1. Introduction

Alveolar echinococcosis (AE) is a severe parasitic disease caused by the infection of Echinococcus multilocularis (Em). Very little is known on the relationship between TGF-β/Smad signaling pathway and Treg/Th17 balance in the infected liver at different periods after Em infection. Using RT-qPCR, immunohistochemistry, flow cytometry and CBA assay, we measured the expression levels of TGF-β, Smad2/3/7, ROR-γt, Foxp3, IL-17, IL-10 and -6 were significantly increased. In the late stage of infection (day 90 to day 270), Treg cells, Foxp3, TGF-β and IL-10 maintained at high levels whereas Th17 cells and IL-17 decreased significantly. TGF-β/Smad signaling pathway was activated during the chronic infection. Our data suggest that there were Treg/Th17 imbalance in the middle and especially in the late stage of Em infection and that Treg/Th17 imbalance may be regulated by TGF-β/Smad signaling pathway. Treg and Th17 subsets may be involved in regulating immune tolerance and tissue inflammation, and facilitating the long-term survival of Em in the host.
and IL-10 increased in Em infected mice, suggesting that IL-17 and IL-10 might be involved in the chronic infection of Em [21,22].

Studies reported that cytokines of TGF-β, IL-6 and IL-23 played critical roles in the differentiation of Treg and Th17 cells [23,24]. TGF-β alone could induce naive CD4⁺ T cells to differentiate into Treg cells [25]. In the infection and inflammation environment, TGF-β in combination with inflammatory factor IL-6 could induce naive CD4⁺ T cells to differentiate into Th17 cells. In addition, TGF-β played an important role in cell proliferation through its downstream signaling pathway. Studies have shown that the main downstream effector protein in the TGF-β signaling pathway was the Smad protein family [26–30]. Through binding with TGF-β type II receptor (TβRII), TGF-β can activate TGF-β type I receptor (TβRI), which further activates Smad2/3 through phosphorylation. Phosphorylated Smad2/3 (phospho-Smad2/3) complex can bind with the receptor Smad4 and then is translocated into the nucleus. In the nucleus, the Smad complex in combination with the nuclear translocation factor ROR-γt (retinoic acid-related orphan nuclear receptor gamma t)/Foxp3 (forkhead box P3), a Th17/Treg cell-specific transcription factor ROR-γt can regulate gene transcription [27,28].

Zhang et al. [11] and Wang et al. [33] found significant changes in the expression levels of TGF-β and Smad proteins in the liver of Em infected mice, suggesting that TGF-β and Smad proteins may mediate the interactions between Em and its host. In this study, we detected the dynamic changes of TGF-β, Smad proteins, ROR-γt, Foxp3, Treg cells, Th17 cells and their related cytokines in the three stages of Em infection. The role of TGF-β/Smad signaling pathway in regulating Treg cells and Th17 cells was also analyzed.

2. Materials and methods

2.1. Animals and reagents

Eighty Balb/c mice (18–22 g, female) were obtained from Experimental Animal Center of Xinjiang Medical University. All animal experiments were approved by the Ethical Committee of First Affiliated Hospital of Xinjiang Medical University (A-20100920002).

RNA extraction kit was purchased from Qiagen (Hilden, Germany). Reverse transcription kit was purchased from Invitrogen (Carlsbad, California, USA). SYBR Green real-time quantitative kit was purchased from Takara (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China). Primers for β-actin, TGF-β, Smad2, Smad3, Smad7, Foxp3 and ROR-γt were retrieved from the GenBank and were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Polyclonal rabbit antibodies for TGF-β, Smad proteins, IL-17, IL-10, phosphor-Smad2/3/7 were purchased from Santa Cruz (California, USA). Biotin labeled secondary antibody and DAB developing kit were purchased from Beijing Zhongshan Biotechnology Co., Ltd. (Beijing, China).

2.2. Animal model

Pathogen-free Balb/c mice (8–10 week old) were randomly divided into the infection group (n = 40) and the control group (n = 40). The former head larva of Em was taken from alveolar hydatid infected hamster and suspended at a concentration of 1 × 10⁵ cells/ml. Mice in the infection group were injected with 0.1 ml former head larva suspension as previously described [11,34], while mice in the control group were injected with 0.1 ml saline. At 2 days, 8 days, 30 days, 60 days, 90 days, 180 days and 270 days after infection, 5 mice in each group were sacrificed. Peripheral blood and liver tissue were collected for further analysis.

2.3. Quantitative real-time RT-PCR analysis (qRT-PCR)

Total RNA extraction was performed according to the instructions provided by the kit. For reverse transcription, 1 μg of total RNA was reverse transcribed into cDNA. The real-time RT-PCR was conducted with the SYBR Green PCR premix following the manufacturer’s protocols. Primer sequences were listed in Table 1. β-Actin was used as an internal control. The 2⁻ΔΔCt method was used to determine the specific Ct value of each target gene. Each experiment was performed 3 times.

2.4. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by density gradient centrifugation. For the analysis of CD3⁺, CD4⁺ and CD8⁺ cells, PBMCs were incubated with antibodies of anti-CD3-APC, anti-CD4-PERCP, and anti-CD8-FITC at 4 °C for 20 min in the dark. For the analysis of natural Treg cells, cells were incubated with anti-CD4-PERCP and anti-CD25-PE-CY7 for 20 min in the dark. Then cells were washed with phosphate buffered saline (PBS) and centrifuged at 3000 r/min for 5 min. The supernatant was discarded. And the cells were resuspended in fixation buffer and incubated for 20 min in the dark. After fixation, cells were washed with permeabilization buffer and centrifuged at 3000 r/min for 5 min. Then cells were incubated with anti-Foxp3-PE or isotype control in permeabilization buffer. After incubation for 30 min, cells were washed with PBS and resuspended in PBS before flow cytometry analysis. For analysis of Th17 cells, the cell suspension was stimulated with 20 ng/ml phorbol 12-myristate-13-acetate and 1 μg/ml ionomycin in the presence of 2 mmol/ml monensin (Sigma-Aldrich, St. Louis, Missouri, USA) in 24-well plates. After 4 h of culturing (37 °C, 5% CO₂), the cells were transferred to tubes and washed once in PBS. The cells were then incubated with anti-APC-CD3 and anti-PerCP-CRD4 at 4 °C for 30 min. After surface staining, the cells were fixed and permeabilized and then stained with anti-human PE–IL-17 or isotype control. For flow cytometry analysis, cells were resuspended in PBS. Data were analyzed by FlowJo software.

2.5. Immunohistochemistry

To block endogenous peroxidase, sections were incubated with 3% H₂O₂ in the dark. Then antibodies were incubated in citrate buffer. After washing with PBS for three times (5 min each time), the sections were incubated overnight at 4 °C with primary antibodies of anti-IL-17, anti-IL-10, anti-TGF-β, anti-TRβ1/2 and anti-phospho-Smad2/3/7. Then the sections were incubated with secondary antibodies at 37 °C for 20 min. Finally, the sections were developed with DAB. For negative control, primary antibodies were replaced with PBS. The sections were observed under the optical microscope (Nikon YS 100, Tokyo, Japan). Five views were randomly selected at ×400. The positive cells were

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Expected size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROR-γt</td>
<td>F: CAGCGCCAACACCTCTCCTTC</td>
<td>247 bp</td>
</tr>
<tr>
<td></td>
<td>R: AGCAGCCGCTGCTGAGC</td>
<td></td>
</tr>
<tr>
<td>Foxp3</td>
<td>F: GCTAGCTGATGTCCTGAGG</td>
<td>112 bp</td>
</tr>
<tr>
<td></td>
<td>R: ACAGCATGGTCGCTGCTT</td>
<td></td>
</tr>
<tr>
<td>Smad2</td>
<td>F: GTGCCGCGGCTTACCA</td>
<td>213 bp</td>
</tr>
<tr>
<td></td>
<td>R: ACACTGACAAAGGCCCCGT</td>
<td></td>
</tr>
<tr>
<td>Smad7</td>
<td>F: AGAGAATCTAGGAGCCAGCA</td>
<td>234 bp</td>
</tr>
<tr>
<td></td>
<td>R: TCAAGCCTTCGTACTCGCG</td>
<td></td>
</tr>
<tr>
<td>Smad3</td>
<td>F: GTCAACCGTGGTGGC</td>
<td>150 bp</td>
</tr>
<tr>
<td></td>
<td>R: GCAGCAGAGCTCCTGCGATA</td>
<td></td>
</tr>
<tr>
<td>TGF-βi</td>
<td>F: GTCTGCGACGACATGGTGGAAA</td>
<td>143 bp</td>
</tr>
<tr>
<td></td>
<td>R: TGGTCTGACGACATGGTGGAAA</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: TGGCGGCTACTTACGACC</td>
<td>233 bp</td>
</tr>
<tr>
<td></td>
<td>R: TGCTCAGCAGACCTGCAC</td>
<td></td>
</tr>
</tbody>
</table>
counted. The positive rate was the number of positive cells to the total number of cells.

2.6. Cytometric bead array (CBA)

The concentrations of IL-10, IL-6, IL-17 and TGF-β in the sera were quantitatively determined by BD™ CBA Mouse Cytokine Kit (BD Biosciences, San Jose, CA, USA). Briefly, the CBA technique was based on 4 bead populations with distinct fluorescence intensities that had been coated with capture antibodies specific for IL-10, IL-6, IL-17 and TGF-β proteins. Four standard curves (ranging from 1 to 5000 pg/ml) were obtained from one set of calibrators and four results were obtained on one test sample. The maximum and minimum limits of detection for all the four cytokines were 5000 pg/ml and 1 pg/ml. CBA was used to measure these cytokines on flow cytometry (ASR II) according to the manufacturer’s instruction. The data were analyzed using FCAP Array software.

2.7. Pathological morphology

The pathological morphology of liver tissue was observed by hematoxylin and eosin staining (H&E staining). Briefly, sections were deparaffinized with xylene and rehydrated in gradual dilutions of ethanol. Then sections were stained with hematoxylin. After rinsing under running water for 2-5 min, sections were differentiated with 1% HCl in ethanol. Finally, sections were counterstained with eosin. The sections were observed under light microscope.

2.8. Statistical analysis

SPSS 13.0 software was used for statistical analysis. Data were expressed as mean ± standard error of mean (SEM). Differences between groups were analyzed by one-way ANOVA and multiple comparison test. Correlation was evaluated by Spearman correlation analysis. P < 0.05 was considered statistically significant.

3. Results

3.1. Pathological morphology of the liver in Em infected mice

Dynamic changes of liver pathological morphology during infection were observed. At 2 days, 8 days, 30 days, 60 days, 90 days, 180 days and 270 days after infection, mice were sacrificed and liver tissue were collected for H&E staining. Representative results were shown in Fig. 1. In liver tissue of the control group, the hepatic lobule structure was intact. Hepatic cells were neatly arranged in cords, with clearly delineated cell borders. A small amount of inflammatory cell infiltration was occasionally seen (Fig. 1A). Meanwhile, liver morphology was changed after Em infection. At 2 days after infection, the pathological morphology of liver tissue showed no significant abnormal change (Fig. 1B). Inflammatory cell infiltration was observed in liver tissue at 30 days after infection (Fig. 1C). At 90 days after infection, the infiltration of inflammatory cells further increased. And there was bile duct hyperplasia. The hepatocytes showed focal necrosis. Proliferation of Kupffer cells was seen. Alveolar hydatid cyst was formed (Fig. 1D). At 180 days after infection, the alveolar hydatid cyst was expanded with fibroblast proliferation and inflammatory cell infiltration. The normal liver structure was disrupted. Focal necrosis of liver cells was also seen. Granulomatous nodules were formed (Fig. 1E). At 270 days after infection, there were diffuse fiber hyperplasia and fibrosis in the hepatic portal area. In liver tissue, massive necrosis, infiltration of lymphocytes and alveolar hydatid cyst could be seen (Fig. 1F). Taken together, these results suggest that liver tissue was damaged by Em infection and that these damages may be mediated by strong immune responses.

3.2. Expression of TGF-β, Smad2, Smad3, Smad7, Foxp3 and ROR-γt at mRNA level in the liver after Em infection

In order to detect the dynamic changes of TGF-β, Smad proteins, Foxp3 and ROR-γt at mRNA level during infection, we performed qRT-PCR assay. Liver tissues were collected at 2 days, 8 days, 30 days, 60 days, 90 days, 180 days and 270 days after infection. The house keeping gene β-actin was used as an internal control. Quantitative results were shown in Fig. 2.

In the infection group, TGF-β mRNA expression increased from day 8 and peaked at day 270 (Fig. 2A). Meanwhile, TGF-β mRNA expression level maintained at a low level at all time points in the control group. Compared with the control group, the levels of TGF-β mRNA expression from day 8 to day 270 were significantly higher in the infection group (P < 0.05).

As shown in Fig. 2B, increased Smad2 mRNA expression was observed in the infection group from day 60 to day 270. Smad2 mRNA expression peaked at day 270. Statistically, the levels of Smad2 mRNA expression from day 60 to day 270 in the infection group were significantly higher than those in the control group (P < 0.05). Similar results were found in the expression levels of Smad3 mRNA (Fig. 2C). In the infection group, increased Smad3 mRNA expression was observed from day 30 to day 270. Smad3 mRNA expression peaked at day 270. Statistically, there were significant differences between the infection group and the control group from day 30 to day 270 after infection (P < 0.05). At 2 days after infection, Smad7 mRNA expression level in the infection group was significantly higher than that in the control group (P < 0.05) (Fig. 2D).

From day 30 to day 270 after infection, Foxp3 mRNA expression levels in the infection group were significantly increased than those in the control group (P < 0.05). Foxp3 mRNA expression reached peak at day 180 after infection (Fig. 2E).

In the infection group, ROR-γt mRNA expression levels increased from day 8 to day 90, with a peak on day 90, and decreased from day 180 to day 270 (Fig. 2F). Statistically, the levels of ROR-γt mRNA expression were significantly higher than those in the control group on day 8, day 30 and day 90 after infection (P < 0.05).

Therefore, these results showed that expression levels of TGF-β, Smad proteins; Foxp3 and ROR-γt at mRNA level were dynamically changed during Em infection. And these results imply that TGF-β/Smad signaling pathway was activated in the middle and late stage of Em infection.

3.3. Levels of T cell subpopulations in mouse peripheral blood after Em infection

Dynamic changes of T cell subpopulations in mouse peripheral blood during infection were analyzed by flow cytometry. Peripheral blood was collected at 2 days, 8 days, 30 days, 60 days, 90 days, 180 days and 270 days after infection. The detected T cell subpopulations included CD3+ T cells, CD4+ T cells, CD8+ T cells, CD4+IL-17+ Th17 cells and CD4+CD25+Foxp3+ Treg cells. Quantitative results were shown in Fig. 3.

First we checked the percentages of CD3+ T cells, CD4+ T cells and CD8+ T cells in mouse peripheral blood. As shown in Fig. 3A, the percentages of CD3+ T cells, CD4+ T cells and CD8+ T cells in the infection group were not significantly different from those in the control group (P > 0.05).

Then the percentages of CD4+CD25+Foxp3+ Treg cells were also detected (Fig. 3B). Compared with the control group, at 90 days and 180 days after infection, the percentages of CD4+CD25+Foxp3+ Treg cells in the infection group were significantly higher (P < 0.05).

Further we measured the percentages of CD4+IL-17+ Th17 cells. The percentages of CD4+IL-17+ Th17 cells increased in the infection group at 60 days and 90 days after infection, significantly higher than in the control group (P < 0.05). At 180 days and 270 days after infection, the
percentages of CD4+IL-17+ Th17 cells decreased to normal levels (Fig. 3C).

In addition, we analyzed the ratio of Treg/Th17 (Fig. 3D). At 60 days and 90 days after infection, the Treg/Th17 ratio in the infection group was significantly lower than that in the control group (P < 0.05). In contrast, at 270 days after infection, the Treg/Th17 ratio in the infection group was significantly higher than that in the control group (P < 0.05).

Collectively, our results indicate that Treg cells and Th17 cells were involved in Em infection at different infection stages.

3.4. Expression of IL-17, IL-10, TGF-β, TGF-β receptors and phosphor-Smad2/3/7 in liver tissue of Em infected mice

In order to further verify the activation of TGF-β/Smad signaling pathway during Em infection, we analyzed the expression and distribution of IL-17, TGF-β, IL-10, phosphor-Smad2/3/7, TβRI and TβRII in liver tissue by immunohistochemistry. At 2 days, 8 days, 30 days, 60 days, 90 days, 180 days and 270 days after infection, liver tissues were collected. Cells with brown stain were positively stained cells. Representative immunohistochemical results were shown in the left part of Fig. 4 and quantitative results were shown in the right part of Fig. 4.

IL-17 expression increased in the infection group. And IL-17 positive expression was mainly distributed in the hepatic sinusoids, the portal areas, the hepatocytes and the inflammatory cells around the alveolar hydatid cysts and the germinal layers. IL-17 expression peaked at 90 days after infection. Statistically, IL-17 expression levels in the infection group were significantly higher than those in the control group at 30 days, 60 days, 90 days, 180 days and 270 days after infection (P < 0.05) (Fig. 4A).

In the infection group, IL-10 was mainly expressed in the hepatocytes, the hepatic sinusoids, the portal areas, the alveolar hydatid cysts and the germinal layers. Compared with the control group, IL-10 expression level was gradually increased with the infection time, with a peak on day 90. Statistically, IL-10 expression levels in the infection group were significantly higher than those in the control group at 30 days, 60 days, 90 days, 180 days and 270 days after infection (P < 0.05) (Fig. 4B).
Expression of TGF-β in the infection group was higher than that in the control group and was primarily located in the cytoplasm of the hepatocytes, the hepatic sinusoids, the granuloma area, the alveolar hydatid cysts and the germinal layers. At 90 days after infection, the highest level of TGF-β expression was observed in the infection group. At 30 days, 60 days, 90 days, 180 days and 270 days after infection, TGF-β expression levels in the infection group were significantly higher than those in the control group (P < 0.05) (Fig. 4C).

Expression of TGF-β receptors (TβRI and TβRII) was similar to that of TGF-β. TβRI and TβRII were expressed at relatively low levels in the control group. Meanwhile, their expression in the infection groups increased with the infection time, peaking at 90 days after infection. The dominant expression areas of TβRI and TβRII in the infection group were significantly higher than those in the control group (P < 0.05) (Fig. 4D).

Phosphor-Smad2/3 expression in the infection group was mainly located in the hepatic cytoplasm, the hepatic sinusoids, the alveolar hydatid cysts and the germinal layers. Statistically, expression of phosphor-Smad2/3 in the infection group was significantly higher than those in the control group at 90 days, 180 days and 270 days after infection (P < 0.05) (Fig. 4E).

Expression levels of phosphorylated Smad7 (phosphor-Smad7) in liver tissue were also detected. Phosphor-Smad7 was mainly expressed in the cytoplasm of hepatic cells in the hepatic sinusoids and the portal area. Statistically, at 8 days after infection, the phosphor-Smad7 expression level in the infection group was significantly higher than that in the control group (P < 0.05) (Fig. 4F).

Thus these data further confirmed that TGF-β/Smad signaling pathway was activated during Em infection.

### 3.5. Serum levels of TGF-β, IL-17, IL-6 and IL-10 after Em infection

As previously described, we have observed dynamic changes of Treg cells and Th17 cells in mouse peripheral blood. Next we detected the dynamic changes of their related cytokines in the serum by CBA kit. The measured cytokines included TGF-β, IL-17, IL-6 and IL-10. Results were shown in Fig. 5. As shown in Fig. 5A, TGF-β levels in the infection group were higher than those in the control group at all time points. Statistically, there were significantly higher levels of TGF-β in the infection group at day 8, day 90, day 180 and day 270 (P < 0.05). Fig. 5B showed the dynamic changes of IL-17 levels in the serum. IL-17 levels in the infection group increased to its highest level at day 60 after infection. Statistically, IL-17 levels in the infection group at day 60 and day 90 after infection were significantly higher than those in the control group (P < 0.05). In addition, IL-6 levels in the control group maintained at low levels at all time points (Fig. 5C). However, in the infection group, IL-6 levels increased to its highest level at day 2 after infection. Then IL-6 levels in the infection group declined along with infection time but were still higher than those in the control group. And there were statistically higher levels of IL-6 in the infection group at day 2, day 8, day 30 and day 90 after infection (P < 0.05). Moreover, IL-10 levels were also detected (Fig. 5D). Similarly, IL-10 levels in the control group were not significantly changed. In the infection group, IL-10 levels increased from day 30 to day 270 after infection. Statistically, from day 30 to day 270 after infection, IL-10 levels in the infection group were significantly higher.
than those in the control group \( (P < 0.05) \). Collectively, these findings indicate that the changes in serum cytokine levels were consistent with the changes in Treg cells and Th17 cells.

3.6. Correlation analysis

To determine whether the expression levels of TGF-β, IL-17 and IL-10 in different tissues have consistent tendency of changing, we performed Spearman correlation analysis. For TGF-β, correlation analysis was performed between relative level of TGF-β mRNA in liver tissue and expression level of TGF-β in peripheral blood. We found that they were positively correlated, with \( p = 0.000 \), \( r = 0.699 \). For IL-17, correlation analysis showed that IL-17 expression on CD4+ T cells and IL-17 expression in liver tissue were positively correlated, with \( p = 0.000 \), \( r = 0.628 \). And, IL-17 expression on CD4+ T cells was positively correlated with IL-17 level in peripheral blood (\( p = 0.000 \), \( r = 0.691 \)). Additionally, there was also positive correlation between IL-17 level in peripheral blood and IL-17 expression in liver tissue, with \( p = 0.000 \), \( r = 0.596 \). Similarly, for IL-10, IL-10 expression in liver tissue and IL-10 expression in peripheral blood were positively correlated, with \( p = 0.000 \), \( r = 0.693 \). Collectively, these results indicate that expression levels of TGF-β, IL-17 and IL-10 in different tissues showed consistent tendency of changing during Em infection.

4. Discussion

In most helminthic infections, Th1/Th2 cell imbalance played an important role in clearing or protecting the parasite. Rogan [13] reported that different cytokines were secreted in different periods of secondary hydatid infections in mice. IFN-γ (secreted by Th1 cells) was primarily produced at the early stage of infection, while IL-4 (secreted by Th2 cells) was mainly produced at the late stage of infection [12,13]. The imbalance between Th17 and Treg cells has been observed in CE patients in recent [21,35]. More importantly, our results further demonstrated that Treg/Th17 imbalance existed in the middle and late stages of *E. multilocularis* infection and might be closely regulated by the activation of TGF-β/Smad signaling pathway. In our study, Th17 cell and its related cytokine IL-17 were up-regulated in the middle stage of Em infection. Meanwhile Treg cells, IL-10, TGF-β were increased brightly in the middle and especially in the late stage of infection. TGF-β/Smad signaling pathway was activated during the chronic infection, thus altering Treg/Th17 transcription factor mRNA patterns, and then influencing the differentiation and function of Treg and Th17 cell in *E. multilocularis* infection (Fig. 6).

Currently, studies found that CD4+CD25+Foxp3+ Treg cells were involved in the immune response during parasite infection [36–38]. Increased numbers of CD4+CD25+ Treg cells have been observed in both human and murine malaria infection [39,40]. Evidence of the role of Treg cells as suppressors of T-cell responses in malaria was initially demonstrated in murine models, where Treg cells are known to be associated with increased or delayed parasite growth. A study of experimental Leishmania infection found that CD4+CD25+ Treg cells can effectively inhibit the host clearance of pathogens, while immune effectors are significantly enhanced after removal of CD4+CD25+ Treg cells [41]. In our study, at 60 days after infection, CD4+CD25+Foxp3+ Treg level in the infection group was significantly increased and maintained...
at high percentage in the middle and late stage of infection. Treg cells could suppress the immune responses through the secretion of TGF-β and IL-10 cytokines [42-46]. TGF-β was usually considered to be an anti-inflammatory factor, which can promote expression of Foxp3 and induce the differentiation of Treg cells [47,48]. Herbert et al. [49] found that TGF-β and IL-10 protected the liver by inhibiting liver inflammation in the acute phase of schistosome infection. In this study, serum TGF-β and IL-10 levels were significantly increased after Em infection. In the liver areas close to lesions, there were strong TGF-β and IL-10 immunostaining present during the middle and late stages of infection. This result suggests that Treg cell and its related cytokines TGF-β, IL-10 might have a potentially promoting role for the growth of *E. multilocularis* in host.

Th17 cells played dual roles in immune response to pathogens and inflammatory mainly by the secretion of IL-17 cytokine. Hong et al. [50] found that the *Eimeria maxima* infection could induce the secretion of many inflammatory cytokines, and IL-17 was the most obviously increased cytokine, with an increase of 1650-fold, which aggravated the illness. Monteiro et al. [51] showed that Th1 cell response weakened whereas levels of IL-17 and TNF-α were significantly up-regulated in the acute phase of *Trypanosoma cruzi* infection. Our results show that in the middle stage of infection, percentages of CD4+ IL-17+ Th17 cells and concentration of IL-17 in the serum of *E. multilocularis*-infected mice. The pathological features of *E. multilocularis*-infected mice. The pathological features of *E. multilocularis*-infected mice. Th17 cells are related both to parasitic growth and to host’s immune response, leading to a large number of inflammatory cells infiltration and necrosis of liver cells, and IL-17 not only could protect the host by eliminating extracellular pathogens but also could cause the autoimmune pathological damage in the body [18,52]. So we suspect that high level of IL-17 may be related to autoimmune inflammation damage in the liver. In the middle stage of infection, Treg/Th17 ratio was significantly lower in the infection group than that in the control group, suggesting that CD4+ T cells tended to polarize to Th17 cell subset; at the late stage of infection, those molecules (TGF-β and IL-10) actively modulate the host’s immune system and shift the Treg/Th17 imbalance to the Treg-dominant suppressive immune response.

In order to explore the relationship between Treg/Th17 cell imbalance and TGF-β/Smad signaling pathway in Em infection, we further analyzed the expression and distribution of TGF-β, TIR I, TIR II, phosphor-Smad2/3, ROR-γt and Foxp3. In this study, Smad7 mRNA expression and phosphorylation were high in the early stage of infection, while expression of ROR-γt and Foxp3 mRNA was very low. Smad7 could interrupt TGF-β signal transduction by preventing the translocation of Smad2/3 complex into the nucleus [30,32,53,54]. Therefore, we hypothesized that Smad7 may suppress the differentiation both of Treg and Th17 cell by inhibiting TGF-β signal pathway in the early stage of Em infection.

In the middle stage of Em infection, levels of TGF-β and TIR I/TIR II were significantly increased. Smad2/3 expression was up-regulated sharply, while Smad7 expression was decreased, and ROR-γt mRNA was up-regulated. These data suggest that TGF-β/Smad signaling pathway was activated by the stimulation of large number of *Echinococcus* cysts. The differentiation of Th17 cells are driven primarily by the cytokines of TGF-β and IL-6 [23,24]. Malhotra et al. and Fantini et al. [53,54] reported that Smad2 was essential in the differentiation Th17 cells, which played an important role in the expression of IL-6 receptor and regulated the transcription of ROR-γt. In our study, the levels of TGF-β and IL-6 were both increased in the peripheral blood and liver tissue.
in the middle stage of infection, and up-regulation of TGF-β signaling occurs via a feed-back mechanism without the inhibition of Smad7, as intracellular signal transducers, Smad2 and Smad3 predominantly mediate TGF-β signaling in Th17 cell differentiation (Fig. 6). In a previous study, Bommireddy and Doetschman[55] reported that Treg cell effects were mediated partly through Smad signaling. In a recent study, Ai-Di et al. [56] revealed that Smad-dependent TGF-β signaling and Smad-independent TGF-β signaling discretely control non-Treg and Treg functions to modulate immune tolerance and immune homeostasis. We also found that Foxp3 mRNA was up-regulated and Treg cell was increased in the middle stage of infection, and these results indicate that Smad could be exploited to induce Treg cell differentiation and may aid in more precisely identifying the TGF-β signaling pathways that drive Foxp3 expression in T cells (Fig. 6).

In the late stage of infection, the number of Treg cells was increased followed by the activation of TGF-β/Smad signaling pathway (Fig. 6). However, Th17 cells and IL-10 were declined; this may be caused by the decrease of IL-6. One study found that TGF-β was strongly expressed by most of the infiltrating lymphocytes in the hydatid cyst and the granuloma [57]. The high levels of TGF-β in hepatic cells and fibroblasts around the hydatid cyst were involved in the formation of fiber wall of hydatid cyst and immune tolerance [33,58]. Our study found high levels of TGF-β in liver tissue and serum in the late stage of infection. The high expression of TGF-β not only was related to liver fibrosis, but also could induce the generation of Treg cells, and these results may benefit to Em for long-term survival.

In conclusion, our data demonstrate that Treg and Th17 subsets may involve in immune tolerance and tissue inflammation in the E. multilocularis infection. The growth of Em may activate TGF-β/Smad signaling pathway. Treg/Th17 imbalance is regulated by this pathway and finally facilitates to the long-term survival of E. multilocularis in the host. Our results require the analysis of blocking or inhibition experiments of TGF-β/Smad pathway in vivo and vitro. Once the effects of TGF-β pathway on Treg/Th17 cell have been ascertained, a promising clinical treatment for human echinococcosis may come true in future.

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References


