Production and Evaluation of a Novel Multi-Epitope Bivalent Vaccine Against *Echinococcus multilocularis* Metacestode

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Abstract

Alveolar *Echinococcosis* is a globally widespread zoonotic disease caused by the larval stage of *Echinococcus multilocularis* (Em) and is seriously harmful to human health. In our previous studies, we found that Em-EMY162 had good prophylactic and therapeutic effects against Em infection and the dominant epitopes of Em-EMY162 and Em-TSP3 were also identified. In this study, a multi-epitope vaccine LTB-ETBM targeting both Em-EMY162 and Em-TSP3 was designed and constructed, and then the immunogenicity and protective efficacy were evaluated in Em infected mice. The results showed that LTB-ETBM could induce high levels of specific IgG against Em-EMY162 and Em-TSP3 and a mixed Th1/Th2 lymphocyte response to LTB-ETBM. Moreover, the LTB-ETBM significantly inhibited the formation of cysts in mice challenged with 1000 Em protoscoleces. In a therapeutic mouse model injected intraperitoneally with 1000 protoscoleces, vaccination with LTB-ETBM using either Freund’s or CpG as an adjuvant significantly decreased the growth of protoscoleces and the formation of cysts. LTB-ETBM may be efficacious for use as a prophylactic or therapeutic agent against Em infection.

Keywords *Echinococcus multilocularis* · Multi-epitope bivalent vaccine · Prevention · Therapeutic

Introduction

*Echinococcosis* is a neglected zoonotic infection disease caused by the larval stage of the genus *Echinococcus*. In humans, *Echinococcosis* is classified as cystic *echinococcosis* (CE) and alveolar *echinococcosis* (AE) depending on the type of *Echinococcus* species that causes infection. AE is a globally widespread zoonotic disease caused by the metacestode of *Echinococcus multilocularis* (Em). The AE disease shows a as a result of the continued parasite proliferation (Wang and Gottstein 2016). Humans are accidentally infected with Em, which commonly has a long incubation period that may be greater than ten years. Once AE symptoms develop, continuous proliferation of lesions can cause disorders of the hepatic tissues, such as fibrosis and abscesses (Cai et al. 2017; Eckert and Deplazes 2004). AE lesions behave like a slowly growing and metastasizing liver cancer, and AE can be lethal if left untreated and the pathogen migrates to other organs like the lung, brain and skeleton (Atanasov et al. 2013; Pang and Chu 2015). Medication has a definite effect on patients if AE is caught early, but it has a negligible efficacy on terminal patients. The use of preventive vaccines for serious diseases, which can allow...
recognition and elimination of the pathogen by the immune system, is widely recognized and accepted. Therefore, it may be possible to achieve protection against Em by triggering immune responses that are different from those induced by natural infection.

In our previous study, we expressed and purified a subunit vaccine LTB-EMY162 against Em. It can protect mice against Em infection and reduce the cyst formation (2/6) (Li et al. 2018). In addition, one study has reported that subcutaneous and intranasal administration of rEm-TSP3 (tetraspanin 3), derived from the Em-TSP3 protein located on the surface of Em cysts, protoscoleces and adult worms, achieved an 81.9% and 62.8% reduction, respectively, in the number of cysts in the liver (Dang et al. 2012a, b). Therefore, EMY162 and TSP3 may be excellent candidate antigens for the development of new vaccines against Em. We recently predicted and detected the dominant Th and B cell epitopes of EMY162 and TSP3 (Pang et al. 2020, 2022).

In this study, we selected four dominant epitopes from EMY162 and TSP3 to construct a bivalent multi-epitope vaccine LTB-ETBM, which is composed of the highly specific B and T cell epitopes from EMY162 and TSP3, and an intramolecular mucosal adjuvant *Escherichia coli* heat-labile enterotoxin B subunit (LTB). The vaccine antigen LTB-ETBM was purified and used to immunize BALB/c mice, and its immunogenicity, prophylactic and therapeutic effects were evaluated.

**Materials and Methods**

**Design of the Multi-Epitope Bivalent Vaccine LTB-ETBM**

Based on our previous study, the dominant epitopes of EMY162 and TSP3 were identified by performing specific ELISA, lymphocyte proliferation, flow cytometry and ELISPOT assays. The four epitopes with the highest specificity in these assays were EMY1627–13, EMY16236–48, TSP333–42 and TSP380–90 (Pang et al. 2020, 2022).

The theoretically optimal sequence consisting of the intra-molecule adjuvant LTB (GenBank: AAL55672.1), linkers, and tandem copies of the Th and B cell epitopes named ETBM was established. The LTB-ETBM sequence was submitted to GenBank (accession number: MT731963). The sequence was analyzed using bioinformatics software for modeling and prediction. For all details, please see our preliminary study (Guo et al. 2014).

**Construction, Expression, and Purification of Vaccine Antigen LTB-ETBM**

To obtain the vaccine antigen LTB-ETBM, a DNA fragment LTB-ETBM was synthesized after reverse translation and codon-optimization. The synthesized LTB-ETBM gene was cloned into the plasmid pCzn1 after digestion (NdeI and XbaI) and connection, generating the expression plasmid pCzn1-LTB-ETBM. The recombinant plasmid pCzn1-LTB-ETBM was transformed into ArcticExpress competent cells (DE3). The vaccine antigen LTB-ETBM was purified by Ni²⁺-IDA-Sepharose CL-6B (Genscript, Nanjing, China), and measured by 12% SDS-PAGE. The purification method of LTB-ETBM was followed by the HUPO proteomics standard initiative (http://www.psidev.info/miape) and publication guidelines. The purified protein was concentrated using a dialysis bag. It is stored at – 80 °C for later use.

**Immunization and Infection**

The BALB/c mice (SPF, male, 4–6 weeks, n = 6) were purchased from Beijing vital river laboratory animal technology company (Beijing, China). All animal procedures were approved by the Animal Ethical and Experimental Committee of Qinghai University (QHDX-2019-09). The mice were immunized with 0.5 mg/mL of LTB-ETBM, rLTB (recombinant LTB purified in our lab), or phosphate buffer solution (PBS) with the same volume of complete Freund’s adjuvant (Sigma, St. Louis, USA) for the first vaccination and incomplete Freund’s adjuvant (Sigma, St. Louis, USA) for the second and third vaccinations. The last booster vaccination consisted of the fusion protein without adjuvant. Mouse antisera were collected after the last booster on the fifth day, and then stored at – 80 °C for later use.

Em protoscoleces were isolated and preserved in our lab as described previously (Li et al. 2018). Em protoscoleces were isolated as follows: mice were sacrificed and aseptically separated the cysts from the abdomen and liver. The cysts were cut into pieces and ground through 300-μm nylon mesh and 900-μm nylon mesh in turn. Protoscoleces were suspended in normal saline (1000 protoscoleces/200 μl) after being obtained on the mesh at the last filtration.

Prophylactic experiments of vaccine were performed as previously (Boubaker et al. 2015; Li et al. 2018). The mice were vaccinated with LTB-ETBM, rLTB or PBS, with six mice in each group. After two weeks, all mice were challenged with protoscoleces (intraperitoneally, 200 μl of normal saline suspension). Vaccinated mice were maintained for four months before the investigation of Em infection. The cysts (including subcutaneous, abdominal and thoracic cysts as well as cysts from the inside or surface of the liver) were
carefully stripped and weighed. The antisera were collected and stored at −80 °C for later use.

Therapeutic experiments were performed as previously (Li et al. 2018). To establish the Em-infected mouse model, mice were challenged with protoscoleces (intraperitoneally, 200 μl with normal saline suspension). After four months, three mice were killed to determine whether they were successfully infected with Em. The infected mice were subcutaneously injected monthly for 4 months with 0.5 mg/mL LTB-ETBM in PBS emulsified with the same volume of Freund’s adjuvant or 15 μg CpG. The rLTB and PBS follow the same protocol as the control group. All mice were sacrificed and aseptically separated the cysts for evaluation of Em infection after two weeks. The cysts (including subcutaneous, abdominal and thoracic cysts and cysts from the inside or surface of the liver) were carefully stripped and weighed. The antisera were collected and stored at −80 °C for later use.

Western Blot Analysis of the Immunoreactivity of the LTB-ETBM Vaccine

The purified EMY162 was separated by 12% SDS-PAGE (Bio-Rad, California, USA) and equilibrated in ice cold transfer buffer, then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, USA) by 200 mA constant current. The PVDF membrane was incubated with mouse polyclonal anti-LTB-ETBM serum (1:2500). The membrane was washed with PBST four times and incubated with HRP-goat anti-mouse IgG (Jackson Immuno Research Lab, West Grove, United States) at a dilution of 1: 10,000. Luminescence ECL detection kits (Thermo Fisher) were used to monitor the positive signals.

The Immunogenicity Assessment of the LTB-ETBM Vaccine

After the last injection, the antisera were analyzed by indirect ELISA. The 96-well microplates were coated with EMY162 or TSP3 overnight at 4 °C. The sera were diluted to 1:8,000 and 1:4,000, respectively. The HRP-goat anti-mouse IgG, IgG1, IgG2a, IgA, IgM and IgE (IgE were purchased from Jackson Immuno Research Lab, West Grove, United States) at a dilution of 1:10,000 were used as secondary antibodies, respectively.

Determination of Specific Antibodies Production After Em Challenge

Serum samples were collected from the mice after prophylactic and therapeutic vaccination. The titers of serum specific antibodies against EMY162 were determined by indirect ELISA. The 96-well microplates were coated with EMY162 overnight at 4 °C. The sera were diluted to 1:8,000 and 1:4,000, respectively. The HRP-goat anti-mouse IgG, IgG1, IgG2a, IgA, IgM and IgE (IgE were purchased from Jackson Immuno Research Lab, West Grove, United States) at a dilution of 1:10,000 were used as secondary antibodies, respectively.

Evaluation of T Lymphocyte Responses

The splenocyte proliferation assay was performed according to the protocol previously described (Guo et al. 2012). Splenocytes were prepared using 70 μm nylon mesh cell strainer (Falcon, Corning, USA) and Lympholyte®-M (Cedarlane, Canada) from mice vaccinated with LTB-ETBM or PBS. The splenocytes were seeded with 2 × 10^5 cells/well and cultured in triplicate in a 96-well plate. Subsequently, the cells were stimulated with 2 μg/well LTB-ETBM, EMY162, TSP3, EMY16236–48, EMY1627–13, TSP380–90, or TSP333–42. The plates were incubated for sixty hours in a cell incubator, then added 20 µl/well of MTS (Promega, Beijing, China). After three hours of incubation, the absorbance was measured at 490 nm. The stimulation index (SI) represents cell proliferation, and the formula is based on our previous study (Li et al. 2018).

Determination of Cytokine Production

Cytokines (IFN-γ, IL-4, IL-17, and IL-10) in serum were measured by its mouse ELISA kit on the basis of the user guide (R&D Systems, Minneapolis, MN, United States) after prophylactic or therapeutic experiments. In our experiment, the PBS control group was immunized with Freund’s
adjuvant, which would affect the cytokine concentration, so we added a normal control group to indicate the cytokine concentration of vaccine LTB-ETBM.

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism 6 software. Data is expressed as mean ± standard deviation (SD). Differences between the two groups were tested using Student’s paired t-tests, and ***p < 0.001, **p < 0.01, *p < 0.05 was considered statistically significant. One-way analysis of variance was used to make statistical comparisons of the IgG antibodies specific for EMY162, it was applied to compare the differences among groups.

**Results**

**Design and Construction of the Multi-Epitope Divalent Vaccine LTB-ETBM**

The fragments EMY1627–13, EMY16236–48, TSP333–42, and TSP380–90 were selected as the components of the multi-epitope divalent vaccine LTB-ETBM. The LTB-ETBM vaccine (shown in Fig. 1) contained tandem copies of the selected epitopes, which were fused with the C-terminus of the intramucosal adjuvant LTB. DPRVPSS was used as a spacer between LTB and the epitopes. KK was selected as the linker between Th cell epitopes and GS as the linker between B cell epitopes. The recombinant plasmid pCzn1-LTB-ETBM was verified by restriction enzyme digestion using Nde I and Xba I and by nucleotide sequencing. After digestion, the DNA band was about 600 bp, consistent with the predicted size of the ETBM gene (Fig. 2a). The sequencing results also confirmed that the pCzn1-LTB-ETBM plasmid was a successful construction.

**Expression and Purification of LTB-ETBM**

The LTB-ETBM fusion protein was expressed in ArcticExpress (DE3) cells. SDS-PAGE analysis indicated that most of the protein was in inclusion bodies. The pure recombination LTB-ETBM was obtained after purification by Ni²⁺-IDA-Sepharose CL-6B (Fig. 2b). Results from Western blot analysis showed that the polyclonal antibody induced by the LTB-ETBM protein could react with EMY162 (Fig. 2b, lane 7). It was demonstrated that LTB-ETBM had specific immunoreactivity against EMY162. The immunoreactivity against the LTB-ETBM protein was also verified by ELISA (Fig. 3a and b).

**Production of Specific Antibodies After Immunization**

The LTB-ETBM-induced specific antibodies against EMY162, TSP3, and Em whole protein was evaluated by indirect ELISA. After immunization with the LTB-ETBM vaccine, the mice had significantly higher titer of IgG antibodies against EMY162 \( F(2, 15) = 99.12; t = 8.73 \) vs. rLTB; \( t = 11.96 \) vs. PBS (Fig. 3a), TSP3 (\( t = 12.54 \) (Fig. 4a) and protoscoleces whole protein (\( t = 4.639 \) and (Fig. 3b) compared with mice immunized with rLTB or PBS. These results confirmed that LTB-ETBM had good immunogenicity and immunoreactivity. Moreover, antigen-specific antibodies against EMY162 and TSP3 were also detected by indirect ELISA. Mice receiving the LTB-ETBM vaccine showed significantly higher titers of IgG1 (\( t = 34.20 \)), IgG2a (\( t = 39.73 \)), IgM (\( t = 10.05 \)), IgE (\( t = 7.52 \)) and IgA (\( t = 3.340 \) antibodies against EMY162 than PBS-immunized mice (Fig. 3c and d). Mice receiving the LTB-ETBM vaccine showed significantly higher titers of IgG1 (\( t = 11.60 \)), IgG2a (\( t = 5.612 \)) and IgM (\( t = 4.977 \)) antibodies against TSP3 than PBS-immunized mice (Fig. 4a and b), the levels of IgE (\( t = 0.9134 \)) and IgA (\( t = 0.9479, p = 0.3655 \) ) antibodies against TSP3.
between LTB-ETBM and PBS-vaccinated mice was not as significant.

**Evaluation of T Lymphocyte Responses**

Here we investigated the lymphocyte responses to the Th epitopes in LTB-ETBM. Splenocytes were separated from mice receiving LTB-ETBM or PBS vaccine, and then stimulated with TSP3, EMY162, LTB-ETBM, EMY16236–48, EMY1627–13, TSP380–90, or TSP333–42. As shown in Fig. 4c, mice receiving LTB-ETBM showed significant proliferation of splenocytes after stimulation with LTB-ETBM (SI = 2.512 ± 0.114). Moreover, after the splenocytes stimulation with EMY162 (SI = 1.805 ± 0.119), TSP3 (SI = 1.890 ± 0.149), EMY16236–48 (SI = 1.673 ± 0.114), EMY1627–13 (SI = 1.703 ± 0.075), TSP380–90 (SI = 1.584 ± 0.07), or TSP333–42 (SI = 1.551 ± 0.076). Splenocytes from mice receiving LTB-ETBM showed significant proliferation compared with that of the PBS-treated mice, but the differences in SI between these treatments and the PBS control were not positive (SI < 2). These results indicated that the multi-epitope bivalent vaccine LTB-ETBM could induce lymphocyte responses against EMY162, TSP3 and Th epitopes.

**Prophylactic Effect of LTB-ETBM**

Four months after being intraperitoneally injected with protoscoleces, the quantity and weight of cysts were evaluated to determine the prophylactic effect of LTB-ETBM. The mice vaccinated with LTB-ETBM showed fewer (LTB-ETBM/rLTB/PBS; 4/6/6) and smaller cysts (Fig. 5a, b) compared with mice vaccinated with PBS or rLTB, indicating that the multi-epitope divalent vaccine LTB-ETBM could reduce the cysts formation against Em.

**Therapeutic Effect of LTB-ETBM**

Two weeks after the last vaccination, the quantity and weight of cysts were evaluated to determine the therapeutic effect of LTB-ETBM. The Em-infected mice treated with LTB-ETBM could reduce the cysts formation (Fig. 6a, b). The weight (Fig. 6a) and the number of cysts (Fig. 6b) were significantly reduced in mice treated with LTB-ETBM compared with mice treated PBS. These results indicated that LTB-ETBM had a certain therapeutic effect.

**Production of Specific Antibodies After Protected and Treated with LTB-ETBM**

The multi-epitope divalent vaccine LTB-ETBM induced different levels of serum IgG (including IgG1 and IgG2a), IgM, IgE, and IgA antibodies after prophylactic or therapeutic vaccination. Compared with the PBS group, the mice immunized with LTB-ETBM, showed higher levels of specific IgG (t = 7.85), IgG1 (t = 9.350), and IgG2a (t = 4.763) antibodies against EMY162 (Fig. 5c, d). The Em-infected mice treated with LTB-ETBM plus Freund’s adjuvant or...
LTB-ETBM plus CpG induced significantly higher levels of specific IgG ($t = 15.16$; $t = 20.22$), IgM ($t = 7.323$; $t = 8.559$), IgE ($t = 10.74$; $t = 19.39$), IgA ($t = 5.234$; $t = 9.389$), IgG1 ($t = 14.99$; $t = 18.75$), and IgG2a ($t = 4.857$; $t = 7.194$) antibodies against EMY162 than mice treated with PBS (Fig. 6c, d).

**Determination of Serum Cytokine Concentration**

Here we investigated the levels of cytokines (IFN-γ, IL-4, IL-17, and IL-10). Mice receiving the LTB-ETBM showed significantly increased levels of IFN-γ ($t = 5.145$ vs. PBS; $t = 6.941$ vs. control) and IL-4 ($t = 2.691$ vs. PBS; $t = 4.352$ vs. control) in LTB-ETBM-immunized mice were significantly increased than those in PBS and normal control mice. The IL-10 ($t = 0.7102$ vs. PBS; $t = 1.725$ vs. control) was still the same, and the IL-17 ($t = 3.373$ vs. PBS; $t = 3.801$ vs. control) (Fig. 5e) was significantly lower than those in the PBS and NC.

The Em-infected mice treated with LTB-ETBM significantly increased levels of IL-4 ($t = 3.697$ vs. PBS; $t = 3.275$ vs control) and IFN-γ ($t = 1.578$ vs. PBS; $t = 3.405$ vs. control) than those treated with PBS or normal control mice, and the IL-17 ($t = 1.707$ vs. PBS; $t = 2.622$ vs. control) and IL-10 ($t = 2.056$ vs. PBS; $t = 2.715$ vs. control) were significantly increased than those in PBS-treated mice (Fig. 6e).

**Discussion**

In this study, four dominant epitopes from Em-EMY162 and Em-TSP3 were selected, and a bivalent multi-epitope vaccine LTB-ETBM was successfully constructed for the
control of Em. The results showed that LTB-ETBM could effectively inhibit the formation of cysts and significantly reduce the number of vesicles, which had a certain prophylactic and therapeutic effects against Em effects.

**Alveolar Echinococcosis** caused by Em is a zoonotic disease with a high disability and mortality in animal husbandry areas. Invading the tissues and organs through intrahepatic vasculature is the pathological characteristic of AE (Yang et al. 2019). In some highly unique cases, distant metastasis to the brain and spine has also been observed (Meinel et al. 2018). For example, in one case report, a patient suffered from AE with liver, lung, and diaphragm involvement; recurrence still occurred 6 years after treatment (Pang and Chu 2015). The most common treatments for AE are surgery or drug therapy, but they do not completely cure it. The recombinant EG95 vaccine for CE caused by *Echinococcus granulosus* infection has been widely used for sheep and cattle, and has achieved good results (Jazouli et al. 2020). However, studies on the antigenic proteins of Em intermediate and terminal hosts have not obtained similar effects.

In our previous studies, we found that Em-EMY162 had good protective and therapeutic effects against Em (Li et al. 2018). EMY162 has been reported to be expressed in all four stages of the worm. It has also been reported that TSP3 has a certain prevention and treatment effect against Em, and TSPs have been reported to be used for vaccines against *Opisthorchis viverrini* and *Schistosomiasis japonicum*. Our previous study found that LTB-EMY162 had preventive and therapeutic effects against Em, but did not achieve full protection (Li et al. 2018). We speculate that this may be due to the poor effect of single antigen protein on the prevention and treatment of complex pathogens such as parasites, it also found that in our other study (Wang et al. 2021).
LTB-ETBM constructed in this study can target multiple antigen proteins of Em. In the study of immune protection, we found that LTB-ETBM had improved protective effects compared with LTB-EMY162, and the number and weight of cysts were reduced more obviously. These results suggested that simultaneous targeting of
multiple antigens against Em could enhance its protective effect. In our previous study, we identified dominant antigen epitopes EMY16236–48, EMY1627–13, TSP333–42, and TSP380–90 by AE patient serum than other predicted epitopes in ELISA, higher proliferation of B and Th cell lymphocytes, and higher levels of cytokines assessed using ELISpot and flow cytometry (Pang et al. 2020, 2022).

**Fig. 6** Effect of LTB-ETBM on cyst formation, change of antibodies and serum cytokine concentrations after therapeutic immunization. The four months AE mice model were immunized with LTB-ETBM plus Freund’s, LTB-ETBM plus CpG, rLTB, or PBS, monthly for 4 months. a Weight of the cysts after therapeutic immunization. b Number of the cysts after therapeutic immunization. c Detection of IgG1 and IgG2a antibodies after therapeutic immunization. The recombinant protein EMY162 coated on a 96-well plate (anti-sera dilution in 1:4000). d Detection of serum antibodies (IgG, IgA, IgM, and IgE) after therapeutic immunization. The recombinant protein EMY162 coated on a 96-well plate (anti-sera dilution in 1:4000). e and f The serum cytokine concentrations of mice after therapeutic vaccination and normal mice.
In this study, a multi-epitope bivalent vaccine LTB-ETBM targeting EMY162 and TSP3 was constructed, which contained the intra-molecular mucosal adjuvant LTB and tandem of Th and B cell epitopes from both EMY162 and TSP3. LT is a thermally unstable enterotoxin secreted by E. coli. LT is composed of A and B subunits. A subunit is the toxic site of LT, while B subunit is non-toxic and is the binding site of LT. LTB is widely used as mucosal immune adjuvant because of its conservative amino acid sequence, high activity and non-toxicity. LTB can recognize GM1 ganglioside and other receptors on the cell surface, and is often used as an antigen carrier, which can be used in combination with antigen to enhance the body's uptake of antigen and enhance the immune response. LTB binding GM1 can act on a variety of immune cells and regulate T cell differentiation. LTB can effectively initiate local and systemic T and B cell immune responses and up-regulate the expression of B cell surface molecules. Many reports have found that LTB can induce good intramolecular adjuvants and regulate immune typing (Lublin et al. 2022; Zerna et al. 2021).

The Em parasite avoids being cleared by the host immune system through an immune escape mechanism, so we speculated that balancing the host immune response may be beneficial in preventing and controlling AE. As for the immune escape mechanism of Em, a previous study found that the levels of IFN-γ increased gradually, at 3 months began to decline, whereas the levels of IL-4 increased after 3 months infect (Ma et al. 2014). These changes in cytokines may lead to the rapid growth of Em in the body. Thus, there is dissonance in the host Th1 and Th2 immune response during Em infection. In our study, LTB-ETBM as a therapeutic vaccine induced high titers of specific IgG, IgA, IgM, and IgE antibodies against EMY162 (Fig. 6c, d), maintained the high levels of IgG1 and IgG2a specific antibodies, and reduced cyst formation (Fig. 6a, b). Thus, we speculated that LTB-ETBM could balance the host immune response and thereby inhibit lesion proliferation.

The rational design of the epitope vaccine is very important for the efficacy of the vaccine. In our previous study, we showed that the linkers DPRVPSS, KK, and GS allowed the immunologic competence of each epitope to be retained while avoiding the production of new epitopes at linkage sites (Guo et al. 2012, 2014). Results from a splenic lymphocyte proliferation assay showed that splenic lymphocytes from mice receiving LTB-ETBM proliferated after stimulation with EMY162, EMY162, EMY162, EMY162, TSP3, TSP3, TSP3, and TSP3, and that the antibodies induced by LTB-ETBM could recognize the EMY162 and TSP3 antigens and protoscoleces whole protein, showing that Th epitopes all retained their functions. Furthermore, LTB-ETBM induced specific IgG antibodies against EMY162 and TSP3 (Figs. 3 and 4). In addition, we found that LTB-ETBM could induce the production IgG1 and IgG2a antibodies and increase the concentrations of the serum cytokines IFN-γ and IL-4 (Fig. 4). Thus, we speculated that these specific antibodies and cytokines could play an important role in controlling Em infection.

In this work, we found that when the multi-epitope divalent vaccine LTB-ETBM was used as a prophylactic vaccine, the levels of IgG, IgG1 and IgG2a were significantly increased than those in the PBS control group, and IFN-γ and IL-4 were significantly increased than those in the PBS and normal control groups. The increases in these specific antibodies and serum cytokines might have important roles in eradicating cyst. The weight of cysts was significantly lower when mice were injected with LTB-ETBM than when mice were injected with PBS or (Fig. 6). The size of cysts was also smaller than that in mice injected with LTB-ETBM with Freund’s adjuvant. Unfortunately, we had to discontinue the treatment because the PBS group mice had difficulty moving and ate less. The cyst size may have been further reduced if the experimental period was lengthened.

Moreover, we used CpG adjuvants in the therapeutic vaccine and obtained more positive results compared with Freund’s adjuvant. It has also been reported that the CpG DNA can stimulate a variety of immune cell activations and the production of a variety of cytokines. CpG DNA induces the production of Th1-type cytokines and IgG2a antibodies, showing a good adjuvant effect, that can enhance both humoral and cellular immune responses, especially cellular immune responses (Bode et al. 2011; Kovacs-Nolan et al. 2009; Sagara et al. 2009; Tengvall et al. 2005). Therefore, LTB-ETBM with CpG adjuvant is worth investigating as a novel vaccine against Em. In ongoing studies, we are looking for other active antigens to increase the efficacy of LTB-ETBM.

Many reports have found that the multi-epitope vaccine has the advantages of more focused antigens and better safety, and the multi-epitope vaccine for two antigens has a better protective effect than the subunit vaccine for a single antigen (Guo et al. 2014). In future studies, more antigenic proteins related to nutrient uptake from the host, development, and nutrient metabolism of Em will be investigated to design multi-epitope vaccines for the prevention and treatment of AE. In ongoing studies, we are looking for other active antigens to increase the efficacy of LTB-ETBM therapy.

Conclusions

In conclusion, a multi-epitope divalent vaccine LTB-ETBM against Em was designed, constructed, expressed and purified. The experimental results showed that LTB-ETBM could significantly reduce cyst formation in an Em-infected...
mouse, and induced specific IgG and IgA antibodies and a mixed Th1–Th2 cell response.

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Author Contributions Feng Tang, Le Guo, Haining Fan and Ri-li Ge conceived and designed research. Runle Li, Kunmei Liu, Lin Feng and Binwen Hu conducted experiments. Jinwei Ma, Mingyuan Xin, Mingquan Pang, Pei Zhou contributed analytical tools and analyzed data. Runle Li wrote the manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature that could be construed as influencing the position presented in the manuscript.

Ethical Approval All animal procedures were approved by were approved by the Animal Ethical and Experimental Committee of Qinghai University (QHDX-2019-09).

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