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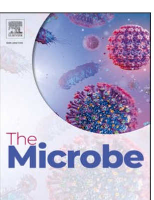

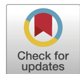

Comparison of two BCG adjuvants in enhancing the immunogenicity of a DNA vaccine encoding the *Toxoplasma gondii* ROP2 gene in BALB/c mice

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A R T I C L E I N F O

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A B S T R A C T

The lack of a definitive treatment against toxoplasmosis and its high prevalence encouraged us to seek ways to expand DNA vaccines. The rhoptry protein 2 (ROP2) of *Toxoplasma gondii* is one of the most important antigens expressed in all three stages of the parasite's life cycle. BCG is an immune stimulator and can act as an important adjuvant for protection against infectious diseases. The recombinant pcROP2 plasmid was constructed, transfected into CHO cells, and protein expression was confirmed by western blotting. The recombinant plasmids (100 μg/100 μL) and two types of BCG adjuvants, namely intact BCG and BCG lysate, were injected into female BALB/c mice three times. Then, immunological factors (total IgG, IgG2a, and IgG1) and two cytokines, including IFN-γ and IL-4, were measured, and the survival rate of the mice after challenge with *T. gondii* tachyzoites was monitored. The group receiving pcROP2 +BCG lysate produced more IgG2a and total IgG than the other groups ($p \leq 0.05$). Evaluation of spleen cytokines IFN-γ and IL-4 showed that IFN-γ levels were significantly higher in mice receiving the recombinant plasmid and BCG lysate compared to the other groups. In mice receiving pcROP2 +BCG lysate, the survival rate was higher than in the other groups, suggesting that pcROP2 +BCG lysate is more effective at inducing cellular immune responses and may be suitable for increasing the lifespan of mice with acute toxoplasmosis.

1. Introduction

Toxoplasma gondii (*T. gondii*), a member of the Apicomplexa phylum and an intracellular parasite that inhabits many tissues of birds and mammals, can cause toxoplasmosis (Zaki et al., 2024; Dubey, 2008). In healthy individuals with a normal immune system, infections are usually asymptomatic, but in immunocompromised individuals, infections can cause severe disease. In these subjects, chronic infection with *T. gondii* can be reactivated and may lead to encephalitis or death (Wang et al., 2017; Weiss and Dubey, 2009). Severe and fatal complications of toxoplasmosis in humans and animals, as well as insufficient available treatments, have prompted researchers to conduct extensive studies to develop effective vaccines against this infection (Antczak et al., 2016; Innes et al., 2019).

Compared with protein vaccines, DNA vaccines are relatively stable, affordable, and safe; they are more capable of stimulating all immune system pathways, especially cellular immunity, and are commonly used against intracellular parasites (Li and Petrovsky, 2016; Ghaffarifar, 2018). In toxoplasmosis, protective immunity is mediated by cellular responses, which include $CD4^{+}$ and $CD8^{+}$ T lymphocytes. A gene-encoding plasmid that can activate both types of T lymphocytes could be a promising candidate for DNA vaccine development (Ghaffarifar, 2018; Khan and Moretto, 2022). *T. gondii* contains many immunogenic antigens, the most important of which are excretory

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antigens that stimulate the cellular immune system and can serve as suitable candidates for vaccination (Rezaei et al., 2019; Wang et al., 2016a).

Rhoptry protein 2 (ROP2) of *T. gondii* is one of the most significant antigens in the ROP family and is expressed during all three stages of the parasite's life cycle. Deletion of ROP2 can disrupt the parasite's infection process and reduce its pathogenicity (El Hajj et al., 2006; Sinai and Joiner, 2001). The complete ROP2 gene expresses a 64 kDa protein that can stimulate humoral immune responses, including IgG and IgM (Daryani et al., 2012; Hosseinian Khosroshahi et al., 2010). In 2011, Sánchez et al. reported that the recombinant ROP2 protein induced protective immunity against *T. gondii* infection (Sanchez et al., 2011). In 2012, Khosroshahi et al. reported that a DNA vaccine containing ROP2 along with other genes and adjuvants such as IL-12 or alum induced immunogenicity against *T. gondii* (Khosroshahi et al., 2012). In 2018, Picchio et al. reported that a recombinant protein cocktail (ROP2 +GRA4 +TgPI-1) promoted protective immunity against chronic toxoplasmosis (Picchio et al., 2018). These characteristics of ROP2 have led to its inclusion in DNA vaccines (Rezaei et al., 2019; Foroutan et al., 2019; Zhang et al., 2023a).

Kim et al. demonstrated that Bacillus Calmette-Guérin (BCG) can act as a potent immune stimulant and protect mice from influenza virus infection by studying the BCG cell wall as an adjuvant (Kim et al., 2021). Similarly, Counoupas et al. reported that the BCG vaccine could enhance specific immune responses and T cell numbers in mice, nearly eliminating disease with minimal inflammation and no detectable virus in the lungs following COVID-19 infection (Counoupas et al., 2021).

ROP2 can stimulate cellular immune response (Hosseinian Khosroshahi et al., 2010), and BCG can augment these responses as well, which is one of the objectives of this study. The aim of this study was to evaluate a DNA vaccine encoding the ROP2 protein using two types of BCG vaccines—intact BCG and BCG lysate—and to compare the effects of these adjuvants on immunological responses and survival rates. In this study, we directly compared intact BCG with BCG lysate as adjuvants for ROP2 DNA vaccination.

2. Materials and methods

2.1. Ethics statement

This project was approved by the Ethical Committee of Tarbiat Modares University, adopting the Declaration of Helsinki (1975) and the Society for Neuroscience Animal Care and Use Guidelines (1998) (Ghaffarifar et al., 2015). (Approval No: IR.MODARES.AEC.1401.075).

2.2. Construction and extraction of recombinant plasmids

In this study, the complete ROP2 gene (accession number Z36906.1, 1686 bp, 64 kDa) containing *Hin*dIII and *Eco*RI restriction sites was cloned using the InsT/A clone™ PCR product cloning kit (Fermentas®), then subcloned into the expression plasmid pcDNA3. The empty pcDNA3 plasmid was transformed into *E. coli* strain TG1. Recombinant plasmids containing the ROP2 gene (pcROP2) and empty pcDNA3 plasmids were prepared at a concentration of 100 μg/100 μL (Hosseinian Khosroshahi et al., 2010; Ghafarifar et al., 2019). The presence of ROP2 in the recombinant plasmids was confirmed by PCR and restriction enzyme digestion. The recombinant plasmid was transfected into CHO cells, and ROP2 expression was confirmed by western blotting.

Primers with restriction enzyme sites used in this study:

Forward: 5´- ATT **AAGCTT** ATG GAA AAC TGT GCG TCG GTC AG- 3´

Reverse: 5´- ATT **GAA TTC** TCA TGC CGG TTC TCC ATC AG- 3´

2.3. Animals and parasites

Healthy female BALB/c mice aged 6–8 weeks and weighing 20–25 g were obtained and maintained under standard laboratory conditions. Tachyzoites of the RH strain of *T. gondii* were stored at −80°C in the Medical Parasitology Department of Tarbiat Modares University. Animals were housed in a pathogen-free facility with ad libitum access to food and water.

2.4. Preparation of the *T. gondii* antigen

Tachyzoites were centrifuged (3000 rpm, 20 min) and resuspended in phosphate-buffered saline (PBS) containing 5 mM phenylmethylsulfonyl fluoride (PMSF). Parasites were lysed using an ultrasonic cell disruptor at a frequency of 42 kHz for 6 min, with 30-second cooling intervals between each sonication cycle. The sample was kept on ice throughout. Cellular debris was centrifuged at 12,000 rpm for 20 min at 4°C. Samples were dialyzed in PBS for 4 h to remove PMSF, filtered through a 0.22 μm filter, and the protein concentration was determined by the Bradford method. Samples were stored at −20°C until use (Chen et al., 2018; Wang and Yuan, 2016)

2.5. Preparation of intact BCG and BCG Lysate

BCG is a dense powder from *Mycobacterium bovis* Pasteur strain 1173P2. After dilution with sodium chloride, BCG can be cultured at 5–30 $\times 10^8$ CFU per vial. Five milligrams of BCG were dissolved in 5 mL of 0.9 % sodium chloride. BCG lysate was prepared using an ultrasonic cell disruptor to produce 5×10^5 CFU. Sonication was performed at 42 kHz for 6 min with 30-second cooling intervals. After centrifugation at 12,000 rpm for 20 min at 4°C, the lysate was prepared for injection at 20 ng/μL (Kim et al., 2021; Counoupas et al., 2021; Zhang et al., 2023b).

2.6. Immunization and challenge

Immunization was performed three times at 2-week intervals (days 0, 14, and 28). Mice were divided into six groups (8 mice per group): 3 mice were used for immunological assays, and 5 mice for survival studies after challenge with the RH strain of *T. gondii*. Group 1 (PBS, 100 μL) and group 2 (pcDNA3, 100 μg/100 μL) were controls; groups 3 (BCG, 5×10^5 CFU/100 μl), 4 (pcROP2, 100 μg/100 μL), 5 (pcROP2 +BCG), and 6 (pcROP2 +BCG lysate) were experimental.

BCG lysate was mixed with an equal volume of Freund's complete adjuvant (prepared from the Razi Vaccine and Serum Research Institute of Iran) for the first injection (1 mL of lysate with a concentration of 20 ng/μL and 1 mL of adjuvant) and with Freund's incomplete adjuvant for the second and third injections. Injection volumes were 100 μL per mouse. Injections were intradermal for the intact BCG group and intramuscular into the quadriceps muscle for other groups. Two weeks after the last injection (day 42), all mice were challenged intraperitoneally with 1×10^5 live RH strain tachyzoites. A lethal dose of the RH strain, used to ensure uniform mortality for comparison. Mice were monitored daily for survival.

2.7. Blood sampling method for BALB/c mice

On days 14 and 42, 3 mice per group were bled via retro-orbital plexus puncture under light anesthesia, as described by Riley (1960) (Riley, 1960). Blood samples were collected in heparinized microtubes for subsequent analysis.

2.8. Antibody assay

Sera were tested for IgG, IgG1, and IgG2a against *T. gondii* using ELISA. Plates were coated with *T. gondii* tachyzoite antigens at 20 μg/mL (100 μL/well) in carbonate-bicarbonate buffer (0.2 M, pH 9.6). Sera were diluted 1:40 in blocking buffer (TBS/Tween, 50 mM Tris, 137 mM NaCl, pH 7.4, 0.5 % Tween-20). Mouse IgG, IgG1, and IgG2a kits (Southern Biotech) were used (Khosroshahi et al., 2012; Foroutan et al., 2020a).

2.9. Cytokine assay

Fourteen days after the last injection (day 42), spleens from 3 mice per group were harvested. Lymphocytes were isolated by crushing spleens in 5 mL PBS, centrifugation (3000 rpm, 10 min), lysis buffer treatment for 6 min at room temperature, followed by washing with PBS + 2 % FBS. Lymphocyte suspensions (3.5×10^6 cells/mL) were cultured in 24-well plates with 10 % FBS + RPMI and stimulated with parasite antigen (50 μg/mL) at 37°C, 5 % CO2 for 72 h. Supernatants were collected and IL-4 and IFN-γ levels were measured by ELISA (Foroutan et al., 2020a, 2020b).

2.10. Statistical analysis

SPSS v21 was used for statistical analyses. Survival rates were analyzed using Kaplan–Meier curves. The Mann–Whitney test was used for pairwise group comparisons. LSD and Tukey tests were applied to detect significant differences among multiple groups, and one-way ANOVA was used to compare mean variables. Microsoft Excel 2021 was used to generate charts. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Confirmation of recombinant plasmid construction by gel electrophoresis, PCR, and western blotting

Colony PCR results showed a 1686 bp band (Fig. 1A) compared with a 1 kb ladder. Agarose gel electrophoresis of intact recombinant plasmids and plasmids digested with *Hin*dIII and *Eco*RI showed a separated 1686 bp band (Fig. 1B). Expression of ROP2 protein in CHO cells was confirmed by western blotting, showing a 64 kDa protein compared with a protein marker ladder (Fig. 1C).

3.2. IgG, IgG1, and IgG2a measurement results

ELISA results indicated that the highest average IgG concentration was observed in the pcROP2 +BCG lysate group, whereas the lowest was in the PBS group. The average total IgG concentrations across groups were compared using the Mann–Whitney test (Fig. 2A). For IgG1, the lowest average OD was observed in the pcROP2 +BCG group, and the highest in the pcROP2 group (Fig. 2B). Regarding IgG2a, the lowest and highest average ODs were found in the PBS and pcROP2 +BCG lysate groups, respectively (Fig. 2C).

3.3. Cytokine production

ELISA results showed that the lowest average concentration of IL-4 was detected in the BCG group, whereas the highest was observed in the pcROP2 group after 72 h of lymphocyte culture (Fig. 3A). For IFN-γ, the lowest average concentration was in the PBS group, and the highest in the pcROP2 +BCG lysate group (Fig. 3B). These results indicate that the recombinant plasmid containing the pcROP2 gene along with BCG lysate effectively stimulated cellular immune responses, inducing high levels of IFN-γ (favoring Th1 responses) and low levels of IL-4 (associated with Th2 responses, humoral immunity, and IgG1 production). The IFN-γ/IL-4 ratio in different experimental groups is shown in Fig. 4.

3.4. Survival rate evaluation in immunized and control groups

Survival assessment after challenge with the RH strain of *T. gondii* showed that deaths in the control groups began on day 3 and all mice died by day 5. In the BCG group, deaths began on day 3, with all mice dead by day 6. In the pcROP2 group, deaths started on day 4, with all mice dead by day 7. In the pcROP2 +BCG group, deaths began on day 5, with all mice dead by day 8. In the pcROP2 +BCG lysate group, deaths began on day 5, and all mice died by day 10. Thus, the lowest survival was observed in the pcDNA3 and PBS groups, while the highest survival was in the pcROP2 +BCG lysate group (Fig. 4). All mice ultimately died due to acute infection with the highly virulent RH strain (Fig. 5).

4. Discussion

T. gondii parasites in humans can cause a wide range of complications, from mild symptoms to severe clinical manifestations. In animals, infection can lead to livestock miscarriage and reduced productivity during pregnancy (Dubey, 2008; Weiss and Dubey, 2009). Therefore, the development of a vaccine against the *Toxoplasma* is necessary for both humans and animals (Innes et al., 2019). The parasite stimulates a

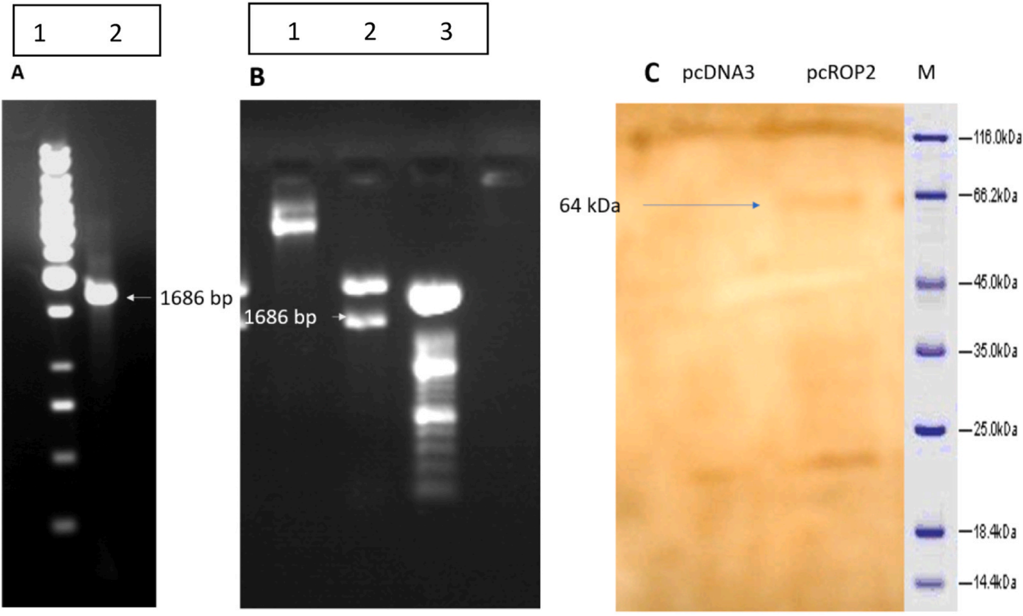
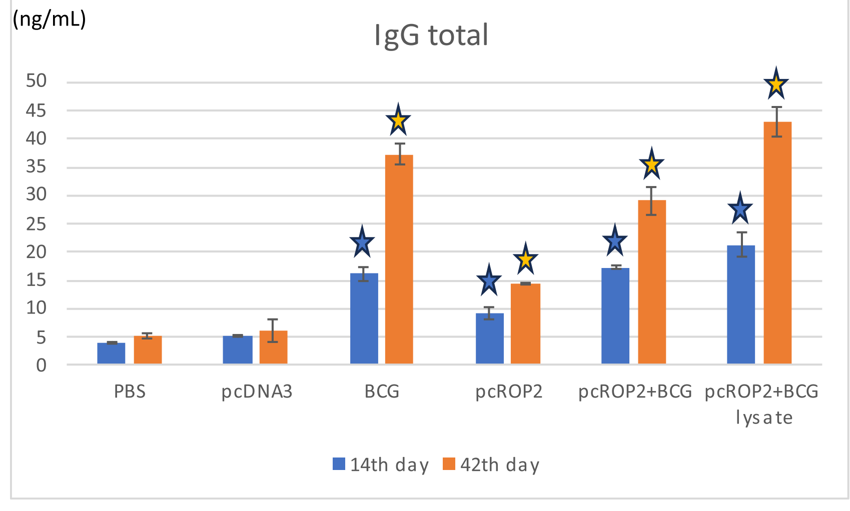


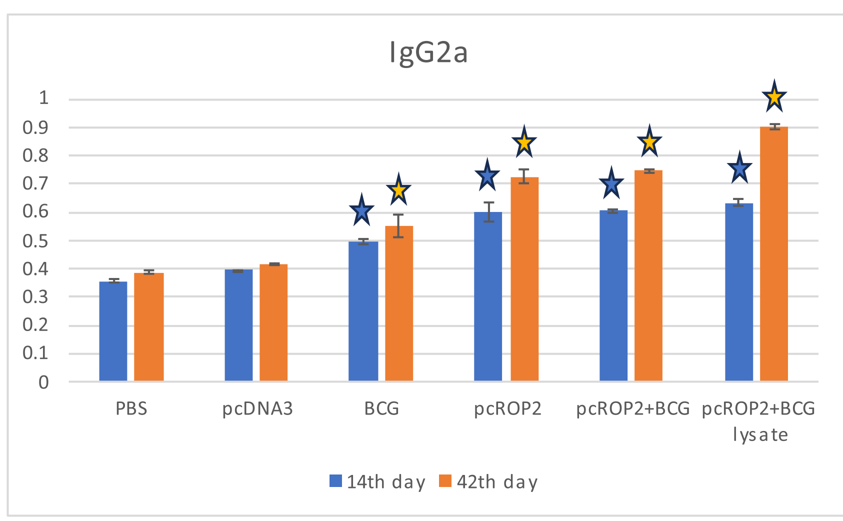
Fig. 1. Construction and Identification of pcROP2. **(A)** Colony PCR for the ROP2 gene showed a 1686 bp band. Lane 1: 1 kb DNA ladder; Lane 2: ROP2 gene; **(B)** Agarose gel electrophoresis of recombinant plasmids. Lane 1: intact recombinant plasmid; Lane 2: plasmid digested with *Hin*dIII and *Eco*RI showing the separated 1686 bp band; Lane 3: 1 kb DNA ladder; **(C)** Western blot analysis of CHO cells transfected with pcDNA3 and pcROP2 compared with the protein marker ladder (M).



A

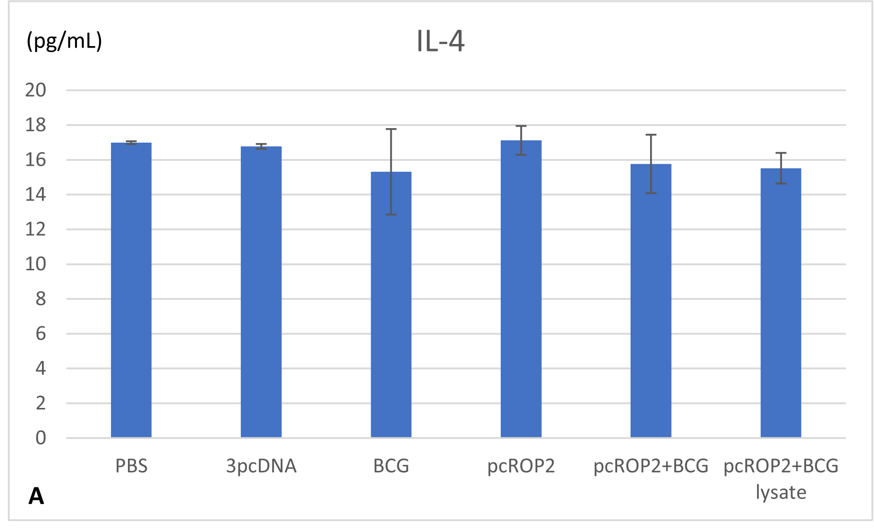
IgG1
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PBS
pcDNA3
BCG
pcROP2
pcROP2+BCG
pcROP2+BCG lysate
14th day
42th day

B



C

Fig. 2. Antibody responses, **(A)** IgG total, **(B)** IgG1, and **(C)** IgG2a (ng/mL) in the sera of immunized and control mice. * There is Statistical differences for IgG and IgG2a in vaccinated groups with control groups ($p < 0.05$). No significant differences were observed for IgG1 among the groups ($p > 0.05$).



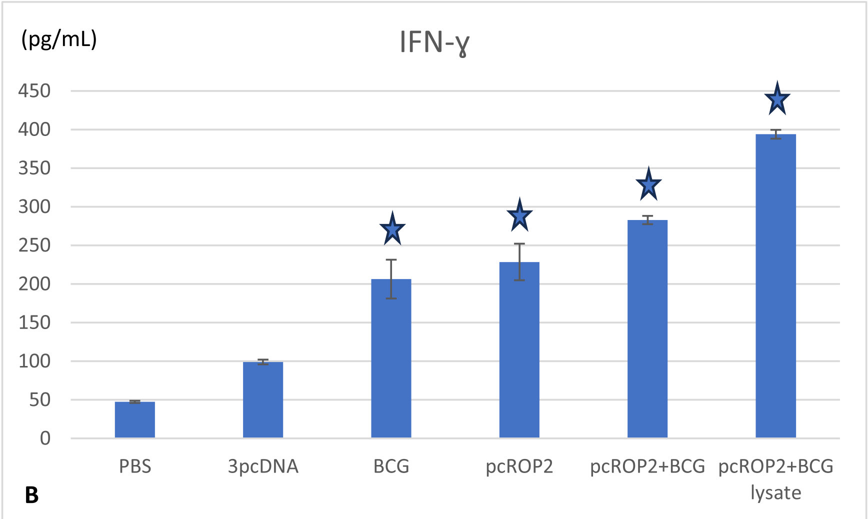


Fig. 3. Average concentrations of IL-4 (A) and IFN-γ (B) (pg/mL) detected by ELISA in splenocyte cultures from immunized mice after stimulation with *T. gondii* antigen. No significant differences were observed for IL-4 among the groups ($p > 0.05$), whereas significant differences were detected for IFN-γ in all vaccinated groups compared with controls ($p < 0.05$).

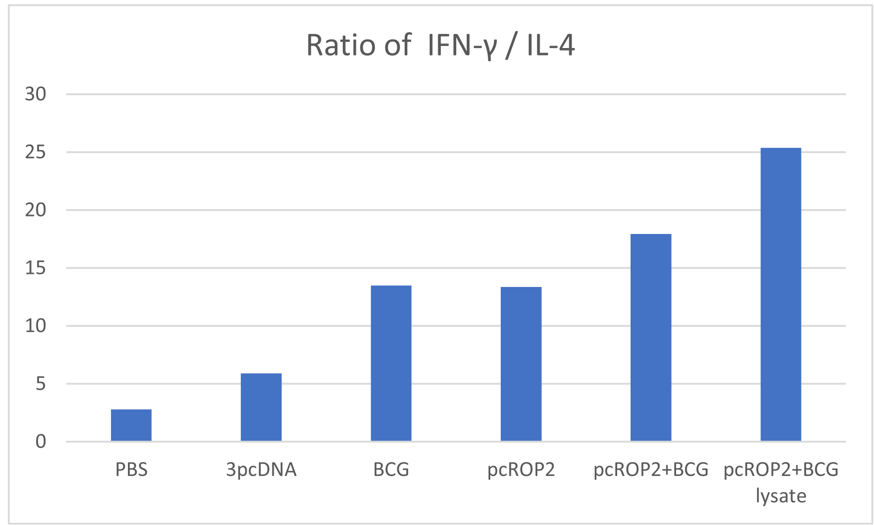


Fig. 4. Ratio of IFN-γ/IL-4 in immunized mice after stimulation with *T. gondii* antigen.

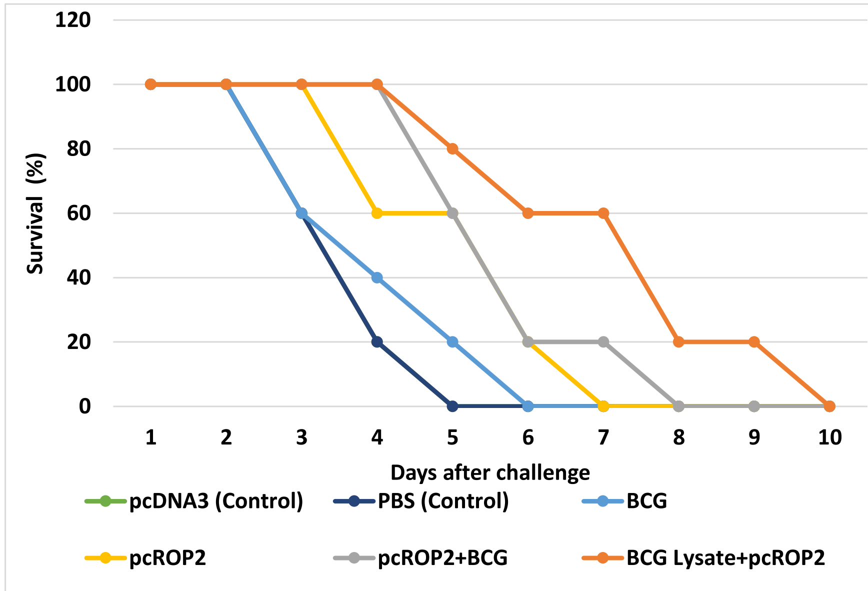


Fig. 5. Survival curves of immunized BALB/c mice after challenge with 1×10^5 tachyzoites of the RH strain of *T. gondii*, two weeks after the last immunization. The survival curve of the pcDNA3.1 group completely overlapped with that of the PBS group.

Th1 immune response and induces high levels of IFN-γ (Khan and Moretto, 2022; El-Kady, 2011), which is reflected in increased IgG2a secretion. Several attenuated *T. gondii* strains, such as live bradyzoite (T-263), and viral vector-based vaccines have been developed for animal immunization (Foroutan et al., 2019, 2020b). The Toxovax live tachyzoite vaccine, for example, reduces miscarriage in goats and sheep; however, no vaccine is currently available for human use. Killed *T. gondii* vaccines induce lower immunological responses and provide insufficient protection compared with live vaccines (Zhang et al., 2013; Wang et al., 2019).

In this study, we evaluated the immunogenicity of the ROP2 antigen of *T. gondii* in combination with a BCG adjuvant as a DNA vaccine. The aim was to investigate whether the BCG adjuvant could enhance the immunogenicity of a DNA vaccine encoding the ROP2 gene in BALB/c mice. The DNA vaccine was administered at a concentration of 100 µg with BCG adjuvant, injected intramuscularly three times at two-week intervals. Humoral and cellular immune responses were measured by ELISA, and a subset of immunized mice was challenged with 1×10^5 live tachyzoites of the RH strain two weeks after the last injection to assess survival. The survival results showed that in control groups (PBS and pcDNA3), deaths began on day 3, with all mice dead by day 5. In the BCG group, deaths began on day 3 and concluded by day 6. In the pcROP2 group, deaths began on day 4 and ended by day 7. In the pcROP2 +BCG group, deaths started on day 5, with all mice dead by day 8. In the pcROP2 +BCG lysate group, deaths began on day 5 and ended by day 10. The lowest survival was observed in control groups, while the highest was in the pcROP2 +BCG lysate group. These results indicate that recombinant plasmids encoding ROP2, either alone or with an adjuvant, can prolong survival, although full protection was not achieved.

These findings are consistent with previous studies on secretory antigens. For example, Khosroshahi et al. reported that mice receiving ROP2 and SAG1 plasmids with adjuvants survived longer than controls (Khosroshahi et al., 2012). In Eslami Rad et al.'s study on the ROP1 antigen, mouse challenge results showed that all mice in the control groups died by day six, whereas mice injected with recombinant plasmids survived longer (Eslami Rad et al., 2011). Similarly, Solhjoo et al. reported that combining the SAG1 antigen with the alum adjuvant enhanced both cellular and humoral immunity in immunized mice compared with control groups. Moreover, mice receiving the recombinant plasmid showed increased lifespan, higher cytokine levels, and elevated humoral antibody responses relative to controls (Solhjoo et al., 2007). Wang et al. demonstrated that a DNA vaccine containing ROP17 induced Th1 immune responses, increased IgG2a and IFN-γ levels, and extended survival up to 21 days compared with 8 days in controls (Wang et al., 2016b). Therefore, in this study, after three injections, two weeks post-immunization, antibody levels were higher in the immunized groups compared with the controls. However, the stimulation of the immune system by the ROP2 gene-encoding plasmid was not sufficient to provide complete protection against the lethal RH strain challenge. Instead, it only prolonged the lifespan of the vaccinated mice and partially reduced the severity of infection. These findings suggest that combining multiple parasite antigens may be required to achieve stronger and more protective immunity. The novelty of this study lies in the first comparison of intact BCG and BCG lysate as adjuvants for a ROP2 DNA vaccine.

According to the ELISA results (Fig. 2), total IgG levels were higher in all vaccinated groups than in the controls at both time points. Due to the adjuvant properties, antibody levels were significantly greater in groups that received the recombinant plasmid together with BCG compared with the plasmid alone. The highest IgG response was observed in the pcROP2 +BCG lysate group, while the lowest response was seen in the PBS group. This indicates that the plasmid combined with BCG adjuvant was able to stimulate the immune system more effectively, and its efficiency appeared to increase with time after vaccination. IgG1 and IgG2a responses followed the same pattern, confirming consistency with previous studies on other secretory antigens (Vazini et al., 2018).

Based on the results of the present study of the recombinant plasmid pcROP2 with BCG adjuvant, it can be concluded that this vaccine can stimulate the immune system and the production of specific antibodies. This study also examined the results of cellular immune assessment, especially IgG2a, in our study and in other studies showing the immune response activity of Th1 cells (Zhang et al., 2014). According to the results of Fig. 3, for the evaluation of IL-4, the lowest average concentration of the cytokine was detected in the BCG group within 72 h after lymphocyte culture, and the highest average concentration was detected in the pcROP2-receiving group. Additionally, according to the results shown in Fig. 3, the lowest average concentration of IFN-γ was detected in the PBS group within 72 h of lymphocyte culture, and the highest average concentration of this cytokine was detected in the

pcROP2 +BCG lysate group. According to the results of the evaluation of IFN-γ levels, cytokine levels in the group that received the recombinant plasmid with adjuvant were greater than those in the other groups, especially in the group receiving pcROP2 +BCG lysate, in which IFN-γ was much higher than in the other groups. Since the adjuvant has an effect on increasing antibody levels, the results obtained from the cytokine evaluation in our study were acceptable, and comparison with other studies also confirmed our findings. According to Chen et al., cytokine levels in the blood serum of mice are high, and IL-4 levels are low (Chen et al., 2009). In a study by Khosroshahi et al. involving a cocktail DNA vaccine, the levels of the cytokines IFN-γ, TNF-α, and IL-12 increased significantly compared to those in the control and other groups (Hosseinian Khosroshahi et al., 2011). Vazini et al. reported that for a DNA vaccine cocktail of GRA7 and pcROP2, IFN-γ levels in the pcROP2 +GRA7 group were much higher than in the other and control groups, and IL-4 levels in this group were also lower than in the other groups (Vazini et al., 2018). In general, in our study, IL-4 levels in all groups were much lower than IFN-γ levels, and as expected, the recombinant plasmid with BCG lysate adjuvant led to the production of cytokines stimulating the Th1-type (IFN-γ) immune response. In a previous study, Yu et al. (2013) demonstrated the use of BCG as a vector to express the TgCyP antigen to develop an effective vaccine to prevent toxoplasmosis (Yu et al., 2013). In 2007, Wang et al. used BCG as a live vector to express the ROP2 gene of *T. gondii*. They found that BALB/c mice immunized with BCG/pMV262-ROP2 exhibited specific immune responses against the ROP2 protein, showing a significant delay in mortality compared with the control group ($P < 0.05$) (Wang et al., 2007).

5. Conclusions

Based on the evaluations performed in this study, the following conclusions can be drawn: A recombinant plasmid containing pcROP2 of *T. gondii* in combination with a BCG lysate adjuvant has the ability to stimulate the cellular immune system more effectively than pcROP2 +BCG, and the induced cellular immune responses produce high levels of IFN-γ, which leads to the development of Th1-type immune responses. Low levels of IL-4 also support these results. IgG2a measurements also indicate the performance of Th1-type immune responses. The plasmid encoding pcROP2 with BCG lysate enhanced Th1-type responses and prolonged survival, though full protection was not achieved.

CRediT authorship contribution statement

Hélder Cortes: Writing – review & editing, Validation, Methodology, Data curation. **Kamy Hosseinian Khosroshahi:** Writing – review & editing, Data curation, Conceptualization. **Hamed Yousefi:** Writing – original draft, Methodology, Investigation. **Fatemeh Ghaffarifar:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Masoud Foroutan:** Writing – review & editing, Supervision.

Ethical approval

This study was approved by the Tarbiat Modares University Ethical Committee (IR.MODARES.AEC.1401.075).

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, we used Chat GPT version 5(o) in order to improve the readability and language of the manuscript. After using this, we reviewed and edited the content as needed and took full responsibility for the content of the publication.

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Declaration of Competing Interest

All the authors declare that they have no conflicts of interest.

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Data availability

Data will be made available on request.

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