Immunoprotective effect and mechanism of E.granulosus recombinant antigen P29 against CD4+T cell deficient mice with Echinococcus multilocularis infection

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Acta Biochimica et Biophysica Sinica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>ABBS-2023-466.R3</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>14-Dec-2023</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Li, Ming Zhu, Yazhou Li, Zihua Song, Jiahui Zhao, Wei; Ningxia Medical University</td>
</tr>
<tr>
<td>Keywords:</td>
<td>CD4+T cell deficient mice, rEg.P29, E. multilocularis</td>
</tr>
</tbody>
</table>
Original Article

Immunoprotective effect and mechanism of *Echinococcus granulosus* recombinant antigen P29 against CD4⁺ T-cell-deficient mice with *Echinococcus multilocularis* infection

Ming Li¹,†, Yazhou Zhu²,†, Zihua Li³, Jiahui Song³, and Wei Zhao²,³,*

¹Department of Hepatobiliary Surgery, Ningxia Medical University General Hospital, Yinchuan 750004, China, ²Department of Medical Immunology and Pathogen Biology, Ningxia Medical University, Yinchuan 750004, China, ³Ningxia Key Laboratory of Prevention and Control Of Common Infectious Disease, Ningxia Medical University, Yinchuan 750004, China.

†These authors contributed equally to this article.

*Correspondence address. Tel: +86-13895112892; E-mail: zw-6915@163.com

Abstract

Alveolar echinococcosis (AE) is a zoonotic parasitic disease caused by infection with the larval stage of *Echinococcus multilocularis* and a major challenge to human public health. Vaccines are the most effective way to prevent and control infectious diseases. We previously revealed that the *Echinococcus granulosus* recombinant protein P29 is a good vaccine candidate against *E. granulosus*. However, the protective and immunological mechanism of rEg.P29 against *E. multilocularis* remain unclear. In this study, CD4⁺ T cell-deficient mice are transferred with spleen CD4⁺ T cells isolated from wild-type mice and subjected to rEg.P29 immunization, and then these immunized mice are infected with *E. multilocularis*. The cyst inhibition rate was calculated by weighing the body and cyst weights. The level of antibody was detected by ELISA. Flow cytometry was used to detect the level of IFN-γ production by CD4⁺ T and CD8⁺ T cells. The cytokines in culture supernatant was detected by ELISA. The expression of CD44 and CD62L on memory T cells was determined by flow cytometry. The results show the cyst inhibition rate is 41.52% after adoptive transfer CD4⁺ T cells. Furthermore, the levels of IgG, IgM, IgA and IgE in serum are significantly increased compared with PBS group. The IFN-γ-secretion by CD8⁺ T cells and the level of IFN-γ in culture supernatant are obviously increased; and the number of CD4⁺ T cells is increased, but the number of IFN-γ producing CD4⁺ T cells have no significant difference compared with PBS group. In addition, the number of CD44⁺CD62L⁻CD8⁺ memory T cells in the spleen is significantly increased while the number of CD44⁺CD62L⁺CD8⁺ memory T cells has no significant difference. Collectively, rEg.P29 can alleviate *E. multilocularis* infection by inducing humoral immune responses and CD8⁺ T cell responses.

Key words: CD4⁺ T cell-deficient mice, rEg.P29, *E. multilocularis*

Introduction

Alveolar echinococcosis (AE) is a sufferable chronic parasitic disease caused by the intrahepatic tumor-like growth of the metacestode of *E. multilocularis* [1-3], and is also a zoonotic disease that seriously endangers human health and life [4]. Humans can serve as aberrant intermediate hosts, obtaining infection by ingestion of eggs by chance [5]. The eggs enter the liver through blood circulation and parasitize the liver tissue. Therefore, AE mainly originates from the
liver. Hepatic AE shows infiltrative growth in the host liver, and can cause liver injury, hepatic coma, and portal hypertension in patients. In addition to the liver tissue, AE can also be found in the brain and other organisms, which can seriously threaten a patient’s life [6, 7]. At present, surgery with drug therapy is the preferred AE treatment choice. However, surgical resection of this hydatid disease is often incomplete, causing patients to relapse easily [8]. Drug therapy has many side effects, and its efficacy varies significantly among different patients [9]. Furthermore, other preventive measures have no distinct effects on AE. Hence, it is very important to determine a timely and effective approach to preventing *E. multilocularis* infection.

Vaccination against *E. multilocularis* may be an alternative or feasible treatment method [10]. In recent years, a large number of *E. multilocularis* antigens have been found to be able to induce adaptive immune responses and have vital roles both in intermediate and definitive hosts, such as EG95 [11], and 14-3-3 [12]. rEg.P29 was a novel 29 kDa antigen of *E. granulosus* [13]. Our previous study found the protective efficacy of rEg.P29 in sheep and mouse models with secondary infection of *E.granulosus* were 94.5% and 96.6%, respectively [14, 15]. In addition, rEg.P29 can induce mice to produce high levels of IgG, IgM, IgE and IgA antibodies, and IFN-γ secreted of CD4+ T and CD8+ T cells were significantly increased [16]. These data suggested rEg.P29 can induce high levels of humoral and cellular immune responses. However, rEg.P29, as a novel antigen of *E. granulosus*, to prevent *E. multilocularis* infection is unclear.

In this study, we constructed a mouse model of rEg.P29 immunization combined with *E. multilocularis* infection, clarified the protective effect of rEg.P29 against *E. multilocularis* infected CD4+ T cell-deficient mice.

**Materials and methods**

1. **Antigen purification and endotoxin removal**
   The expression and purification of rEg.P29 were performed following previously published methods [17]. In brief, in order to expressed rEg.P29, *E. coli* was induced in the presence of 50 μg/mL isopropyl β-D-1-thiogalactopyranoside (IPTG; Invitrogen, Carlsbad, USA) for 10 h at 37°C. Then, according to the manufacturer’s instructions for the His Purification kit (Merck, Darmstadt, Germany), the rEg.P29 was purified. The purified rEg.P29 was identified by 10% SDS-PAGE, and the concentration of rEg.P29 was tested by a CBA kit (KeyGEN Biotech, Nanjing, China). Finally, the endotoxin was removed by an endotoxin removal kit (GenScript Biotech Corporation, New Jersey, USA), and the endotoxin concentration was detected by an endotoxin detection kit (GenScript Biotech Corporation).

2. **Isolation and vitality identification of protocephalic larvae**
   Protoceolcs (PSCs) were isolated from *E. multilocularis*-infected gerbils. Briefly, cysts were isolated from gerbils and stripped tissue components of mice. Sterile PBS buffer was used to wash cyst samples. Then, the cyst tissue was shredded and placed on a 300-mesh iron filter for grinding and rinsed with PBS while grinding. The filtrate was filtered through a 200-mesh sieve and naturally settled for 5 minutes. The supernatant was discarded, and the sample was washed 2 times with PBS. PSCs were stained with 1% eosin (Beyotime, Shanghai, China) to identify their viability. PSCs inocula with a vitality over 95% confirmed by 1% eosin exclusion were used for portal injection in mice.

3. **Identification of CD4+ T cell-deficient mice**
   DNA extraction was performed according to the manufacturer’s instructions for the DNA extraction kit (TIANGEN, Beijing, China). In brief, 1–2 mm of mouse tail was cut off, and DNA
was extracted. Then, polymerase chain reaction (PCR) was performed in a 25 μL reaction mixture containing 12.5 μL of PCR master mix (Vazyme, Nanjing, China), 1.5 μL of template DNA, 1 μL of each primer, and 9 μL of nuclease-free water (TIANGEN). The sequences of the forward primers were 5′-CTAGCATGAATGAGGAGGATGGG-3′ and 5′-CCTCACGCTTGTCTGACC-3′, the sequences of the reverse primers were 5′-CTGCGAGGTTCCTTCTCAGTG-3′, and PCR amplification was undertaken using the following protocol: 94°C for 3 min; 94°C for 30 s (denaturation), 60°C for 35 s (annealing), 72°C for 35 s (extending), for 35 thermal cycles; 72°C for 5 min, hold at 4°C. The amplified products were analysed and confirmed by agarose gel electrophoresis.

4. CD4⁺ T cell isolation from wild-type mice and adoptive transfer

Spleen cells of wild-type mice were isolated immediately by pushing the spleen through a 70-μm strainer in Hank’s balanced salt solution, followed by Ficoll-Hypaque (Tianjin HaoYang Biological Manufacture, Tianjin, China) density gradient centrifugation at 450 g for 20 min. Mononuclear cells were collected, washed twice with buffer, and divided into three groups in 300μL/tubes. Sorting CD4⁺ T cells, according to manufacturer’s instruction of CD4⁺ T cell magnetic bead isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, staining for APC-labelled anti-CD4 (BD Biosciences, San Jose, USA) markers was performed, including incubation at 4°C in the dark for 30 min. After washing twice, the cells were suspended in 2 mL of PBS with 10% FBS and sorted by flow cytometry (BD FACSAria™ III; BD, New Jersey, USA). The purification of CD4⁺ T cells was >99%. Finally, CD4⁺ T cells were collected for use in later experiments.

5. Animal immunization and infection

Wild C57BL/6 mice were obtained from the Experimental Animal Center of Ningxia Medical University. C57BL/6N-Cd4emleyagen (strain number: KOCMP-12504-Cd4-B6N-VA) was purchased from Cyagen Biosciences (Cyagen, Suzhou, China). The study was approved by the Experimental Animal Ethics Committee of Ningxia Medical University (No. KYLL-2021-765), and experiments were carried out in strict accordance with national and institutional guidelines. Eighteen identified homozygous mice were randomly divided into 6 groups: PBS (subcutaneously injected with 100 μL PBS buffer)+Infection (intraperitoneally injected with 2000 PSCs/200 μL PBS); CpG (subcutaneously injected with 20 μg CpG ODN 1826)+Infection; rEg.P29 (subcutaneously injected with 20 μg rEg.P29)+CpG+Infection; PBS+CD4⁺ T (adoptive transfer of wild-type mouse CD4⁺ T cells)+Infection; CpG+CD4⁺ T+Infection; rEg.P29+CpG+CD4⁺ T+Infection. For subcutaneous immunization, the emulsified mixture was suspended in PBS and injected into the lower quadrant of the abdomen (100 μL/mouse). The immunization and infection scheme is shown in Figure 1.

6. Sample collection and cell culture

Three months later, the mice were anaesthetized with isoflurane, and blood samples and spleens were collected from each mouse. Spleen cells were isolated immediately by pushing the spleen through a 70-μm strainer in balanced buffer (PBS with 10% calf serum), followed by Ficoll-Hypaque (Haoyang, Tianjin, China) density gradient centrifugation at 450 g for 20 minutes. Then, monocytes were collected and washed twice with buffer. Finally, monocytes were resuspended at a final concentration of 2×10⁶ cells/mL in complete RPMI 1640 medium (HyClone, Logan, USA) including 10% calf serum (Gemini Bio, West Sacramento, USA), 100 U/mL penicillin/streptomycin (Pricella, Wuhan, China), 2 mM L-glutamine (Nuwei Biotec, Beijing, China), and 50 μM 2-mercaptoethanol (Gibco, Grand Island, USA).

7. ELISA
A total of 200 μL of cell suspension was plated into each well of a round-bottom 96-well plate and stimulated with or without rEg.P29 protein (10 μg/mL) in the presence of anti-CD28 (1 μg/mL) at 37°C with 5% CO₂ for 3 days. Cytokines (IFN-γ, TNF-α, IL-2, IL-4, and IL-6) in the supernatants were detected using BD OptEIA Mouse ELISA Sets (BD Biosciences) according to the manufacturer’s instructions. H₂SO₄ (1 M) was used to stop the reaction, and the absorbance of each well was measured at 450 nm using a microplate reader (Thermo Fisher, Waltham, USA). According to standard serial dilutions of cytokines, the concentrations of each sample were calculated.

For antibody detection of each sample, according to the requirements of the reagent instructions, the antibodies in the serum were detected by enzyme-linked immunosorbent assay (ELISA). In brief, 10 μg/mL rEg.P29 was coated on the ELISA plate and placed at 4°C overnight. The plates were washed five times with PBST (PBS containing 0.05% Tween 20) and blocked with 5% skim milk powder in PBST at 37°C for 1 h. After washing five times with PBST, the plates were incubated with mouse serum (1:500) in 5% skim milk powder in PBST for 2 h and washed five times with PBST for 3 min. Horseradish peroxidase (HRP)-conjugated anti-mouse IgM, IgG, IgA (100 μL each; Abcam, Scottsdale, USA) and IgE (100 μL; Invitrogen) were added to the enzyme plates and incubated at 37°C for 1 h. After washing, 100 μL TMB Single-Component Substrate solution (Solar bio, Beijing, China) was added for 8–10 min, and the reaction was stopped by 2 M H₂SO₄. The absorbance was measured at 450 nm using an ELISA reader (Thermo Fisher, Massachusetts, USA).

8. Flow cytometry

To measure the level of intracellular cytokines, a 2×10⁶ cells/ml cell suspension was diluted to 1×10⁶ cells/ml with RPMI 1640 and stimulated with or without rEg.P29 (10 μg/mL) in the presence of anti-CD28 (1 μg/mL) at 37°C with 5% CO₂ for 20 h. In addition, brefeldin A (Sigma-Aldrich, St Louis, USA) was added to the culture at a concentration of 10 μg/mL. The cells were washed twice with buffer 1 (PBS with 10% FCS). Then, fluorochrome-conjugated monoclonal antibodies (mAb) were used to stain for phenotyping for 30 min at 4°C in the dark. Then, the cells were washed with buffer, fixed with 4% paraformaldehyde and permeabilized with buffer 2 (PBS with 10% FCS and 0.1% saponin) overnight at 4°C. Intracellular cytokines were detected with fluorochrome-conjugated mAbs staining for 30 min at 4°C in the dark. Cells were washed with buffer and measured using a FACS Celesta (BD Biosciences) for data collection. Data were analysed using FlowJo 10 (Tree Star, San Carlos, USA).

For memory cell detection, cells were washed with buffer and then stained with CD3, CD8, CD62L and CD44 mAbs (BD Biosciences) for 30 min at 4°C in the dark. The cells were washed twice with buffer and measured using a FACS Celesta (BD Biosciences) for data collection. Data were analysed using FlowJo 10 (Tree Star, San Carlos, USA).

9. Statistical analysis

All data were analysed using Statistics Software SPSS 20.0 and GraphPad Prism 8.0. Unpaired Student’s t-test was used for comparisons between two groups, and one-way or two-way ANOVA was used for comparisons among more than two groups. Data are presented as the mean or the mean ± SD. P<0.05 was considered statistically significant.

Results
1. CD4+ T cell preparation and identification

The CD4+ T cells of the spleen were sorted by a magnetic bead isolation kit, and the purity of CD4+ T cells was detected by flow cytometry. The results showed that the proportion of CD4+ T cells in the spleen was 18.8% before isolation, and was increased to 99.4% after isolation, as shown in Figure 2A,B. These data confirmed that the sorted CD4+ T cells can be used for adoptive transfer.

2. rEg.P29 alleviated E. multilocularis infection in CD4+ T cell-deficient mice

Our previous data showed the immune protection of rEg.P29 against E. granulosus-infected mice and sheep was 94.3% and 98.6% [14, 15], respectively. However, the protection and mechanism of rEg.P29 against E. multilocularis remain unclear. Here, CD4+ T cell-deficient mice with or without CD4+ T cells from wild-type mice were immunized with rEg.P29 and then subjected to E. multilocularis infection. The cyst inhibition rate analysis showed the immune protection of rEg.P29 against E. multilocularis infection in CD4+ T cell-deficient mice was 35.74% compared with the control; after adoptive transfer of CD4+ T cells from wild-type mice, the protection of rEg.P29 against E. multilocularis infection in CD4+ T cell-deficient mice was 41.52% compared with the control (Table 1).

Twelve weeks after infection, the autopsy results showed that the size and weight of cyst in the abdominal cavity of rEg.P29+infection group were significantly decreased compared with PBS+infection group and CpG+infection group (Figure 3A-C). After transfer adoptive CD4+ T cells, the size and weight of cyst in the abdominal cavity of CD4+ T+ rEg.P29+CpG+infection group were also reduced compared with CD4+ T+PBS+infection group and CD4+ T+CpG+infection group (Figure 3D-F). These data suggested that rEg.P29 alleviated E. multilocularis infection in CD4+ T cell-deficient mice.

3. rEg.P29 induced an antibody response in CD4+ T cell-deficient mice with E. multilocularis infection

To further reveal the mechanism of the protective effect, we analyse the antibody response against rEg.P29 in CD4+ T cell-deficient mice. The antibody subtypes IgG, IgM, IgA and IgE in serum samples were tested by ELISA. As shown in Figure 4, the results showed that the levels of rEg.P29-specific IgG, IgM, IgA and IgE in serum samples were increased in rEg.P29+CpG+infection compared with PBS group and CpG group. The levels of rEg.P29-specific IgG, IgM, IgA and IgE of CpG+infection group had no significant difference compared with PBS group (Figure 4A-D). After adoptive transfer CD4+ T cells, the levels of IgG, IgM, IgA and IgE in serum of rEg.P29+CpG+infection were significantly increased compared with CD4+T+PBS+infection and CD4+T+CpG+infection group. The levels of IgG, IgM, IgA and IgE of CD4+T+CpG+infection group had no significant difference compared with CD4+ T+PBS group (Figure 4E,F). These data suggested rEg.P29 induced specific antibodies could prevent E. multilocularis infection in CD4+ T cell-deficient mice.

4. rEg.P29 induced CD8+ T cells response in CD4+ T cell-deficient mice, but did not induce CD4+ T cell response during E. multilocularis infection

To understand the roles of the cellular response in the process of E. multilocularis infection, specific cytokines produced by lymphocytes in the spleen of CD4+ T cell-deficient mice in each group before or after adoptive transfer CD4+ T cells were detected to identify the level and type of cellular response against E. multilocularis. The results suggested that IFN-γ secretion by CD8+ T cells was significantly increased in rEg.P29+CpG+infection group with rEg.P29 stimulation when compared with unstimulation. The IFN-γ secretion by CD8+ T cells of PBS+infection group and...
CpG+infection group with rEg.P29 stimulation had no difference compared with unstimulation. However, the IFN-γ secretion by CD4+ T cells of rEg.P29+CpG+infection group with rEg.P29 stimulation had no difference compared with unstimulation and the number of CD4+ T cells had no difference compared with PBS+infection group and CpG+infection group (Figure 5A–D). After adoptive transfer CD4+ T cells, the results showed that IFN-γ secretion by CD8+ T cells of CD4+T+rEg.P29+CpG+infection group with rEg.P29 stimulation was significantly increased when compared with unstimulation. The IFN-γ secretion by CD8+ T cells of CD4+T+PBS+infection group and CD4+T+CpG+infection group had no difference with rEg.P29 stimulation compared with unstimulation. In addition, The number of CD4+ T cells were significantly increased, but the IFN-γ secretion by CD4+ T cells of CD4+T+rEg.P29+CpG+infection group had no difference with rEg.P29 stimulation compared with unstimulation (Figure 5E–H).

Moreover, the cytokines in the culture supernatant were detected by ELISA. The results showed that the level of IFN-γ of rEg.P29+CpG+infection group was significantly increased, but the level of IL-2, IL-6, TNF-α, and IL-10 had no difference, compared with PBS+infection group and CpG+infection group (Figure 6A–E). After adoptive transfer CD4+ T cells, the levels of IFN-γ and IL-2 of CD4+T+rEg.P29+CpG+infection group were significantly increased, but the levels of TNF-α, IL-6, and IL-10 had no difference compared with CD4+ T+PBS+infection group and CD4+ T+CpG+infection (Figure 6F–J). These data suggested that rEg.P29 can induce CD8+ T cell response to alleviate E. multilocularis.

5. rEg.P29 induced CD8+ memory T cells in the spleen

The vaccine induced the formation and maintenance of memory cells, which play a critical role in protecting against parasite infection. Spleen lymphocytes were used to detect CD44+CD62L+CD8+CD8+ memory cells by flow cytometry on the twelve week after priming and boost immunization. As shown in Figure 7, the results suggested that the proportion of CD44+CD62L+CD8+ memory cells (Tcm) was significantly decreased in rEg.P29 immunized group compared with PBS or CpG group (Figure 7A,B), while the proportion of CD44+CD62L+CD8+ memory cells (Tem) was not significantly changed compared PBS and CpG groups (Figure 7A,C). After adoptive transfer CD4+ T cells, the CD44+CD62L+CD8+ T cells of rEg.P29 plus infection were decreased than PBS plus infection and CpG plus infection, the proportion of CD44+CD62L+CD8+ T cells were not significantly different (Figure 7D–F).

Discussion

AE is a zoonotic parasitic disease. Humans are infected by accidental oral uptake of viable eggs, and the eggs parasitize the liver tissue as the blood circulates and form cysts in the liver. The alveolar cyst causes maliging tumor-like lesions with infiltrative, proliferative and destructive characteristics that are primarily located in the liver and then metastasize to other organs. Surgery is the main therapeutic option, but its outcomes are compromised by postoperative complications. Albendazole may have a certain therapeutic effect, but it should be administered continuously and cause toxic side effects; therefore, the current cure rate of AE is only 30%-40% per year [18].

The incidence of AE is approximately 18,200 cases per year worldwide, resulting in approximately 666,000 disabilities every year, and it is significant to determine that 91% of new cases occur in China [19, 20]. Vaccines are the most economical and effective way to prevent and control infectious diseases [21]. In recent years, studies have suggested that vaccination is an effective way to control and prevent E. multilocularis infection, and immunization with recombinant proteins of parasites, such as leucine aminopeptidase (LAP), EMY162, EM95 and tetraspanin (TSP),
could protect against *E. multilocularis* infection and infiltration in host livers [22]. The study showed that the REMY162 antigen induced a significant level of protection for the host (74.3%) in experimental infection with *E. multilocularis* eggs in mice [23]. Vaccination with rEm-LAP significantly decreased both the number and size of cysts in an *E. multilocularis* metacestode-infected mouse model [24]. Our research results suggested that the protection of rEg.P29 against CD4+ T cell-deficient mice with *E. multilocularis* was 35.74%, rEg.P29 can also induce CD4+ T cell-deficient mice to produce high levels of antibody responses and CD8+ T-cell immune responses. These results clarified that rEg.P29 induced antibody response and CD8+ T cell response to prevent *E. multilocularis*. The mechanisms of immunity include parasite-neutralizing antibodies that can inhibit parasite motility in the liver at the site of infection and in the bloodstream during transit to the hepatocyte host cell and block interaction with host cell receptors on hepatocytes [25].

According to the secretion of cytokines, CD4+ T cells are divided into different subpopulations and play various functions in a large number of diseases, such as Th1, Th2, Th17 and Th9. Th1 cells mainly secrete the cytokine IFN-γ, while the cytokine IL-4 is induced by Th2 lymphocytes. Th17 lymphocytes can secrete IL-17 cytokines [26], while IL-9 is induced by Th9 lymphocytes [27]. During the early stage of *E. multilocularis* development, the research results suggested that IFN-γ secreted by Th1 lymphocytes was able to eliminate metacestodes at the initial stages of development. With the development of infection, the Th2 immune response gradually becomes dominant in an infective *E. multilocularis* mouse model [28]. In this article, we found that the protection of rEg.P29 against *E. multilocularis* was 41.52% after adoptive transfer of CD4+ T cells from wild-type mice and rEg.P29 induced high levels of IgG, IgM, IgA and IgE antibodies and IFN-γ-secreting CD8+ T cells, and the number of CD4+ T cells was significantly increased, but IFN-γ-secreting CD4+ T cells were not significantly different after adoptive transfer. We infected 2×10^6 CD4+ T cells from wild mice via the tail vein. Only a small portion of CD4+ T cells resided in the spleen tissue, and the other portion of CD4+ T cells reached the infection site or other organisms in the blood circulation and played a role in anti-*E. multilocularis* infection. In addition, we used bioinformatics to compare the amino acid sequences and protein spatial structure of the P29 proteins of *E. multilocularis* and *E. granulosus* and found that there are four amino acid changes between *E. granulosus* and *E. multilocularis* (Supplementary Figure S1), and the position of the amino acid mutation may be an antigen epitope and cause the rEg.P29 antigen not being able to recognize the epitope of *E. multilocularis*. The accessibility of an epitope combined with the right amino acid sequences to form resistant complexes with MHC class II can establish dominance over the other epitopes by being presented at higher quantities to T cells [29]. Spatial structure comparison showed that the structure of the P29 protein is basically the same between *E. granulosus* and *E. multilocularis* (Supplementary Figure S2).

During the vaccine development process, selecting appropriate adjuvants can significantly increase the effectiveness of the vaccine. In our priming and boost schedule, adjuvant CpG ODNs promoted the immunogenicity of rEg.P29. CpG ODNs improve the function of professional antigen-presenting cells and boost the generation of humoral and cellular vaccine-specific immune responses [30]. In our previous research, CpG ODNs could not induce independently specific immune responses without rEg.P29, but it can enhance the immune effect without inducing a specific immune response [31].

In conclusion, the protection of rEg.P29 against *E. multilocularis* was 35.74% without CD4+ T cells. After adoptive transfer CD4+ T cells of wild mice, the protection of rEg.P29 was 41.52%.
It can be seen from this that CD4+ T cells also play critical roles during anti-
E. multilocularis infection.

Supplementary Data

Supplementary data is available at Acta Biochimica et Biphysica Sinica online.

Funding

This work was supported by the grant from the Ningxia Hui Autonomous Region Key Research
Program (No. 2022BEG02037).

Conflict of Interest

The authors declare that they have no conflict of interest.

References


Figure legends:

Figure 1. Scheme of immunization and sampling protocol  C57BL/6 N-Cd4emlyagen mice (n=3/group) transferred CD4+ T cells from wild-type mice to CD4+ T cell-deficient mice (−5) and were immunized following the prime-boost scheme. Two boosts (−2 and −1) were performed in the process. After one week, 2000 PSCs/200 μL were intraperitoneally injected into each mouse (0). Spleen samples and serum samples were collected and tested at the 12th week (12).

Figure 2. Anatomical view of intraperitoneally infected protoscolex mice in each group  At the 12th week after E. multilocularis infection, the mice were anaesthetized with isoflurane, and the infection status of Echinococcus multilocularis in the abdominal cavity of mice in each group was observed.

Figure 3. The level of antibody in serum was detected by ELISA  Twelve weeks after E. multilocularis infection, serum was collected to determine the antibody level. (A–D) Before adoptive transfer, the change in antibodies in serum was detected. (E-H) After adoptive transfer, the change in antibodies in serum was detected. (A,E) The level of IgG in serum. (B,F) The level of IgM in serum. (C,G) The level of IgA in serum. D and H: The level of IgE in serum. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Flow cytometry was used to detect the proportion of CD4+ T cells before and after sorting  The spleen was collected from wild-type C57BL/6 N mice, and lymphocytes were isolated. LCM was used to sort CD4+ T cells T cells. (A) The proportion before isolation. (B) The proportion after isolation.

Figure 5. The expression of IFN-γ in CD4+ T cells and CD4+ T cells before or after adoptive transfer of CD4+ T cells from wild-type mice was detected by flow cytometry  (A,E) The expression of IFN-γ in CD8+ T cells before or after adoptive transfer was detected by flow cytometry. (B,F) The expression of IFN-γ in CD4+ T cells before or after adoptive transfer was detected by FCM. (C,D) Frequencies of IFN-γ-producing CD8+ T cells in each group are shown. (G,H) Frequencies of IFN-γ-producing CD4+ T cells in each group are shown. *P<0.05, **P<0.01, ***P<0.001.

Figure 6. Cytokines were detected in the culture supernatant by ELISA  Twelve weeks after E. multilocularis infection, lymphocytes were collected and stimulated with rEg.P29 protein, ELISA analysis of the changes in cytokines of each group before or after adoptive transfer. (A–E)
Cytokines (IFN-γ, IL-2, TNF-α, IL-6 and IL-10) were detected before adoptive transfer. (F,J) Cytokines were tested after adoptive transfer. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

**Figure 7. Memory phenotype of CD8$^+$ T cells** At the twelve week after *E. multilocularis* infection, the spleen was collected, and lymphocytes were isolated. Splenocytes were collected to measure the memory phenotype of CD8$^+$ T cells before or after adoptive transfer of CD4$^+$ T cells from wild-type mice. (A,D) Representative contour plot analysis of gated CD8$^+$ T cells expressing CD44 and CD62L in the spleen. Number of CD8$^+$ T cells presenting the cell surface phenotype (B,E) CD44$^+$CD62L$^-$ cells and (C,F) CD44$^-$CD62L$^+$ cells before or after adoptive transfer of CD4$^+$ T cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$. 
### Priming & Boosting scheme

<table>
<thead>
<tr>
<th>S.C.</th>
<th>Adoptive transfer</th>
<th>Priming</th>
<th>Boosting</th>
<th>Infection</th>
<th>Sample testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS+infection</td>
<td>-5</td>
<td>-4</td>
<td>-3</td>
<td>-2</td>
<td>-1</td>
</tr>
<tr>
<td>CpG+infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rEG.P29+CpG+infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS+CD4+T+infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CpG+CD4+T+infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rEG.P29+CpG+CD4+T+infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

180x41mm (300 x 300 DPI)
For Peer Review

50x40mm (600 x 600 DPI)

CD4

CD3

Before isolation

18.8

After isolation

99.1

75x40mm (600 x 600 DPI)
A  PBS+Infection  B  CpG+Infection  C  rEg.P29+ CpG+Infection
D  CD4^+T+ PBS+Infection  E  CD4^+T+ CpG+Infection  F  CD4^+T+rEg.p29 CpG+Infection

128x150mm (300 x 300 DPI)
170x118mm (300 x 300 DPI)
162x105mm (300 x 300 DPI)
Table 1. The body and cyst weights of each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Cyst weight (g)</th>
<th>Cyst inhibition rate a</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS+Infection</td>
<td>29.81 ± 4.45</td>
<td>5.99 ± 2.55</td>
<td></td>
</tr>
<tr>
<td>CpG+Infection</td>
<td>36.66 ± 1.86</td>
<td>8.62 ± 0.90</td>
<td></td>
</tr>
<tr>
<td>rEg.P29+CpG+Infection</td>
<td>28.38 ± 2.59</td>
<td>3.85 ± 3.33*</td>
<td>35.74%</td>
</tr>
<tr>
<td>PBS+CD4 T+Infection</td>
<td>41.96 ± 1.61</td>
<td>9.10 ± 5.30</td>
<td></td>
</tr>
<tr>
<td>CpG+CD4 T+Infection</td>
<td>38.92 ± 4.52</td>
<td>8.37 ± 1.22</td>
<td></td>
</tr>
<tr>
<td>rEg.P29+CpG+CD4 T+Infection</td>
<td>35.64 ± 2.86</td>
<td>5.34 ± 1.44*</td>
<td>41.52%</td>
</tr>
</tbody>
</table>

*Cyst inhibition rate (%)=[(cyst weight of PBS group–cyst weight of rEg.P29 immunization group)/cyst weight of PBS group]×100%. *P<0.05 vs PBS+Infection group; #P<0.05 vs PBS+CD4 T+Infection group.
Supplementary Figure S1. P29 sequences were blasted between *Echinococcus granulosus* and *Echinococcus multilocularis*. The sequences of EgP29 and EmP29 were obtained from NCBI Database and run blast.

<table>
<thead>
<tr>
<th>Range</th>
<th>Query</th>
<th>Sbjct</th>
<th>Score</th>
<th>Expect</th>
<th>Method</th>
<th>Identities</th>
<th>Positives</th>
<th>Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-238</td>
<td>AHA85399.1</td>
<td>60</td>
<td>480 bits (1236)</td>
<td>7e-180</td>
<td>Composition matrix adjusted</td>
<td>234/238 (99%)</td>
<td>236/238 (99%)</td>
<td>0/238 (0%)</td>
</tr>
</tbody>
</table>
Supplementary Figure S2. The spatial structure of EgP29 and EmP29 were constructed

SWISS-MODEL was used to predict the spatial structure of EgP29 and EmP29. (A) The structure of Eg P29. (B) The structure of Em P29.
Echinococcus granulosus recombinant protein P29 is a good vaccine candidate against *E. granulosus*. However, the protective and immunological mechanism of rEg.P29 against *Echinococcus multilocularis* remain unclear. In this study, we constructed a mouse model of rEg.P29 immunization combined with *E. multilocularis* infection, clarified the protective effect of rEg.P29 against *E. multilocularis* infected CD4$^+$ T cell-deficient mice.

1. The recombinant protein P29 of *E. granulosus* alleviates *E. multilocularis* infection by inducing the production of specific antibodies and CD8$^+$ T cell responses in CD4$^+$ T cell-deficient mice.

2. After adoptive transfer of CD4$^+$ T cells, the protection of the recombinant protein P29 against Echinococcus multilocularis-infected mice was significantly improved.
450x350mm (300 x 300 DPI)