

Toxoplasma gondii α -amylase deletion mutant is a promising vaccine against acute and chronic toxoplasmosis

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Summary

Individuals with inhibited immunity may develop lethal toxoplasmosis; thus, a safe and effective vaccine is urged to be developed. *Toxoplasma gondii* (*T. gondii*) α -amylase (α -AMY) is one of the enzymes responsible for starch digestion. In the present study, we first generated a ME49 $\Delta\alpha$ -amy mutant and discovered that loss of α -AMY robustly grew *in vitro* but contributed to significant virulence attenuation *in vivo*. Therefore, we established a mouse model to explore the protective immunity of $\Delta\alpha$ -amy mutant against acute and chronic toxoplasmosis. The results indicated that the survival rates of short-term or long-term immunized mice re-infected with the tachyzoites of multiple *T. gondii* strains were nearly 100%. ME49 $\Delta\alpha$ -amy not only could provide protective immunity against tachyzoites infection but also could resist the infection of tissue cysts. Furthermore, we detected that ME49 $\Delta\alpha$ -amy vaccination could effectively eliminate the proliferation of parasites in mice and prevent the formation of cysts. The significant increases of Th1-type cytokines, Th2-type cytokines and specific total IgG and IgG subclasses (IgG2a and IgG1) confirmed efficiency of a combination of cellular and humoral immunity against infection. In conclusion, ME49 $\Delta\alpha$ -amy attenuated strain

can produce strong immune responses to provide efficient protection against toxoplasmosis, which signifies that ME49 $\Delta\alpha$ -amy mutant may be a potential vaccine candidate.

Introduction

Toxoplasma gondii (*T. gondii*), a vital obligate intracellular parasitic protozoan, is the causative agent of toxoplasmosis (Kato, 2018). The characteristics of it are worldwide distribution and broad host range that can infect all warm-blooded animals including humans (Prandovszky *et al.*, 2018; Coutermarsh-Ott, 2019). *T. gondii* is considered as a considerably successful parasitic organism, which can persist in the host for the host's entire lifetime and present opportunistic pathogenicity after successful infection (Dubey and Jones, 2008; Montazeri *et al.*, 2018a,b). *T. gondii* usually can be controlled by immunocompetent hosts. Nevertheless, individuals with inhibited immunity may develop severe clinical symptoms such as encephalitis, ophthalmia, abortion and even death (Williams, 1979; Miedema *et al.*, 2013; Yang *et al.*, 2018). The main epidemic strains of *T. gondii* are classified as types I, II and III: type I, a robust virulence in mice, type II, predominant strain of human infections in Europe and North America, and type III, known for its low virulence in hosts (Howe and Sibley, 1995; Howe *et al.*, 1997). The diversified population structure of *T. gondii* strains, complexity of life cycle and diversity of transmission routes result in the difficulties in toxoplasmosis control (Howe and Sibley, 1995; Dubey, 2009; Shwab *et al.*, 2018). At present, drugs against toxoplasmosis are restricted to combinations of pyrimethamine and sulfadiazine. However, this treatment may contribute to numerous side effects, drug residues and no effect on chronic infection (Rajapakse *et al.*, 2013; Montazeri *et al.*, 2015, 2018a,b).

In recent years, important progresses have been made in the development of vaccines against toxoplasmosis, especially in live-attenuated vaccines, DNA vaccines, recombinant protein vaccines and live vector-based vaccines (Wang *et al.*, 2019). Surface proteins (SAGs), dense granules (GRAs), rhoptries (ROPs) and micronemes (MICs) are core component of DNA vaccines and recombinant protein vaccines, which play crucial roles in development of *T. gondii* and manipulation of the host's immune responses (Zhang *et al.*, 2015; Pan *et al.*, 2017;

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Zhang *et al.*, 2018). Although above vaccines alleviated *T. gondii* infection, none of these were able to effectively eliminate the formation of cysts and provide enough protection. Therefore, it is imperative to develop a safe and efficient vaccine for *T. gondii*. Live-attenuated vaccine is one of the most effective approaches to provide immune protection, although numerous attempts have been made to produce attenuated *T. gondii* vaccines, only one commercial vaccine, Toxovax[®] based on the live-attenuated tachyzoite S48 strain, is licensed (Buxton *et al.*, 1991).

The energy metabolism of bradyzoites is different from tachyzoites, and the most typical feature is massive amylopectin accumulated in the cytoplasm (Guimaraes *et al.*, 2003). *T. gondii* can store glucose in the form of amylopectin that can be found in several life stages (Guimaraes *et al.*, 2003; Guerardel *et al.*, 2005). It has been hypothesized that amylopectin granules may be an energy reserve for the conversion of bradyzoites to tachyzoites (Coppin *et al.*, 2003). Recent studies have proved that a Ca²⁺-dependent protein kinase (CDPK2), as a key regulator in amylopectin metabolism, can bind to amylopectin granules and phosphorylate some enzymes involved in amylopectin metabolism. And the deletion of *CDPK2* leads to an imbalance of digestion and storage of amylopectin, thereby effecting the development of bradyzoites (Uboldi *et al.*, 2015; Sugi *et al.*, 2017). α -amylase (α -AMY) is one of the enzymes responsible for amylopectin digestion, and it may be phosphorylated by CDPK2 (Uboldi *et al.*, 2015). However, the specific biological function of α -AMY in *T. gondii* is rarely reported.

In recent years, genome editing techniques have been applied to develop attenuated *Toxoplasma* vaccines, and some studies have indicated that genetically attenuated strains produced by deletion of certain genes could provide excellent effect against toxoplasmosis (Ismael *et al.*, 2006; Lagal *et al.*, 2015; Wang *et al.*, 2016). In the present study, we first constructed a $\Delta\alpha$ -amy mutant designated ME49 $\Delta\alpha$ -amy and then proved the ability of α -AMY to degrade amylopectin and detected that the virulence of the mutant *in vivo* was significantly alleviated. Therefore, we established a mouse model to explore the immune responses of $\Delta\alpha$ -amy mutant against acute infection and chronic infection caused by multiple strains. The results obtained from this study revealed that ME49 $\Delta\alpha$ -amy mutant might be a potential vaccine candidate against various *T. gondii* strains infections.

Results

Toxoplasma α -AMY is involved in amylopectin digestion and disruption of α -AMY attenuates virulence *in vivo* but grows robustly *in vitro*

The α -AMY gene deletion was successfully established using CRISPR/CAS9 genome editing technique. *T. gondii*

α -AMY gene was completely knocked out in ME49 strain, and the pyrimethamine-resistant marker *DHFR** was inserted by CRISPR/CAS9-mediated homologous recombination (Fig. 1A). After the α -AMY-targeted CRISPR plasmid and the homology template were co-transferred into the ME49 strain, the single clones were screened by diagnostic PCRs to verify the deletion of the α -AMY gene and the correct insertion of homologous recombination fragments (Fig. 1B). In addition, the lack of α -AMY protein expression in ME49 $\Delta\alpha$ -amy strain was confirmed by Western blotting, which further demonstrated that α -AMY was successfully knocked out in the ME49 strain (Fig. 1C).

To identify the function of α -AMY in amylopectin digestion, the formation of amylopectin was detected by periodic acid-Schiff (PAS) staining. The uneven and slight accumulation of amylopectin granules was distributed in α -AMY knockout tachyzoites, whereas the accumulation was further enhanced by bradyzoite culture conditions (Fig. 2A and B). The plaque assay was performed to evaluate the growth of disruption of α -AMY mutant, and results in Figure 2C and D showed that there was no difference between parental strain and α -AMY deletion mutant. To explore the effect of ME49 strain loss of α -AMY on virulence, ICR mice were intraperitoneally injected with 100 tachyzoites of parental strain ME49 and α -AMY deletion mutant respectively. The survival of infected mice was monitored for 30 days. As shown in Figure 2E, mice infected with ME49 began to show irreversible death from day 11, and the final mortality rate reached 70%. However, the survival of mice infected with ME49 $\Delta\alpha$ -amy was as high as 90%, and mice did not show significant clinical symptoms in form of locomotor activity and depressive symptoms 30 days post-observation (Mahmoud *et al.*, 2017). In addition, α -AMY deletion resulted in significant reduction in the formation of brain cysts (Fig. 2F).

To determine whether these phenotypic changes were directly caused by α -AMY deletion, we randomly inserted a C-terminally Ty-tagged complementary fragment in ME49 genome (Fig. 1A). Screening PCRs and immunostaining demonstrated the desired integration of the complementing construct and the successful expression of protein (Fig. 1B and D). Phenotypic analysis of the complementation strain indeed rescued the defects in $\Delta\alpha$ -amy mutant (Fig. 2). Together, these results indicated that disruption of α -AMY had no effect on growth *in vitro* but contributed to virulence attenuation and cysts reduction in mice.

ME49 $\Delta\alpha$ -amy vaccination protects mice from tachyzoites infection

Considering low virulence of α -AMY deletion mutant in mice, this feature may indicate that α -AMY deletion mutant has potential to be a good vaccine candidate. Thus, ICR mice were first vaccinated with 100

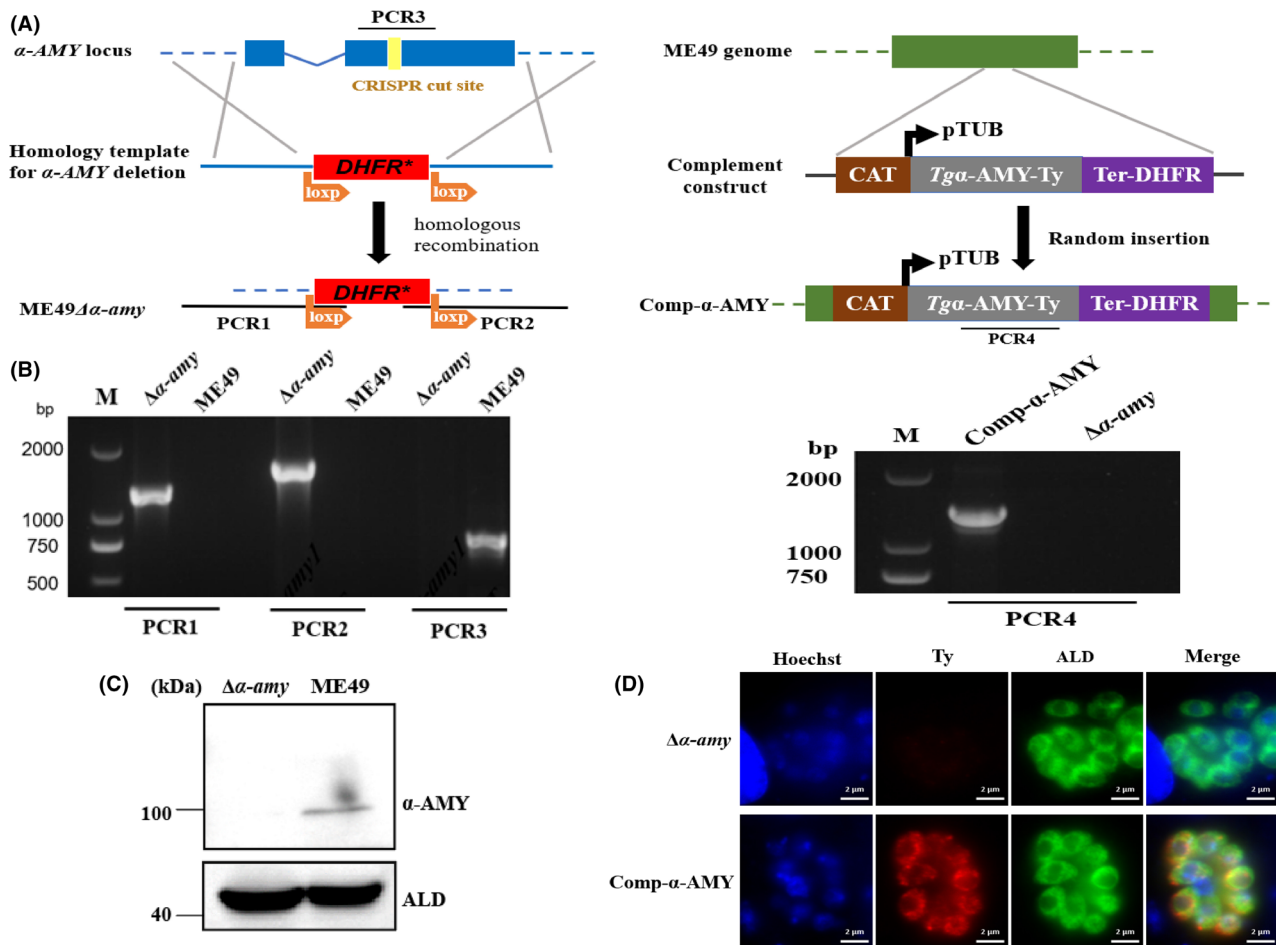


Fig. 1. Construction of α -AMY knockout and complementation strains.

A. Schematic illustration of generation of the ME49 $\Delta\alpha$ -amy and Comp- α -AMY strains via CRISPR/CAS9-mediated technique.

B. Diagnostic PCRs on a $\Delta\alpha$ -amy: *DHFR** and Comp- α -AMY clones.

C. Western blotting confirmed disruption of α -AMY in transgenic parasites. Parasite lysates were incubated with anti- α -AMY antibody, ALD served as the loading control.

D. The presence of α -AMY was confirmed by IFA. Samples were stained with mouse anti-Ty and rabbit anti-*T. gondii* ALD.

ME49 $\Delta\alpha$ -amy tachyzoites to evaluate the immune protection. After 30 days, the vaccinated mice were challenged with 10^3 tachyzoites of RH Δ hxgp r t, ME49, 10^4 tachyzoites of VEG, C7719 or TgPIG-WH1 by intraperitoneal injection for 30 days. As expected, non-immunized mice all died within 9 days after infection with RH Δ hxgp r t, and ME49, VEG and C7719 infection of mice led to 80%, 40% and 60% mortality rates respectively. Nevertheless, the survival rates of all vaccinated mice were upon 100% (Fig. 3A–D), which showed significant increases when compared with non-vaccinated mice. These above results illustrated that α -AMY deletion mutant was able to provide effective protection against acute infection caused by the tachyzoites of *T. gondii*.

To verify whether ME49 $\Delta\alpha$ -amy produced longer-term immune protection against acute toxoplasmosis in mice,

ICR mice were immunized with 100 tachyzoites of ME49 $\Delta\alpha$ -amy for 75 days; then, they were intraperitoneally injected with 10^3 tachyzoites of type I strain RH Δ hxgp r t, type II strain ME49, 10^4 tachyzoites of type III strain VEG or DB#3 isolated strain TgPIG-WH1. Being monitored 30 days later, the survival rates of vaccinated mice re-infected RH Δ hxgp r t, ME49 and VEG were 100%, which were consistent with the results of 30 days post-immunization (Fig. 3E–G). While the mortality rate of non-vaccinated mice infected attenuated strain VEG was only 20%, there was no significant difference from the immunized group despite the deaths. And both naïve group and vaccinated group re-infected TgPIG-WH1 all survived (Fig. 3H). These aforementioned results demonstrated that ME49 $\Delta\alpha$ -amy could exert short-term and long-term protective immunity against acute infection.

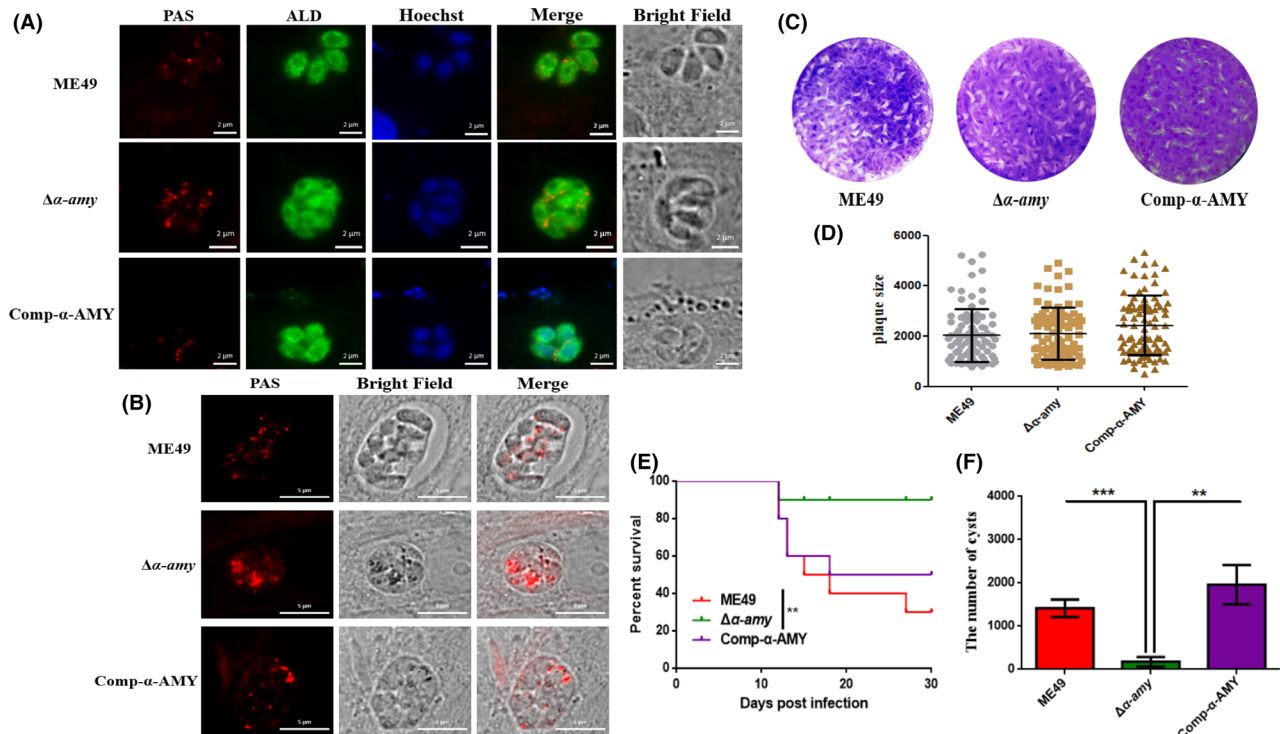


Fig. 2. Phenotypic analysis of α -AMY knockout and complementation strains. A and B. Amylopectin accumulation was detected with PAS staining. Parasites were inoculated to HFF host cells and incubated for 24 h under normal culture conditions for tachyzoites (A) or incubated for 2 h to allow the parasites to invade host cells, followed by incubation under conditions of pH 8.2 medium without CO₂ for 4 days for bradyzoites (B). C. Plaque assay of parasites *in vitro*. Purified indicated tachyzoites were used to infect HFF cells, and 200 parasites were cultured for 12 days before staining to determine the plaque size. D. Relative size of plaques in Fig. 2C. E. Survival curves of mice infected with tachyzoites of indicated strains. ME49 strain and α -AMY-deficient mutant were used to infect ICR mice (100 parasites per mouse, 10 mice for each group) by intraperitoneal injection, and the survival of mice was monitored for 30 days. F. Brain cysts of ICR mice infected with corresponding strains. * P < 0.05, Student's *t*-test. ** P < 0.01, Mantel-Cox log-rank test. The data are presented as the mean \pm SEM of three independent experiments.

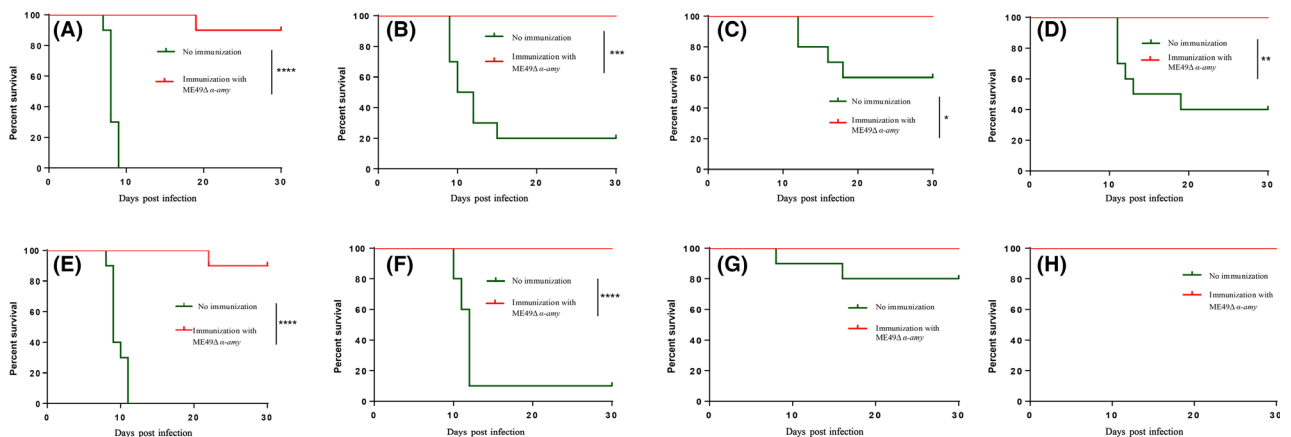


Fig. 3. ME49 $\Delta\alpha$ -amy vaccination protected mice from multiple *T. gondii* tachyzoites infection. Survival curves of ME49 $\Delta\alpha$ -amy-vaccinated mice, 30 days post-vaccination, intraperitoneally challenged with 10³ tachyzoites of RH Δ hxgprt (A), 10³ tachyzoites of ME49 (B), 10⁴ tachyzoites of VEG (C) or 10⁴ tachyzoites of C7719 (D) strains. 75 days post-vaccination, vaccinated mice were intraperitoneally challenged with 10³ tachyzoites of RH Δ hxgprt (E), 10³ tachyzoites of ME49 (F), 10⁴ tachyzoites of VEG (G) or 10⁴ tachyzoites of TgPIG-Wh1 (H). All mice were monitored for 30 days, and non-vaccinated mice were included as control. **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05, Mantel-Cox log-rank test.

ME49 $\Delta\alpha$ -amy vaccination protects mice from bradyzoites infection

Since ME49 $\Delta\alpha$ -amy could effectively resist acute infection induced by tachyzoites of *T. gondii*, we further explored whether it could also take effect against bradyzoites infection that was induced by *T. gondii* cysts. 30 days immunized later, either naïve mice or immunized mice were orally infected with 50 cysts of ME49 strain. After 30 days of continuous observation, the survival of mice was recorded. Consistent with the results of protective immunity against tachyzoites infection, the final survival rate of naïve mice infected with cysts was only 20%, and yet all vaccinated mice survived after infection (Fig. 4). These results further signified that $\Delta\alpha$ -amy mutant could provide good immune protection against bradyzoites infection.

ME49 $\Delta\alpha$ -amy vaccination contributes to decrease of parasite burden in peritoneal fluids

Due to ME49 $\Delta\alpha$ -amy vaccination performed efficient protection against acute infection, we probed the possible mechanism of this phenomenon and examined the tachyzoites growth in both naïve mice and immunized mice. After infection with the tachyzoites of RH Δ hxgp rt for 6 days, the peritoneal fluids were harvested, and parasite burden was measured by qPCR. As shown in Figure 5, abundant parasites were detected in non-vaccinated infected mice, which almost developed more than 10^8 parasites. In contrast, the low levels of parasites burden appeared in vaccinated mice infected with RH Δ hxgp rt ($\approx 10^2$ per mouse), which nearly decreased by 10^6 -fold compared with non-immunized control group, suggesting that vaccinated with ME49 $\Delta\alpha$ -amy could effectively eliminate the proliferation of parasites in mice.

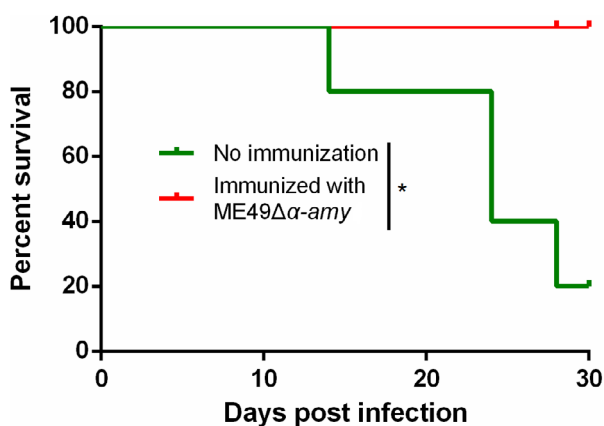


Fig. 4. ME49 $\Delta\alpha$ -amy vaccination protected mice from bradyzoites infection. ME49 $\Delta\alpha$ -amy vaccination were infected with 50 fresh brain cysts of ME49 strain by oral infection, and all mice were monitored for 30 days (5 mice for each group). * $P < 0.05$, Mantel-Cox log-rank test.

ME49 $\Delta\alpha$ -amy immunization provides protection against chronic infection

The typical trait of chronic infection was the persistence of tissue cysts containing bradyzoites, which was considered to be the primary approach of exposure to *T. gondii* (Pittman and Knoll, 2015; Montazeri *et al.*, 2018a,b). Therefore, study was performed to validate whether $\Delta\alpha$ -amy mutant vaccination could provide protection against chronic infection. TgPIG-WH1, a less virulent strain and prone to forming cysts, was used to re-infect the immunized mice (Xia *et al.*, 2018a). 30 days post-infection, the survival rates of both naïve mice and immunized mice infected with TgPIG-WH1 were 100% (Fig. 2H). The brain tissues of four mice that were randomly picked out from each group were harvested, and the brain cysts number was counted using DBA staining under fluorescence microscope, subsequently. The number of cysts in vaccinated mice group significantly decreased compared with non-vaccinated mice, although a small amount of brain cysts could still be detected in vaccinated mice, which might be produced by the ME49 $\Delta\alpha$ -amy infection. Thus, results in Figure 6 revealed that ME49 $\Delta\alpha$ -amy immunization could effectively prevent the formation of cysts, which demonstrated that ME49 $\Delta\alpha$ -amy could also exert protective immunity against chronic infection.

Levels of cytokines and *T. gondii* specific IgG antibodies after ME49 $\Delta\alpha$ -amy immunization

To elucidate the potential mechanism of protective immunity against *T. gondii* infection provided by $\Delta\alpha$ -amy

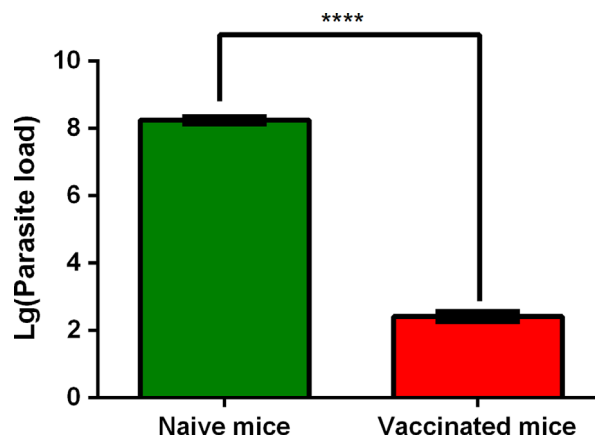


Fig. 5. ME49 $\Delta\alpha$ -amy vaccination effectively reduced the replication of challenging parasites in mice. Naïve or vaccinated mice were challenged with 10^5 tachyzoites of RH Δ hxgp rt (5 mice for each group) by intraperitoneal injection, and the parasite loads in peritoneal fluids 6 days post-infection were determined by quantitative PCR. The data are presented as the mean \pm SEM of three independent experiments, **** $P < 0.0001$, Student's *t*-test.

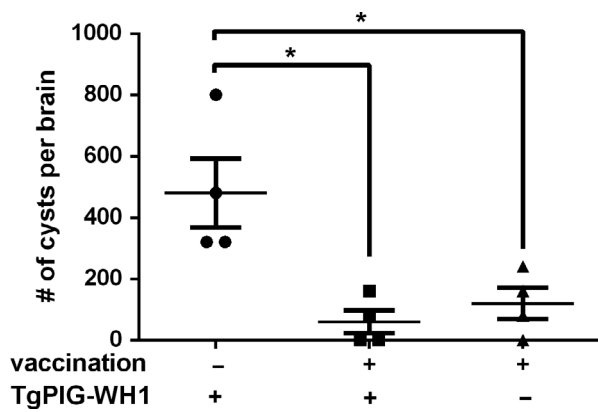


Fig. 6. ME49 $\Delta\alpha$ -*amy* vaccination prevented mice from the formation of cysts. The survived mice in both naïve and immunized groups challenged with TgPIG-WH1 were sacrificed, and the number of *Toxoplasma* cysts in the brain was determined by DBA-FITC staining under fluorescent microscopy. Data from the analysis of four mice in each group were graphed, * $P < 0.05$, Student's *t*-test.

mutant, the levels of cytokines in serum were measured by ELISA 30 days or 75 days after ME49 $\Delta\alpha$ -*amy* vaccination. The results showed that the levels of Th1-type cytokines (IFN- γ , IL-12 and TNF- α) significantly increased than those in naïve mice (Fig. 7A–C). Similarly, the level of Th2-type cytokine (IL-4) of immunized mice at 30 days was notably higher than that of non-

immunized mice. Although the level of IL-4 in the serum of vaccinated mice still increased after 75 days, there was no significant difference compared with the non-vaccinated group (Fig. 7D). In addition, we determined the presence of *T. gondii* specific IgG levels and found that ME49 $\Delta\alpha$ -*amy* vaccination elicited high levels of IgG, which developed an incremental trend with the extension of time (Fig. 7E). Then, the levels of specific IgG subclasses (IgG1 and IgG2a isotypes respectively) were detected to evaluate Th1 and/or Th2 response in immunized mice. As shown in Figure 7E, the expression levels of IgG1 and IgG2a 30 and 75 days post-immunization were significantly increased in vaccinated mice. Together, these results illuminated that vaccination with ME49 $\Delta\alpha$ -*amy* in mice was likely to stimulate organism to elicit a mix of Th1/Th2 immune response against the infection.

Cytokine production by splenocytes of immunized mice

Splenocytes were collected to further assess cellular immune responses and antigen-specific memory responses induced by soluble *Toxoplasma* antigen (TSA) in immunized mice. 145 days post-immunization, the cytokine production of serum samples was measured, and results revealed that there was no significant difference of cytokine levels between immunized mice

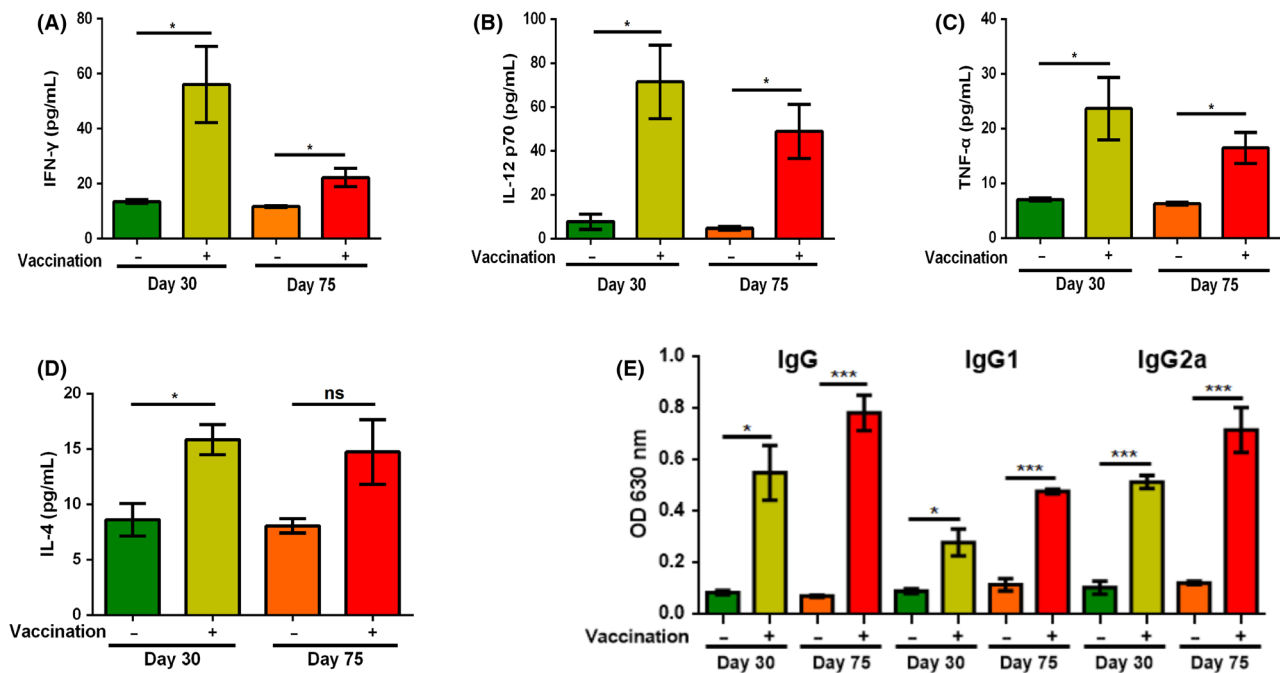


Fig. 7. Levels of cytokines and *Toxoplasma*-specific IgG in the serum of mice 30 and 75 days post-vaccination. A–D. Levels of IFN- γ , IL-12 p70, TNF- α and IL-4 were measured by ELISA. E. Levels of *Toxoplasma*-specific total IgG and IgG subclass (IgG1 and IgG2a) measured by indirect ELISA. Serum samples of naïve mice were determined as controls, and three mice from each group were analysed. The data are presented as the mean \pm SEM of three mice from each group. *** $P < 0.001$, * $P < 0.05$, 'ns' indicated as not significant, Student's *t*-test.

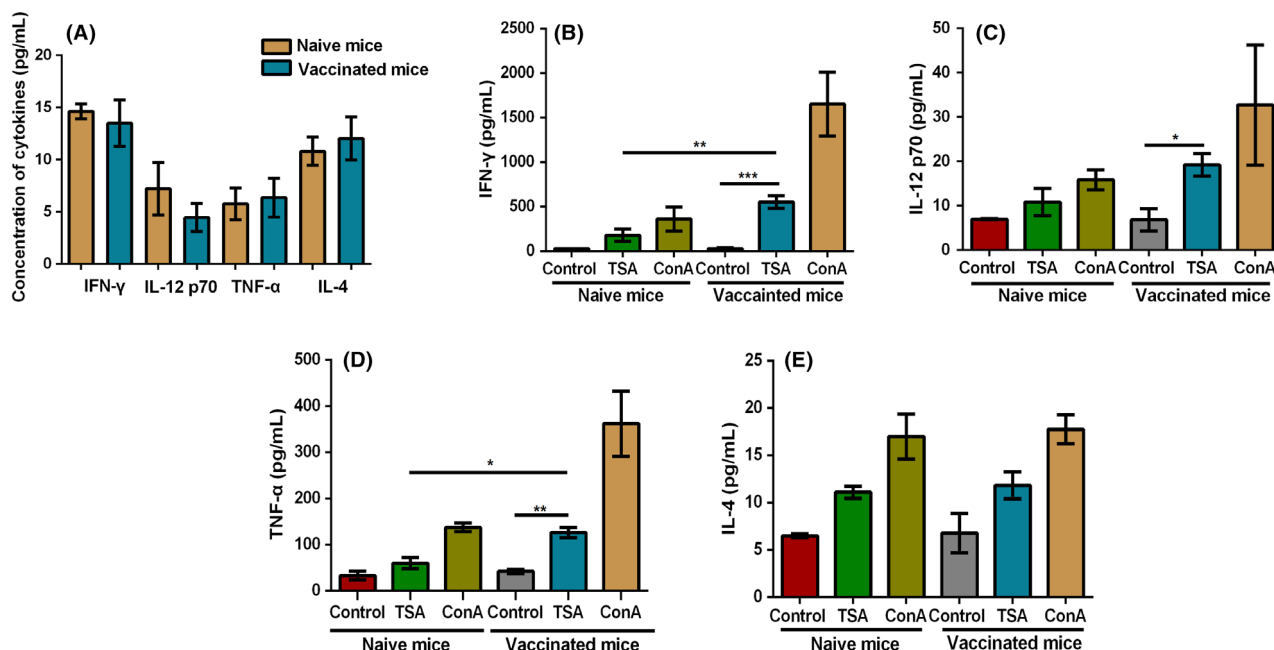


Fig. 8. Cytokines production by splenocytes of naïve mice and vaccinated mice after *Toxoplasma* antigen stimulation. A. The serum samples of naïve mice and immunized mice were collected to measure the indicated cytokines 145 days post-immunization. B–E. The splenocytes of vaccinated mice were harvested 145 days post-vaccination. Control is the group treated with foetal bovine serum, TSA is the group treated with total *Toxoplasma* antigen, and ConA is the group treated with concanavalin A as a positive control. The levels of IFN- γ , IL-12 p70, TNF- α and IL-4 were measured by ELISA. Three mice from each group were analysed, and each treatment condition was repeated three times, *** P < 0.001, ** P < 0.01, * P < 0.05, Student's t -test.

and naïve mice (Fig. 8A). In contrast, IFN- γ , TNF- α , IL-12 levels of splenocytes stimulated by TSA markedly increased in immunized mice compared with that in non-immunized mice, although the production of IL-4 showed an increase, there was no significant difference compared with naïve mice (Fig. 8B–E). Taken these results together, Th1-type cells-mediated immune responses triggered robust protection against secondary stimulation.

Discussion

T. gondii can cause persistent infection in both animals and humans as a worldwide distributed intracellular protozoan, and severe toxoplasmosis generally occurs in immunocompromised hosts (Di Cristina *et al.*, 2017; Wang *et al.*, 2017a). The main clinical symptoms of toxoplasmosis are mental illnesses and congenital impairment (Elsheikha, 2008; Sutherland *et al.*, 2015). Currently, the most commonly used drugs for toxoplasmosis cure are pyrimethamine and sulfadiazine; however, these therapeutic drugs take no effect on chronic infection and there may exist risk of side effects in the treated hosts (Pramanik *et al.*, 2019). Recent research has demonstrated that vaccines may be the best choice for toxoplasmosis treatment, and a commercial vaccine

for preventing abortion in sheep is available (Dunay *et al.*, 2018). Nevertheless, numerous drawbacks still present in current studies, and it is urgent to excavate a safe and efficient vaccine. In the present study, a disruption of α -AMY strain was successfully constructed, and then, a mouse model vaccinated was established to study the protective immunity provided by ME49 $\Delta\alpha$ -amy against acute and chronic infection.

Amylopectin granule is considered to be an important energy storage material for bradyzoites formation (Coppin *et al.*, 2003; Tomavo, 2015), and α -AMY is an enzyme involved in the amylopectin metabolism pathway of *T. gondii*, which may be phosphorylated by CDPK2 (Uboldi *et al.*, 2015; Sugi *et al.*, 2017). However, the specific biological function of α -AMY is rarely reported. In this study, CRISPR/Cas9 gene editing technology was applied to generate deletion of α -AMY gene, and results in our research revealed that α -AMY could degrade amylopectin in both tachyzoites and bradyzoites conditions. In view of a low dose (100) of ME49 $\Delta\alpha$ -amy tachyzoites contributing to the attenuation of the virulence in mice, thus this dose was used for all subsequent experiments to evaluate the possibility of $\Delta\alpha$ -amy mutant resistance to *T. gondii* challenge.

One of the most effective means of provision of protective immune responses are live-attenuated vaccines

due to the mimic natural infection that can built an approximate microenvironment for antigen processing and presentation (Wang *et al.*, 2019). Based on the phenotypes of effective growth *in vitro* and low proliferation ability *in vivo*, α -AMY-deficient ME49 strain was thought to be a potential live-attenuated vaccine against *T. gondii*. The rapid replication of tachyzoites throughout hosts' body can cause the outbreak of acute infection, and the severity of an acute infection is affected by environmental factors, the strains of *T. gondii* and the route of infection (Pittman and Knoll, 2015). The main epidemic strains of *T. gondii* are classified as types I, II and III, which exert different acute virulence in mice (Howe and Sibley, 1995; Howe *et al.*, 1997), and the main route of host infection with *Toxoplasma* is to ingest contaminated water and food (Esch and Petersen, 2013). As shown by the results, mice immunized with ME49 $\Delta\alpha$ -amy appeared no clinical symptom and all survived when challenged with tachyzoites of type II, type III and other natural isolates by intraperitoneal infection, as well as re-infection with cysts by oral infection, even the protection rate reached 90% and parasite burden in peritoneal fluids dropped to an extremely low level against type I strain challenge, indicating that ME49 $\Delta\alpha$ -amy vaccination could trigger effective protection to significantly improve survival rates and reduce parasite burden caused by acute *T. gondii* infection. Tissue cysts can facily be killed by physical methods, while antiparasitic drugs have no effect on cysts (Coombs and Muller, 2002; Hill and Dubey, 2002). Consequently, the aim of developing an efficient vaccine is to prevent tissue cysts formation. Mice immunized with ME49 $\Delta\alpha$ -amy could dramatically reduce the formation of brain cysts. Compared with other previously reported vaccines, ME49 $\Delta\alpha$ -amy could provide a long-term protective immune response against both acute infection and chronic infection (Huang *et al.*, 2019; Zheng *et al.*, 2019). However, there are some disadvantages of it, such as incomplete reduction of cysts and a certain virulence threat (Fox and Bzik, 2010).

The B-cell response can be activated and produce numerous specific IgG, which can neutralize the adhesion of *T. gondii* and then inhibit its ability of infection (Sayles *et al.*, 2000). In the study presented here, we measured specific anti-*T. gondii* IgG antibody titres and IgG isotypes in both vaccinated mice and non-vaccinated mice. Consistent with other attenuated *T. gondii* vaccines, ME49 $\Delta\alpha$ -amy vaccination indeed upregulated the production of IgG, IgG1 and IgG2a, which signified that ME49 $\Delta\alpha$ -amy immunization induced a mixed Th1/Th2-type immune response. CD8⁺ T and CD4⁺ T cells play a crucial role against *T. gondii* as effector T lymphocytes (Suzuki and Remington, 1988). IL-12 produced by macrophages, neutrophils and dendritic cells can induce proliferation and differentiation of Th1-type T cells, which

can stimulate natural killer cells and T cells to produce IFN- γ , a major cytokine against *T. gondii* (Gazzinelli *et al.*, 1993). In addition to the effective elimination of tachyzoites, IL-12 and IFN- γ are also essential for maintaining chronic infection (Yarovinsky, 2014). Other cytokines such as TNF- α , IL-2, IL-4 and IL-6 play a protective role in *T. gondii* infection. Then, we analysed the levels of cytokines and found that both Th1-type cytokines (IFN- γ , IL-12 and TNF- α) and Th2-type cytokines (IL-4) significantly increased. Above results further supported the inference that ME49 $\Delta\alpha$ -amy immunized could simultaneously activate Th1 and Th2 immune responses to secrete cytokines against *T. gondii*. Regarding patterns of cytokines measured in splenocytes harvested from naïve mice or immunized mice after 145 days, a time point, cytokines recovered to normal levels. When the cytokines production of splenocytes isolated from immunized mice strongly increased after stimulation with *Toxoplasma*-specific antigen; moreover, these cytokines are mainly based on Th1-type cytokines. These results potentially suggest that ME49 $\Delta\alpha$ -amy vaccination can induce a combination of Th1/Th2 immune response, whereas Th1-type cell-mediated immune response, especially IFN- γ -mediated activation, plays a leading role.

In conclusion, the present study demonstrated that α -AMY was able to degrade amylopectin and deletion of α -AMY mutant attenuated virulence *in vivo* but normal proliferation *in vitro*, which were the bases of a potential vaccine candidate. In addition, a safe dose, 100 tachyzoites of ME49 $\Delta\alpha$ -amy vaccination provided both short-term and long-term protective immunity against challenge with multiple *T. gondii* classic strains and nature isolates. The results of extreme reduction in the parasite cyst burden in the brain of immunized mice illustrated that vaccination of ME49 $\Delta\alpha$ -amy could also effectually prevent form chronic infection. And the administration of ME49 $\Delta\alpha$ -amy was able to stimulate a combination of humoral immune response and cellular immune response to protect animals from further infections of various *T. gondii* strains. Our findings may reveal the potential of ME49 $\Delta\alpha$ -amy as an efficient and safe live-attenuated vaccine candidate. Despite these, the protection of ME49 $\Delta\alpha$ -amy needs to be improved based on its above deficiencies. It has been proved that the deletion of *CDPK2* would lead to abnormal accumulation of abundant amylopectin, which may prevent the establishment of the chronic toxoplasmosis in the mouse model (Uboldi *et al.*, 2015). In addition, live-attenuated Pru: Δ *cdpk2* strain could also develop a protective immune response against acute, chronic and congenital toxoplasmosis (Wang *et al.*, 2018). Therefore, in order to abolish the formation of brain cysts in mice, we will consider the deletion of *CDPK2* based on ME49 $\Delta\alpha$ -amy strain in the future studies, making it a safer and more efficacious

vaccine in the clinical application. In addition, in biological security of this vaccine requires to be further studied, and the protective efficiency also needs to be assessed in large animals, such as sheep.

Experimental procedures

Animals and parasites

8-week female ICR mice were acquired from the Hubei Provincial Centre for Diseases Control and Prevention (Wuhan, China). All mice were co-housed under specific pathogen-free conditions at 25°C under a 12 h light–dark cycle (Wuhan, China). All experimental procedures were approved by the Scientific Ethic Committee of Huazhong Agricultural University (HZAUMO-2019-005), and all efforts were made to minimize the suffering of animals.

Tachyzoites of *T. gondii* type I strain RH Δ hxgprt, type II strain ME49, the mutant strain ME49 Δ α -amy, type III strain VEG, RFLP genotype ToxoDB #3 strain TgPIG-WH1 and ToxoDB #9 strain C7719 were propagated in human foreskin fibroblast cells (obtained from ATCC, USA) which were cultured in DMEM supplemented with 2% FBS and were incubated at 37°C and 5% CO₂.

Generation of α -AMY knockout and complementation strains

CRISPR/CAS9-mediated homologous gene replacement system was used to knockout α -AMY gene (TGME49_246690) of *T. gondii*, and the methods were described previously (Mahmoud *et al.*, 2017). Briefly, the α -AMY targeting CRISPR plasmid and 5H-DHFR-3H homologous templates for replacing α -AMY were co-transfected into purified tachyzoites of ME49 and then selected with 1 μ M pyrimethamine. Positive single clone was identified by diagnostic PCR. The construction of complementation strain was based on the processes of p α -AMY:: α -AMY-CAT fragment being transfected into ME49 Δ α -amy and selected with 30 μ M chloramphenicol, and diagnostic PCR (PCR4) was used to identify positive clones. All primers and plasmids used in this study were listed in Table S1. In addition, a series of techniques used to determine protein expression and parasites growth, such as Western blot, IFA, plaque assays and periodic acid-Schiff (PAS) staining, were all executed as previously described (Shen and Sibley, 2014; Sugi *et al.*, 2017; Xia *et al.*, 2018a).

Virulence tests in mice

8-week-old female ICR mice were infected with 100 freshly egressed tachyzoites of ME49 or ME49 Δ α -amy by intraperitoneal injection. Then, all mice were monitored daily to assess mortality for 30 days, and the

serum samples of surviving mice were collected for MIC3-based ELISA test after 30 days post-infection. The anti-MIC3 negative mice were left out in subsequent analysis.

Immune protection against tachyzoites infection and bradyzoites infection

Eight-week female ICR mice were first immunized with ME49 Δ α -amy, 30 and 75 days post-vaccination; then both immunized mice and naïve mice were challenged with 10³ tachyzoites of RH Δ hxgprt or ME49, 10⁴ tachyzoites of VEG, C7719 or TgPIG-WH1 by intraperitoneal injection to test protective immunity against tachyzoites infection (10 mice/parasite strain), or 50 fresh brain cysts of ME49 by oral infection to test efficacy against bradyzoites infection (5 mice/group). Subsequently, clinical symptoms and survival of all mice were monitored daily for another 30 days.

Determination of parasite burden in peritoneal fluids by ME49 Δ α -amy vaccination

Additionally, vaccinated mice and naïve mice of the same age were intraperitoneally injected with 10⁵ tachyzoites of RH Δ hxgprt. After 6 days post-infection, all the mice were euthanized by inhalation of CO₂, and peritoneal fluid was harvested from ME49 Δ α -amy-immunized mice infected with RH Δ hxgprt and non-immunized mice infected with RH Δ hxgprt, and total genomic DNA was extracted from peritoneal fluid using the EasyPure Genomic DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's protocols. The method of calculating parasite burden in peritoneal fluids was performed as described previously (Xia *et al.*, 2018b). Briefly, CT value of each sample was measured by quantitative real-time PCR using corresponding genomic DNA samples, and β -tubulin gene-specific quantitative RT-PCR primers were listed Table S1. Quantitative RT-PCR was performed on ABI Step one plus real-time PCR instrument using TB Green qPCR Master Mix (TaKaRa, Japan). Parasite burden in peritoneal fluids was calculated from a standard curve using CT values and Lg (tachyzoite numbers).

The formation of brain cysts after vaccination

Mice immunized with ME49 Δ α -amy and naïve mice were infected with 10⁴ tachyzoites of TgPIG-WH1, and mice immunized but not TgPIG-WH1 challenged were considered as a control group. After 30 days post-infection, seropositive mice were euthanized and brain tissues of them were homogenized to measure the number of cysts by DBA-FITC (Vector Laboratories, Burlingame,

CA, USA) staining as described previously (Huskinson-Mark *et al.*, 1991).

Preparation of soluble *Toxoplasma antigen* (TSA)

The method of preparing soluble tachyzoite antigens was performed as previously described (Mineo *et al.*, 1993; Bastos *et al.*, 2016). Briefly, freshly egressed *T. gondii* ME49 tachyzoites were collected, washed in PBS and then lysed by sonication on ice for 30 min. Subsequently, the samples after sonication treatment were centrifuged at 12 000 rcf for 10 min at 4°C. The supernatants were collected as TSA that were stored at -80°C before use, and the TSA concentration was determined BCA Protein Assay Kit (Beyotime Biotechnology, Beijing, China).

Vaccine-induced cytokines and antibody levels measurements

Serum samples of immunized mice and naïve mice were gathered for cytokines and antibody levels detection 30 or 75-days post-infection. The effect of vaccine-induced cytokine production was measured using mouse IFN- γ , TNF- α , IL-12p70 and IL-10 ELISA kits (BioLegend, San Diego, CA, USA) according to the manufacturer's protocols. The levels of *T. gondii* specific total immunoglobulin G (IgG) and subclasses of IgG antibodies IgG2a and IgG1 as indicators of type 1T-helper (Th1) and type 2T-helper (Th2) responses, respectively, were analysed using ELISA assay as described previously (Wang *et al.*, 2017a; Huang *et al.*, 2019). A total of 100 μ l soluble tachyzoite antigens (5 μ g ml⁻¹) were coated in a 96-well plate and incubated at 4°C overnight. Then, the plate was washed 3 times and blocked with 1% BSA at 37°C for 1 h. After washing 3 times, 100 μ l serum samples were added to appropriate wells at 37°C for 45 min. Subsequently, 100 μ l of HRP conjugated goat anti-mouse IgG secondary antibodies (1:5000 dilutions, Beyotime Biotechnology, Beijing, China), anti-mouse IgG1 (1:10 000, Abcam, Cambridge, UK) and anti-mouse IgG2a (1:10 000, Abcam) were added to each well and incubated at 37°C for 30 min. Finally, tetramethylbenzidine as substrate was incubated to visualize the immunoreaction, and the OD value was determined at 630 nm using a microplate reader.

Cytokines in splenocyte supernatants after *Toxoplasma antigen* treatment

The spleens and serum samples of naïve mice and vaccinated mice were harvested 145 days vaccination later, and the splenocyte suspensions were acquired as previously described (Elsheikha, 2008; Wang *et al.*, 2017b).

In brief, spleens were washed in Roswell Park Memorial Institute medium (RPMI) 1640 (Life Technologies, Rockville, MD, USA), and then the spleen was pushed through a nylon net. Subsequently, red cell lysis solution (Biosharp, Beijing, China) was added into the compound to remove the red blood cells. The purified suspensions were plated in 96-well plates and cultured in RPMI 1640 supplemented with 10% FBS. The suspensions in either non-vaccinated mice or vaccinated mice were treated in groups as follows: (i) control: suspensions without any treatment as negative controls; (ii) TSA: suspensions were stimulated with total *T. gondii* soluble antigens (TSA, final concentration 50 μ g ml⁻¹); (iii) ConA: suspensions were stimulated with 5 μ g ml⁻¹ concanavalin A (Life Technologies, Rockville, MD, USA) as positive control. After 3 days treatment, the supernatants were gathered to measure the cytokine production using ELISA.

Statistical analysis

All data were performed with GRAPHPAD PRISM 6 (GraphPad Software, La Jolla, CA, USA) and presented as the mean \pm SEM. The statistical differences between groups were determined using Student's *t*-test or two-way analysis of variance as indicated in the figure legends. A value of $P < 0.05$ was defined as statistically significant. Cumulative mortality was graphed as Kaplan–Meier survival plots and analysed by Mantel–Cox log-rank test.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primers used in the study.