Vaccine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Partial protective immunity against toxoplasmosis in mice elicited by recombinant *Toxoplasma gondii* malate dehydrogenase

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ARTICLE INFO

Article history:
Received 21 June 2015
Received in revised form
28 September 2015
Accepted 9 October 2015
Available online xxx

Keywords:
Toxoplasma gondii
Malate dehydrogenase
Mucosal immunity
Nasal vaccine
Mice

ABSTRACT

Toxoplasma gondii can infect humans and wildlife, sometimes causing serious clinical presentations. Currently, no viable vaccine or effective drug strategies exist to prevent and control toxoplasmosis. *T. gondii* malate dehydrogenase (TgMDH) is a crucial enzyme in cellular redox reactions and has been shown to be an immunogenic compound that could be a potential vaccine candidate. Here, we investigate the protective efficacy of recombinant TgMDH (rTgMDH) against *T. gondii* infection in BALB/c mice. All mice were vaccinated via the nasal route. We determined the optimal vaccination dose by monitoring systemic and mucosal immune responses. The results showed that mice vaccinated with 30 μg of rTgMDH produced the highest antibody titers in serum, a strong lymphoproliferative response, marked increases in their levels of IL-2 and IFN-γ, and significantly greater levels of specific secretory IgA (sIgA) in mucosal washes. In addition, the vaccinated mice were orally challenged with tachyzoites of the virulent *T. gondii* RH strain 2 weeks after the final vaccination. Compared to the control group, we found that vaccination with rTgMDH increased the survival rate of infected mice by 47% and also significantly reduced the tachyzoite loads in their liver (by 58%) and brain (by 41%). Therefore, the rTgMDH protein triggers a strong systemic and mucosal immune response and provides partial protection against *T. gondii* infection.

1. Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular protozoan that lives in various tissues of humans and other warm-blooded animals. Toxoplasmosis is globally distributed, with prevalence ranging from less than 10% in China, through 10–30% in America, up to 80% in Brazil [1–3]. Asymptomatic infections of *T. gondii* can occur in immunocompetent hosts. However, in immunocompromised hosts, *T. gondii* infections more commonly develop into symptomatic pathologies, such as lymphadenopathy, chorioretinitis and encephalitis [4]. *T. gondii* can also be transmitted vertically from mother to fetus via the placenta causing miscarriage or congenital toxoplasmosis [1,5]. Vertical transmission of *T. gondii* leads to significant economic losses in livestock farming [6]. Currently, there are no treatments that prevent toxoplasmosis. Some medications have been used to treat congenital and acute toxoplasmosis, albeit with serious side effects reported [7,8]. A vaccine would be an ideal tool to improve the control of toxoplasmosis [9].

Infection with *T. gondii* generally occurs orally. Protective immunity against toxoplasmosis is triggered through the mucosal surface of the intestine [10]. Secretory IgA (sIgA) secreted by gut-associated lymphoid tissue plays a protective role against *T. gondii* by preventing tachyzoites from invading the host [11]. Since all mucosal surfaces have the same mucosal immune system, the nasal route presents an interesting candidate vaccination route [12,13]. The nasal route requires a smaller amount of antigen than the oral route, as it generates both mucosal and systemic immune responses to an antigen [14,15]. Previous studies have shown that vaccination

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http://dx.doi.org/10.1016/j.vaccine.2015.10.067
0264-410X/

with *T. gondii*-soluble tachyzoite antigen (STAg) via the nasal route induced strong mucosal and systemic immune responses and conferred partial protection against *T. gondii* infection in BALB/c mice [16]. However, STAg contained numerous proteins and effective antigens. Detailed examination of STAg with 2-dimensional electrophoresis, mass spectrometry, and western blot analysis revealed that malate dehydrogenase (MDH) could be a potential vaccine candidate against *T. gondii* [17].

MDH exists in the form of a multimeric enzyme and plays a crucial role in the tricarboxylic acid cycle of prokaryotic and eukaryotic cells [18]. MDH may catalyze the reversible conversion between malate and oxaloacetate: a process that provides power for redox reactions [19]. MDH is also involved in energy generation in mitochondria, reactive oxygen species metabolism, and substance transfer among cells [20,21]. Hence, changes of *T. gondii* MDH (TgMDH) activity could lower the survival rate of the pathogen by diminishing the oxidation power of the tricarboxylic acid cycle and the metabolism of sugars, lipids and proteins [22]. In previous experiments, we cloned and expressed TgMDH in a prokaryotic expression system. We then used western blotting methods to reveal that the recombinant TgMDH protein (rTgMDH) could be recognized by murine anti-rTgMDH serum and rabbit anti-*T. gondii* serum. This indicated that rTgMDH was immunogenic and could be a potential candidate for an anti-*T. gondii* vaccine [23].

In this study, we investigated the protective efficacy of rTgMDH (administered intranasally) against *T. gondii* infection in BALB/c mice. Specifically, we determined the optimal vaccination dose by monitoring specific antibodies, cytokine levels and lymphocyte proliferation in the vaccinated mice, calculated the abundance of tachyzoites in the brain and liver of the mice, and recorded the survival time of the mice following infection with chronic and lethal doses of *T. gondii*.

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. All animal protocols were approved by the Laboratory Animal Use and Care Committee of the Shanxi Medical University (Permit Number: SXMU-2011-16) and the Ethics Committee on Animal Research of the Shanxi Medical University (Protocol #: 20110320-1).

2.2. Parasites

T. gondii tachyzoites (RH strain) used to infect mice were provided by Peking University Health Science Center (Beijing, China). The parasites were maintained and collected from serial intraperitoneal passaging in BALB/c mice, as previously described [16].

2.3. Preparation of the recombinant protein

The rTgMDH was expressed in *E. coli* BL21/DE3 as a soluble protein and was purified by affinity chromatography using Nickel-nitrilotriacetic acid resin (Ni-NTA; Qiagen, Germany). Full details of the methods used to prepare the recombinant protein are given in the paper [23]. Below we present a brief summary. Total RNA was extracted from the tachyzoites of *T. gondii* (GenBank accession No. AY650028). The coding region of TgMDH was then amplified with a pair of specific primers. The product of the RT-PCR was then digested with a double restriction enzyme and ligated into a pET-30a(+) vector. The recombinant pET-30a(+)-TgMDH plasmid was then transformed into *E. coli* DH5α with positive clones confirmed by double restriction enzyme digestion, PCR, and sequencing. The correct plasmid was transformed into *E. coli* BL21/DE3 and induced by IPTG. The expressed proteins were purified with Ni-NTA affinity chromatography and analyzed by SDS-PAGE. Western blotting with murine anti-rTgMDH serum and rabbit anti-*T. gondii* serum was used to analyze rTgMDH antigenicity. The endotoxin was removed with the ToxinEraser^{TM} Endotoxin Removal Kit and measured by the Chromogenic End-point Endotoxin Assay Kit (Chinese Horseshoe Crab Reagent Manufactory, Xiamen, China). Less than 0.1 EU/mL was detected in the recombinant protein. rTgMDH was dialyzed against phosphate-buffered saline (PBS) and stored at −70 °C.

2.4. Mice and vaccination schedule

Female BALB/c mice aged six weeks were purchased from the Institute of Laboratory Animals, Chinese Academy of Medical Science (Beijing, China). All mice were maintained under specific-pathogen-free (SPF) conditions and provided with food and water ad libitum. Prior to experiments, the mice were acclimatized for one week. A total of 40 female BALB/c mice were divided at random into five groups (N = eight mice per group). The mice in each of the four treatment groups were intranasally administered with 10, 20, 30 or 40 μg of rTgMDH on days 0, 14 and 21. Each dose of rTgMDH was diluted in PBS at a final volume of 20 μL and injected into the nostrils with a micropipettor (10 μL per nostril). Mice in the fifth control group were intranasally administrated three times with PBS as described above.

2.5. Collections of blood samples and mucosal washes

Prior to collecting the samples, the mice were deprived of food for 8 h to deplete their intestinal contents. Blood samples were collected from the retro-orbital plexus of mice that had been anesthetized with sodium pentobarbital. Blood samples were collected 2 weeks after the last vaccination and stored at −70 °C for further analysis. Nasal, intestinal and vaginal washes were collected as previously described [24]. For the collection of vesical washes, the evacuated bladder was exposed surgically. A micropipette tip was inserted into the opening of the bladder. A total of 100 μL of PBS was injected into the bladder then pulled back into the micropipette for three cycles. Each mouse was flushed in this way six times (100 μL each time). All the fluid was collected in an Eppendorf tube and immediately centrifuged at 12,000 × g for 30 min at 4 °C. Samples were stored at −20 °C.

2.6. Measurement of antibody titers

Antigen-specific antibody levels IgG and IgA in serum, and sIgA in the mucosal washes, were measured by enzyme-linked immunosorbent assay (ELISA). Flat-bottomed wells of 96-well plates were coated with rTgMDH (100 μL/well) at 5 μg/mL overnight at 4 °C. The plates were washed with PBS containing 0.05% Tween 20 and then blocked with PBS containing 5% fetal bovine serum for 40 min at 37 °C. Thereafter, the serum samples (diluted 1:200 in PBS) and mucosal washes were incubated in different wells (100 μL/well) for 2 h at 37 °C. After washing, the wells were incubated with 100 μL of either horseradish-peroxidase (HRP) conjugated goat anti-mouse IgG (AbD Serotec; diluted 1:2500 in PBS) or IgA (Sigma; diluted 1:1000 in PBS) for 2 h at 37 °C. The plate was washed extensively, and incubated with 100 μL of substrate solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 3.7 mM o-phenylenediamine, 30% H_2O_2). The reaction was stopped by adding 50 μL of 2 M H_2SO_4 at which point the absorbance at 492 nm was determined with a microplate reader

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(Epoch Multi-Volume Spectrophotometer System, Biotek, USA). Each sample was assayed twice and average values were calculated.

2.7. Lymphocyte proliferation assay

Spleens were surgically removed from the mice 2 weeks after the third vaccination, as described previously [24]. The crushed spleens were then filtered through stainless steel meshes. After the red blood cells were removed using erythrocyte lysis buffer (0.15 M NH_4Cl, 1.0 M $KHCO_3$, 0.1 mM EDTA, pH 7.2), splenocytes were re-suspended in 2 mL of PBS. The cells were underplayed with isometric lymphocytes separation medium (Shanghai Heng Chemical Reagents, Ltd., China). After centrifugation at 450 × *g* for 10 min, the splenic lymphocytes were washed twice in PBS and then re-suspended in RPMI 1640 medium.

The cells were then plated at a density of 5×10^5 cells per well, in duplicate, in 96-well microtiter plates. Cells were cultured for 72 h at 37 °C with 5% CO_2 with either rTgMDH (10 μg/mL) or concanavalin A (Con A; 5 μg/mL; Sigma) for the positive control or medium alone for the negative control. Then 10 μL of CCK-8 reagent (Dojindo Laboratories; Kumamoto, Japan) was added to each well and incubation continued for a further 3 h. The absorbance at 450 nm was then measured in the cultures to quantitatively evaluate cell viability. We then calculated the stimulation index (SI) for each group as follows:

$$\mathrm{SI} = \frac{\text{average } \mathrm{OD}_{450} \text{ values from stimulated cultures}}{\text{average } \mathrm{OD}_{450} \text{ values from non-stimulated cultures}}$$

2.8. Cytokine analysis

Cytokines were measured according to previously described methods [25]. Briefly, spleen cells were obtained (as described above) and cultured at a density of 1.5×10^6 cells per well, in duplicate, in 24-well microtiter plates. Cell-free supernatants were harvested after 24, 72 or 96 h of stimulation with rTgMDH (10 μg/mL) and assayed for different cytokines: interleukin-2 (IL-2) and IL-4 at 24 h; IL-10 at 72 h, and gamma interferon (IFN-γ) at 96 h. Cytokine concentrations were determined using a commercial ELISA kit (PeproTech, USA) according to the manufacturer's instructions and by reference to standard curves constructed with known amounts of mouse recombinant IL-2, IL-4, IL-10 and IFN-γ. The sensitivity limits for the assays were 16 pg/mL for IL-2 and IL-4, 47 pg/mL for IL-10, and 23 pg/mL for IFN-γ.

2.9. Challenge infection

Two groups of female BALB/c mice (20 mice per group) were vaccinated intranasally with either 20 μL PBS (control group) or 30 μg of rTgMDH suspended in 20 μL of sterile PBS (treatment group). The rTgMDH vaccination dose was based on the results of the above-mentioned experiment. All the mice received two 'booster' doses (of either PBS or the vaccine) on days 14 and 21. Two weeks after the third vaccination, 12 mice from each group were challenged orally with a feeding needle containing an acute dose of *T. gondii* tachyzoites (4×10^4), while the remaining eight mice from each group received a chronic dose (administered orally) of *T. gondii* tachyzoites (1×10^4). Mortality of the mice was observed twice daily for 30 days post-infection. On day 30 post-infection, the chronically infected mice were euthanized and their livers and brains were removed. The tachyzoite loads in the liver and brain tissue were detected using a real-time quantitive PCR assay based on the single-copy SAG1 gene, as previously described [26]. The number of parasites in the samples was calculated from the qPCR threshold cycle (Ct) value according to a standard curve obtained with DNA samples from a range of serial 10-fold dilutions of tachyzoites.

2.10. Statistical analyses

We used one-way ANOVA to test for differences in antibody responses, lymphocyte proliferation and cytokine assays amongst the treatment and control groups. Significant differences in pathogen survivorship within vaccinated and unvaccinated mice were detected using Kaplan–Meier curves. All statistical analyses were performed using the SPSS software, and a significance level of $P < 0.05$ was adopted. The data in each group were analyzed for normality using the Shapiro–Wilk test ($P > 0.10$).

3. Results

3.1. Systemic immune response induced by rTgMDH

We observed that the IgG and IgA antibody levels of individuals in all the treatment groups (i.e. for all administered doses of rTgMDH) were significantly higher than those of the individuals in the control group (Fig. 1). IgA antibody levels were elevated but not significantly so for mice administered a 10 μg dose of rTgMDH (Fig. 1B). For both antibodies the highest responses were seen in individuals administered a 30 μg dose of rTgMDH (Fig. 1). In addition, we found that splenocytes obtained from vaccinated mice in all but the 10 μg rTgMDH group exhibited significantly greater lymphocyte proliferation than did mice in the control group (Table 1). Splenocytes from all the treatment groups and the control groups exhibited a similar lymphocyte proliferation response to ConA (data not shown). We also observed that splenocytes from all the vaccinated mice secreted significantly greater amounts of IL-2 and IFN-γ than did unvaccinated mice, with the 30 μg dose of rTgMDH eliciting the strongest cell-mediated immune response (Table 1). The levels of IL-4 and IL-10 secreted by the vaccinated mice were slightly higher than the control group; however, there were no statistically significant differences in these findings (Table 1).

3.2. Mucosal immune response induced by rTgMDH

Vaccination with all doses of rTgMDH elicited significantly greater levels of sIgA antibodies in nasal and vaginal washes of mice than were observed for mice in the control group, with the 30 μg dose of rTgMDH eliciting the strongest responses (Fig. 2A and C). Similar results were observed for sIgA antibody titer in the intestinal and vesical washes, though the difference was not significant for mice administered the lowest dose of rTgMDH (Fig. 2B and D).

3.3. Immuno-protection against oral challenge

The results of our acute infection challenge experiment revealed that the survival rate of mice vaccinated with a 30 μg dose of rTgMDH was 47% greater than that of unvaccinated mice (Fig. 3A). All the mice in the control group were dead within 13 days of infection, whereas 47% of the vaccinated mice remained alive 30 days post-infection, indicating that rTgMDH induced partial protection against challenge with the highly virulent RH strain of *T. gondii* (Fig. 3A). The results of our chronic infection challenge experiment revealed that mice vaccinated with a 30 μg dose of rTgMDH had significantly fewer tachyzoites than did unvaccinated mice (Fig. 3B). Vaccination reduced the abundance of tachyzoites by 58% in liver and in the brain by 41% compared to the control group, indicating that rTgMDH also provides protection against chronic *T. gondii* infections (Fig. 3B).

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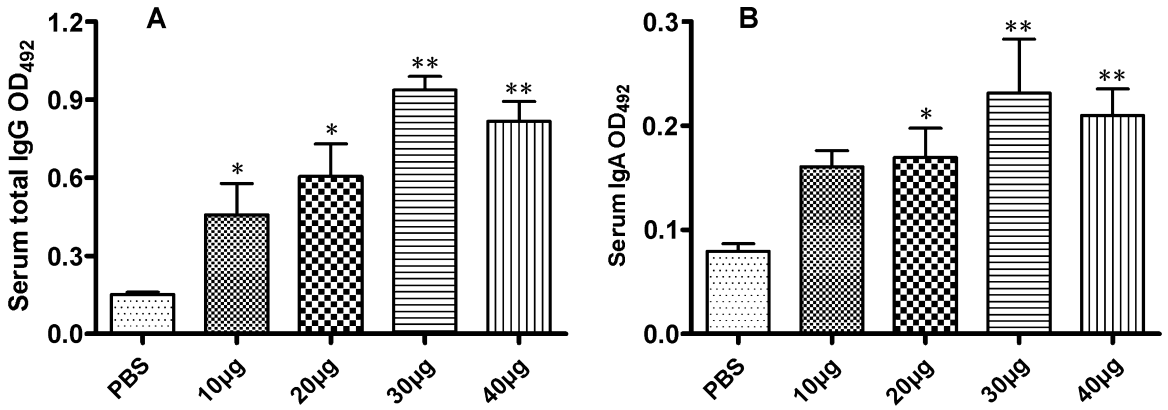

Fig. 1. Intranasal vaccination induces rTgMDH-specific antibody responses in blood serum. Titers of (A) total IgG and (B) total IgA antibodies in the serum of BALB/c mice administered either with PBS (control group) or different doses of rTgMDH. Titers were obtained two weeks after the third vaccination, and were the average of duplicate readings. Results shown here are the mean of the OD_{492} (±SD; $N = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$ (vaccinated vs. PBS group).

Table 1
Lymphoproliferation and cytokine production by splenocytes of mice after stimulation either with rTgMDH (vaccinated mice) or PBS (control mice).

Vaccination dose[a]	Cytokine production (pg/mL)[b]				SI[c]
	IL-2	IFN-γ	IL-4	IL-10	
PBS (Control)	127.53 ± 28.59	191.06 ± 90.43	182.66 ± 7.07	105.54 ± 5.26	1.1575 ± 0.1490
10 μg rTgMDH	505.91 ± 19.54**	637.39 ± 49.81**	214.97 ± 18.78	148.86 ± 9.31	1.1742 ± 0.1438
20 μg rTgMDH	837.79 ± 36.74**	907.95 ± 94.73**	234.00 ± 17.38	192.55 ± 10.43	2.0266 ± 0.1637**
30 μg rTgMDH	1312.57 ± 102.16**	1474.59 ± 89.00**	251.45 ± 18.45	171.79 ± 10.93	2.1337 ± 0.2250**
40 μg rTgMDH	1009.01 ± 71.14**	1409.84 ± 48.84**	220.97 ± 14.34	141.20 ± 13.89	1.5723 ± 0.0986**

[a] Mice ($n = 8$ per group) were vaccinated intranasally on days 0, 14 and 21 with either PBS (control group) or different doses of rTgMDH.
[b] The splenocytes taken from mice two weeks after the third vaccination were examined for cytokine production by ELISA. Values for IL-2 and IL-4 were obtained after 24 h, those for IL-10 after 72 h, and those for IFN-γ after 96 h. Results are presented as the means ± SD. $^{**}P < 0.01$ (vaccinated vs. control group).
[c] The results of proliferation assays are expressed as the stimulation index (SI). SI is the average OD_{450} values from splenocytes stimulated with varying concentrations of rTgMDH divided by the average OD_{450} values of the control group.

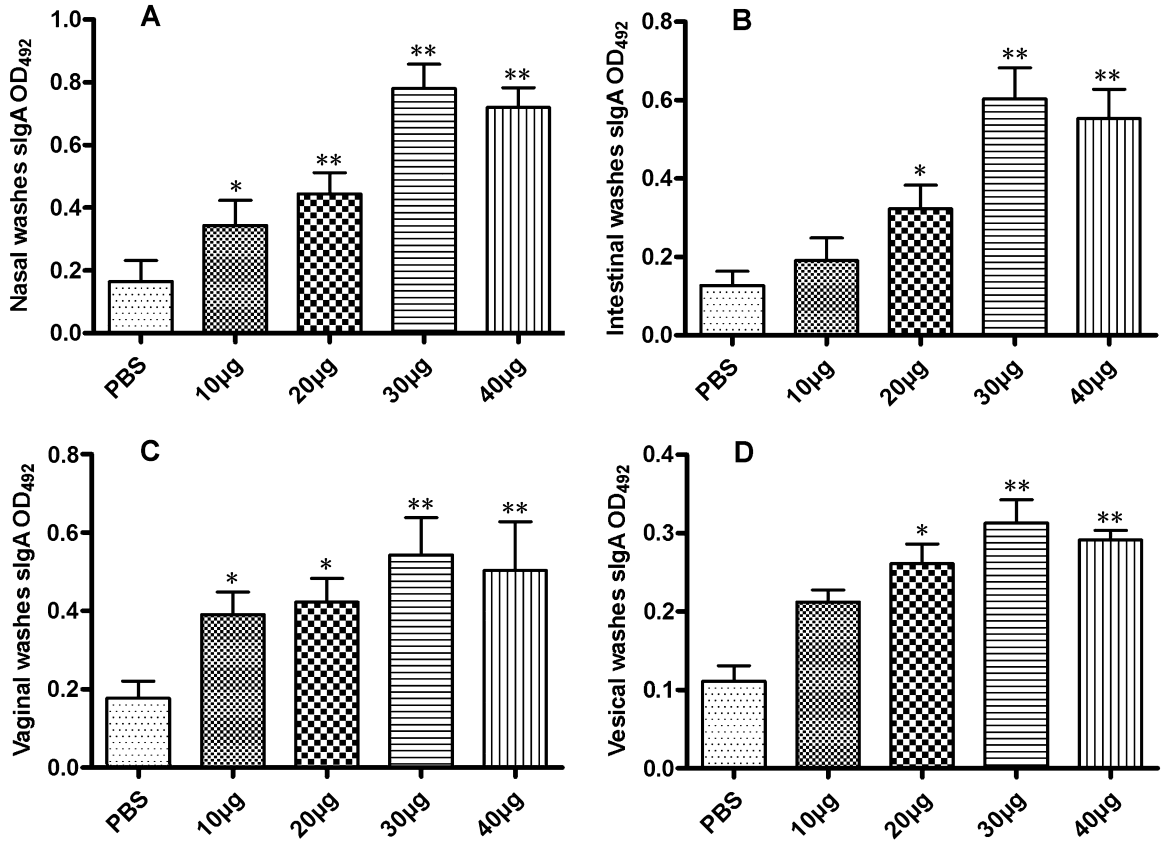

Fig. 2. Intranasal vaccination induces rTgMDH-specific sIgA responses in mucosal washes from the (A) nose, (B) intestine, (C) vagina and (D) the vesica. The sIgA antibodies in mucosal washes of BALB/c mice administered either with PBS (control group) or different doses of rTgMDH were the average of duplicate readings. Results are expressed as the mean of the OD_{492} (±SD; $N = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$ (vaccinated vs. PBS group).

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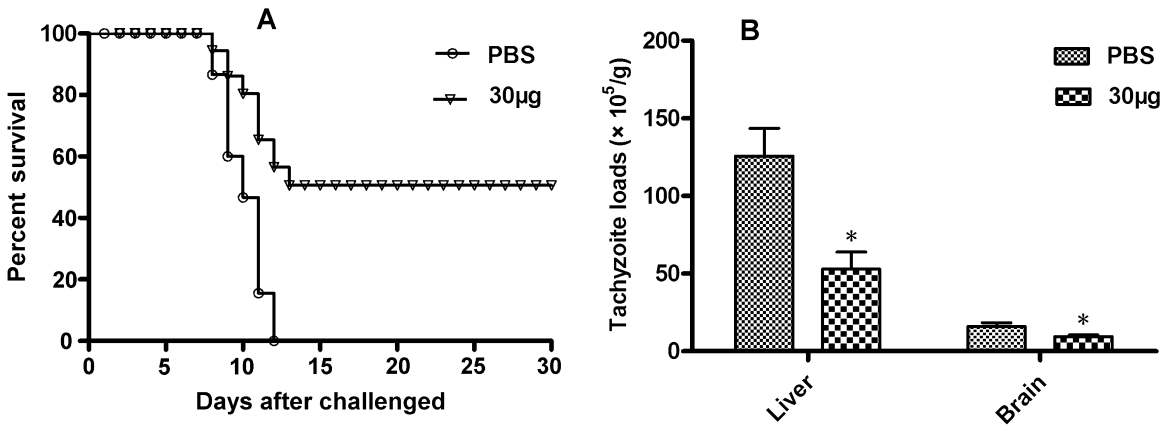

Fig. 3. (A) Daily mortality of vaccinated (received 30 μg of rTgMDH, $N = 12$) and unvaccinated (received PBS; $N = 12$) following acute infection with the *T. gondii* pathogen (challenged with 4×10^4 tachyzoites). (B) Abundance of tachyzoites in brain and liver of vaccinated (received 30 μg of rTgMDH, $N = 8$) and unvaccinated (received PBS; $N = 8$) mice following chronic infection with the *T. gondii* pathogen (challenged with 1×10^4 tachyzoites 30 days after infection). Values are means (±SD). $^*P < 0.01$ (vaccinated vs. PBS group).

4. Discussion

Immunological changes and protection efficiency of TgMDH delivered as a DNA vaccine have been evaluated in previous studies, and revealed that the recombinant protein (such as GRA1) vaccine conferred a better immune response and prolonged survival against acute toxoplasmosis compared to DNA vaccinations [27,28]. TgMDH was an important tool to provide energy for the parasite. Therefore, an investigation of the rTgMDH protein vaccine candidate against *T. gondii* infection to extend the survival time in mice model is significant.

Most of the candidate vaccines have been examined using intramuscular immunization strategies [27,29]. Compared to this or the parenteral route, the nasal route is an exciting alternative for vaccination that has the potential to induce not only a systemic immune response but also a local mucosal immune response [30]. For toxoplasmosis, a systemic immune response protects the host against intracellular infection, while a mucosal immune response prevents the initial invasion of *T. gondii* into the intestinal epithelium.

In this study, mice were vaccinated with different dose of rTgMDH via the nasal route. All vaccinated groups presented high titers of IgG antibodies in serum compared to the control group. Along with macrophages, specific IgG antibodies can protect hosts from chronic *T. gondii* infections by preventing reactivation and inhibiting the attachment of the parasite to host cell receptors [31]. Our results support this idea, as vaccinated mice that were challenged with low doses of *T. gondii* (i.e. chronic infections) exhibited significantly fewer tachyzoites than did unvaccinated infected mice. These results indicate that specific antibodies provide partial protection for mice against *T. gondii* penetration.

The success of a new antigen as a candidate vaccine depends on the type of Th cells induced (Th1 or Th2). Th1 cells mediate cellular immunity against intracellular parasites, bacteria and viruses through secretion of interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β) [32]. Th2 cells provide protection against extracellular infectious organisms through the secretion of IL-4, IL-5, IL-10 and IL-13 [33]. Toxoplasmosis is mediated predominately by Th1 cells [34], with IL-2 promoting T cell proliferation and stimulating the production of IFN-γ [35]. IFN-γ is the key mediator of resistance to *T. gondii* and promotes multiple complex intracellular mechanisms to kill the parasite and inhibit its replication [36].

As expected, vaccination with rTgMDH elicited strong Th1 type immune responses in our experiments. We found that intranasal vaccination with rTgMDH induced particularly high levels of IL-2 and IFN-γ in splenocytes cultures, but lower levels of IL-4 and IL-10. Thus, we observed that intranasal vaccination of mice with a 30 μg dose of rTgMDH elicited an ideal cellular immune response. These cytokines can activate macrophages and other partial immune cells to envelope antigen and engulf and clear *T. gondii* and are also associated with the observed reduction in the number of tachyzoites in brain and liver tissues.sIgA antibodies are the main component of the mucosal immune system. The most important functions of these antibodies are 'immune exclusion' and 'immune elimination', which is achieved by binding to surface antigens on potential pathogens [37]. The increase of sIgA antibody in nasal wash observed in this study reflected that rTgMDH was effectively processed and presented. This process lead to the activation of T-cells that helped B cells to secrete sIgA at the inductive site [38]. Once induced, mature B cells migrate to the effector sites (including the respiratory tract, the gastrointestinal tract and genital tract) that may be located quite far from the inductive site [39,40]. The inductive and effector sites work together to maintain the mucosal barrier. Our results support previous work regarding intranasally vaccinated mice with rTgACT [24].

The main purpose of vaccine is to generate a protective immune response against *T. gondii* infection. In a previous study, immunizing mice with DNA vaccines (pTgMDH) increased their survival times, but not extensively, as all individuals still died within 18 days of infection [27]. In this study, we found that vaccination with rTgMDH increased the survival rate of infected mice by almost 50% and also significantly reduced the tachyzoite loads in their tissue. These results suggest that the host's resistance to *T. gondii* infection after intranasal vaccination with rTgMDH restrains the proliferation of tachyzoites in the body.

In summary, our results demonstrated that rTgMDH is a promising protein vaccine candidate. Intranasal vaccination with rTgMDH can prolong animal survival times and reduce tachyzoite loads in tissues challenged with *T. gondii*. This protection stems from the systemic and mucosal immune responses triggered by rTgMDH. The strong systemic immune responses may inhibit the activity and proliferation of tachyzoites in the host. Mucosal immune responses may develop barriers that prevent the invasion of tachyzoites.

Authors' contributions

Conceived and designed the experiments: ZL. Performed the experiments: ZL, FY, YY, LY. Analyzed the data: ZL, YL, JC. Contributed reagents/materials/analysis tools: KZ, LY, JC, YW. Wrote

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and revised the paper: ZL, JC, YW. All authors read and approved the final version of the manuscript.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 81371841 to JC), the Laboratory of Parasite and Vector Biology, MOH, China (No. WSBKTKT201402 to ZL) and the Shanghai Municipal Health Bureau, China (No. 20124Y038 to YW). The funders had no role in study design, data collection and analysis, decision to publish, or preparation.

Conflict of interests. The authors declare they have no competing interests.

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Please cite this article in press as: Liu Z, et al. Partial protective immunity against toxoplasmosis in mice elicited by recombinant *Toxoplasma gondii* malate dehydrogenase. Vaccine (2015), http://dx.doi.org/10.1016/j.vaccine.2015.10.067