Ginseng Stem-and-Leaf Saponin (GSLS)-Enhanced Protective Immune Responses Induced by *Toxoplasma gondii* Heat Shocked Protein 70 (HSP70) Against Toxoplasmosis in Mice

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GINSENG STEM-AND-LEAF SAPONIN (GSLS)–ENHANCED PROTECTIVE IMMUNE RESPONSES INDUCED BY *TOXOPLASMA GONDII* HEAT SHOCKED PROTEIN 70 (HSP70) AGAINST TOXOPLASMOSIS IN MICE

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ABSTRACT: *Toxoplasma gondii* is an obligate intracellular protozoan parasite and is able to infect birds and mammals

approved by the Zhejiang University Experimental Animal Ethics Committee (Permit No. ZJU201308-1-10-072).

Tachyzoites of *T. gondii* RH strain were harvested from African green monkey kidney (Vero) cell monolayer. The parasites were washed twice in phosphate-buffered saline (PBS) and centrifuged at 1,500 *g* for 5 min. Soluble tachyzoite antigen (STAg) was then prepared as previously described (Qu et al., 2013). The genomic DNA of *T. gondii* was extracted using a Universal Genomic DNA Extraction Kit (TaKaRa, Shanghai, China) according to the manufacturer's instructions.

Preparation of recombinant HSP70 protein

Genomic DNA of *T. gondii* was used as template for PCR to amplify the TgHSP70 using the following specific primers: 5′-CGGGATCCTATGGCGGACTCTCCTGCTGTGGGTATT-3′ and 5′-CCCAAGCTTTTAATCAACTTCCTCCACGGTGG-3′ based on the published sequence (GenBank U85648.1). *Bam*-HI and *Sac*-I restriction sites were introduced to the amplified product, which was then digested and cloned into the expression vector pET-28b (Novagen, Madison, Wisconsin) to obtain the recombinant plasmid pET28-HSP70. The plasmid was then transformed into *Escherichia coli* BL21 (DE3) cells (Novagen), and gene expression was induced by adding 1.0 mM isopropyl-β-D-thiogalactopyranoside. HIS fusion protein rTgHSP70 was purified using a nickel-nitrilotriacetic acid agarose column system (Qiagen, Shanghai, China), and the endotoxins were removed through a Endotoxin Removal Kit (GenScript, Shanghai, China). The purity and antigenicity of rTgHSP70 were analyzed and confirmed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot. Briefly, the purified recombinant protein rTgHSP70 was separated in a 12% polyacrylamide gel and then electroplated onto a nitrocellulose membrane, which was incubated with the *T. gondii*–positive serum of mice at a 1:400 dilution as the primary antibody and followed by anti-mouse IgG-alkaline phosphatase conjugate horseradish peroxidase (dilution, 1:5,000) (Sigma-Aldrich, Shanghai, China) as the secondary antibody. Finally, the membrane was soaked in Enhanced Chemiluminescence substrates (Thermo Scientific, Shanghai, China) for signal development.

Immunization and infection

A total of 120 female ICR mice were randomly divided into 6 groups: Group A, PBS control; Group B, mice were subcutaneously injected with 50 μg GSLS; Group C, mice were subcutaneously injected with 100 μg rTgHSP70 and PBS; and Groups D–F, mice were subcutaneously injected with 100 μg rTgHSP70 and GSLS of dosages 25 μg, 50 μg, and 100 μg, respectively. Mice were immunized 3 times with the same components at the same dosage every 2 wk. All mice were intraperitoneally (i.p.) challenged with 500 tachyzoites of *T. gondii* RH strain 1 wk after the final inoculation. Survival data of mice in different groups were observed and recorded daily.

Determination of antibodies

The levels of antibodies against *T. gondii* in mouse sera were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates (Corning, Shanghai, China) were coated overnight at 4 C with 10 μg/ml purified rTgHSP70 diluted in 100 mM carbonate-bicarbonate buffer (pH 9.6). After 3 washes, the wells were blocked with 10 mM PBS containing 5% skim milk for 1 hr at 37 C. After 3 washes, 100 μl of serum samples from mice diluted 1:1,000 in PBS-5% milk were added to each well and incubated for 1 hr at 37 C. After washing, goat anti-mouse IgG, IgG1, and IgG2a-horseradish peroxidase conjugate antibodies (dilution, 1:1,500) were added as the secondary antibody at 37 C for 1 hr. After washing, the immune complexes were developed by the addition of 100 μl tetramethylbenzidine chromogenic substrate (Sigma-Aldrich) for 20 min, and stopped by adding 50 μl of 2 M H_2SO_4. The optical densitiy was measured at 450 nm with an ELISA reader (Chromate 4300; Awareness Technology, Palm City, Florida) 3 times for each sample.

Lymphoproliferation asssy

Splenocyte proliferation was assayed as described previously (Qu et al., 2013). Briefly, splenocyte suspensions were obtained from mice in each group by pushing the spleens through a steel mesh. Splenocytes were resuspended in complete medium (RPMI 1640 containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS) after the red blood cells (RBCs) were removed by using RBC lysis solution (Sigma). Cells were then seeded in 96-well coaster plates with a density of 1×10^4 cells per well and incubated with rTgHSP70 (10 μg/ml) or concanavalin A (ConA) (5 μg/ml; Sigma) as control at 37 C with 5% CO_2. After 68 hr, 50 μl of methyl thiazolyl tetrazolium (MTT) solution (2 mg/ml) was added to each well. After incubating for 4 hr, the plates were centrifuged at 1,400 g for 5 min, and the untransformed MTT solution was carefully removed. Finally, 200 μl of DMSO working solution (192 μl DMSO with 8 μl 1M HCl) was added to each well, and the absorbance was measured at 570 nm with an ELISA reader 3 times for each well after developing for 15 min.

Flow cytometry analysis of T cell subsets

Flow cytometry was performed to determine the levels of $CD4^+$ and $CD8^+$ T cell subsets in the splenocytes of mice from all groups. Splenocytes were suspended with a density of 1×10^6 cells and stained with FITC-conjugated anti-mouse CD4 (eBioscience, Shanghai, China) and APC-conjugated anti-mouse CD3 (eBioscience) or PE-conjugated anti-mouse CD8a (eBioscience) and APC-conjugated anti-mouse CD3 for 30 min at 4 C in the dark. Cell subsets were analyzed on a FACScan flow cytometrer (Becton Dickinson, San Jose, California) with a total of 5,000 cells of light scattering properties. Lymphocytes were determined and gated by forward and side scatter profiles.

Determination of the expression of IL-4 and IFN-γ in splenocytes

Splenocytes (1×10^7) of mice from all groups were lysed in 1 ml of Trizol reagent (Invitrogen, Shanghai, China), and total RNA was isolated according to the manufacturer's instructions. RNA was reverse transcribed to cDNA in a DEPC-treated tube by using a Reverse Transcription Kit (Takara). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed with the SYBR Green Realtime PCR Master Mix (2 ×, TOYOBO) on the ABI 7500 (PE Applied Biosystems, Foster City, California). All

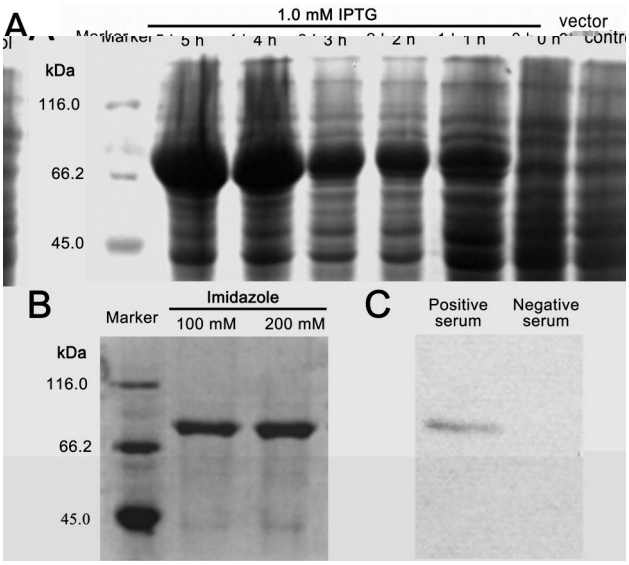

FIGURE 1. Expression and identification of recombinant HSP70 of *Toxoplasma gondii*. (**A**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of rTgHSP70 expressed in *Escherichia coli* using 12% acrylamide gel. Lanes 1–5: Expression of rTgHSP70 at 5 hr, 4 hr, 3 hr, 2 hr, and 1 hr after induction by IPTG, respectively; Lane 6: induced control culture of cells without IPTG; Lanes 7: induced control culture of cells with empty vector. (**B**) SDS-PAGE analysis of the purification of rTgHSP70 by Ni^{2+}-charged agarose column system. (**C**) Western blot detection of rTgHSP70 by serum of mice as primary antibodies, Lane 1: *T. gondii*–positive serum; Lane 2: *T. gondii*–negative serum.

samples were run in triplicate and normalized to β-actin. The PCR primers were as follows: IL-4 forward: 5′-GTCATCCTG CTCTTCTTTCTC-3′, reverse: 5′-GTGGACTTGGACTCATTC ATG-3′; IFN-γ forward: 5′-CTCTGAGACAATGAACGC TAC-3′, reverse: 5′-TTCTTCCACATCTATGCCACT-3′; β-actin forward: 5′-GATGGTGGGAATGGGTCAGA-3′, reverse: 5′-TACGACCAGAGGCATACAGG-3′.

Statistical analysis

All statistical analysis was performed using the SPSS 20.0 software package (SPSS Inc., Chicago, Illinois), and the statistical significance between all groups was tested by one-way ANOVA. The result was considered significantly different if P value was less than 0.05.

RESULTS

Expression and Western blot of recombinant HSP70 protein

The fragment of TgHSP70 was cloned into expression vector pET-28b and then transformed into *E. coli* as described in the Methods section. Recombinant HSP70 protein (76 kDa, theoretically) was successfully inducted by the addition of 1 mM IPTG (Fig. 1A). Ni^{2+}-charged agarose column system was applied to purify the protein, which was analyzed by SDS-PAGE as shown in Figure 1B. In order to detect the immunity of rTgHSP70, the result of Western blot indicated that rTgHSP70 could interact with the *T. gondii*–positive serum of mice and could not be recognized by the negative control sera (Fig. 1C).

Specific IgG induced by vaccination

Serum samples of mice from all groups were collected weeks 0, 1, 3, and 5, and then antibodies against rTgHSP70 were detected by ELISA. No specific IgG was found in those mice injected with PBS or GSLS throughout the test period, as shown in Figure 2A. Anti-rTgHSP70 antibodies of mice from groups C-F were detectable as early as 1 wk post-immunization at a significant high level compared to control groups ($P < 0.05$). Co-immunization of rTgHSP70 with GSLS (50 μg and 100 μg) induced a significant higher specific antibody than that with 25 μg GSLS or rTgHSP70 alone at 3 and 5 wk after the first immunization ($P < 0.05$).

The IgG subclass of sera were analyzed by using STAg as coating antigens, and both IgG1 and IgG2a were produced in immunized ICR mice as shown in Figure 2B. The mice vaccinated with rHSP70 and GSLS (50 μg and 100 μg) induced notably higher specific IgG1 and IgG2a antibodies than the other groups ($P < 0.05$), and mice in the PBS control group did not generate any antibody response.

Detection of cellular immune response

The splenocytes from immunized mice were prepared at weeks 1 and 5 to assess the proliferative immune responses to rTgHSP70. Splenocytes from mice injected with rTgHSP70 present a significant higher proliferative response level to rTgHSP70 compared to that from mice injected with PBS or GSLS alone at both week 1 and week 5 ($P < 0.05$) (Fig. 3). A strong increase of the splenocyte proliferative response to HSP70 was observed from mice immunized with rTgHSP70 and GSLS (50 μg and 100 μg) compared with groups immunized with

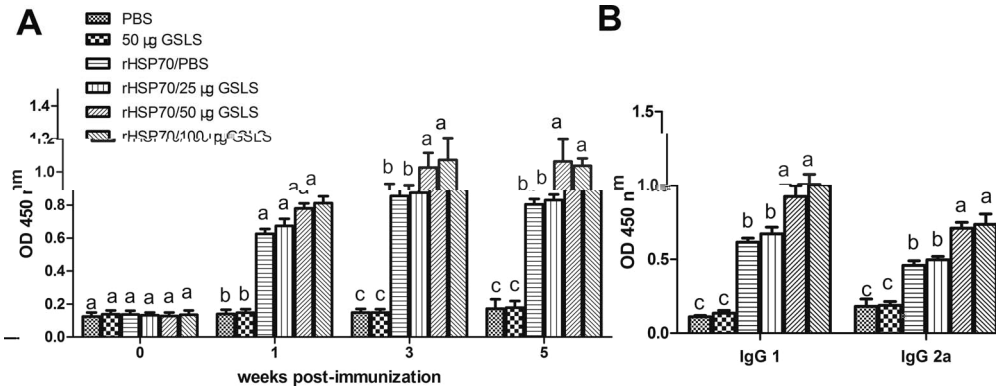

FIGURE 2. The effect of immunization on the antibody response detected by ELISA. (**A**) Detection of total IgG antibodies. Serum samples were collected at weeks 0, 1, 3, 5 after immunization for IgG analysis. (**B**) Determination of the specific anti-rTgHSP70 IgG subclass profile. Sera were collected at week 5 after first immunization. The values were presented as means of OD450 ± SD (n = 5 mice per group), Columns with different letters present statistical difference ($P < 0.05$).

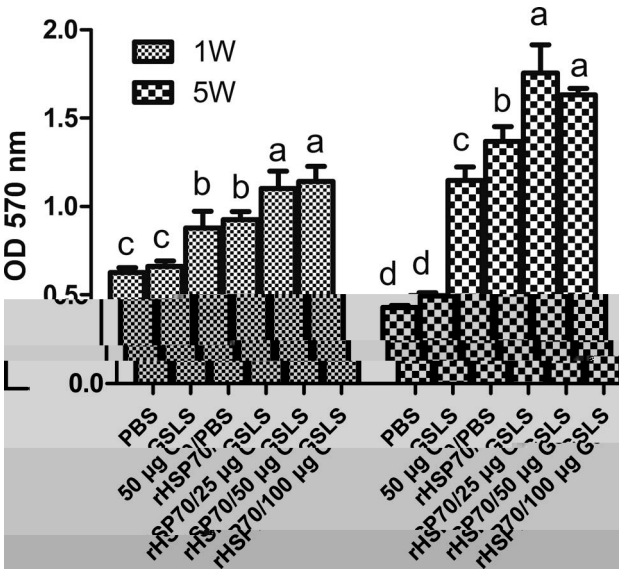

FIGURE 3. Splenocyte proliferation responses to rTgHSP70. Splenocyte suspensions were obtained from mice in each group at weeks 1 and 5 after the first immunization and cultured with rTgHSP70. Splenocyte proliferation was assessed by the MTT method as described in the text. The values were presented as means of OD570 ± SD (n = 5 mice per group), Columns with different letters present statistical difference ($P < 0.05$).

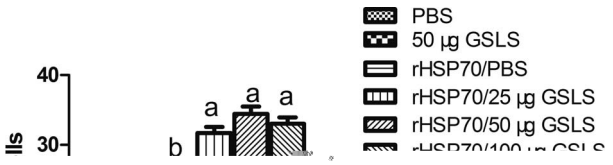

FIGURE 4. Effect of rHSP70-induced activation of $CD4^+$ and $CD8^+$ T cells in the spleen by flow cytometry. One week after the final immunization, the percentage numbers of $CD4^+$ and $CD8^+$ T cells in the spleens were evaluated and expressed as means ± SD (n = 5 mice per group), Columns with different letters present statistical difference ($P < 0.05$).

rTgHSP70 alone or rTgHSP70 + 25 μg GSLS ($P < 0.05$), while there was no significant difference among the control groups.

Effect of rTgHSP70-induced activation of $CD4^+$ and $CD8^+$ T cells

To investigate the effect of rTgHSP70 and GSLS on the activation of $CD4^+$ and $CD8^+$ T cells, splenocytes of mice were collected at 1 wk after the last inoculation and were then evaluated by flow cytometry. Percentages of $CD4^+$ and $CD8^+$ T cells in the spleens of mice immunized with rTgHSP70 and GSLS (25 μg, 50 μg, and 100 μg) were remarkably higher than that in mice immunized with rTgHSP70 alone ($P < 0.05$). In addition, mice immunized with rTgHSP70 presented significant high level of $CD4^+$ and $CD8^+$ T cells compared with control groups ($P < 0.05$) (Fig. 4).

Expression of IL-4 and IFN-γ by splenocytes

RNA was isolated from mouse splenocytes at 1 wk after the final immunization in order to evaluate the effect of rTgHSP70 and GSLS on cytokine mRNA expression based on the qRT-PCR. Compared to nonimmunized controls, splenocytes from rTgHSP70-vaccinated mice presented significantly higher expression levels of IL-4 and IFN-γ compared to control groups ($P < 0.05$) (Fig. 5). Dramatically IFN-γ expression levels of spleen cells in mice co-administration with rTgHSP70 and GSLS (50 μg and 100 μg) were observed compared with the groups immunized with rTgHSP70 alone or rTgHSP70 + 25 μg GSLS ($P < 0.05$).

Protective effect of immunized mice against challenge with *T. gondii* RH strain

To assess whether the vaccination of rTgHSP70 and GSLS could induce effective protection against the lethal *T. gondii* RH strain infection, mice from all groups were intraperitoneally injected with 500 tachyzoites 1 wk after the last immunization and observed daily to record the survival rate. Mice from 50 μg GSLS group or rTgHSP70/PBS group lived slightly longer, but no significant difference was observed when compared with PBS control group, which died out within 6 days post-injection (Fig. 6). Substantially significantly longer survival times were observed in mice co-immunized with rTgHSP70 and GSLS (50 μg and 100 μg) compared with the control groups ($P < 0.05$). Although all experimental mice died eventually within 15 days, the total survival time (117 days) of the mice immunized with rTgHSP70 + 50 μg GSLS was longer than any other groups.

DISCUSSION

Adjuvant is widely applied in vaccines as an innate immunity enhancer by augmenting the interaction between antigens and

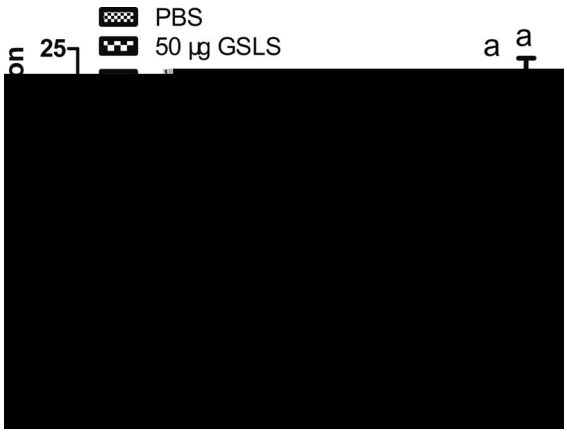

FIGURE 5. The effect of rTgHSP70 and GSLS on mRNA expression of IFN-γ and IL-4 in splenocytes. Total mRNA of splenocytes were prepared 1 wk after the final immunization, and then the expression levels of IFN-γ and IL-4 were determined by RT-PCR. The values were presented as means ± SD (n = 5 mice per group). Columns with different letters present statistical difference ($P < 0.05$).

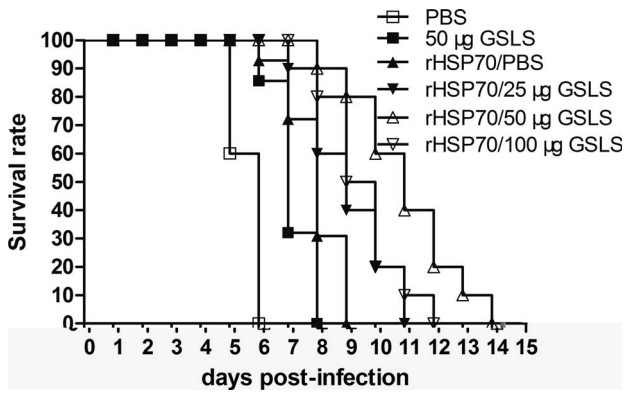

FIGURE 6. Survive rate of ICR mice after challenge of *Toxplasma gondii*. Mice (n = 10/group) were intraperitoneally challenged with 500 *T. gondii* RH strain tachyzoites 1 wk after the last immunization and observed daily for mortality.

antigen-presenting cells and stimulating high levels of antibody (Sun et al., 2007; Zhang et al., 2008). Our team has tried to search for effective adjuvants in assisting vaccines against toxoplasmosis. We demonstrated that ginsenoside Rg1 (Qu et al., 2011) and ginsenoside Re (Qu et al., 2013) had positive effects in assisting the recombinant protein TgSAG1 and TgROP18 vaccines, respectively, which prolonged the survival time of *T. gondii*–infected mice. Ginseng stem-and-leaf saponins (GSLS) contained ginsenosides Rg1 8.0%, Re 20.3%, Rb1 1.4%, Rb2 4.8%, Rf 0.3%, Rc 3.7%, and Rd 11.9% and was much cheaper and easier to obtain than ginsenoside Rg1 and ginsensoside Re, making it more suitable in clinical application. Even though GSLS alone provide limited protection, Zhai reported that oral administration of GSLS could produce better vaccination against avian influenza (Zhai et al., 2011a), Newcastle disease (Zhai et al., 2011b), and infectious bursal (Zhai et al., 2014) diseases in chickens. All these properties make GSLS a promising effective adjuvant. Some vaccine studies had already revealed the protective effect of HSP70 against toxoplasmosis, Makino et al. (2011) found that DNA vaccine with *Tghsp70* gene induced DC activation and Th1 polarization, and Kikumura et al. (2010) reported that *Tghsp70* gene vaccine induced protective immunity and prolonged survival time of *T. gondii*–infected mice.

In the present study, the immunogenicity and protection potency of rTgHSP70 in assistance with GSLS were comprehensively evaluated in mice model. Mice immunized with rTgHSP70 developed a significantly high level of specific IgG antibodies compared with the PBS control group as measured by ELISA ($P < 0.05$), and those administrated with rTgHSP70 + GSLS (50 μg, 100 μg) generated notable higher IgG antibody level than that of the rTgHSP70/PBS group ($P < 0.05$). In addition, the mice vaccinated with rTgHSP70 and GSLS (50 μg and 100 μg) induced significant higher specific IgG1 and IgG2a antibodies than the other groups ($P < 0.05$). We found a tendency that the antibody level rose along with the increase of the vaccination dosage of GSLS. A similar tendency was observed when assessing the proliferative immune response of splenocytes from mice immunized with rTgHSP70 and GSLS, and these 2 groups co-administrated with rTgHSP70 and GSLS (50 μg and 100 μg) presented a distinctly high level of immune response compared with the other groups at 1 and 5 wk after primary immunization. $CD4^+$ and $CD8^+$ T lymphocytes are essential for host in long-term resistance against *T. gondii* infection probably through the production of IFN-γ, which is known as the central cytokine responsible for the control of toxoplasmosis (Suzuki et al., 1988; Dupont et al., 2012, 2014). $CD3^+CD4^+CD8^-$ is the surface marker of T helper cells that is involved in the adaptive immune responses, while $CD3^+CD4^-CD8^+$ locates on the cytotoxic T cells that play an important role in immune systems (Montoya et al., 1996; Goldszmid and Sher, 2010). In this study, we observed a notable difference in the percentage of $CD4^+$ and $CD8^+$ T cells between the vaccinated and control groups evaluated by flow cytometry. We found a significant increase in both of these components in rTgHSP70 immunized mice ($P < 0.05$), and those administrated with GSLS (25 μg, 50 μg, and 100 μg) presented a slighter but significantly higher level compared to the rTgHSP70/PBS group, suggesting that immunization with rTgHSP70 and GSLS induced the activation of both $CD4^+$ and $CD8^+$ T lymphocytes. Compared with the rTgHSP70/PBS group, immunization with rTgHSP70 and GSLS (50 μg and 100 μg) enhanced the Th1 immune responses by inducing dramatically high levels of IFN-γ ($P < 0.05$). In addition, a slight but significant increase of cytokine IL-4, a factor of Th2 immune response, was observed in spleen cells from mice vaccinated with rTgHSP70 + GSLS (25 μg, 50 μg, 100 μg) compared with control groups ($P < 0.05$). These results were in accordance with that of the splenocyte proliferative responses. Several studies had reported a similar tendency of Th1 response to recombinant protein vaccines against toxoplasmosis (Dziadek et al., 2012; Qu et al., 2013; Yu et al., 2013; Pinzan et al., 2015).

To evaluate the protection efficacy of the recombinant antigen rTgHSP70 vaccine, all mice were intraperitoneally challenged with 500 tachyzoites of *T. gondii* RH strain. Results showed that rTgHSP70 and GSLS (50 μg, 100 μg) could prolong the survival time of *T. gondii*–infected mice when compared with the control group, demonstrating that this vaccination could afford partial protection against *T. gondii* RH strain. Data we gathered here were in accordance with a few *Tghsp70* gene vaccine studies. Makino et al. (2011) found that DNA vaccine with *Tghsp70* gene induced DC activation and Th1 polarization and was able to limit parasite loads in *T. gondii*–infected mice, and Kikumura et al. (2010) reported that *Tghsp70* gene vaccine could induce protective immunity to prolong survival time of *T. gondii*–infected mice.

In conclusion, the present study evaluated the immunogenicity and protective efficacy of rTgHSP70 in assisting with different dosages of GSLS for the first time. The vaccine of rTgHSP70 + GSLS was able to elicit remarkable immune responses and prolong the survival time of *T. gondii*–infected mice. Our results suggested that rTgHSP70 and GSLS could provide partial protection against *T. gondii* RH strain, and that GSLS could be treated as a promising adjuvant in immunization against *T. gondii*.

ACKNOWLEDGMENTS

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