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Vaccination with recombinant adenoviruses expressing *Toxoplasma gondii* MIC3, ROP9, and SAG2 provide protective immunity against acute toxoplasmosis in mice

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ABSTRACT

Toxoplasmosis is a worldwide zoonosis caused by the protozoan parasite *Toxoplasma gondii*, an obligate intracellular parasite. Currently, no viable vaccine or effective drug strategies exist to prevent and control toxoplasmosis. *T. gondii* microneme protein 3 (MIC3), rhoptry protein 9 (ROP9), and surface antigen 2 (SAG2) are related to active invasion of the parasite. Hence, we constructed recombinant adenoviruses expressing *Tg*MIC3, *Tg*SAG2, or *Tg*ROP9 and evaluated the recombinant adenoviruses as potential vaccines against acute *T. gondii* infection in BALB/c mice. Mice immunized with the recombinant adenoviruses were measured the antibody levels, percentages of lymphocytes and actived T lymphocytes, cytokine productions, and the survival rates and time to evaluate the protective efficacy. Results showed that immunization with the bivalent or trivalent recombiant adenoviruses could strongly stimulate humoral and cellular immune responses in mice, resulting in effective immune protections against lethal challenge with the tachyzoites of *T. gondii* RH. These results indicated that the divalent and trivalent adenoviruses, especially Ad-ROP9-MIC3-EGFP, may be promising vaccine candidates against acute *T. gondii* infection.

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1. Introduction

Toxoplasma gondii is an obligatory intracellular protozoan parasite [1] that can infect a wide-range of warm-blooded vertebrates, including humans and birds [2,3]. Although *T. gondii* typically causes asymptomatic toxoplasmosis in humans, as an opportunistic pathogen, primary infections during pregnancy can lead to miscarriage or congenital toxoplasmosis [4]. Furthermore, infections may manifest as severe symptoms in immunosuppressed patients, such as those infected with HIV [3,5]. In addition, *T. gondii* infection also causes abortion, stillbirth, and death of neonates in anamals (e.g., sheep, goats and pigs), leading to great financial losses in livestock and food industry [6].

Recently, drug-resistant *T. gondii* have emerged and chronic toxoplasmosis couldn't be completely eliminated with typical treatments, such as pyrimethamine and sulfadiazine [7,8]. The problem of parasites eradication, disease reactivation, toxic effects and emerging drug resistance in parasites makes drug treatment unreliable for long term treatment [6,9]. Vaccines can be used as an effective means of controlling and preventing infectious disease. By now, inactived vaccine, subunit vaccine, and DNA vaccine have been developed to fight against *T. gondii* [10]. Unfortunately, there is no vaccine currently approved for human use, and live attenauted S48 strain is the only commercial vaccine, was used as veterinary vaccine in some countries [11]. Therefore, the development of a novel, effective, and safe vaccine against *T. gondii* is urgently required.

Previous studies have demonstrated adenovirus vector has the similar effects with adjuvant to improve the innate immune [12,13]. In addition, the recombinant adenovirus was used to express antigens and safely tested in various immunization protocols with a wide range of hosts, including humans, resulting

Abbreviations: Tg, Toxoplasma gondii; SAG2, surface antigen 2; MIC3, microneme protein 3; ROP9, rhoptry protein 9; GRA, dense granule antigen; Ad, Adenovirus; HEK293A, human epithelial kindey 293A; FBS, foetal bovine sera; PBS, phosphate buffer saline; ELISA, enzyme-linked immunosorbent assay; IgG, Immunoglobulin G; PFU, plaque forming unit; CTL, Cytotoxic T lymphocyte; Th, T helper.

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in different degrees of immune response and protection [14–16]. In recent years, many reports have shown that adenoviruses expressing *T. gondii* antigens can induce the protective efficacy against acute and chronic toxoplasmosis in mice [17–19]. *T. gondii* invasion depends on several secretory proteins, including microneme proteins (MICs), rhoptry proteins (ROPs), dense granule antigens (GRAs), and surface antigens (SAGs) [20,21]. Studies have also demonstrated that *Tg*MIC3, *Tg*ROP9, and *Tg*SAG2 are related to active invasion of the parasite [22–24] and confer different degrees of protection against acute *T. gondii* infection in different vaccination strategies [25–27].

In the present study, we developed the recombinant adenoviruses expressing *Tg*MIC3, *Tg*ROP9, or *Tg*SAG2 and explored a prime-boost protocol using the recombiant adenoviruses. Our aimed to access whether vaccination with the mutilant recombiant adenoviruses expressing *T. gondii* invasion-related proteins could stimulate immune responses and provide higher levels of protection against acute toxoplasmasis in BALB/c mice.

2. Materials and methods

2.1. Ethical statement and animals

All experiments were carried out in accordance with the guidelines established by Jilin University and were approved by the Animal Welfare and Research Ethics Committee of Jilin University. The ethical clearance number for this study is KZ2010-01. Six- to eight-week-old specific pathogen free grade female BALB/c mice were maintained in an enriched environment in all of the experiments.

2.2. Cells and parasites

HEK293A cells were maintained in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Switzerland), supplemented with 10% foetal bovine sera (FBS) (Gibco, Australia), 100 μg/mL of streptomycin, and 100 IU/mL of penicillin at 37 °C in 5% CO2 humidified incubator. *T. gondii* RH and ME49 tachyzoites were harvested from the peritoneal fluids of infected BALB/c mice.

2.3. Generation of specific antibodies rabbit anti-MIC3/SAG2/ROP9-His IgG

Genes encoding MIC3 (GenBank: AJ132530.1), ROP9 (GenBank: AJ401616.1), and SAG2 (GenBank: M33572.1) were cloned from *T. gondii* RH strain using specific primers (Suppmentary Table 1) by reverse transcription PCR, respectively. Obtained genes were cloned into pET-28a (Novagen, Dusseldorf, Germany) vectors, and the His-tagged (MIC3/SAG2/ROP9-His) recombinant proteins were expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIPL. Polyclonal rabbit anti-MIC3/SAG2/ROP9-His IgG were respectively generated as described previously [28,29]. The purified polyclonal rabbit anti-MIC3/SAG2/ROP9-His IgG were identified by recognizing *T. gondii* native proteins (MC3, ROP9, SAG2) from the tachyzoites of *T. gondii* RH and ME49 strains. The purified polyclonal rabbit anti-MIC3/SAG2/ROP9 IgG and rabbit IgG (Negative) were used as the primary antibodies, respectively and an AP-labelled goat anti-rabbit IgG (1:5000, Beyotime, Shanghai, China) was used as the secondary antibody. BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China) was used to color.

2.4. Construction and packaging of recombinant adenoviruses

The target genes (MC3, ROP9, and SAG2) were amplified using specific primers (Supplementary Table 1) as described above, respectively. The amplified genes were incorporated into the pHBAD-EF1-MCS-CMV-EGFP shuttle vector using restriction enzymes and T4 DNA ligase to generate the recombinant plasmids. Subsequently, the recombinant shuttle plasmids and pBHGlox (delta) E1,3Cre genome plasmid were co-transfected into the HEK293A cells using lipofectamine reagent. Recombinant adenoviruses (i.e., Ad-EGFP, Ad-SAG2-EGFP, Ad-ROP9-EGFP, Ad-MIC3-EGFP, Ad-SAG2-ROP9-EGFP, Ad-SAG2-MIC3-EGFP, Ad-ROP9-MIC3-EGFP, and Ad-SAG2-ROP9-MIC3-EGFP) were produced in HEK293A cells after about 14 days.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.12.044.

2.5. Co-expression genes in vitro determined by Western blot

HEK293A cells infected with the recombinant adenoviruses were harvested and prepared to detect the target proteins (MIC3, ROP9, and SAG2) by Western blot. Mouse anti-β-actin IgG (1:5000, Abcam, Shanghai, China) and the rabbit anti-MIC3/SAG2/ROP9-His IgG were used as primary antibodies, and AP-labelled goat anti-rabbit IgG (1:5000, Beyotime, Shanghai, China) or AP-labelled goat anti-mouse IgG (1:5000, Beyotime, Shanghai, China) were used as the secondary antibodies. BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China) was used for color development.

2.6. Immunization of mice

270 female BALB/c mice were randomly divided into nine groups of 30 mice each, and immunized with 50 μL purified recombinant adenoviruses (10^9 PFU), Ad-EGFP (10^9 PFU), or PBS via intramuscular injection at two-week intervals. All groups were immunized twice on Days 0 and 14. Immunized mice were weighed weekly and observed daily after the vaccination.

2.7. Measurement of anti-Toxoplasma antibodies by ELISA and Western blot

Serum samples were collected from the vein plexus of the mice tails on Days 0, 14, and 28. The levels of anti-*Toxoplasma* IgG antibodies were detected using a commercial ELISA kit (LifeSpan BioSciences, Inc., North America) in accordance with the manufacturer's instructions. Briefly, 10 μL serum sample from each mouse and 100 μL sample diluent were mixed and added to well, and the plates were incubated for 30 min at 37 °C. Subsequently, the liquild was aspirated from each well and washed 4 times, and the wells were incubated with 100 μL of horseradish-peroxidase (HRP) conjugated anti-mouse IgG for 20 min at 37 °C, respectively. After washing, plates were then incubated with 100 μL substrate buffer in the dark for 10 min at 37 °C and subsequently 50 μL stop solution was added to each well. The absorbance was measured at 450 nm using ELISA reader (TECAN sunrise, Switzerland). All samples were assayed in triplicate.

For detection of anti-ROP9/MIC3/SAG2 IgG in the mouse sera, total tachyzoite lysate antigen (TLA) of *T. gondii* RH strain was desolved in 12% polyacrylamide gels under denaturing conditions and transferred onto PVDF (polyvinylidene fluoride) membranes. Membranes were blocked in TBST (1 M Tis-HCL pH8.0, 8.8 g NaCl and 500 μL TritonX-100 were added to 1 L water) supplemented with 5% skim milk, incubated with individual serum samples of immunized or control mice (1:1000), washed thoroughly and then incubated with AP-labelled goat anti-mouse IgG (1:5000). BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China) was used for color development.

2.8. Lymphocytes and cytokine assays

Two weeks after the booster immunization, 10 mice from each group were sacrificed. Splenocytes were isolated to detect lymphocytes as proviously described [30]. Briefly, Murine spleens were cut into pieces, minced and pressed into a 70 μm cell strainer. Red blood cells (RBCs) were depleted with a RBC lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.11 mM $EDTA.Na_2$, pH 7.2). After washing, cells were stained with Zombie NIR™ Fixable Viability dye (Biolegend, San Diego, CA) and then pre-incubated with a purified anti-mouse CD16/32 antibody to reduce nonspecific binding. Cells were incubated with specific antibodies or isotype controls following the manufacturers' guidance. The antibodies used in this study were as follows: Pacific Blue™ anti-mouse CD45, APC anti-mouse CD3, FITC anti-mouse CD8a, PerCP anti-mouse CD4, and PE-Cy7 anti-mouse CD226 (all obtained from Biolegend, San Diego, CA). CD226 was used as marker of T lymphocytoes activation [31,32]. Cells were detected and analyzed using a FACS Aria III flow cytometer (BD Biosciences, San Jose, CA, United States), and positive cells were defined using an isotype control or a fluorescence minus one (FMO) control.

The blood were intraorbitally collected from the mice to analyze the level of cytokines in the serum using a mouse Th cytokine panel (13 plex) (Biolegend, US) with FACS Aria III flow cytometer (BD Biosciences, San Jose, CA, United States) in accordance with the manufacturer's instructions.

2.9. Protection against T. gondii challenge

Two weeks after the booster immunization, 10 immunized mice per group were intraperitoneally challenged with a low dose of 1×10^2 *T. gondii* RH tachyzoites, and the same number mice were intraperitoneally challenged with a high dose of 1×10^3 *T. gondii* RH tachyzoites in parallel. The mice were observed daily for 30 days post-challenge, and the survival time was recorded for each mouse.

2.10. Statistical analysis

Differences in antibody titers and bodyweight between each group were analyzed by a two-way ANOVA followed by a Bonferroni post-test. Differences in the spleen index, cytokine productions, and lymphocytes percentages were assessed by a one-way ANOVA followed by a Turkey's multiple comparison test. The survival data were analyzed by Kaplan-Meier analysis followed by log-rank test. All statistical analysis were performed using GraphPad Prism. version 5.0 or SPSS Statistics version 17.0 softwares. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Generated specific antibodies

The purified ploYclonal rabbit anti-MIC3/ ROP9/SAG2-His IgG could specifically recognize *Tg*MIC3, *Tg*ROP9, and *Tg*SAG2 from the tachyzoites of *T. gondii* RH and ME49 strains. Western blot results showed the band sizes were corresponded to the predicted molecular weight of SAG2, MIC3 and ROP9, 22 kDa, ~38 kDa, and ~36 kDa respectively (Supplementary Fig. 1).

3.2. Recombinant adenoviruses expressing products

The recombinant adenoviruses were generated in HEK293A cells about 14 days. The generated recombinant adenoviruses (Fig. 1A) could express enhanced green fluorescence protein (EGFP) and target proteins (MIC3, ROP9, and SAG2). Target proteins were successful detected by Western blot, and the band sizes were corresponded to the predicted molecular weight of target proteins (Fig. 1B).

3.3. Specific IgG antibody response in immunized BALB/c mice

To assess the systemic humoral immune responses induced following immunization, serum samples were collected from each mouse of different groups, and anti-*T. gondii* IgG antibody was detected by ELISA. On Day 14 after the primary and booster immunization, mice immunized with the recombinant adenoviruses induced extremely significantly higher *T. gondii*-specific IgG antibody levels compared to the mice injected with PBS or Ad-EGFP (Fig. 2; $p < 0.001$). However, there was no statistical difference between the PBS and Ad-EGFP groups (Fig. 2; $p > 0.05$).

Two weeks after booster immunization, the sera obtained from all group of mice were detected using TLA by Western blot, respectively. Supplementary Fig. 2 showed that sera from different group of mice reacted with the band sizes corresponding to the predicted molecular weight of target proteins.

3.4. Bodyweight changes of the immunized mice

There was no significantly differences ($p > 0.05$) in bodyweight between the experimental groups and control groups (Table 1). Moreover, daily monitor results did not show any changes in clinical signs of all of the groups.

3.5. Cytokine productions

For the detection of cytokines, FACS analysis was performed using mouse sera. The level of IL-6, TNF-α, and IL-22 production from the experimental group of mice was significantly higher ($p < 0.05$, $p < 0.01$, or $p < 0.001$) compared to that of the control groups (Fig. 3A and B). The level of IF-γ production from the experimental group of mice was significantly higher ($p < 0.05$, $p < 0.01$, or $p < 0.001$) compared to that of the PBS group (Fig. 3A), however, compared to the control group immunized with Ad-EGFP, significant differences ($p < 0.05$ or $p < 0.01$) were found with groups immunized with Ad-SAG2-EGFP, Ad-ROP9-EGFP, and Ad-ROP9-MIC3-EGFP (Fig. 3A). A significant increase of IL-17A ($p < 0.05$) was observed in the Ad-SAG2-MIC3-EGFP and Ad-ROP9-MIC3-EGFP groups compared to the PBS group (Fig. 3B). Moreover, IL-10 produced by the control groups was dramatically lower ($p < 0.05$, $p < 0.01$, or $p < 0.001$) compared to that of the experimental groups, except for the Ad-SAG2-EGFP group (Fig. 3B). As expected, no significant production of these cytokines was observed between the PBS and Ad-EGFP groups (Fig. 3A and B). In addition, no significant changes of the levels of IL-2, IL-4, IL-5, IL-9, IL-13, IL-17F, and IL-21 production were found among all groups ($p > 0.05$; the data are not showed).

3.6. Cellular immune response induced by recombinant adenoviruses

To determine lymphocytes and actived T lymphocytes responses in immunized mice, FACS analysis was performed using mouse spleen cells. After 14 days booster immunization, the percentage of T lymphocytes in the spleen of the experimental groups increased significantly ($p < 0.05$, $p < 0.01$, or $p < 0.001$) compared to those of the PBS group (Fig. 4A). Notably, among all of the groups, the percentage of non-T lymphocytes was the lowest in the spleen of mice immunized with Ad-ROP9-MIC3-EGFP, and T lymphocytes was the highest (Fig. 4A).

Importantly, the percentage of activated Th lymphocytes in the spleen of all the experimental groups was significantly higher ($p < 0.05$, $p < 0.01$, or $p < 0.001$) compared to that of the PBS group

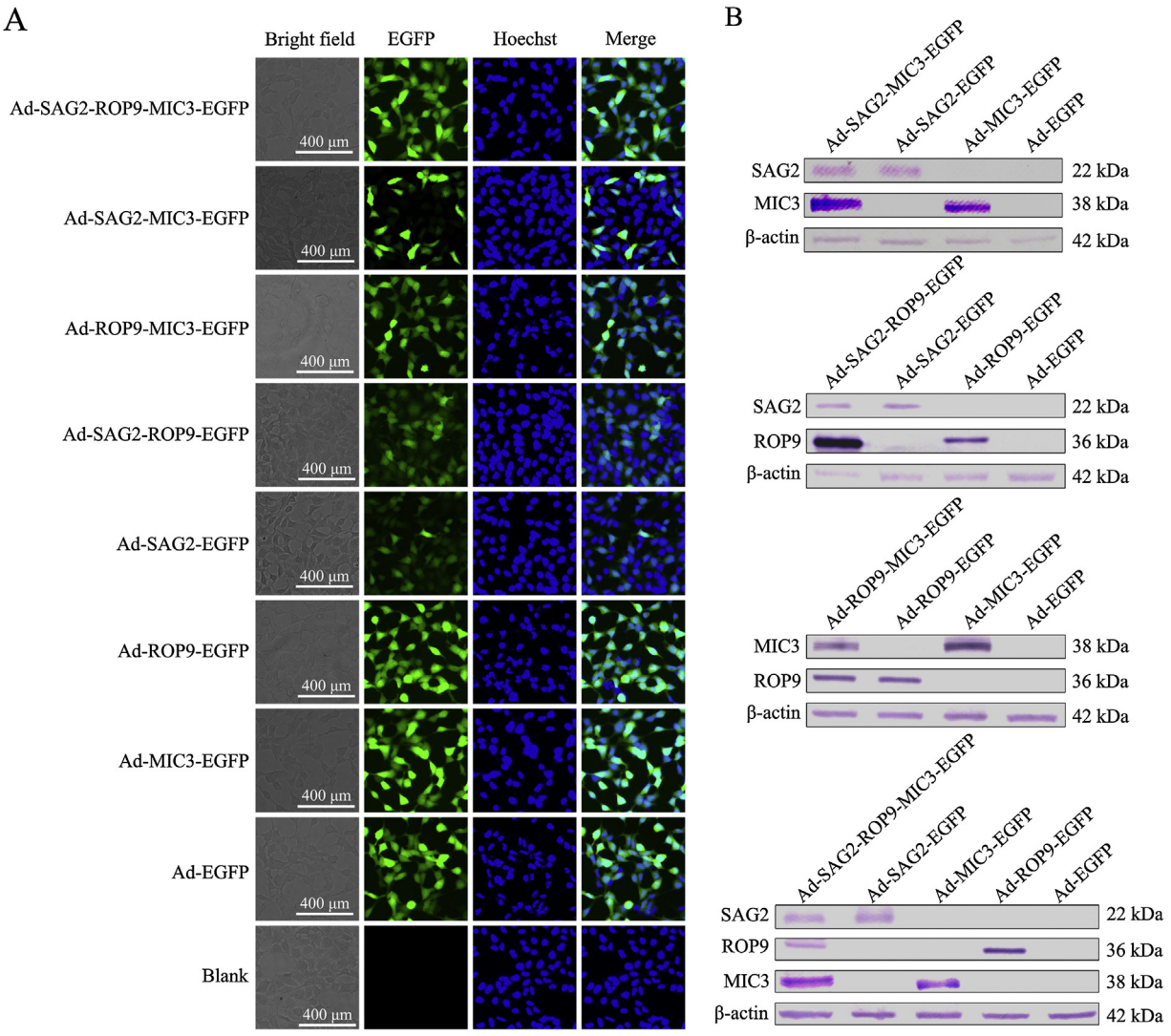

Fig. 1. Recombinant adenoviruses expressed the target proteins. (A) Recombinant adenoviruses infected HEK293 cells. (B) The infected HEK293A cells were detected by Western blot. β-actin was used as a control with a molecular weight of 42 kDa.

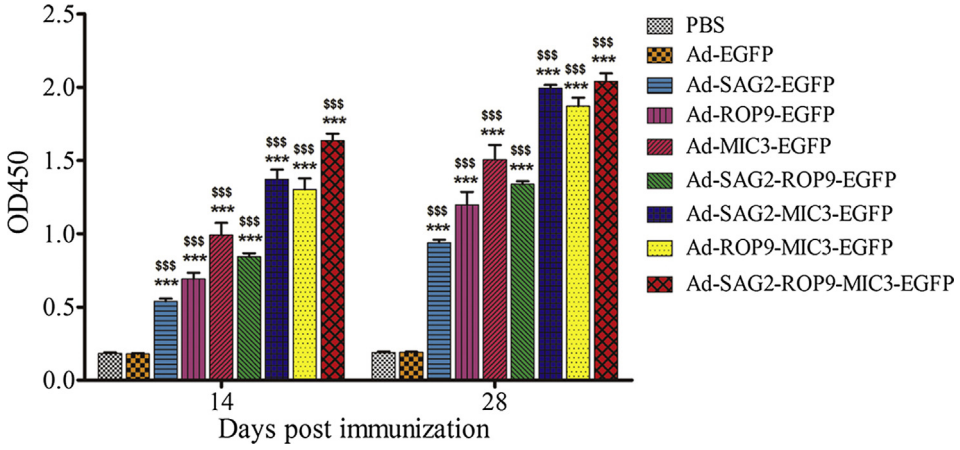

Fig. 2. Levels of mean anti-*Toxoplasma* IgG antibody in the immunized mice. Results are indicated as the mean ± SD for 10 mice in each group. $^{***,\$\$\$}P < 0.001$. Asterisks denote comparisons with the PBS group, and dollar signs denote comparisons with the Ad-EGFP group.

(Fig. 4B). Moreover, bivalent and trivalent adenovirus groups increased significantly ($p < 0.05$ or $p < 0.01$) compared to the Ad-EGFP group (Fig. 4B). The percentage of activated CTLs in the spleen of the Ad-SAG2-MIC3-EGFP, Ad-ROP9-MIC3-EGFP, and Ad-SAG2-ROP9-MIC3-EGFP groups increased significantly ($p < 0.05$ or $p < 0.001$) compared to those of the control groups (Fig. 4C).

Table 1
Mean bodyweight changes.

Group/*P*	Bodyweight (g)		
	Day 0	Day 14	Day 28
PBS	18.29 ± 0.27	20.52 ± 0.81	21.42 ± 0.50
Ad-EGFP	18.39 ± 0.30	20.54 ± 054	21.57 ± 0.91
Ad-SAG2-EGFP	18.43 ± 0.46	20.51 ± 0.65	21.65 ± 072
Ad-ROP9-EGFP	18.34 ± 0.19	20.69 ± 0.50	21.51 ± 0.62
Ad- MIC3-EGFP	18.25 ± 0.41	20.86 ± 0.64	21.73 ± 0.58
Ad-SAG2-ROP9-EGFP	18.30 ± 0.14	20.63 ± 0.29	21.67 ± 0.41
Ad-SAG2-MIC3-EGFP	18.15 ± 0.38	20.50 ± 0.71	21.49 ± 0.80
Ad-ROP9-MIC3-EGFP	18.29 ± 0.24	20.68 ± 0.66	21.50 ± 0.81
Ad-SAG2-ROP9-MIC3-EGFP	18.45 ± 0.32	20.67 ± 0.44	21.58 ± 0.62
Significance (*P*)	NS	NS	NS

Notes: Data presented as mean ± SD (n = 10); NS: Not significant ($P > 0.05$).

3.7. Protective effects of recombinant adenoviruses against acute T. gondii infection

To determine the protective efficacy of recombinant adenoviruses, immunized mice and control mice were challenged with lethal *T. gondii* RH strain (1×10^2 or 1×10^3 tachyzoites) at 2 weeks after boost immunization. Following a low dose challenge with 1×10^2 *T. gondii* RH tachyzoites, the survival rate was 80% in the Ad-ROP9-MIC3-EGFP group, 50%-70% in the Ad-SAG2-ROP9-EGFP, Ad-SAG2-MIC3-EGFP, and Ad-SAG2-ROP9-MIC3-EGFP groups, and 20−50% in the Ad-SAG2-EGFP, Ad-ROP9-EGFP, and Ad-MIC3-EGFP groups (Fig. 5A). However, the mice injected with PBS or Ad-EGFP only survived 8–11 days (Fig. 5A). The survival time and survival rates were significantly longer or higher compared to that of the control group (PBS and Ad-EGFP) for all groups immunized with the recombinant adenoviruses (Fig. 5A; $p < 0.001$ or $p < 0.01$). There was no significant difference between the PBS and Ad-EGFP groups (Fig. 5A; $p > 0.05$).

Following a high dose challenge with 1×10^3 *T. gondii* RH tachyzoites, the survival time of the Ad-SAG2-EGFP (11.30 ± 1.25 days), Ad-ROP9-EGFP (12.80 ± 1.75 days), and Ad-MIC3-EGFP (13.10 ± 2.08 days) groups were substantially prolonged compared to the PBS (7.10 ± 0.32 days) and Ad-EGFP groups (7.20 ± 0.42 days) (Fig. 5B). The survival rate was 40% in the Ad-ROP9-MIC3-EGFP group, 30% in the Ad-SAG2-ROP9-MIC3-EGFP and Ad-SAG2-MIC3-EGFP groups, and only 10% in the Ad-SAG2-ROP9-EGFP group (Fig. 5B). The survival time and surviving rates were significantly longer or higher compared to that of the control group (PBS and Ad-EGFP) for all groups of mice immunized with the recombinant adenoviruses (Fig. 5B; $p < 0.001$). There was no significant difference between the PBS and Ad-EGFP groups (Fig. 5B; $p > 0.05$).

4. Discussion

Vaccination is considered the most effective way to reduce the burden of infectious diseases and prevent subsequent economic loss [33,34]. Viruses are considered as promising vectors to develop the success of recombinant vaccines [11,35]. As an extensively employed vector system [36], adenoviruses can infect a broad range of host cells and produce high titers [37]. Furthermore, recombinant adenoviruses have been successfully used to express different proteins [38–40]. In the present work, we generated recombinant adenoviruses expressing *T. gondii* invasion-related proteins (MIC3, ROP9, and SAG2) and explored immunogenicity and protective efficacy induced by the recombinant adenoviruses.

Previous investigations found that immunization with *T. gondii* SAG2, ROP9, or MIC3 elicited protective immunity against acute or chronic toxoplasmosis in mice using different strategies [26,41–43]. In one study, Caetano *et al.* obtained 50%-60% reduction in brain cysts after immunization in BALB/c mice with recombinant adenovirus expressing SAG2, and splenocytes from immunized mice secreted IFN-γ after *in vitro* stimulation with tachyzoite lysate antigen [41], Whereas in an experiment by Machado et al., mice immunized twice with Ad-SAG2 displayed 72% reduction of parasite burden when compared to control group [42]. Other studies showed mice immunized with DNA vaccine pVAX-*Tg*ROP9 were induced high level of antibody and elicited IFN-γ production, and prolonged survival time against lethal challenge with tachyzoites of the *T. gondii* RH [26]. In addition, Nie et al. reported that mice immunized with recombinant pseudorabies virus expressing *Tg*MIC3 could stimulate significant humoral and cellular Th1 immune responses, and 50% survival rates against lethal challenge with 100 tachyzoites of the *T. gondii* RH were obtained [43].

Previous studies have revealed that *T. gondii*-specific CTL, particularly in synergy with Th lymphocytes, contribute to limiting *T. gondii* dissemination [44,45]. Additionally, other studies have demonstrated that a multivalent vaccine may evoke higher T lymphocyte responses compared to a vaccine expressing single antigen [10,46]. In the present study, we found that the percentage of activated $CD4^+$ T lymphocytes in mice immunized with bivalent or trivalent adenoviruses was significantly higher ($p < 0.05$, $p < 0.01$, or $p < 0.001$) compared to that of the control groups (PBS and Ad-EGFP). The percentage of the activated CD8 + T lymphocytes in mice immunized with bivalent or trivalent adenoviruses was extremely significantly higher ($p < 0.001$) compared to that of the PBS group, and when compared to Ad-EGFP group, the percentage was also significantly higher ($p < 0.05$) for bivalent or trivalent adenoviruses, except for the Ad-SAG2-ROP9-EGFP group. These data strongly demonstrated that multivalent adenoviruses could induce potent effector T cell responses.

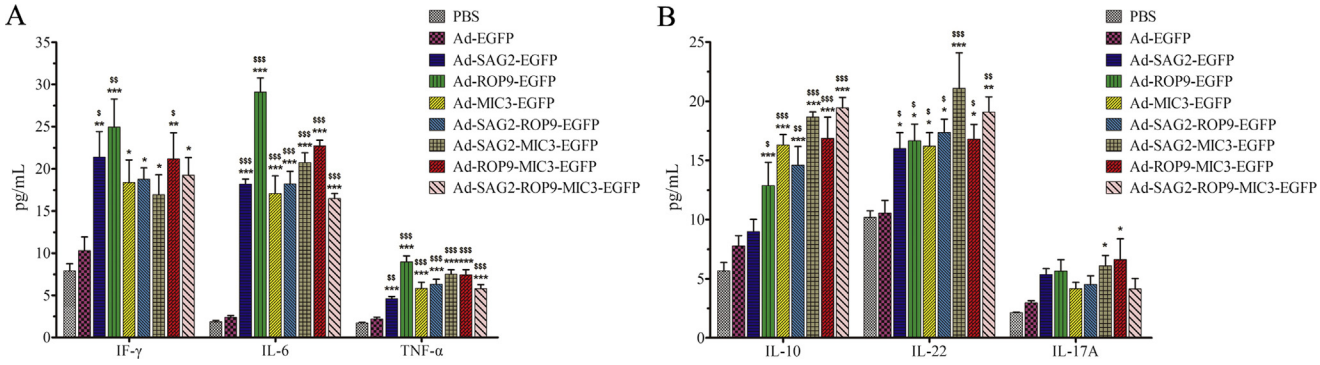

Fig. 3. Cytokines from the sera of immunized mice. All the results are presented as the mean ± SD (n = 10). $^{*,\$}P < 0.05$; $^{**,\$\$}P < 0.01$; $^{***,\$\$\$}P < 0.001$. Asterisks denote comparisons with the PBS group, and dollar signs denote comparisons with the Ad-EGFP group.

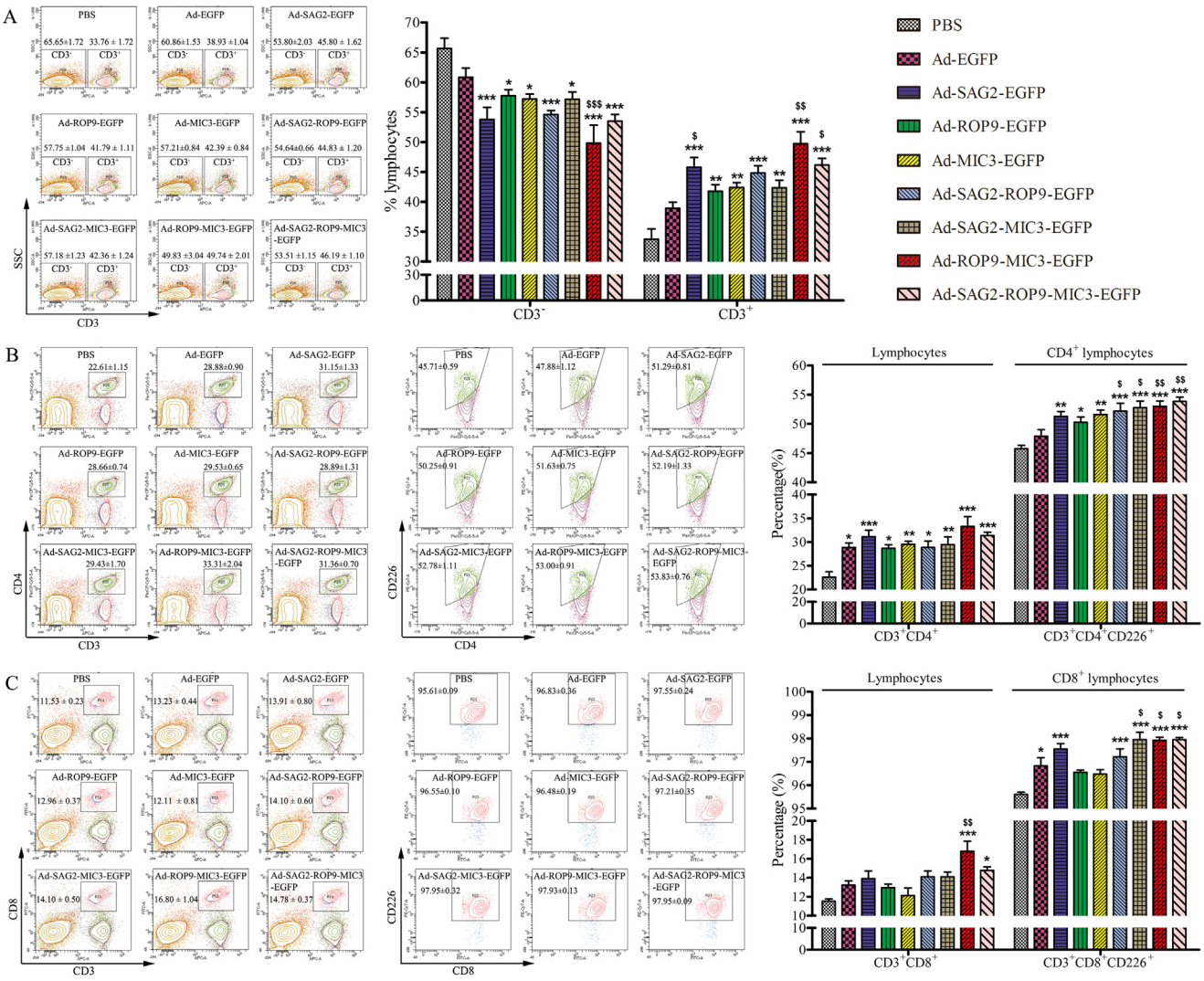

Fig. 4. Multicolor flow cytometry analysis of the lymphocytes and T lymphocyte subpopulations responses from the spleens of immunized mice in each of different groups. (A) The percentage of non-T ($CD3^-$) and T lymphocytes ($CD3^+$). (B) The percentage of Th ($CD3^+CD4^+$) and activated Th lymphocytes ($CD3^+CD4^+CD226^+$). (C) The percentage of CTL ($CD3^+CD8^+$) and activated CTL ($CD3^+CD8^+CD226^+$). Data are presented as the mean ± SD (n = 10). $^{*,\$}P < 0.05$; $^{**,\$\$}P < 0.01$; $^{***,\$\$\$}P < 0.001$. Asterisks denote comparisons with the PBS group, and dollar signs denote comparisons with the Ad-EGFP group.

Pro-inflammatory cytokines (e.g., IFN-γ, TNF-α, and IL-6) produced by Th1 lymphocytes are associated with a protective immune response against the *T. gondii* infection. For example, IFN-γ can activate macrophages and CTL responses to kill *T. gondii* [47], and IFN-γ synergized with TNF-α has been shown to mediate parasite resistance in chronic toxoplasmosis [48]. Additionally, reports showed that in the absence of IL-6, mice were unable to initiate a rapid pro-inflammatory response against *T. gondii* [49]. In our study, notably higher ($p < 0.01$ or $p < 0.001$) levels of TNF-α and IL-6 were produced in mice immunized with recombinant adenoviruses compared to the control groups (PBS and Ad-EGFP); significantly higher ($p < 0.05$, $p < 0.01$, or $p < 0.001$) level of IFN-γ was produced in mice immunized with recombinant adenoviruses compared to the PBS group. The immune response produced by other Th subtypes were also analyzed (e.g., IL-22 and IL-17A), which were variably increased in the mice immunized with the different adenoviruses. However, compared with the control groups, the level of IL-10 produced by Th2 cells was obviously enhanced in the mice immunized with the recombinant adenoviruses ($p < 0.05$, $p < 0.01$, or $p < 0.001$), with the exception of Ad-SAG2-EGFP group. Zheng B et al. have reported similar findings, in that the level of IL-10 produced by the mice in the experimental group was higher ($p < 0.05$) than that of the control groups [50].

Besides cellular immunity, humoral immunity resulting in the production of antigen-specific IgG antibodies also appears to be important for controlling *T. gondii* infection [7]. Our study showed that mice immunized with recombinant adenoviruses significantly increased the level of *T. gondii*-specific antibody in comparison to mice injected with Ad-EGFP or PBS ($p < 0.001$). These results also suggest that the target genes can be highly efficiently expressed in the adenovirus vector. Additionally, it is equally important that no abnormal clinical symptoms and bodyweight differences were observed in the mice immunized with the recombinant adenoviruses, in accordance with previous reports [51–53].

Challenge dose may be a vital factor in evaluating the immune protection of vaccines, which focus on survival rate or prolonged time. In our study, the immunized mice were respectively challenged with a high dose (1×10^3) and low dose (1×10^2) trachyozites of *T. gondii* RH. As predicted, higher survival rates and longer survival time in the mice immunized with the bivalent or trivalent adenoviruses in comparison with those of the single adenoviruses, Ad-EGFP, and PBS.

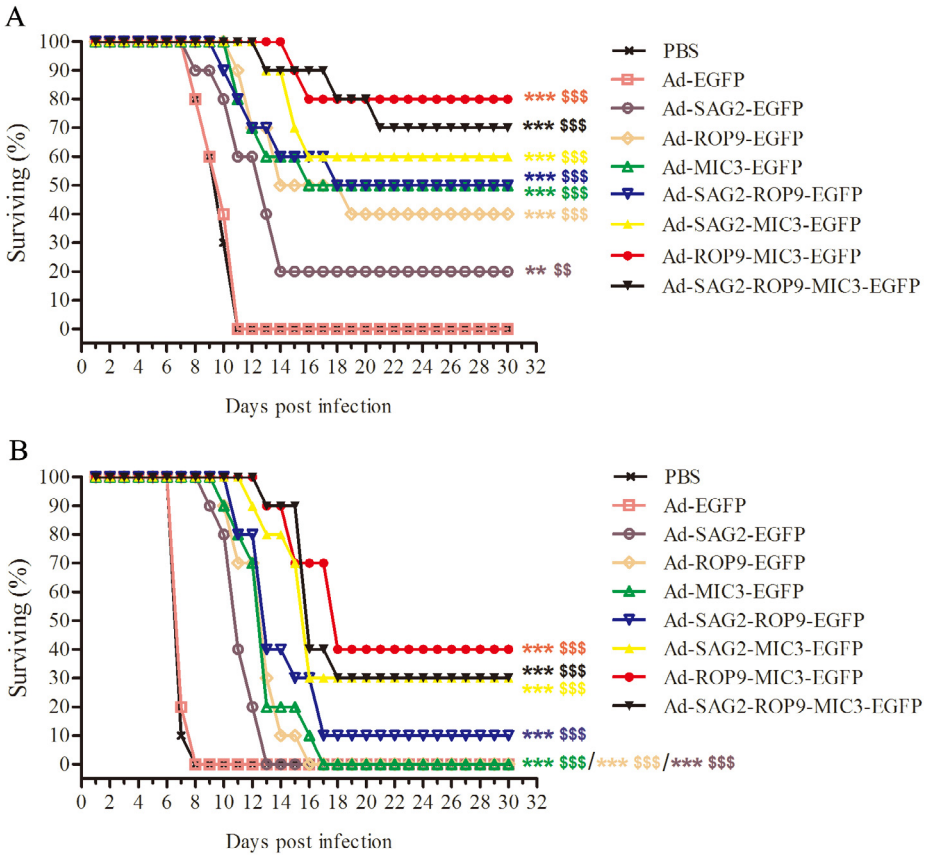

Fig. 5. Immunization with the recombinant adenoviruses generated protective immunity against *T. gondii* RH. Nine groups of BALB/c mice were immunized by using a prime-boost strategy. (A) Survival curves of the immunized mice challenged with a low dose of *T. gondii* RH. Immunized mice were challenged with 1×10^2 tachyzoites two weeks after the booster immunization. (B) Survival curves of the immunized mice challenged with a high dose *T. gondii* RH. Immunized mice were challenged with 1×10^3 tachyzoites two weeks after the booster immunization. Each group was comprised of 10 mice and the survival time was monitored daily for 30 days after challenge.

5. Conclusions

In summary, the recombinant adenoviruses expressing *Tg*MIC3, *Tg*SAG2, or *Tg*ROP9 were successfully developed. Immunization with the bivalent or trivalent recombiant adenoviruses could stimulate humoral and cellular immunity in mice, resulting in effective immune protections against lethal challenge with tachyzoites of *T. gondii* RH. Therefore, these recombiant adenoviruses may be promising vaccine delivery system against acute toxoplasmosis.

Conflict of interests

All authors have declared that there is no conflict of interest.

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Author contributions

Chen Q and Jiang N conceived and designed the study. Zhang D and Jiang N carried out the experiments and analyzed the data. Zhang D drafted the manuscript and Chen Q revised the manuscript. Funding were provided by Chen Q and Jiang N. All authors reviewed and approved the submitted version of the manuscript.

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