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Production and evaluation of a novel multi-epitope bivalent vaccine against *Echinococcus multilocaularis* metacestode

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Abstract

Alveolar *Echinococcosis* is a globally widespread zoonotic disease caused by the larval stage of *Echinococcus multilocularis* (*E.m.*) and is seriously harmful to human health. In our previous studies, we found that Em-EMY162 has good protective and therapeutic effects against *E.m.* and the dominant epitopes of Em-EMY162 and Em-TSP3 were also identified. In this study, a mucosal immunity multi-epitope vaccine LTB-ETBM targeting both Em-EMY162 and Em-TSP3 was designed and constructed. Furtherly the immunogenicity and immunoprotection were evaluated in *E.m.* infected mice model. LTB-ETBM could induce the mice generating high levels of specific IgG against Em-EMY162 and Em-TSP3. Furtherly a Th1/Th2 mixed lymphocyte responses to LTB-ETBM was identified. Moreover, the LTB-ETBM significantly inhibited the formation of cysts in mice challenged with 1000 *E.m.* 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 activating the immune system and for use as a prophylactic or therapeutic agent against *E.m.* infection.

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* (*E.m.*). The AE disease shows a chronically progressing hepatic damage as a result of the continued parasite proliferation. Humans are accidentally infected with *E.m.*, 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 *E.m.* 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 *E.m.* It can protect mice infected *E.m.* (2/6) and reduce the cyst formation(Li et al., 2018). Another group found that subcutaneous and intranasal administration of rEm-TSP3, derived from the Em-TSP3 protein located on the surface of *E.m.* 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; Dang et al., 2012b). Therefore, EMY162 and TSP3 may be excellent candidate antigens for the development of a vaccine against *E.m.* We

recently predicted and detected the EMY162 and TSP3 dominant Th and B cell epitopes(Pang et al., 2020).

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

Materials And Methods

Design of the multi-epitope bivalent vaccine LTB-ETBM

Based on our previous study, the dominant epitopes of each protein were identified by performing specific ELISA, lymphocyte proliferation, flow cytometry and ELISpot assays. The four epitopes with the highest specificity in these assays were $EMY162_{7\text{-}13}$, $EMY162_{36\text{-}48}$, $TSP3_{33\text{-}42}$ and $TSP3_{80\text{-}90}$ (Pang et al., 2020).

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 the LTB-ETBM

To construct the fusion protein 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 (*Nde*I and *Xba*I) and connection, generating the expression vector pCzn1-LTB-ETBM. The recombinant plasmid was transformed into ArcticExpress competent cells (DE3). The fusion protein LTB-ETBM was purified by Ni^{2+}-IDA-Sepharose CL-6B (Genscript, Nanjing, China), and measured by 12% SDS-PAGE. Methods of LTB-ETBM purification were 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℃ 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). The animal experiments on E. multilocularis 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 from *E. coli* BL21 ArcticExpress competent cells [DE3] transformed with the expression vector pCzn1-LTB), 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. The mice antisera were collected after the last booster on the fifth day. It is stored at -80℃ for later use.

E.m. protoscoleces were isolated and preserved in our lab as described previously (Li et al., 2018). *E.m.* 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.

The protocol of vaccine protective effect was 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, and the protocol was the same as for immunization. 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 *E.m.* 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 mice antisera were collected and stored at -80℃ for later use.

The protocol for vaccine therapeutic effect was performed as previously(Li et al., 2018). To establish the *E.m.*-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 *E. multilocularis*. 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 15μg CpG. The rLTB and PBS follows the same protocol as the control group. All mice were sacrificed and aseptically separated the cysts for evaluation of *E.m.* 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 mice antisera were collected and stored at -80℃ for later use.

Western blot analysis of the immunoreactivity of the LTB-ETBM vaccine

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 mice 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.

Measurement of immunogenicity of the LTB-ETBM vaccine

After the last injection, the anti-serum was analyzed by indirect ELISA. The 96-well plates were coated with EMY162 or TSP3 overnight and blocked with 5% (w/v) bovine serum albumin (BSA) at room

temperature for 4 h. After three times washes, 100 µl diluted serum (1:2500) were added to the corresponding well for 1 h at 37℃. The plate was washed with PBST three times, then 100 µl HRP-goat anti-mouse IgG (1:10,000, IgG1 (1:2,000), IgG2a (1:2,000), IgM (1:2,000), IgE (1:2,000) or IgA (Santacruz, Dallas, USA; 1:6,000) was added to the corresponding well for 1 h at 37℃. Then, the substrates were incubated in 100 µl TMB for 10 min at room temperature and the reaction was stopped by the addition of 50 µl 2 M H_2SO_4. The optical density (OD) was measured at 450 nm by a microplate reader (TECAN, Switzerland).

E.m. whole protein-specific antibodies were measured by an ELISA assay as follows: 96-well microplates were coated with *E.m.* protoscoleces whole protein (1 µg/well, the protein extraction in accordance with the general protocol) at 4 °C for all night. The plate washed with PBST three times, and then added with the anti-serum (1:500) for 1 h at 37℃. The plate was washed with PBST three times, then 100 µl HRP-goat anti-mouse IgG (1:10,000) was added to the corresponding well for 1 h at 37℃. Then, the substrates were incubated in 100 µl TMB for 10 min at room temperature and the reaction was stopped by the addition of 50 µl 2 M H_2SO_4. Tests of the ELISA method were described as mentioned above.

Determination of specific antibody production after challenge *E.m.*

Blood and 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 was 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 [10]. Splenocytes were prepared using 70µm 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, $EMY162_{36-48}$, $EMY162_{7-1}3$, $TSP3_{80\text{-}90}$, or $TSP3_{33\text{-}42}$. 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 immunization, prophylactic and therapeutic vaccine. 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 $EMY162_{7\text{-}13}$, $EMY162_{36\text{-}48}$, $TSP3_{33\text{-}42}$, and $TSP3_{80\text{-}90}$ were selected as the components of a multi-epitope divalent vaccine, ETBM. The multiple epitope divalent vaccine LTB-ETBM (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 proved 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 Ni^{2+}-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 to 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 *E.m.* whole protein was evaluated by indirect ELISA. After immunizing with 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. It was testify to LTB-ETBM had good immunogenicity and immunoreactivity. Moreover, the LTB-ETBM induced antigen-specific antibodies against EMY162 and TSP3 were 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 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, $EMY162_{36-48}$, $EMY162_{7-13}$, $TSP3_{80-90}$, or $TSP3_{33-42}$. 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, the splenocytes stimulation with EMY162 (SI = 1.805 ± 0.119), TSP3 (SI = 1.890 ± 0.149), $EMY162_{36-48}$ (SI = 1.673 ± 0.114), $EMY162_{7-13}$ (SI = 1.703 ± 0.075), $TSP3_{80-90}$ (SI = 1.584 ± 0.07), or $TSP3_{33-42}$ (SI = 1.551 ± 0.076) resulted in proliferation compared with PBS 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 protective effect of LTB-ETBM. The mice vaccinated with LTB-ETBM then challenged with protoscoleces showed fewer (LTB-ETBM/rLTB/PBS; 4/6/6) and smaller cysts (Fig. 5a-b) compared with PBS and rLTB. Thus indicating that the multi-epitope divalent vaccine LTB-ETBM can reduce the cysts formation against *E. m.*

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 *E.m.*-infected mice treated with LTB-ETBM reduce the cysts formation (Fig. 6a-b). The weight (Fig. 6a) and the number of cysts (Fig. 6b) were significantly reduced between mice treated with LTB-ETBM compared with rLTB or PBS. This result indicates that LTB-ETBM has 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 protected 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 *E.m.*-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 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 = 2.970 *vs*. PBS; t = 8.823 *vs*. control) compared with vaccination with PBS or no vaccination (normal control). IL-17 (t = 4.824 *vs.* PBS; t = 4.059 *vs*. control) was down-regulated in mice immunized with LTB-ETBM compared with those immunized with PBS and normal control mice (Fig. 4d).

The mice vaccinated with LTB-ETBM then challenge protoscoleces, the IFN-γ (t = 3.322 *vs*. PBS; t = 6.130 *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 *E.m.*-infected mice treated with LTB-ETBM significantly increased level 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 in the PBS-treated and 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 (Fig. 6e).

Discussion

In this study, two dominant epitopes of Em-EMY162 and Em-TSP3 were selected, and a bivalent multi-epitope vaccine, LTB-ETBM, was successfully constructed for the control of *E.m.* The results showed that LTB-ETBM could effectively inhibit the formation of cysts and significantly reduce the number of vesicles, which has a postive effect on the prevention and control of *E.m.*

Alveolar Echinococcosis caused by *E.m.* is a zoonoticdisease causing 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 cured 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. However, studies on the antigenic proteins of *E.m.* intermediate and terminal hosts have not obtained similar effects.

In our previous studies, we found that Em-EMY162 has good protective and therapeutic effects against *E.m.*(Li et al., 2018). EMY162 has been reported to be expressed in all four stages of the worm. It has also been reported that tetraspanin 3(TSP3) has a certain prevention and treatment effect against *E.m.*, 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 *E.m.*, but did not achieve full protection(Li et al., 2018). We speculate that this may be due to the poor effect of single antigen proteins on the prevention and treatment of complex pathogens such as parasites.

LTB-ETBM constructed in this study targeted multiple antigen proteins of *E.m.* 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 suggest that simultaneous targeting of multiple antigens against *E.m*. can enhance its protective effect.

In our previous study, we identified dominant antigen epitopes $EMY162_{36-48}$, $EMY162_{7-13}$, $TSP3_{33-42}$, and $TSP3_{80-90}$ 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).

In this study, a multi-epitope bivalent vaccine LTB-ETBM targeting EMY162 and TSP3 was constructed, which contains 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.

The *E.m.* parasite avoids being cleared by the host immune system through an immune escape mechanism, so we speculate that balancing the host immune response may beneficial in preventing and controlling AE. As for the immune escape mechanism of *E.m.*, 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 (Ali-Khan, 1978; EMERY et al., 1996; Ma et al., 2014). These changes in cytokines may lead the *E.m.* growth rapidly in the body. Thus, there is dissonance in the host Th1 and Th2 immune response during *E.m.* infection. In our study, LTB-ETBM as a therapeutic vaccine induced high titers of specific IgG, IgA, IgM, and IgE antibodies specific for EMY162 (Fig. 6c-d), maintained the high level of IgG1 and IgG2a specific antibodies, and reduced cyst formation (Fig. 6a-b). Thus, we speculate that LTB-ETBM balanced the host immune response and thereby inhibited 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; Guo et al., 2014). Results from a splenic lymphocyte proliferation assay showed that splenic lymphocytes from mice receiving LTB-ETBM proliferated after stimulation with $EMY162_{36-48}$, $EMY162_{7-13}$, $TSP3_{33-42}$, and $TSP3_{80-90}$, 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 (Fig. 3 and Fig. 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 speculate that LTB-ETBM induced the change of antibodies and cytokines play an important role in *E.m.* prevention and treatment.

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 concentrations 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 protoscoleces. The weight of cysts was significantly lower when mice were injected with LTB-ETBM than when mice were injected with PBS or rLTB (Fig. 6). The size of cysts was also smaller than that in mice injected with LTB-ETBM with Freund's adjuvant. It was pity that 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 (Bauer et al., 2001; Bode et al., 2011; Cooper et al., 2004; 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 *E.m.* In ongoing studies we are looking for other active antigens to increase the efficacy of LTB-ETBM therapy.

Many previous 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 involved in this study 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 *E.m.* can be used 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 *E.m.* was designed, constructed, expressed and purified. The immunogenicity and protective efficacy showed that LTB-ETBM could significantly reduce cyst formation in an *E.m.*-infected mouse, and induced specific IgG and IgA antibodies and a mixed Th1–Th2 cell response.

Declarations

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Ethics approval

The animal experiments on E. multilocularis were approved by the Animal Ethical and Experimental Committee of Qinghai University (QHDX-2019-09).

Conflict of Interest statement

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.

Author contribution

Tang F, Guo L, Fan HN and Ge RL conceived and designed research. Li RL, Liu KM, Feng L and Hu BW conducted experiments. Ma JW, Xin MY, and Zhou P contributed analytical tools. Pang MQ and Li RL analyzed data. Li RL wrote the manuscript. All authors read and approved the manuscript.

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Figures

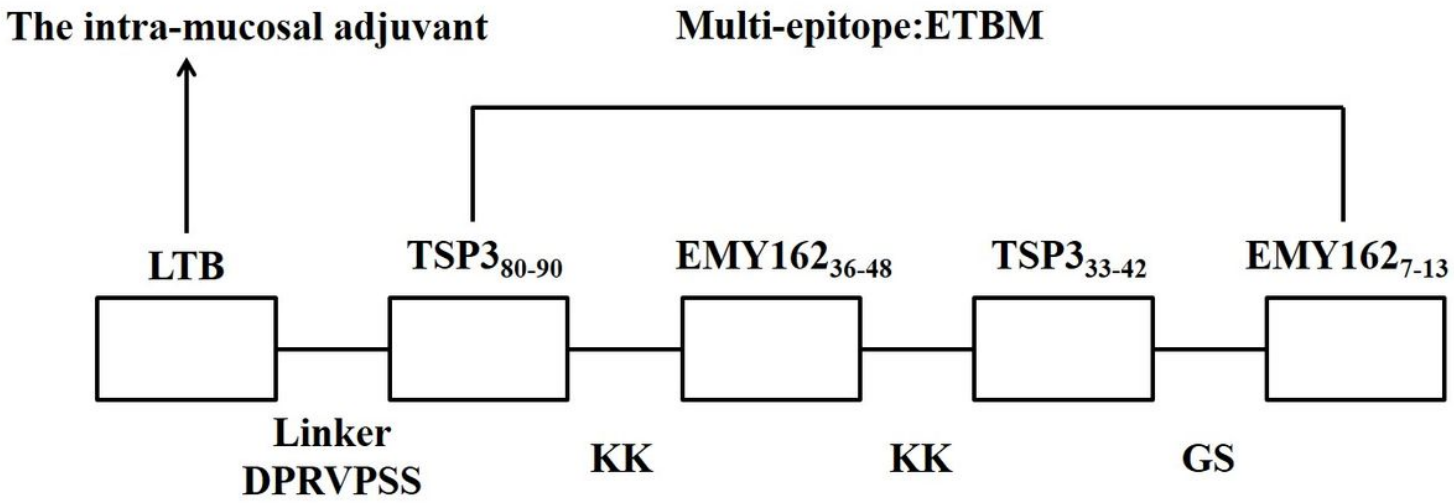

Figure 1

The multi-epitope bivalent vaccine LTB-ETBM. The multi-epitope peptide (ETBM) contains tandem copies of four different epitopes: Th cell epitopes TSP380-90 and EMY16236-48 and B cell epitopes TSP333-42

and EMY1627-13 from the EMY162 and TSP3 antigens. The DPRVPSS, KK, and GS were use as linker.

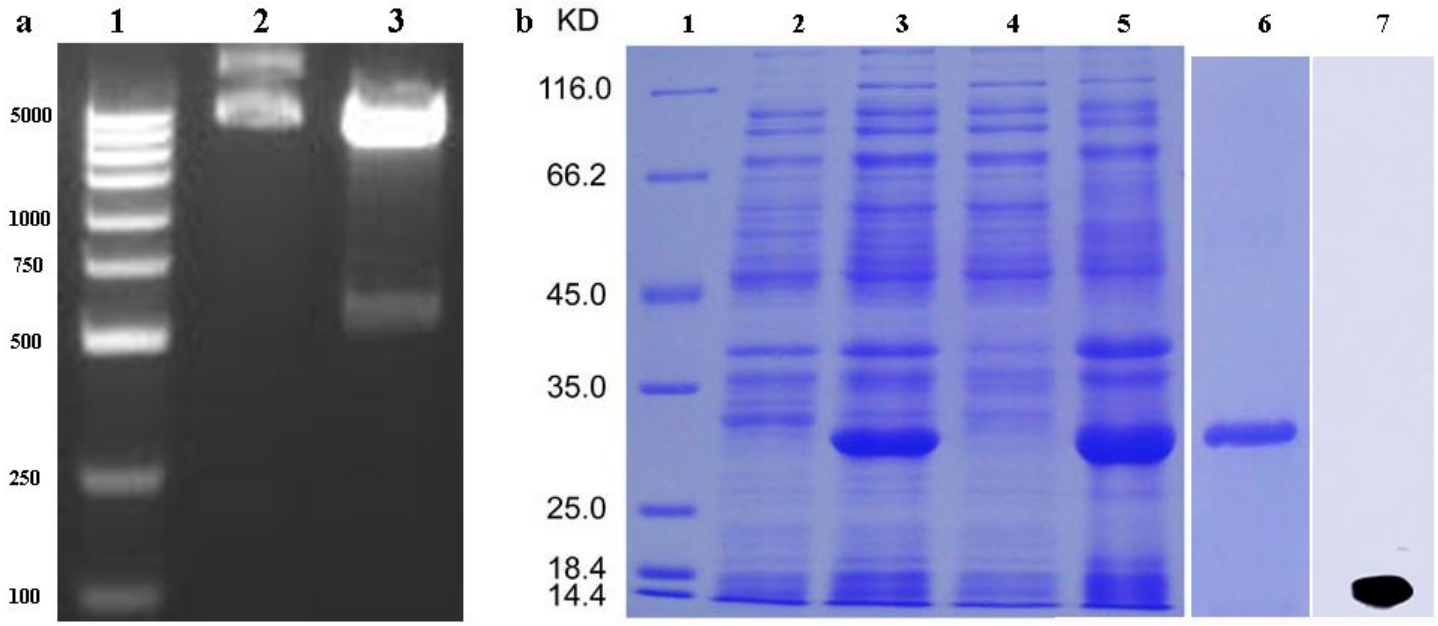

Figure 2

Construction, identification expression, and of purification LTB-ETBM. a Double endo-nuclease restriction was utilized to verify the recombinant pCzn1-LTB-ETBM plasmid. Lane 1: DNA Marker; Lane 2: pCzn1-LTB-ETBM plasmid. Lane 3: pCzn1-LTB-ETBM double digested with Nde I and Xba I. b Expression of LTB-ETBM was detected by SDS-PAGE. Lane 1: Protein marker. Lane 2: proteins from un-induced ArcticExpress (DE3) cells. Lane 3: ArcticExpress (DE3) cells expressing LTB-ETBM (26 kDa) induced with IPTG. Lane 4: soluble protein of induced cells (no target protein detected). Lane 5: inclusion protein of induced cells (LTB-ETBM has a mass of 26 kDa). Lane 6: LTB-ETBM purified by Ni2+-IDA-Sepharose CL-6B. Lane 7: Antigenicity of LTB-ETBM detected by western blotting. Mouse anti-LTB-ETBM serum was used to evaluation the antigenicity against recombinant EMY162.

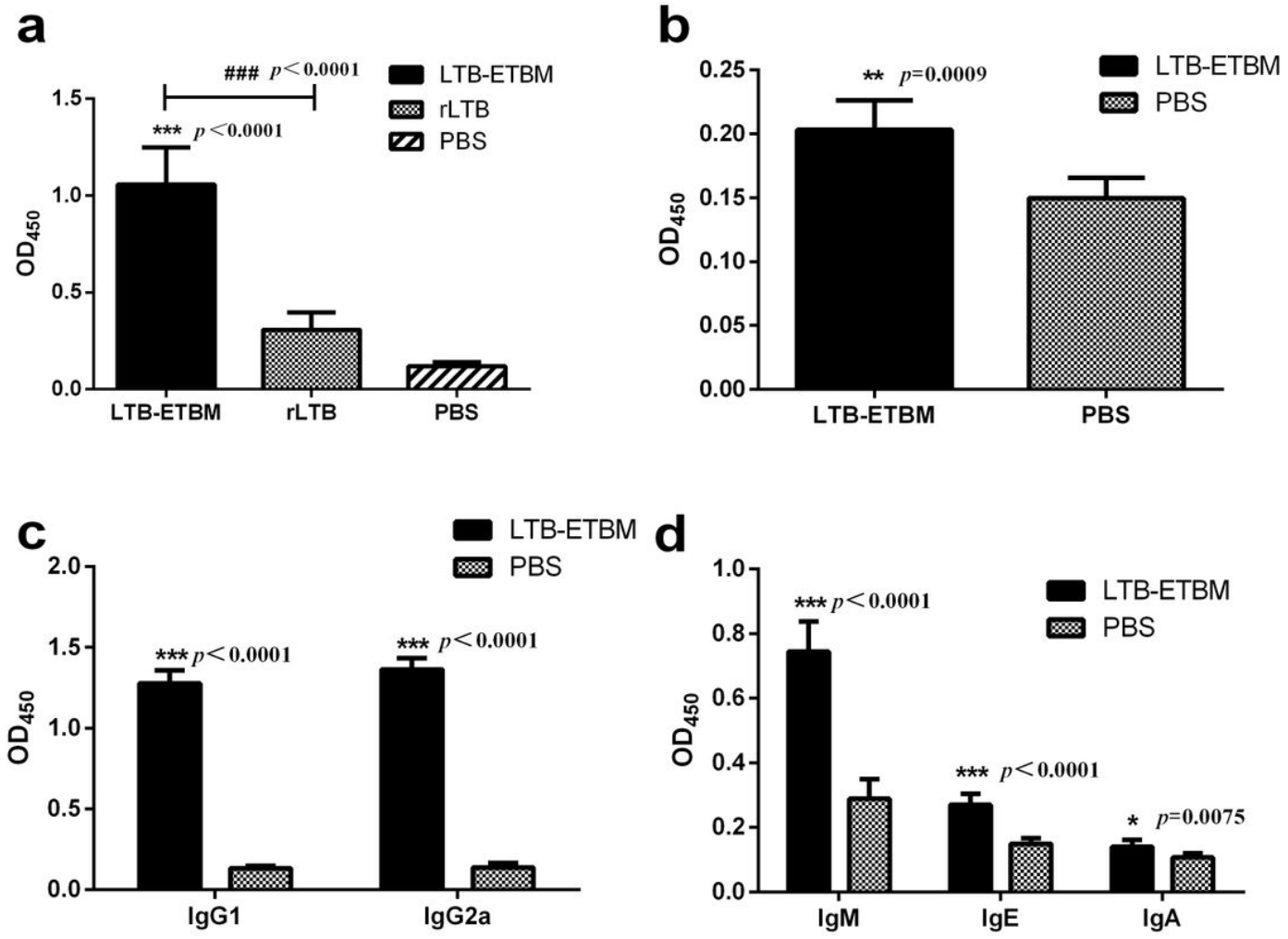

Figure 3

Level of specific antibodies after immunized with LTB-ETBM against EMY162. Balb/c mice were immunized with LTB-ETBM, LTB or PBS, respectively. a Detection of IgG antibodies specific for EMY162 by ELISA. The recombinant protein EMY162 coated on a 96-well plate (anti-sera dilution in 1:2500). b Detection of IgG antibodies specific for E.m. whole protein by ELISA. The protoscoleces whole protein coated on a 96-well plate (anti-sera dilution in 1:500).c Detection of IgG1 and IgG2a isotype antibodies. The recombinant protein EMY162 coated on a 96-well plate (anti-sera dilution in 1:2500). d Detection of IgM, IgE, and IgA antibodies. The recombinant protein EMY162 coated on a 96-well plate (anti-sera dilution in 1:2500).

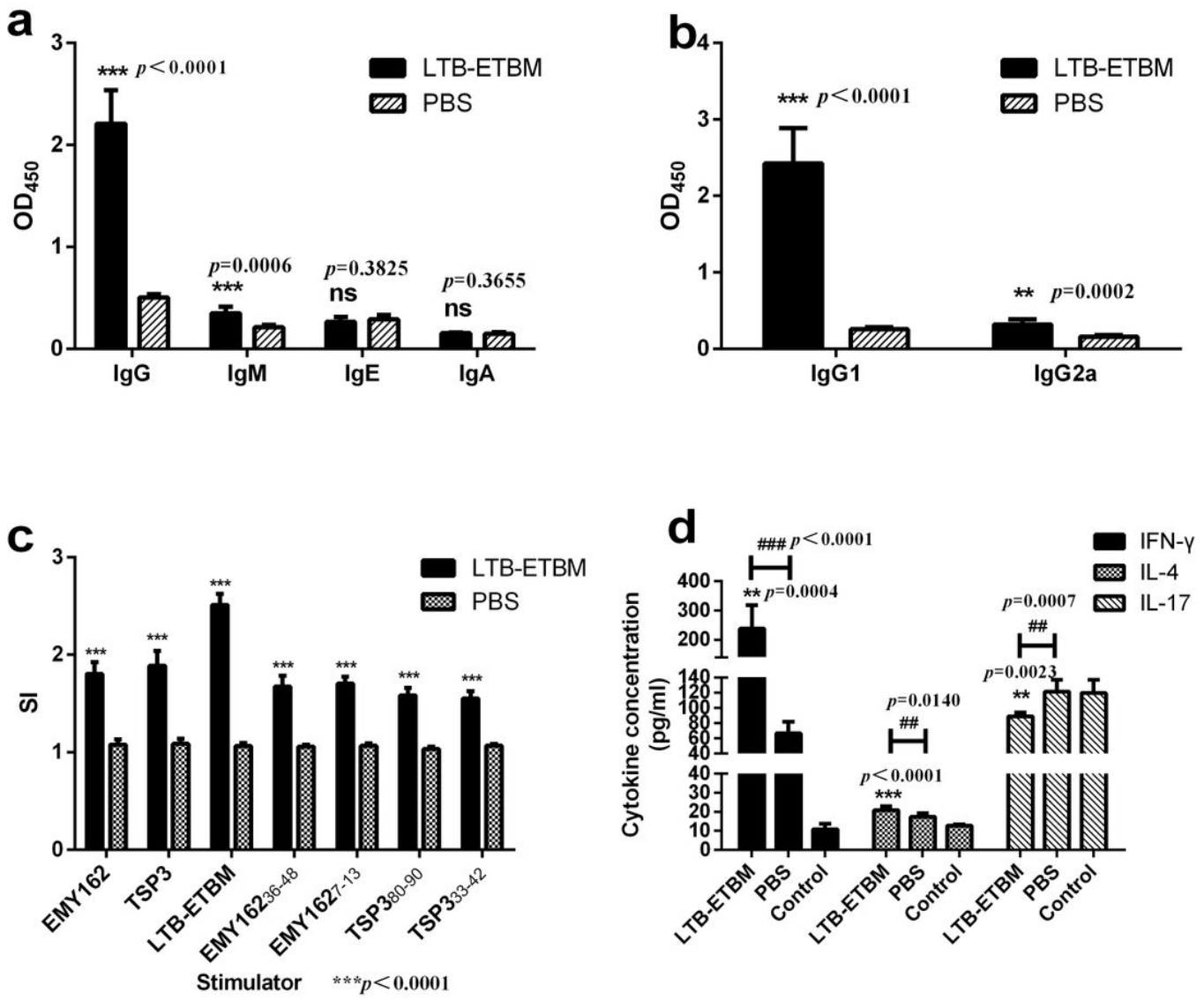

Figure 4

Level of specific antibodies against TSP3, T lymphocyte responses and serum cytokine concentrations after immunized with LTB-ETBM. a Detection of IgG, IgM, IgE, and IgA antibodies. The recombinant protein TSP3 coated on a 96-well plate (anti-sera dilution in 1:2500) b. Detection of IgG1 and IgG2a isotype antibodies. The recombinant protein TSP3 coated on a 96-well plate (anti-sera dilution in 1:2500).c Evaluation of T lymphocyte responses. Splenic lymphocytes were stimulated with EMY162, TSP3, LTB-ETBM, $EMY162_{36-48}$, $EMY162_{7-13}$, $TSP3_{80-90}$, or $TSP3_{33-42}$ peptides, which were separated from the mice immunized with LTB-ETBM or PBS. After a 60-h incubation the cell was detected by MTS. SI show T lymphocyte proliferation rate. d The serum cytokine concentrations of LTB-ETBM, PBS or normal control mice.

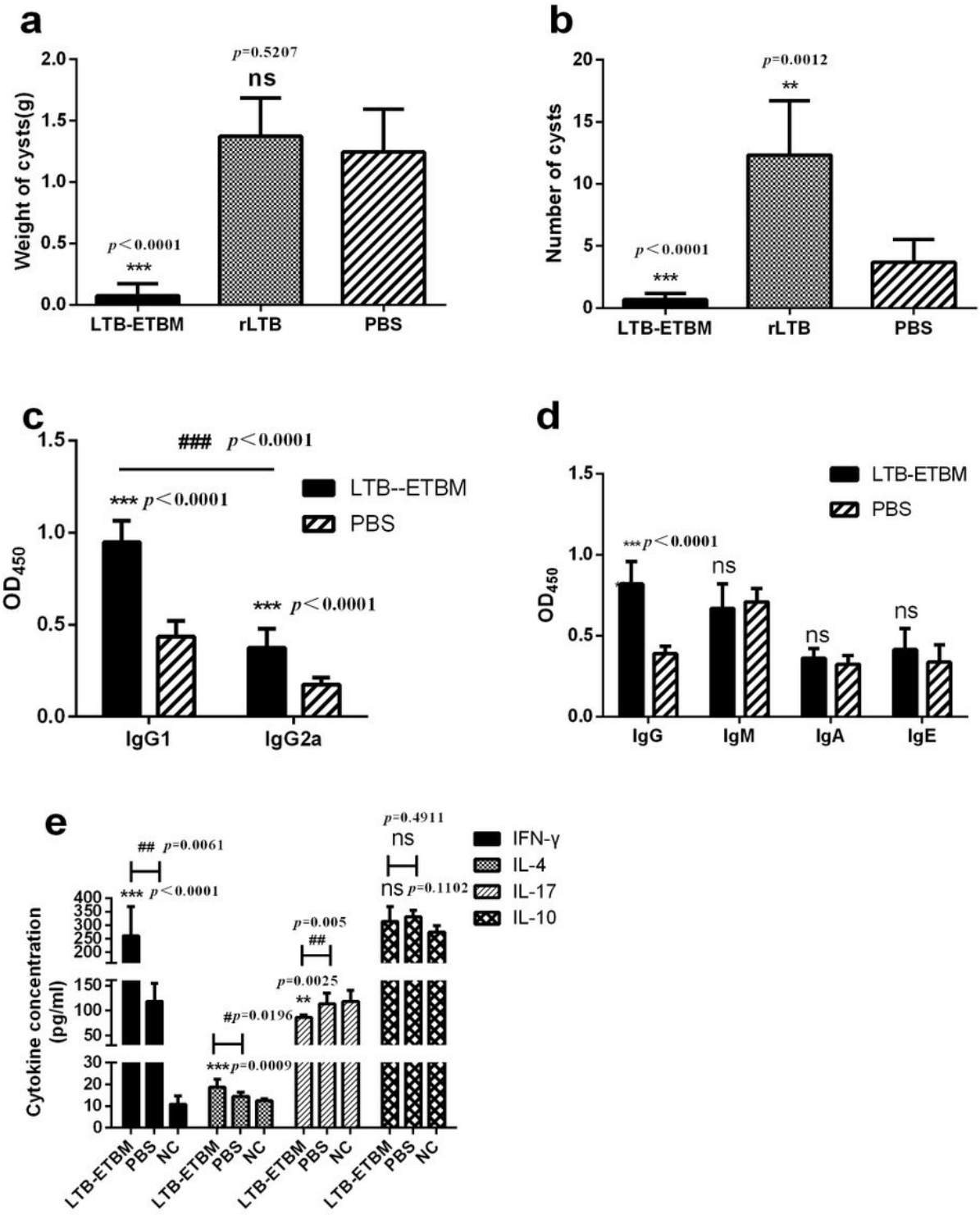

Figure 5

Effect of LTB-ETBM on cyst formation, the change of antibodies and serum cytokine concentrations after prophylactic immunization. The LTB-ETBM or PBS immunized mice were challenged with 1000 protoscoleces. a Weight of the cysts after prophylactic immunization. b Number of the cysts after prophylactic immunization. c Detection of of IgG1 and IgG2a antibodies after prophylactic immunization. The recombinant protein EMY162 coated on a 96-well plate (anti-sera dilution in 1:8000). d Detection of

IgG, IgM, IgE, and IgA specific antibodies after prophylactic experiment. The recombinant protein EMY162 coated on a 96-well plate (anti-sera dilution in 1:6000). e The serum cytokine concentrations of mice after prophylactic vaccination (LTB-ETBM or PBS) and NC mice.

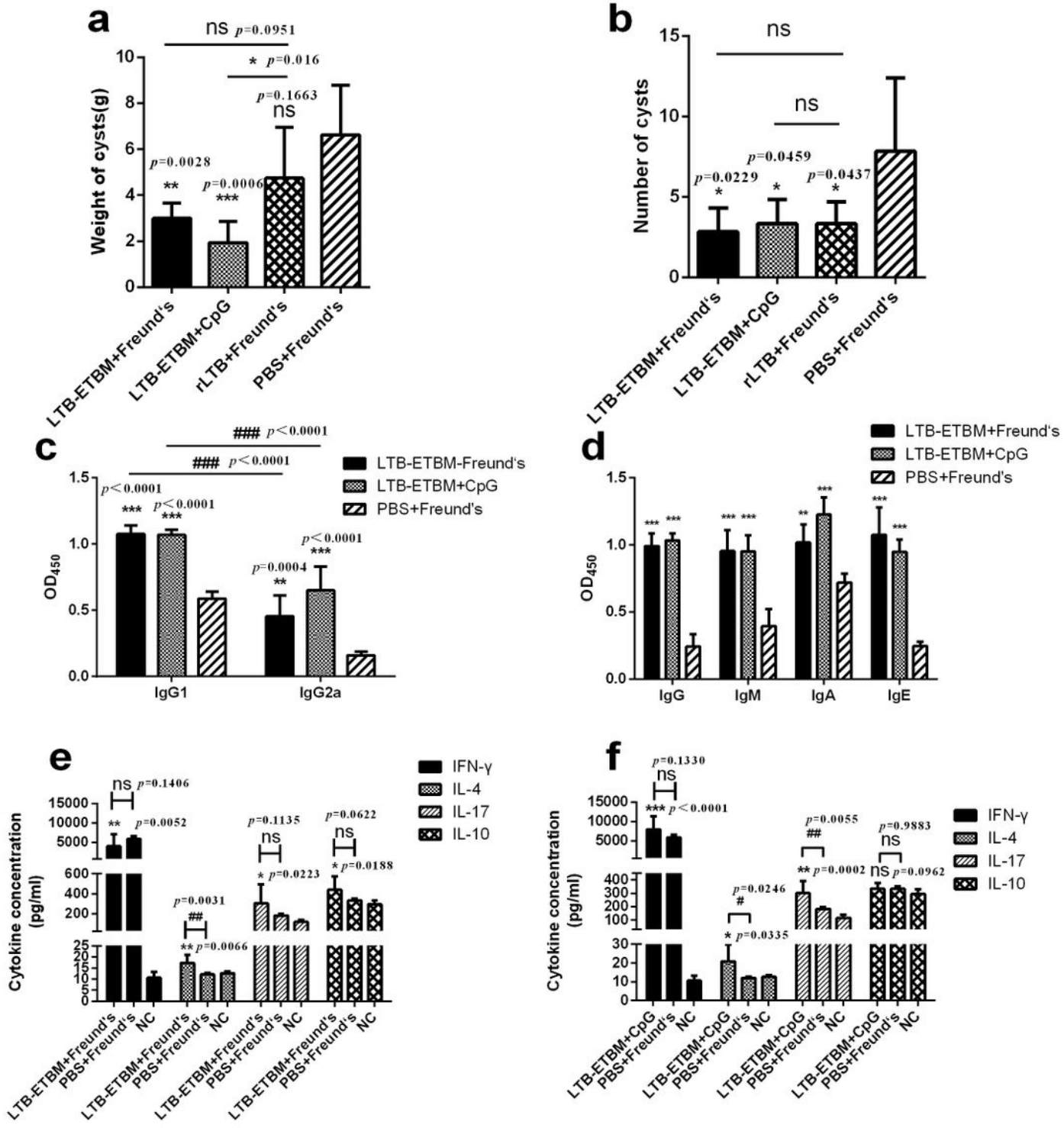

Figure 6

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 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 g The serum cytokine concentrations of mice after therapeutic vaccination and NC mice.