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Original Article

Immunization of BALB/c Mice with Killed Tachyzoites of *Toxoplasma gondii* against Acute Toxoplasmosis

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Abstract

Background: *Toxoplasma gondii* with widespread distribution infects over one third of human populations in the world and can cause serious life-threatening diseases especially for the immunodeficient patients in acute toxoplasmosis. As the clinical pharmaceutical drugs with severe side effects for treatment and non-ideal extant vaccines for prevention, more work starves to be done for keeping advantages in the athletics.

Methods: Aluminum adjuvant and hybrid formaldehyde-killed tachyzoites of *T. gondii* RH and GT1 isolates were prepared to intramuscularly immunize BALB/c mice for five times at 0, 3, 7, 14 and 21 days post first injection. The triggered humoral and cellular immune responses at two weeks post the last immunization and the survival times of infected mice were examined for the hybrid immunization scheme judgement.

Results: The anti-RH and anti-GT1 specific antibodies were both increased at one week prior to challenge ($P < 0.05$), and the survival times of immunized mice (7.33 ± 0.71 d for RH, 7.22 ± 0.97 d for GT1) against acute toxoplasmosis were significantly prolonged by the immunizations performed in the study compared to blank control (6.67 ± 0.50 d for RH, 6.33 ± 0.71 d for GT1; $P < 0.05$), with the higher IFN-γ, IL-2 and IL-12p70 in sera, the elevated $CD3e^+CD4^+$ T and $CD3e^+CD8a^+$ T cells, and the enhanced lymphocyte proliferation in spleen ($P < 0.05$).

Conclusion: The hybrid killed tachyzoites with aluminum adjuvant induced humoral and cellular immune responses of mice, and offered mildly protective efficacy against acute toxoplasmosis.

Introduction

The obligate intracellular protozoan *Toxoplasma gondii* with worldwide distribution, one of the most successful pathogens capable to infect and persist in all the warm-blooded animals (1), results in more than one third of human population infection in the world mainly through mucosal contact, vertical transmission in pregnancy, and/or consumption of the food and water contaminated by this parasite (2-4), with higher exposure rates between 40% and 60% especially in Africa, Southeast Asia, South America and Central-Southern Europe (5). Besides significant neuropsychiatric and behavioral disorders according to previous reports based on human diseases and experimental rodent models (6, 7), exposure to *T. gondii* can also cause serious life-threatening toxoplasmosis for patients with HIV or organ transplants, and for the immunosuppressed individuals, especially in the acute infections (8, 9).

To resist the epidemically protracted pathogens such as SARS-CoV-2 currently prevalent in the global (10), basic works are commonly forced in two footpaths, i.e., treatments using medicines and prevention with vaccines, but for *T. gondii* infection, which dramatically seems to be forceless because of the pharmaceutical drugs that have severe side effects and do not act for cysts in clinic (11, 12), and no approved extant vaccines for human beings and most livestock for prevention (13, 14). Obviously, more work needs continuously to be done to defeat this parasite in the fierce athletics.

In the past few years, the purified antigen, killed or irradiated tachyzoites of *T. gondii* were used for immune (15-17), and the antibody, brain cyst burden, lymphocyte activation, and survival were detected to evaluate the immunological efficacy against toxoplasmosis such as ME49 (Genotype II) or RH (Genotype I) infection (18, 19). Given the consequences less than satisfaction, it is so necessary to explore novel immunization scheme to keep advantages in the athletics. In addition, the protective efficacy of immunization might be too weak to examine or not statistically significant when usage of single element such as one strain of *T. gondii* (20). The hybrid mixed with equal formaldehyde-killed RH and GT1 tachyzoites (Genotype I) and the aluminum adjuvant were therefore prepared in the study, and employed to immunize the female BALB/c mice using novel immunization scheme and more progressive technology for examination. We aimed at evaluating whether the mixture could be as an excellent assistant in future to intercept the acute infections caused by *T. gondii* virulent tachyzoites.

Materials and Methods

Ethics statement

The animal experiments were strictly performed following the guidelines of the Animal Ethics Procedures of the People's Republic of China. The procedures including immunization and challenge with parasites in the study were approved by the Experimental Animal Ethics Committee of Guilin Medical University (Approval No. GLMC202203073).

Animals and tachyzoites

4-6-week-old female BALB/c mice in clean grade were purchased from the Laboratory Animal Center of Guilin Medical University (Guilin, China). To keep rigorous, the mice were resided in the High-density Touch Screen Mouse IVC System (Qingdao, China), and their bedding materials and food were supplied throughout by Guangxi Zhuoyi Biotechnology Co. Ltd. (https://www.zoeybio.com). Tachyzoites of *T. gondii* RH and GT1 strains passaged *in vitro* using Vero cell lines were used to prepare hybrid-killed parasites and to challenge mice for evaluating the protective efficacy of immun-

ization scheme performed in the study. *Toxoplasma* lysate antigen (TLA) was prepared following the previous description (21).

Hybrid formaldehyde-killed parasite preparation

When about 80% of Vero cells were rived by *T. gondii* RH or GT1 tachyzoites in vitro, the culture products were carefully scraped, fully mixed and broken, and purified using the density gradient centrifugation of Percoll (GE Healthcare, Uppsala, Sweden) (22, 23). The obtained tachyzoites were washed thrice with centrifugation at 5 000 rpm for 3 min, counted by a hemacytometer, and adjusted to 2.0×10^6 parasites per milliliter for RH or GT1 strain using stroke-physiological saline solution (SPSS). The mixture containing 1.0×10^6 tachyzoites/ml for each strain and 4% of formaldehyde in analytical reagent grade (Macklin, Shanghai, China) was placed at 4 °C for four weeks when the mixing was performed more than three times a day with at least 20 perversions for each time. The killed tachyzoites were washed thrice as above and adjusted to 4.0×10^6 parasites/ml for the following immunization.

Immunization scheme

After feeding for one week to reduce stress reaction, three BALB/c mice were randomly selected for blood collection seen as below and other mice were allocated into three groups (30 mice each group; Table 1) and intramuscularly immunized for five times at 0, 3, 7, 14 and 21 days post the first immunization. The grouped mice include: Group I without any treatment served as blank control; Group II injected with 100 µl of aluminum adjuvant alone named $Al(OH)_3$; Group III injected with 50 µl of aluminum adjuvant and 50 µl of SPSS containing equal formaldehyde-killed RH and GT1 tachyzoites (i.e., 1×10^5 parasites each) named RH&GT1+$Al(OH)_3$. The spleen tissues of three mice each group were aseptically collected after blood collection prior to challenge (Table 1), and were cut into pieces for the single splenocyte preparation described as previously (24).

Table 1: Details of treatments performed in BALB/c mice

Group	*Treatments (of 100 µl SPSS)*	*Total size*	*Route of administration*	*Mice number in HIR and CIR*	*Mice number in challenges*
I	Blank control	30	-	3^a, 3^b, 3^c, 3^d	9^e, 9^f
II	100 µl $Al(OH)_3$	30	Thigh muscle	3^a, 3^b, 3^c, 3^d	9^e, 9^f
III	50 µl $Al(OH)_3$ + 2×10^5 RH&GT1 parasites	30	Thigh muscle	3^a, 3^b, 3^c, 3^d	9^e, 9^f

[a, b, c, d] For evaluating humoral immune response (HIR), blood samples were collected from three mice each group using eyeball enucleation at 14, 21, 28 and 35-day post first immunization, respectively.

[d] For evaluating cellular immune response (CIR), spleen tissues were aseptically removed from three mice each group after blood collection at 35-day post first immunization.

[e] Two weeks post the final immunization, mice in all the groups were challenged intraperitoneally with 1×10^3 virulent tachyzoites of *T. gondii* RH strain.

[f] Two weeks post the final immunization, mice in all the groups were challenged intraperitoneally with 1×10^3 virulent tachyzoites of *T. gondii* GT1 isolate

Aluminum adjuvant preparation

Aluminum hydroxide dry powder (Macklin) (5.5 g) was mixed into 6.0 ml of SPSS followed by slow addition with 6.0 ml of the concentrated sulfuric acid stored in our laboratory. When the solution was brown, ddH_2O about 80 ℃ was added up to 100 ml with continuous stirring. The obtained mixture was

used to react with equal volume of 8.0% sodium hydroxide (about 75 ℃) (Macklin) in another acid-resistant container, and its pH value was stabilized to 6.9 when the cure was finished. After washed for five times through centrifugation at 8 000 rpm for 5 min, the final product diluted into 260 ml of SPSS was used as the adjuvant for the immunization.

Antibody surveillance

As shown in Table 1, blood samples from three mice each group (only three mice for pre-immunization seen as above) were randomly collected using eyeball enucleation at 0, 14, 21, 28 and 35-day post first immunization to prepare sera for surveilling changes of specific anti-*Toxoplasma* IgG antibodies. The obtained sera were diluted as 1:4 using SPSS before measurement, and ELISA plates were coated with 100 μl of borate buffered saline (BBS, pH 8.0) containing 1.0 μg of TLA prepared using *T. gondii* RH or GT1 tachyzoites for specific antibody assays at 4°C for at least 12 h in a humidified atmosphere. Antibody measurement was performed using the SBA Clonotyping ™ System/HRP (SouthernBiotech, Birmingham, USA) based on the manufacturer's guidance (https://resources.southernbiotech.com/tech bul/ 5300-05.pdf). The optical density of each well was recorded at 450 nm at 10 min after substrate addition by an ELISA reader (Bio-Rad, Hercules, USA).

Cytokine measurement

To character the fluctuation of cytokines in the original sera separated prior to challenge at 35-day post first immunization, the levels of IFN-γ, IL-2, IL-6, IL-10 and IL-12p70 in the above diluted sera were examined by the Legend Max ™ ELISA Kits (Biolegend, California, USA), and were multiplied by four. The minimum detectable concentration of the kit is 8.0 pg/ml for IFN-γ, 0.9 pg/ml for IL-2, 2.0 pg/ml for IL-6, 2.7 pg/ml for IL-10, and 0.5 pg/ml for IL-12p70, respectively.

Lymphocyte proliferation assay

The lymphocyte proliferation analysis was carried out as the previous report using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) (Promega, Madison, USA) with some modifications (24). In brief, the single splenocytes separated prior to challenge were cultured at a concentration of 5×10^4 cells/well in 100 μl of DMEM medium (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (Invitrogen), 10 units/10 μg of penicillin/streptomycin (Invitrogen), and 0.5 μg/0.5 μg of RH/GT1 TLA prepared before for 72 h at 37 °C in a 5% CO_2 atmosphere. The well without TLA addition was performed as control. The optical absorbance each well was read at 490 nm using an ELISA reader, and the stimulation index (SI) defined as the ratio of the stimulated well to its control, was used to evaluate the efficacy of the performed immunization scheme.

Flow cytometry assay

To complementally assess effect of the inactivate tachyzoite mixture on cellular immune responses of immunized mice, the percentages of $CD3e^+CD4^+$ T and $CD3e^+CD8a^+$ T cells in the single splenocytes separated from three mice each group prior to challenge were analyzed using a FACScan flow cytometer with SYSTEM II software (BD Bio-sciences, Burlingame, USA) as previously described (24).

Challenge and survival time record

Aiming to estimate immunizations with the hybrid killed tachyzoites against acute toxoplasmosis, the rest of mice were intraperitoneally injected with 1×10^3 virulent *T. gondii* tachyzoites (nine mice each group for RH, and nine for GT1; Table 1) at 14 days post the last immunization. The survival times of all the challenged mice were daily recorded until all of them were dead.

Statistical analysis

The experimental data presented as mean +/- standard deviation (i.e., Mean+/-SD), was analyzed using the SPSS PASW Statistics v18.0 Data Editor (SPSS Inc., Illinois, USA). The statistical difference between was measured using the student's *t*-test, and was marked with a star if *P* value less than 0.05, and two stars if $P < 0.01$.

Results

Higher specific anti-Toxoplasma antibodies revealed in sera

To monitor the changes of humoral immune response triggered by the hybrid-killed tachyzoites, blood samples were randomly collected from three experimental mice each group (only three for pre-immunization) at five-time points post first immunization, and the obtained sera were diluted as 1:4 for analyzing the levels of specific anti-*Toxoplasma* IgG antibodies. The results showed that at one week prior to challenge both anti-RH and anti-GT1 specific antibodies were detected statistically higher than their controls (anti-RH: $P = 0.0330$ for blank control *vs* RH&GT1+Al(OH)$_3$ at 28 day-, and $P = 0.0276$ for Al(OH)$_3$ at 35 day-; anti-GT1: $P = 0.0412$ for Al(OH)$_3$ at 28 day-, and $P = 0.0196$ for Al(OH)$_3$ and $p = 0.0483$ for blank control at 35 day-post immunization) (Fig. 1).

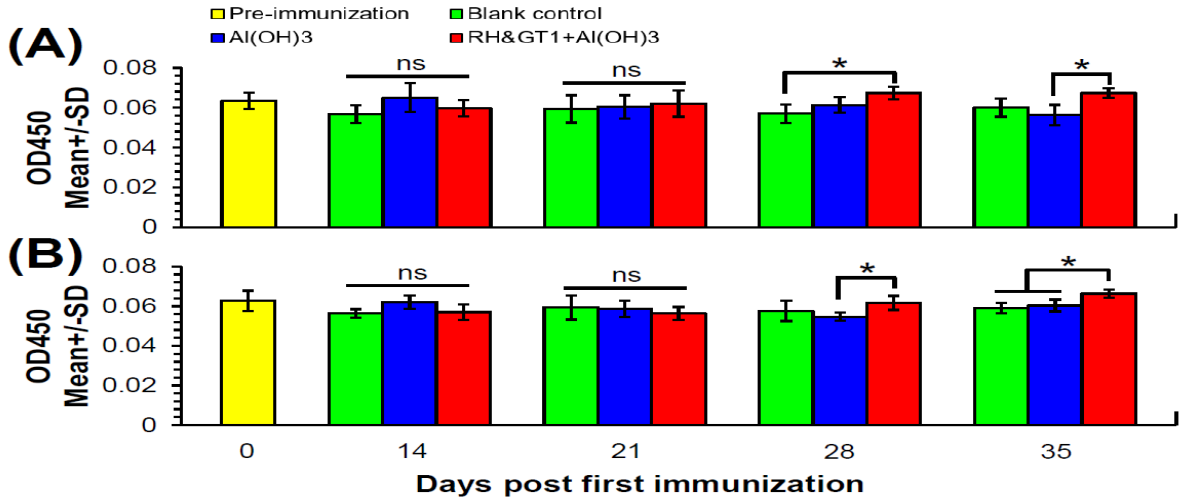

Fig. 1: Levels of specific anti-*Toxoplasma* antibodies in sera of immunized mice. The sera separated at 0, 14, 21, 28 and 35 days post first immunization were diluted as 1:4 using SPSS, and were next employed to read optical density at 450 nm for evaluation of specific anti-RH (A) and anti-GT1 (B) IgG antibodies in sera. No statistical difference between was detected except for anti-RH and anti-GT1 IgG antibodies at 28- and 35-day post first immunization. *$P < 0.05$; ns indicates no significance

Immunization enhanced cytokine production and lymphocyte proliferation

For cytokine examination, IL-6 and IL-10 without statistical difference among all the experimental groups, and three kinds of significantly increased cytokines were determined in sera collected prior to challenge from the mice immunized with RH&GT1+Al(OH)3 in comparison with controls (P = 0.0200 with Al(OH)3 and $P = 0.0427$ with blank control for IL-2, $P = 0.0458$ and $P = 0.0410$ for IL-12p70, and $P = 0.0219$ and $P = 0.0461$ for IFN-γ, respectively) (Fig. 2A).

The results of lymphocyte proliferation showed stimulation index of splenic lymphocytes separated from RH&GT1+Al(OH)3-immunized mice was statistically elevated compared to controls ($P = 0.0321$ for

Al(OH)3, and $P = 0.0459$ for the blank control) (Fig. 2B), suggesting vaccination with the killed RH&GT1 mixture induced the proliferation responses to TLA for lymphocytes in spleen.

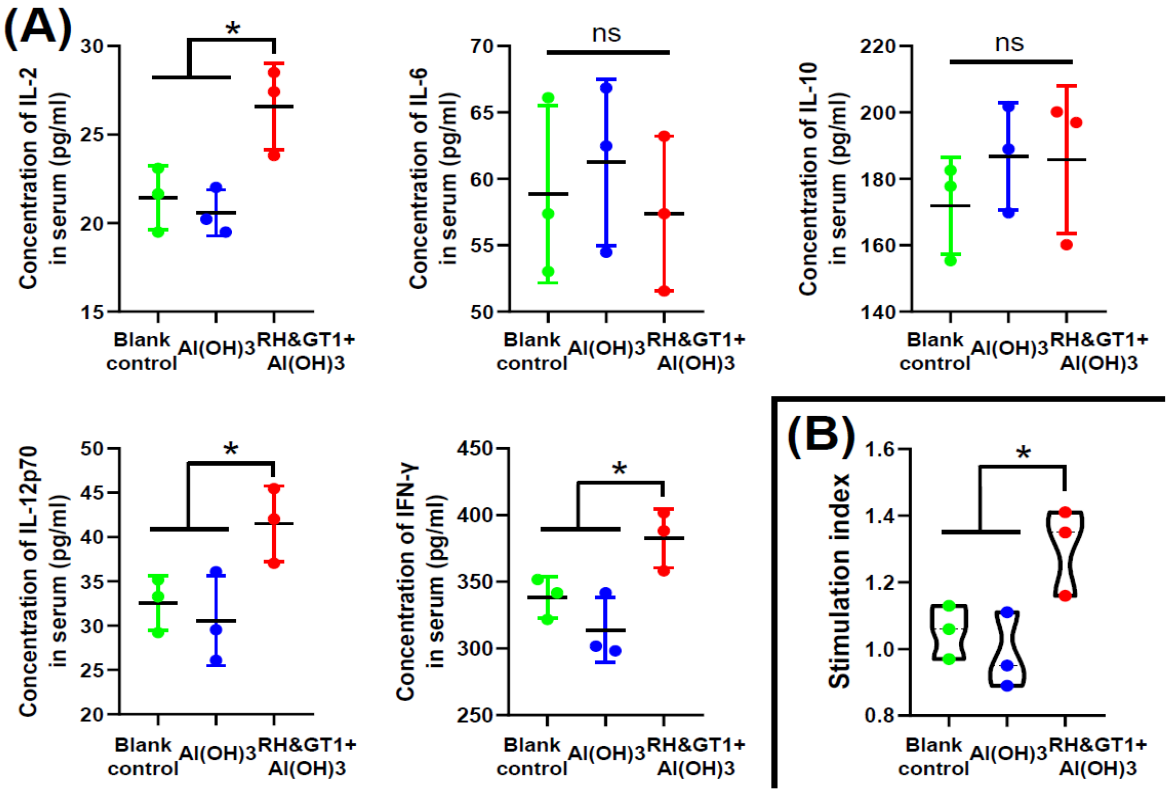

Fig. 2: Cytokines and lymphocyte proliferation analyses. Cytokines (A) including IL-2, IL-6, IL-10, IL-12p70 and IFN-γ in sera separated at two weeks after the last immunization were detected by ELISA kits. Spleens of mice were picked for preparation of the sterile single splenocyte and the following lymphocyte stimulation index (SI) using MTS method (B). IL-2, IL-12p70 and IFN-γ in sera, and SI in RH&GT1+Al(OH)3 group were significantly elevated in comparison with controls. *$P < 0.05$; ns indicates no significance

Hybrid killed tachyzoites induced elevation of T lymphocyte subclasses

Two subclasses of lymphocytes ($CD3e^+CD4^+$ T and $CD3e^+CD8a^+$ T cells) in spleen prior to challenge were measured by analyzing the proportions in the prepared single splenocytes using flow cytometry for evaluating the effect of immunization with the hybrid killed *T. gondii* tachyzoites on cellular immune responses of mice. The percentages of T cells and the statistical significance between presented with different capital letters were marked in Q2 and Q2-1 regions in Fig. 3, which indicated the cellular immune responses of mice were awakened with significant elevation of $CD3e^+CD4^+$ T and $CD3e^+CD8a^+$ T lymphocytes in spleen via immunizations with the hybrid killed parasites.

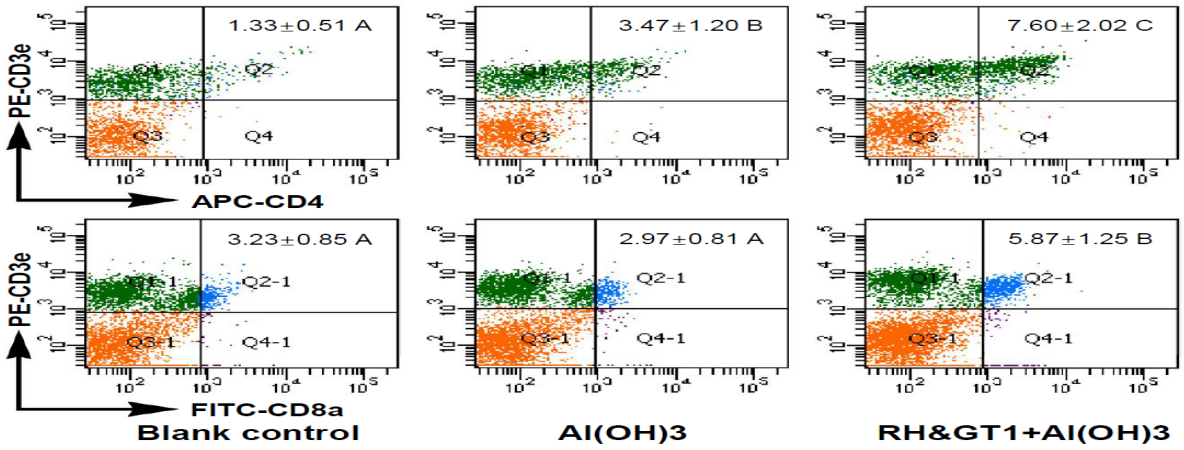

Fig. 3: Flow cytometry assay for subclasses of T lymphocytes in prepared splenocytes. The proportions of CD3e+CD4+ T and CD3e+CD8a+ T cells (Mean+/-SD) were respectively marked in Q2 and Q2-1 regions, and the following capital letters were used to reflect the statistical difference between (i.e., no difference if the same, vice versa)

Immunization prolonged survival time of mice against acute toxoplasmosis

Finishing the five booster immunizations with killed RH&GT1 tachyzoite mixture, all the groups of mice were intraperitoneally challenged with 1×10^3 vigorous tachyzoites of *T. gondii* RH or GT1 strain at two weeks post the final immunization, and the survival times of them were daily monitored to assess the protection of vaccine against acute toxoplasmosis.

The data showed that in comparison with blank control (6.67 ± 0.50 d for RH, and 6.33 ± 0.71 d for GT1), immunization with the killed tachyzoites statistically prolonged the survival time of mice (7.33 ± 0.71 d for RH, and 7.22 ± 0.97 d for GT1, respectively, $P < 0.05$), but no difference was detected in $Al(OH)_3$ (6.56 ± 1.01 d for RH, and 6.44 ± 1.42 d for GT1) both for blank control and the immunized group (Fig. 4).

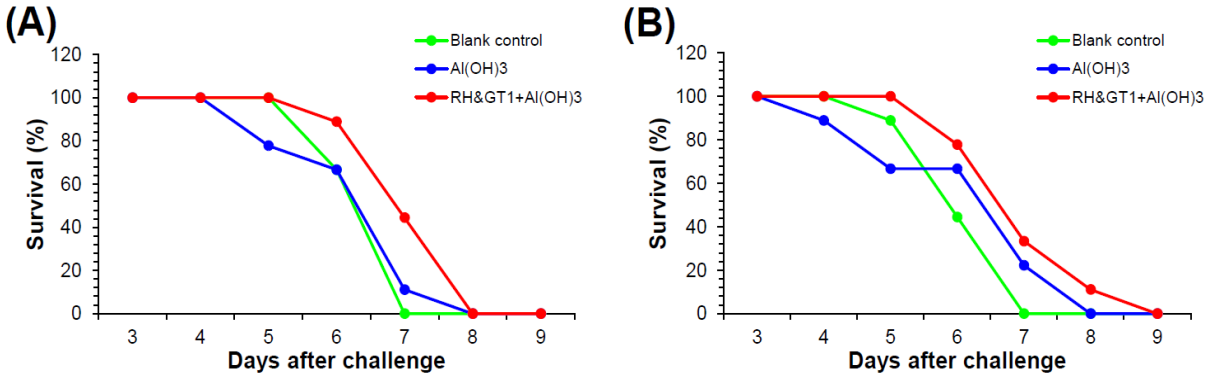

Fig. 4: Survival times of mice prolonged by formaldehyde-killed *Toxoplasma* tachyzoites. All the experimental mice were intraperitoneally injected with 1 × 103 virulent tachyzoites of *T. gondii* RH or GT1 strain at two weeks post the last immunization, and their survival times were daily recorded until all were dead. For RH infection (A), the survival time of RH&GT1+Al(OH)3-immunized mice was significantly prolonged in comparison with blank control ($P < 0.05$), and no difference was detected between Al(OH)3 group. The same phenomenon was uncovered in GT1 infection (B)

Discussion

For preventing *Toxoplasma* infection, the vaccine in veterinary seems more progressive than medical as the S48 strain Toxovax® live vaccine has been developed originally for use in sheep, but by now there was no available vaccine for human (25), reflecting exploring safety vaccine is most important not only for animals but also for human beings. Towards this target, a range of veterinary vaccines in candidate were designed in the past few years to help control *T. gondii* acute and/or chronic infections through eliminating cysts in brain and muscle tissues (26, 27), preventing congenital toxoplasmosis (28), and reducing oocysts shedding in cats (29), but for the protective efficacy all of them were not satisfactory.

To salvage and rectify the situation, besides whether the candidate can induce efficient specific immune responses in host and what is the associated system for delivery, the recent works are mainly focused on (i) the appropriate route for immunization and (ii) immunization with which antigen(s) (30). Additionally, to exclude the inconspicuously protective efficacy probably triggered in host or not satisfactory for usage with a *T. gondii* strain (20), the mixture containing equal killed RH and GT1 tachyzoites were therefore prepared in the study and used with aluminum adjuvant for the next immunizations, aiming to assess whether the hybrid is able to activate specific immune responses in hosts, and whether can be used as an assistant to abolish acute *T. gondii* infection that can cause life-threatening toxoplasmosis for special population of human being.

We found that the survival time of immunized mice against acute *T. gondii* infection was significantly prolonged in comparison with blank control both for RH and GT1 tachyzoites, with increased specific antibodies in sera separated prior to and one week prior to challenge, indicating that the immunization scheme performed in the study, i.e., intramuscular injections with 1×10^5 RH and equal GT1 killed tachyzoites at 0, 3, 7, 14 and 21 days post first immunization, can help mice to elevate humoral immune responses against *T. gondii* acute infection.

To further explore the changes in cellular immune response, the elements associated with *T. gondii* infections were examined subsequently, including the lymphocyte proliferation and the $CD3e^+CD4^+$ T and $CD3e^+CD8a^+$ T cells in spleen (24), and the cytokines in sera including IL-2 (31), IL-6 (32), IL-10 (33), IL-12p70 and IFN-γ (34). Our data revealed the lymphocyte proliferation was enhanced, $CD3e^+CD4^+$ T and $CD3e^+CD8a^+$ T cell subclasses in spleen were elevated, and IL-2, IL-12p70, and IFN-γ but not for IL-6 and IL-10 in sera separated from the immunized mice prior to challenge were increased in comparison with controls, suggesting the cellular immune responses of mice can also be activated by the immunization scheme. Notably, the significantly different anti-*T. gondii* antibodies could not be detected between control and immunized mice at 14 and 21 days post first immunization, indicating that the immunization scheme performed in the present study is inadequate or not perfect enough for prevention of *T. gondii*, which needs to be further optimized in the future studies.

Conclusion

The successive immunizations with hybrid formaldehyde-killed parasites performed in the study triggered the humoral and cellular immune responses and prolonged the survival time of mice against acute *T. gondii* infections, and can be as a candidate to frustrate the acute toxoplasmosis through immunization scheme optimization.

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Conflict of interest

The authors declare that there is no conflict of interest.

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