40 cycles of 95°C for 5 s, 58°C (or other) for 30s, and 72°C for 30s. The results were analyzed using 2^{-ΔΔCt} method to quantify the target genes.

In vitro experiments

Liver mononuclear cells were incubated with PBS or *E. multilocularis* vesicle fluid (Em-VF) (1 mg/mL). There was no endotoxin in the Em-VF as evaluated by the Limulus

amebocyte assay kit (Dana Biotechnology, Tianjin, China; detection range: 0.02-5.00 EU/mL) for 72 h. Galectin-9 level in the supernatant was detected with CBA assay.

Liver CD4 + T cells were adjusted to a cell concentration of 1 × 10⁸ cells/mL and seeded to a 96-well plate. Then, they were incubated with PBS, Em-VF, anti-Tim-3 (20 mg/mL; Cat# 566,346, eBioscience), and Galectin-9 (1.0 μg/m; Catalog #3535-GA-050, R&D Systems) for 72 h. IL-4 and IFN-γ levels in the supernatant were detected with CBA assay.

Cytometric bead array (CBA) assay

The concentrations of IL-4, IFN-γ and Galectin-9 were quantitatively determined by BD™CBA Mouse Cytokine Kit (BD Biosciences, San Jose, CA, USA). Cytokine standards were prepared, mixed with capture beads, vortexed and aliquoted into 50 μL. The samples were diluted and added to each sample tubes. The diluted standards (50 μL) were added to the standard tubes. All test tubes were added with 50 μL mouse cytokine PE detection reagent and incubated in dark at room temperature for 3 h. Mouse PE anti-Tim-3 was purchased from eBioscience Co. (San Diego, CA, USA). After centrifugation at 200 g for 5 min, the supernatant was carefully removed and the beads were re-suspended. The CBA samples were freshly prepared on the day of detection.

Data analysis

The statistical analysis was performed with the statistical software SPSS 17.0 (IBM Co., NY, US). Data are expressed as mean ± standard deviation (SD). Independent samples *t*-test was used for comparison between two groups (such as the difference in Tim-3 + CD4 + T cells). Multiple comparisons were performed with one-way ANOVA. *P* value *P* < 0.05 was considered as statistically significant.

Results

The mRNA expressions of *Tim-3*, *Galectin-9*, *IFN-γ* and *IL-4* in the *E. multilocularis* infected mice

To determine the expression of *Tim-3* and *Galectin-9* mRNAs in the mice infected by *E. multilocularis* at different stages, qRT-PCR was performed. After 2 to 8 days of infection, the mean expressions of both *Tim-3* (day 2: 1.26 ± 0.54; day 8: 1.48 ± 0.46) and *Galectin-9* mRNAs (day 2: 0.98 ± 0.35; day 8: 1.08 ± 0.20) (Fig. 1 A and 1B) in lymphocytes showed no significant changes compared to the control group (*Tim-3*, day 2: 0.98 ± 0.35, day 8: 1.08 ± 0.20; *Galectin-9*, day 2: 0.98 ± 0.35, day 8: 1.06 ± 0.34) (*P* > 0.05).

Table 1 The primers used in quantitative RT-PCR

Primer	Sequence	Size (bp)	Tm (°C)
Tim-3	F:TTAGACATCAAAGCAGCCAAGG	226	58.3
	R:CAGCAGAGACTCCCCTCCAAT		
Galectin-9	F:CTCTTCAGTGCCAGTCTCC	132	60.0
	R:AACCTCTGGTAGTCCCCTGGA		
IFN-γ	F:CTGCTGATGGGAGGAGATGT	176	57.3
	R:TTTGTCAATTCGGGTGTAGTCAC		
IL-4	F: TTGTCATCCTGCTCTTCTTTCTC	164	59.1
	R: GGTGTTCTTCGTTGCTGTGAG		
GAPDH	R:TTTGTCAATTCGGGTGTAGTCAC	157	59.8
	F:CCTGCTTACCACCTTCTTGA		

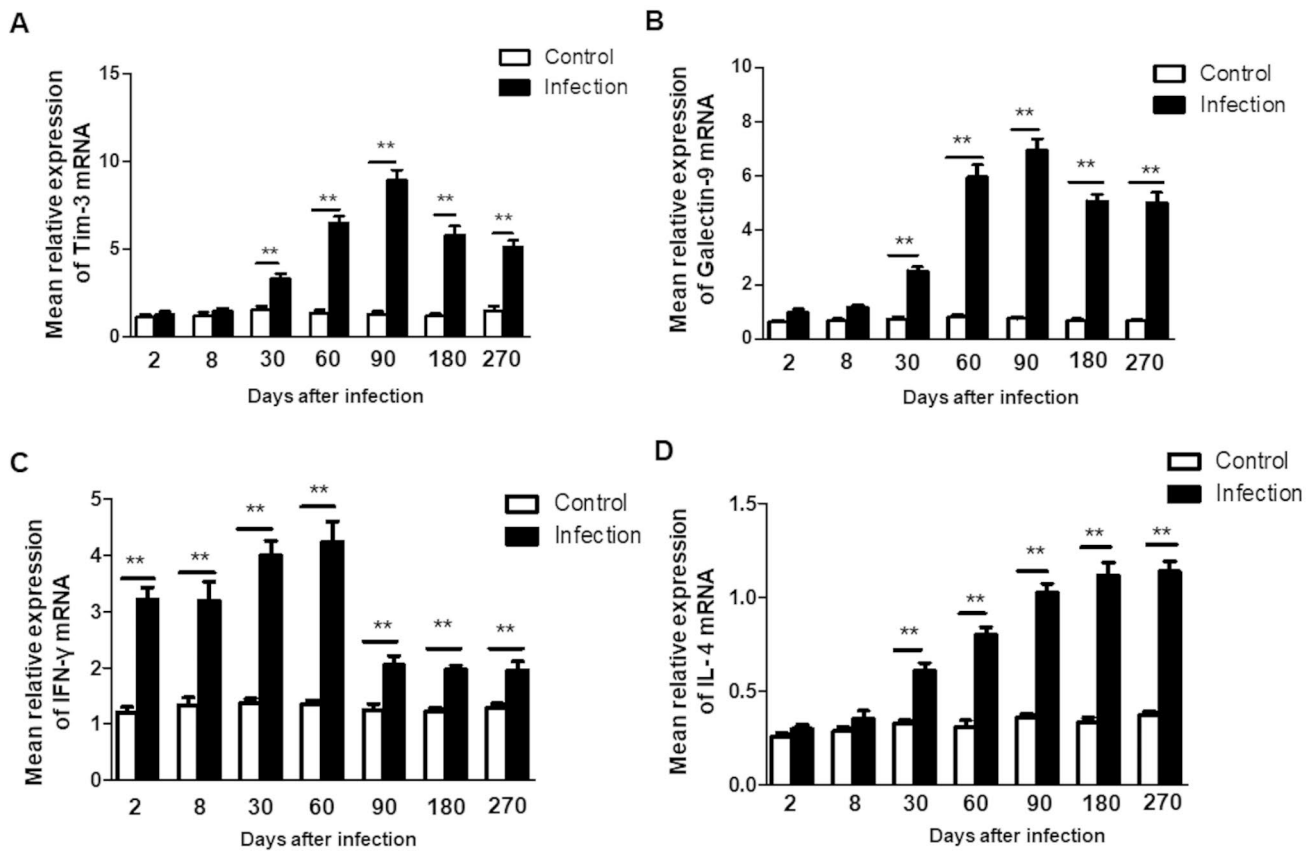


Fig. 1 Analysis of mRNA expression. The mRNA expression levels in the lymphocytes of *E. multilocularis*-infected or non-infected mice were detected with qRT-PCR, using GAPDH as the control. The mean relative expression of each gene was normalized to that of GAPDH. Relative expression of *Tim-3* (A), *Galectin-9* (B), *IFN-γ* (C), and *IL-4* (D) mRNA. At each time point, an independent sample *t* test was used to analyze the difference between the infection and control groups. ** $P < 0.01$

After 30 days of infection, *Tim-3* and *Galectin-9* mRNAs in the lymphocytes began to increase (*Tim-3*: 3.31 ± 0.84 ; *Galectin-9*: 2.51 ± 0.40) ($P < 0.05$). After 60 to 90 days of infection, the mean expressions of *Tim-3* and *Galectin-9* mRNAs significantly increased and reached the peak ($P < 0.05$) (*Tim-3*, day 60: 6.51 ± 1.02 , day 90: 8.91 ± 1.70 ; *Galectin-9*, day 60: 5.98 ± 1.23 , day 90: 6.95 ± 1.22). After 180 to 270 days of infection, the expressions of *Tim-3* and *Galectin-9* mRNAs decreased but still maintained at high levels, which were significantly higher than those in the control group ($P < 0.05$) (*Tim-3*, day 180, infection group: 5.79 ± 1.42 ; control group, 1.17 ± 0.38 ; day 270, infection group: 5.09 ± 1.14 ; control group: 1.48 ± 0.76 ; *Galectin-9*, day 180, infection group: 5.09 ± 0.74 ; control group: day 270: 5.04 ± 1.02 ; control group: 0.67 ± 0.13).

To determine the expressions of *IFN-γ* and *IL-4* mRNAs in the mice infected by *E. multilocularis* at different stages, qRT-PCR was performed. After 2 days of infection, the *IFN-γ* mRNA was 3.21 ± 0.62 , the *IFN-γ* mRNA after 8 days of infection was 3.19 ± 0.96 . After 30 to 60 days of infection, *IFN-γ* mRNA expression continued to increase from

4.00 ± 0.72 to 4.24 ± 1.03 , which has statistically significant difference from those of the corresponding time points in the control group ($P < 0.05$). After 90 days of infection, the *IFN-γ* mRNA was 2.05 ± 0.46 , which has statistically significant difference from those of the corresponding time points in the control group ($P < 0.05$). After 180 to 270 days of infection, the expressions of *IFN-γ* mRNA had no significant changes compared to that at 90 days but were still higher than that in the control group ($P < 0.05$, Fig. 1 C).

After 2 to 8 days of infection, the mean expression of *IL-4* mRNA began to increase from 0.26 ± 0.05 to 0.29 ± 0.06 , but the increase was not significant ($P > 0.05$). After 30 days of infection, the *IL-4* mRNA was 0.61 ± 0.12 . The *IL-4* mRNA after 60 days infection was 0.80 ± 0.11 . It showed an increasing trend over time, and it was significantly higher than that of the control group ($P < 0.05$). After 180 days of infection, the *IL-4* mRNA was 1.12 ± 0.19 . The *IL-4* mRNA after 270 days infection was 1.14 ± 0.15 . There was no significant difference in the *IL-4* mRNA at these two time points in the infection group ($P > 0.05$), but they were significantly higher

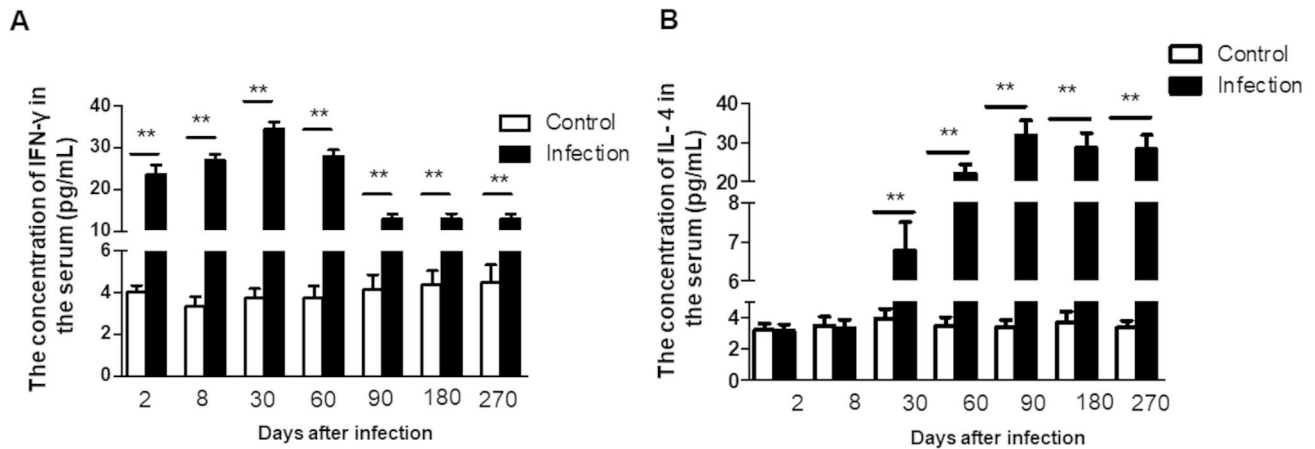


Fig. 2 Analysis of cytokine levels. Serum cytokine levels in *E. multilocularis*-infected or non-infected mice were measured by CBA assay. (A) IFN- γ serum level. (B) IL-4 serum level. At each time point, an independent sample *t* test was used to analyze the difference between the infection and control groups. ** $P < 0.01$

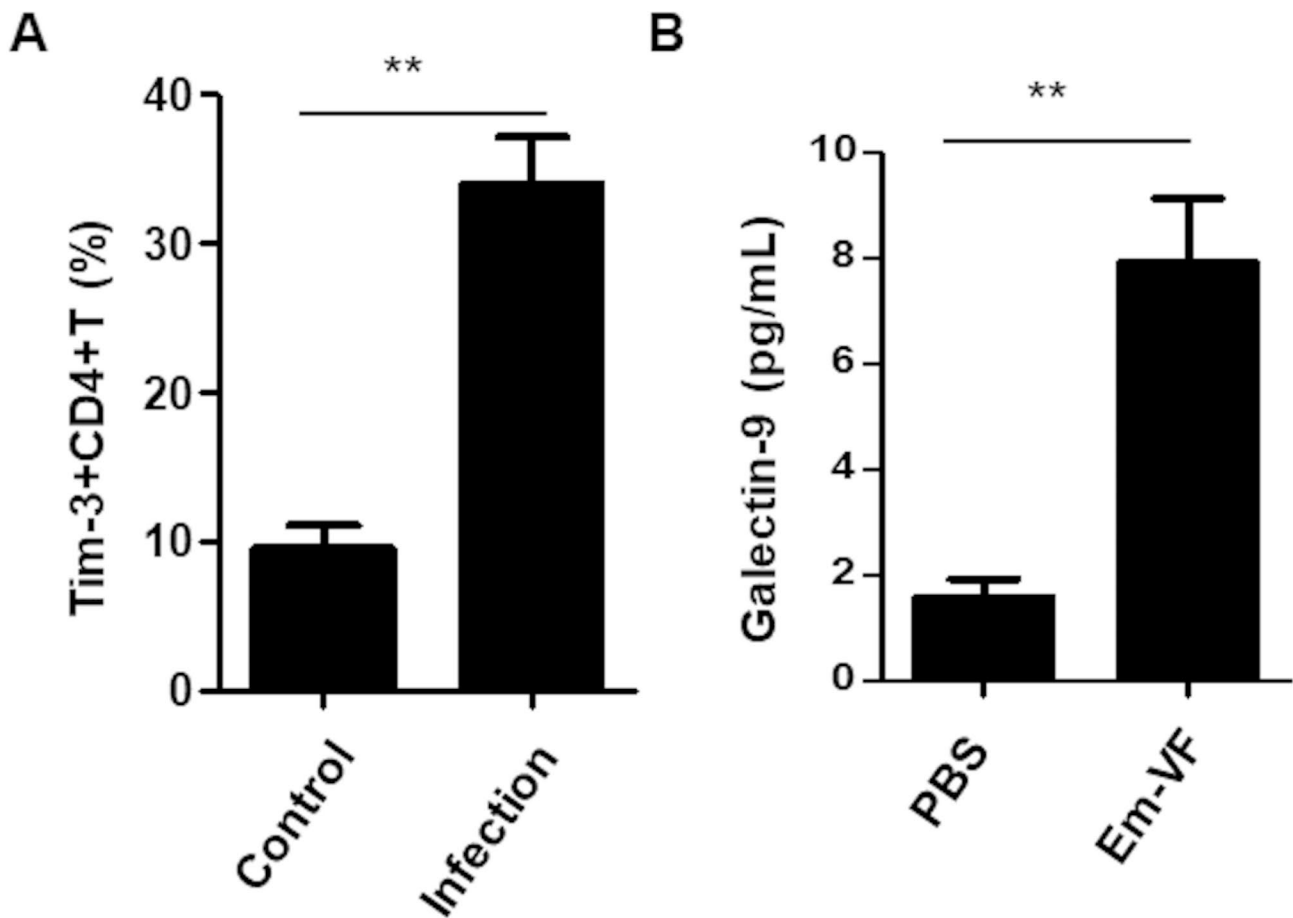


Fig. 3 Tim-3 expression on liver CD4+T cells and Galectin-9 secretion by liver mononuclear cells. (A) Liver lymphocytes of control and *E. multilocularis* infected mice at 90 days of infection were detected with flow cytometry. Quantitative results of Tim-3 + CD4 + T in liver lymphocytes were shown. (B) Liver mononuclear cells were incubated with PBS or Em-VF for 72 h. Then, Galectin-9 level in the supernatant was detected with CBA assay. Independent sample *t* test, ** $P < 0.01$

than those of the control group at the corresponding time points ($P < 0.05$, Fig. 1D).

These results indicate that *IFN- γ* mRNA expression increases significantly in the mice at early infection of *E. multilocularis* while it decreases at late stages of infection. The *IL-4* mRNA expression keeps increasing after the infection of *E. multilocularis*.

Serum cytokine levels in mice infected with *E. multilocularis*

In order to determine serum *IFN- γ* and *IL-4* levels in mice after infected with *E. multilocularis* at different stages, CBA assay was performed. After 2 to 8 days of infection, the serum *IFN- γ* level significantly increased ($P < 0.05$). After 30 to 60 days of infection, serum *IFN- γ* level was further increased, and it was significantly higher than that in the control group ($P < 0.05$). After 90 days of infection, the *IFN- γ* level was significantly lower than before ($P < 0.05$). After 180 to 270 days of infection, the *IFN- γ* level decreased but had no significant changes compared to that of the 90th day ($P < 0.05$, Fig. 2 A).

After 2 to 8 days of infection, the *IL-4* level did not change and showed no statistical difference from that in

the control group ($P > 0.05$). After 30 days of infection, the *IL-4* level increased significantly ($P < 0.05$). The *IL-4* level continued to increase over time and was significantly higher than that in the control group ($P < 0.05$). After 90 days of infection, the serum *IL-4* level reached the peak ($P < 0.05$). After 180 to 270 days of infection, the serum *IL-4* level maintained steady, and it was significantly higher than that in the control group ($P < 0.05$, Fig. 2B).

These results indicate that peripheral level of *IFN- γ* increases significantly at early infection of *E. multilocularis* while decreases in the late infection. The *IL-4* level continuously increases after infection.

SPercentage of Tim-3 + CD4 + T cells in mice infected with *E. multilocularis* and Galectin-9 level in the supernatant

Since the level of *Tim-3* mRNA were the highest in the liver of *E. multilocularis* infected mice at day 90, we isolated Tim-3 + CD4 + T cells from the liver of mice with infection for 90 days. Flow cytometry was used to detect the percentage of Tim-3 + CD4 + T cells. As shown in Fig. 3 A, the *E. multilocularis* infected mice had significantly higher percentage of Tim-3 + CD4 + T cells than control mice

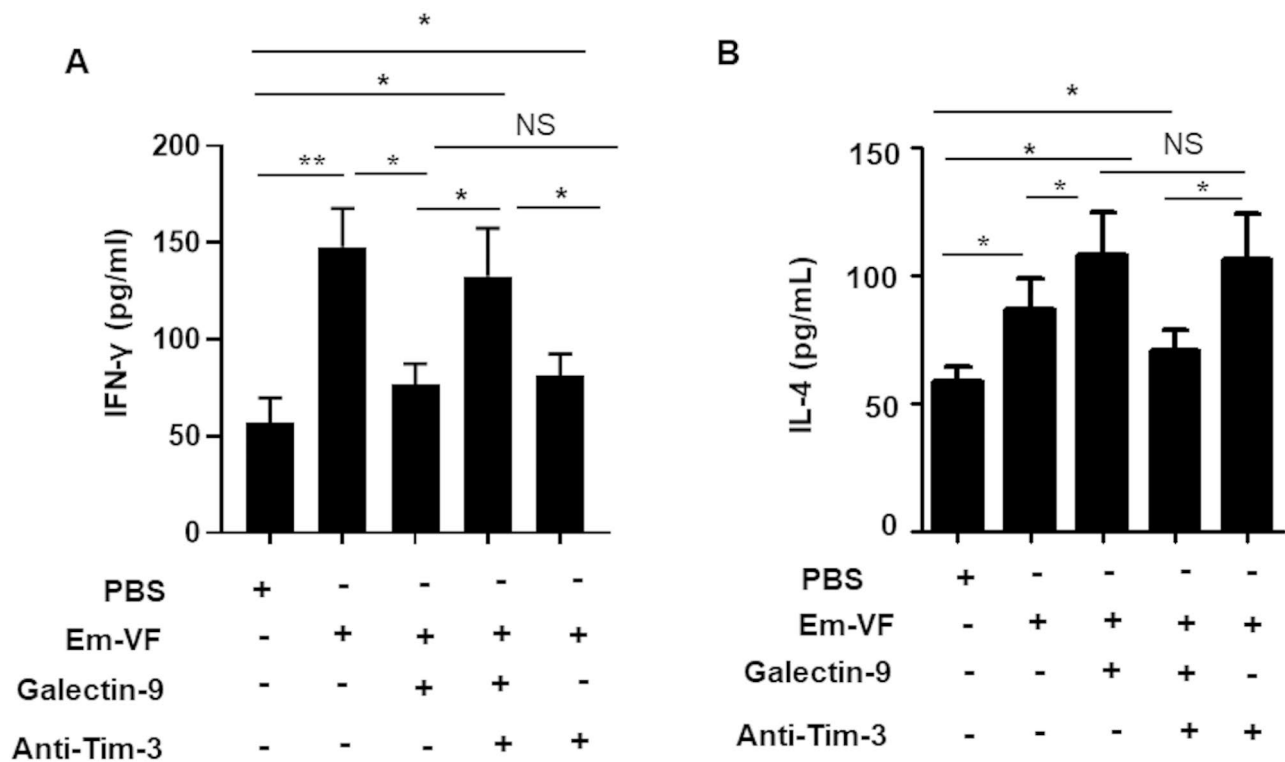


Fig. 4 Cytokine changes caused by Tim-3 and Galectin-9. The liver CD4+T cells of the *E. multilocularis* infected mouse were sorted and incubated respectively with PBS, Em (*E. multilocularis*)-VF, Galectin-9, and anti-Tim-3 mAb for 72 h. Then the cytokine levels in the supernatant were detected by CBA. (A) *IFN- γ* ; (B) *IL-4*. One-way ANOVA, * $P < 0.05$, ** $P < 0.01$

($P < 0.01$). Moreover, the isolated liver mononuclear cells were incubated with PBS or Em-VF for 72 h. Then, Galectin-9 level in the supernatant was detected with CBA assay. The result showed that Galectin-9 level in the supernatant was significantly increased after Em-VF stimulation (Fig. 3B).

Regulation of Tim-3/Galectin-9 on production of IFN- γ and IL-4

To investigate the effect of the Tim-3/Galectin-9 on the level of CD4+T cell-associated cytokines in *E. multilocularis* infection, the isolated CD4+T cells were respectively incubated with PBS, Em-VF, anti-Tim3 and Galctin 9 for 72 h, and then the levels of IL-4 and IFN- γ in the culture supernatant were measured with CBA assay. The results showed that the levels of IFN- γ in the Em-VF and Em-VF + Anti-Tim3 + Galctin 9 culture groups were significantly higher than those in the PBS group and Em-VF + Galectin-9 group ($P < 0.05$) (Fig. 4A). IFN- γ level in Em-VF + anti-Tim3 group was significantly higher than that in PBS group ($P < 0.05$). The highest IL-4 level was found in Em-VF + Galectin-9 group and Em-VF + Anti-Tim-3, significantly higher than other groups ($P < 0.05$) (Fig. 4B). Em-VF and Em-VF + Anti-Tim3 + Galctin 9 groups had significantly higher IL-4 level than PBS control group ($P < 0.05$). These data indicate that the Tim-3/Galectin-9 pathway regulates the production of IFN- γ and IL-4.

Discussion

In this study, for the first time, we report that elevated Tim-3/Galectin-9 participates in the persistent infection of AE, and induces the imbalance of the Th1/Th2-related cytokines IFN- γ and IL-4 in the development of AE.

A previous study has found that immune responses differed significantly in different stages of AE infection [14], but it is unclear whether this difference is related to negative regulation of immune response. Based on this, this study investigated the changes of the negative regulatory molecules Tim-3 and Galectin-9 at different stages. The infection stages were designed according to previous study [14], with small modification. We found that the expressions of Tim-3 and Galectin-9 increased at the early infection stage and decreased slightly at the late stage of infection. Negative co-stimulatory molecules, Tim-3 and Galectin-9, can inhibit or down-regulate the activation of T cells, and promote immunosuppression [26–28]. Previous studies of Tim-3/Galectin-9 focused mainly on autoimmune diseases, transplant immunology and infectious diseases. In chronic hepatitis C virus patients, Tim-3 was highly expressed in CD8+T cells,

which may contribute to the mechanism of infection chronicity [29]. In patients with SLE (Systemic Lupus Erythematosus), the expression of Tim-3 in CD4+ and CD8+ T cells and the serum Galectin-9 level were also higher than those in the healthy controls [30]. Further studies demonstrated that the immune responses of peripheral blood mononuclear cells induced by CD3 in patients with SLE were inhibited by Galectin-9 blocking antigens. Moretto et al. [31] also showed increased Tim-3 expression in peripheral T cells in patients infected with chronic Toxoplasmosis.

This study investigated the role of Tim-3 in the pathogenesis of AE by comparing the expression of Tim-3 in the liver lymphocytes of normal mice and mice at different infection stages. Tim-3 was almost not expressed in the control group. For mice infected with *E. multilocularis*, the expression of Tim-3 changed accompanying with the progression of liver infections. According to the literature, most of the inflammatory cells surrounding the liver lesion during AE infection are T-lymphocytes [32, 33]. In this study, the expression of *Tim-3* mRNA in the liver lymphocytes of *E. multilocularis* infected mice was increased after 30 days of infection, indicating that *E. multilocularis* infection can lead to increased expression of Tim-3 in liver lymphocytes.

Galectin-9, a natural ligand of Tim-3, can specifically bind to the glycosyl side chain of Tim-3 molecule that is expressed on the surface of activated Th1 cells and thus plays an immunomodulatory role [34–36]. Galectin-9 induces intracellular calcium efflux and Th1 cell aggregation and death in a Tim-3-dependent manner *in vitro*, while it results in selective reduction of IFN- γ , thereby suppressing the immune response of Th1-type cells *in vivo* [37].

Dembele et al. [38] found that Galectin-9 significantly increased in malaria infection and the increasing level was associated with the stages of malaria infection. In mouse malaria infection, the expression of Galectin-9 also increased [39]. Steichen et al. [40] revealed that the expression of Galectin-9 increased in the lungs of mouse infected with *Francisella novicida*, and the inflammatory response was enhanced after the infected mouse was administered with Galectin-9. In this study, the expression of *Galectin-9* mRNA in the liver lymphocytes of *E. multilocularis* infected mice was increased after 30 days of infection, indicating that *E. multilocularis* infection can also lead to increased expression of Galectin-9 in liver lymphocytes. Consistently, *in vitro* experiments showed that Galectin-9 level was significantly increased in the supernatant of liver mononuclear cells after Em-VF stimulation.

Studies also show that inhibition of the interaction between Tim-3 and Galectin-9 can significantly increase the clinical manifestations of immune system diseases, which is achieved by regulating cellular immunity [41]. Hou et al. showed that block of Tim-3 can recover the exhausted

lymphocytes in plasmodium infection and enhance the clearance of Malaria. This study found that mice with *E. multilocularis* infection had altered expressions of Th1 and Th2-related cytokines. In the early stage of immune response, the growth and development of Th1 and Th2 cells are impacted by surrounding environment, in which IL-4 plays dominant roles [42–44]. IL-4 can induce Th0 to Th2 cell differentiation [45–47]. The qRT-PCR results of this study showed that the expressions of *IFN- γ* mRNA in mice significantly increased at the early infection of *E. multilocularis*. At the middle stage of infection, *IFN- γ* mRNA levels remained high. However, at the late stage of infection, the expression of *IFN- γ* mRNA significantly decreased. For the expression of *IL-4* mRNA, the results showed no change at the early stage of infection, it began to increase at the middle stage and was maintained at high level in the late stage. CBA assay showed that the changes of *IFN- γ* and *IL-4* were similar to those of the qRT-PCR results. We suppose that *IFN- γ* may be involved in the inhibition of the growth of *E. multilocularis* in the early stage. However, in the late stage, *IL-4* may play a major role inhibiting a robust immune response and promoting the growth of *E. multilocularis* *in vivo* while also producing anti-inflammatory effects. Binding of Galectin-9 to Tim-3 can lead to intracellular calcium loss and Th1 cell aggregation and death *in vitro*, while it can result in the reduction of *IFN- γ* *in vivo* [48]. In this study, the low expressions of Tim-3 and Galectin-9 and the elevated Th1 type cytokines such as *IFN- γ* with inflammatory reaction of liver tissues were observed in the early stage of *E. multilocularis* infection. In the late stage of infection, the expressions of Tim-3 and Galectin-9 significantly increased. The level of *IFN- γ* decreased and the level of Th2 cytokine *IL-4* significantly increased. Thus, it is hypothesized that Tim-3 and Galectin-9 could regulate *IFN- γ* and *IL-4* immune function. To verify whether Tim-3 and Galectin-9 can affect the level of CD4+ T cell-associated cytokines *in vitro*, CD4+ T cells were isolated from the liver of *E. multilocularis* infected mouse and then co-cultured with Em-VF, anti-Tim-3, and Galectin-9 for 72 h. The results showed that the Tim-3/Galectin-9 pathway led to the changes in cytokines during *E. multilocularis* infection.

Conclusions

In conclusion, Tim-3/Galectin-9 signaling pathway caused the changes in *IFN- γ* and *IL-4*, which may play important roles in the process of AE. This study provides important information on the Th1/Th2 imbalance theory of parasitic infection and the immunoregulatory mechanism of AE.

Authors' contributions JBD and HNF conceived and designed the experiments. SYL, FBZ, YJZ and YJL performed the experiments. HNF,

NNP and MTA analyzed the data. SYL, FBZ, HNF Fan and JBD wrote the manuscript.

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Availability of data and material The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethics approval All animal experiments were conducted according to the ethical guidelines of the Animal Care and Use Committee and were approved by the Ethical Committee of First Affiliated Hospital of Xinjiang Medical University (License number: A-20130216-155).

Consent to participate Not applicable.

Consent for publication Not applicable.

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