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Original Article

Assessment of Immunogenicity of Multi-Epitope DNA Vaccine Encoding CDPK3, ROP22 & MIC8 of *Toxoplasma gondii* Adjuvanted with IL-12 against Acute and Chronic Toxoplasmosis in BALB/c Mice

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Abstract

Background: A non-allergenic, immunogenic recombinant DNA plasmid encoding CDPK3, MIC8, and ROP22 epitopes was evaluated as a DNA vaccine against *Toxoplasma gondii* in BALB/c mice, used in combination with IL-12 as a genetic adjuvant, to assess protection against both acute and chronic toxoplasmosis.

Methods: BALB/c mice were immunized three times at two-week intervals with the DNA vaccine combined with IL-12. Humoral immune responses were measured by total IgG, IgG1, and IgG2a levels, while cellular responses were evaluated through interferon-γ production and lymphocyte proliferation assays. Protective efficacy was assessed using challenge models with *T. gondii*: the virulent RH strain for acute infection survival and the non-virulent Tehran strain for chronic infection, based on reduction of brain tissue cysts.

Results: Vaccinated mice exhibited strong antigen-specific immunity, with elevated total IgG, IgG1, and IgG2a titers. Enhanced IFN-γ production and lymphocyte proliferation confirmed cellular activation. These responses correlated with reduced brain cyst counts after infection with the avirulent Tehran strain and prolonged survival with decreased parasite burden following virulent RH strain challenge, indicating improved protection.

Conclusion: Co-delivery of IL-12 enhanced both the immunogenicity and protective efficacy of the CDPK3–MIC8–ROP22 multi-epitope DNA vaccine by inducing a Th1-biased response that conferred protection against acute and chronic *T. gondii* infection. These findings support this vaccination strategy as a promising approach for toxoplasmosis control and highlight the need for further evaluation in diverse animal models and clinical settings to confirm safety, efficacy, and long-term immunity.

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Introduction

Toxoplasma gondii, an obligate intracellular protozoan of the phylum Apicomplexa, has a global distribution and poses health risks to humans and animals (1, 2). It infects most warm-blooded animals as intermediate hosts, while felids serve as definitive hosts supporting sexual reproduction in the intestine (3). About one third of adults worldwide have been exposed to *T. gondii*.

Although clinical disease is uncommon, toxoplasmosis can cause severe outcomes in immunocompromised individuals and congenital infection may lead to miscarriage, stillbirth, or lasting fetal deficits, highlighting the need for effective preventive strategies (4-6). In livestock, *T. gondii* causes reproductive disorders like abortion and stillbirth, leading to economic losses, and poses a human health risk via consumption of undercooked or raw meat containing tissue cysts (7). Several studies have identified *T. gondii* infection as a potential risk factor for schizophrenia (8, 9), multiple sclerosis (10), whereas others have independently reported that latent toxoplasmosis can modify host personality traits (11, 12).

T. gondii infection is diagnosed using serology (IgG/IgM), PCR, histopathology with immunohistochemistry, or bioassay/parasitic isolation depending on host and study goals (13-15). Due to its medical and veterinary importance, significant efforts have focused on developing a *T. gondii* vaccine. Effective immunity requires both humoral and cellular responses; with Th1-biased, IFN-γ–mediated cell-mediated immunity being crucial for controlling infection (16). DNA and subunit vaccines are promising due to better safety than live-attenuated vaccines but often need optimization, while multi-epitope constructs can enhance immunogenicity and broad protection (17). For instance, constructs encoding combinations of *Toxoplasma* virulence-associated proteins have induced robust humoral and cellular immunity in murine models. Among promising vaccine candidates, calcium-dependent protein kinases (CDPKs) are parasite-specific signaling molecules that regulate microneme secretion, gliding motility, egress, and host cell invasion, and their absence in fungal and mammalian host cells makes them safe and selective vaccine targets. Among them, CDPK3 is especially critical for calcium-dependent signaling controlling egress and motility, and its disruption reduces parasite dissemination and virulence (18). Additionally, rhoptry proteins (ROPs) are key virulence factors secreted during host cell invasion, modulating host signaling, immune responses, and gene expression. Among these, ROP22, a rhoptry kinase-like protein, supports parasite survival and enhances fitness, contributing to immune evasion (19). Moreover, microneme proteins (MICs) are calcium-dependent secreted adhesins essential for parasite attachment, gliding motility, and host cell invasion. MIC8, in particular, is critical for efficient entry and lytic cycle maintenance, and its suppression reduces virulence, making it a strong candidate for multicomponent vaccines (20).

The presence of these features together highlights their complementary roles and justifies their inclusion in multi-antigen vaccines (21, 22), and when adjuvants are used, they can lead to the induction of strong and long-lasting Th1 responses (23). Recent DNA vaccines combined with cytokine adjuvants, including IL-12 (24) and IL-33(25) have shown potential to enhance adaptive immunity, protection, and antitumor responses. IL-12 induces IFN-γ production and Th1 differentiation, boosting resistance to toxoplasmosis (26). Together, CDPK3, MIC8, and ROP22 constitute functionally distinct yet biologically interconnected proteins that operate at complementary stages of *T. gondii* invasion and intracellular development.

The key biological roles of these antigens highlight their potential as targets for next-generation toxoplasmosis vaccines; therefore, we investigated the immunogenicity and protective efficacy of an IL-12–adjuvanted multi-epitope DNA vaccine encoding CDPK3,

ROP22, and MIC8 in BALB/c mice, focusing on its ability to induce humoral and Th1-oriented cellular immunity and protect against acute and chronic toxoplasmosis.

Materials and Methods

Experimental animals

Female BALB/c mice (6–8 weeks, 20–25 g) were maintained under standard conditions (12-h light/dark cycle, 22 ± 2 °C) with free access to food and water. All procedures complied with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran.

Preparation of DNA Antigens

In-silico analysis of immunogenic proteins could reduce experimental failure. Accordingly, *T. gondii* CDPK3, ROP22, and MIC8 were analyzed using computational methods, including sequence accessibility, physicochemical properties, transmembrane domains, subcellular localization, phosphorylation and acylation sites, secondary structure, and 3D model construction with validation. B-cell (linear and conformational) and MHC-I/II epitopes were also predicted (27). After confirming immunogenicity and non-allergenicity in silico (our unpublished data), the recombinant multi-epitope plasmid was transfected into *Escherichia coli* DH5α and used in immunogenicity experiments.

Transformation of DNA plasmid into susceptible bacteria

Plasmid DNA was prepared as previously described. Chemically competent *E. coli* DH5α cells were generated by calcium chloride treatment and used immediately or stored in glycerol at −70 °C. Recombinant plasmids in Tris-EDTA (TE) buffer were introduced via heat-shock (42 °C, 90 s), recovered in antibiotic-free Luria-Bertani (LB) medium, and selected on ampicillin plates. Positive colonies were expanded in selective LB broth, and large-scale cultures were harvested for plasmid extraction. The procedure was repeated over one week to obtain sufficient plasmid DNA for immunization (28, 29).

Plasmid DNA extraction from transformed bacterial cells and purity assessment of plasmid DNA

Plasmid DNA was extracted from transformed *E. coli* DH5α using a modified alkaline lysis method. (30).

T. gondii proliferation and tachyzoite lysate antigen preparation

Tachyzoites of the RH strain of *T. gondii* were extracted from BALB/c mice and lysed for *Toxoplasma* lysate antigen (TLA) preparation via needle passage, washing, freeze-thaw cycles, inhibitor treatment, and sonication. Following centrifugation, the supernatant was collected, concentrated, dialyzed, sterilized, and its protein concentration was determined (31).

Immunization schedule

Eighty female BALB/c mice (6–8 weeks) were randomly assigned to groups 1–12 to evaluate acute and chronic toxoplasmosis. Groups 1–6 assessed immune responses before challenge with virulent RH strain, while groups 7–12 evaluated protection against chronic infection with avirulent Tehran strain, receiving the same treatments but with fewer animals. Formulations were administered subcutaneously on days 1, 14, and 28. Blood was collected for serology, splenocytes were isolated for lymphocyte proliferation and cytokine assays, and remaining animals were used for challenge experiments or kept as reserves (Table 1).

Table 1: BALB/c mice groups subcutaneous injection protocols and doses

Acute phase groups and injections	Chronic phase groups and injections
Group 1. DNA Ag + Adj, including 13 mice Receiving 50 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag + 50 µL IL-12	Group 7. DNA Ag + Adj, including 7 mice Receiving 50 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag + 50 µL IL-12
Group 2. DNA Ag alone, including 13 mice Receiving 100 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag	Group 8. DNA Ag alone, including 7 mice Receiving 100 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag
Group 3. TLA alone, including 6 mice Receiving 100 µL TLA	Group 9. TLA alone, including 4 mice Receiving 100 µL TLA
Group 4. Adj IL-12 alone, including 6 mice Receiving 100 µL IL-12	Group 10. Adj IL-12 alone, including 4 mice Receiving 100 µL IL-12
Group 5. pcDNA vector alone, including 6 mice Receiving 100 µL pcDNA 3.1	Group 11. pcDNA vector alone, including 4 mice Receiving 100 µL pcDNA 3.1
Group 6. PBS alone, including 6 mice Receiving 100 µL PBS	Group 12. PBS alone, including 4 mice Receiving 100 µL PBS

Splenic lymphocyte proliferation assay

Four weeks after the final immunization, and according to standard protocols, five mice from groups 1 and 2, and three mice from groups 3 to 6, were selected. Blood and spleen samples were collected following anesthesia, and splenocytes were prepared and cultured (1×10^5 cells/well) with TLA or medium alone. After 48 h incubation, proliferation was evaluated using the MTT assay, optical density was measured at 540 nm, and results were expressed as the stimulation index (SI) (17, 31).

Cytokine assay

Splenocytes were cultured as described for the lymphocyte proliferation assay, with incubation extended to 72 h. Supernatants were collected and stored at −80 °C (31, 32). IFN-γ levels were measured using a commercial mouse ELISA kit according to the manufacturer's instructions.

Antibody assay

Tachyzoite lysate antigen (5 µg/mL) was coated onto 96-well plates and incubated overnight at 4 °C. Plates were washed, blocked with 5% bovine serum albumin (BSA), and incubated with serum samples (1:100), followed by HRP-conjugated goat anti-mouse IgG, IgG1, or IgG2a (1:5000). After substrate addition and reaction termination, absorbance was measured at 450 nm (33). Results were expressed as mean ± standard error of the mean (SEM), and statistical analysis was performed using Student's t-test or one-way ANOVA with $P < 0.05$ considered significant (31).

Challenge of immunized mice

For acute toxoplasmosis, five mice per group were challenged subcutaneously with 1×10^3 RH strain tachyzoites on day 28 post-final immunization, and survival was monitored for three weeks. For chronic toxoplasmosis, groups 7–12 were intraperitoneally inoculated with 1×10^3 Tehran strain tachyzoites, and tissue cyst formation was assessed eight weeks later to evaluate vaccine efficacy.

Statistical analysis

Data were analyzed by one-way ANOVA for lymphocyte proliferation, cytokines, and antibody responses. Survival after RH strain challenge was assessed by Kaplan–Meier analysis. Analyses used SPSS version 21 (IBM Corp., Armonk, NY, USA); graphs were made with GraphPad Prism 6. Data are shown as mean ± SD; $P < 0.05$ was considered significant (31).

Results

Splenocyte proliferation assay (MTT)

The splenocyte proliferation assay showed significant differences in stimulation index (SI) among groups (Fig. 1). The DNA vaccine with IL-12 induced the highest SI ($P < 0.001$), while the DNA vaccine alone also showed significantly higher SI than TLA, IL-12 alone, pcDNA, and PBS groups ($P < 0.01$–0.001). TLA induced moderate proliferation ($P < 0.05$), whereas IL-12 alone, pcDNA, and PBS groups showed low or negligible responses.

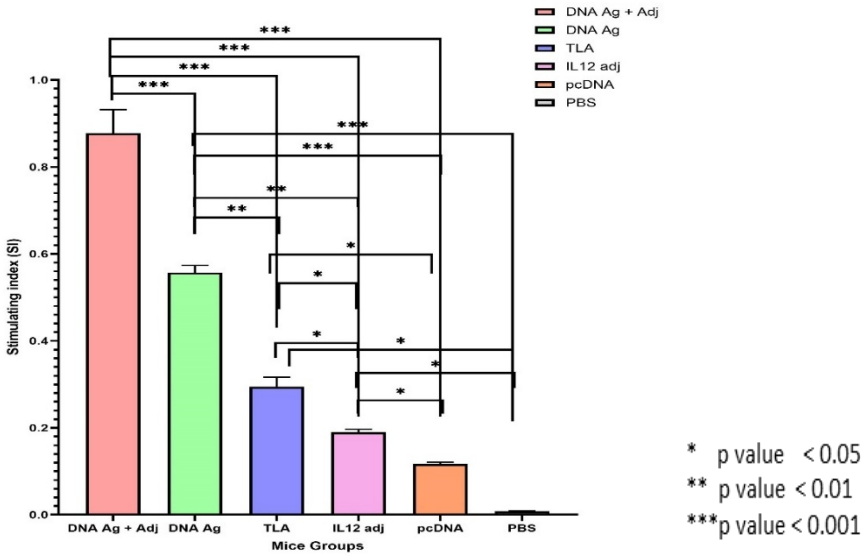

Fig. 1: Effect of administering CDPK3–ROP22–MIC8 multi-epitope DNA Ag, TLA and IL-12 adjuvant on splenic lymphocytes proliferation.
DNA Ag + Adj: Receiving 50 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag + 50 µL IL-12, DNA Ag: Receiving 100 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag, TLA: Receiving 100 µL TLA, Adj IL-12: Receiving 100 µL IL-12, pcDNA: Receiving 100 µL pcDNA 3.1, PBS: Receiving 100 µL PBS, *: *P* value <0.05, **: *P* value <0.01, ***: *P* value <0.001

IFN-γ production

Splenocytes from immunized mice showed significant differences in IFN-γ levels among groups (Fig. 2). The IL-12–adjuvanted DNA vaccine induced the highest IFN-γ ($P < 0.001$), while the DNA vaccine alone also elevated IFN-γ compared with TLA, IL-12 alone, pcDNA, and PBS ($P < 0.001$). TLA induced a moderate increase ($P < 0.01$), whereas IL-12 alone, pcDNA, and PBS showed minimal production.

Assay of IgG subclasses

As shown in Fig. 3a–c, serum TLA-specific antibody responses differed among groups. The IL-12–adjuvanted DNA vaccine induced the highest total IgG, whereas the DNA vaccine alone also significantly increased total IgG compared with TLA, IL-12 alone, pcDNA, and controls ($P < 0.01$–0.001). For IgG1, the DNA vaccine alone elicited the highest levels, with co-administration of IL-12 producing a moderate increase, while TLA induced moderate responses and IL-12 alone, pcDNA, and controls showed minimal production. IgG2a was highest in mice receiving DNA vaccine plus IL-12, followed by DNA vaccine alone; TLA induced moderate levels, IL-12 alone low but significant, and pcDNA and control minimal ($P < 0.01$–0.001). In compairing IgG1/IgG2a ratio among various groups, group 1 showed a ratio of 0.52, jndicating a clear prodominance of IgG2a and a Th1 based responce, while group 2,

demonstrated a >1.0 ratio (1.12), reflecting a shift toward IgG1 dominance and an IL-12-dependent differential immune polarization among the experimental groups.

Challenge and survival test

Kaplan–Meier analysis showed that mice receiving the DNA vaccine + IL-12 had the highest survival, remaining at 100% until day 16 post-infection, followed by gradual decline. DNA vaccine alone also prolonged survival compared with control groups, though less than the adjuvanted group. Mice immunized with TLA, IL-12 alone, pcDNA or unimmunized controls exhibited rapid disease progression, with complete mortality by days 8–16 post-infection (Fig. 4).

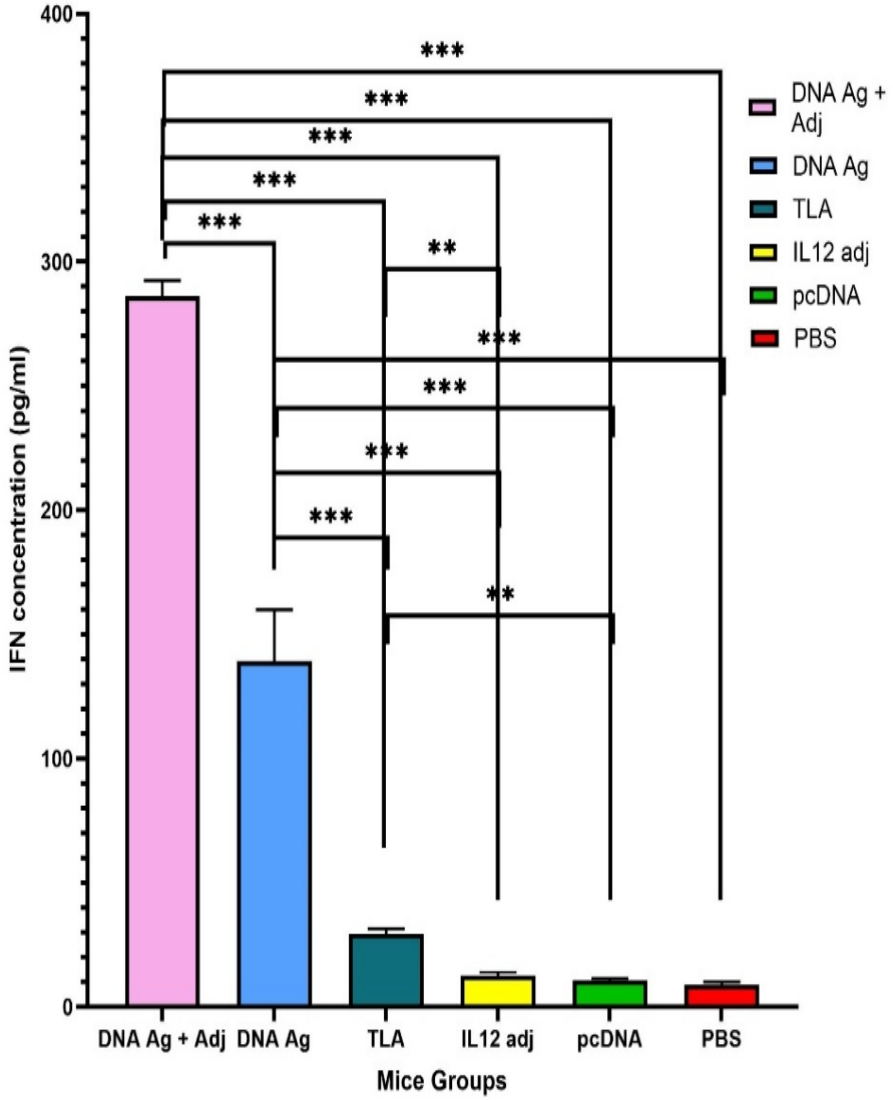

Fig. 2: Effect of administering CDPK3–ROP22–MIC8 multi-epitope DNA Ag, TLA and IL-12 adjuvant on splenic IFN-γ production.

DNA Ag + Adj: Receiving 50 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag + 50 µL IL-12, DNA Ag: Receiving 100 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag, TLA: Receiving 100 µL TLA, Adj IL-12: Receiving 100 µL IL-12, pcDNA: Receiving 100 µL pcDNA 3.1, PBS: Receiving 100 µL PBS. *: *P* value <0.05, **: *P* value <0.01, ***: *P* value <0.001

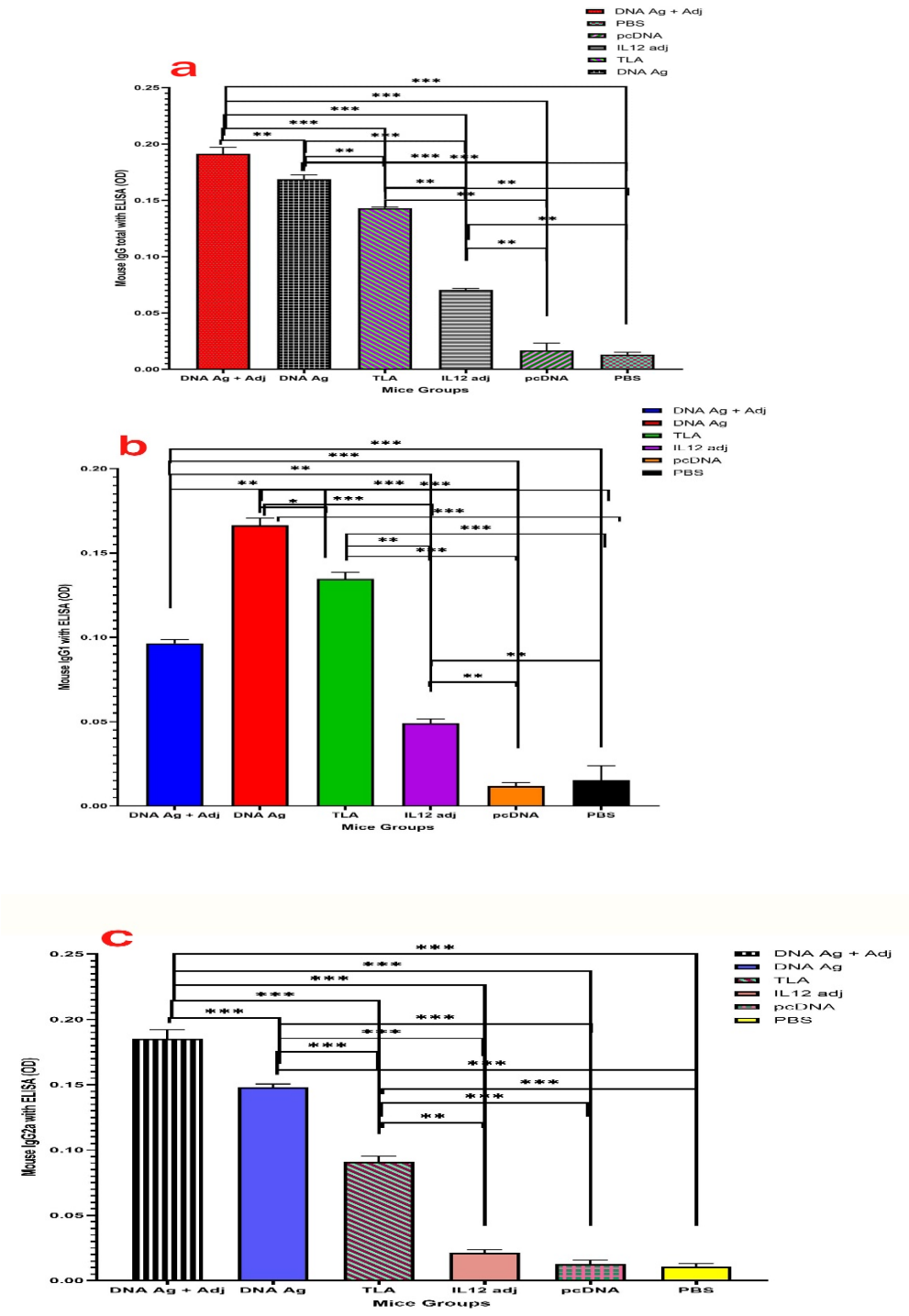

Fig. 3a-c: Effect of administering CDPK3–ROP22–MIC8 multi-epitope DNA Ag, TLA and IL-12 adjuvant on the mice serum TLA-specific IgG total(a), IgG1(b) and IgG2a (c) production.

DNA Ag + Adj: Receiving 50 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag + 50 µL IL-12, DNA Ag: Receiving 100 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag, TLA: Receiving 100 µL TLA, Adj IL-12: Receiving 100 µL IL-12, pcDNA: Receiving 100 µL pcDNA 3.1, PBS: Receiving 100 µL PBS. *: *P* value <0.05, **: *P* value <0.01, ***: *P* value <0.001

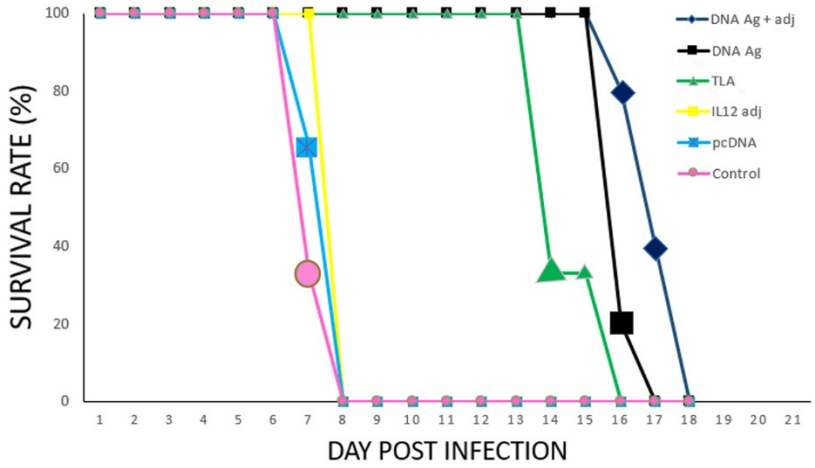

Fig. 4: Survival time of five mice from each group after challenge with lethal dose of *T. gondii* RH strain. DNA Ag + Adj: Receiving 50 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag + 50 µL IL-12, DNA Ag: Receiving 100 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag, TLA: Receiving 100 µL TLA, Adj IL-12: Receiving 100 µL IL-12, pcDNA: Receiving 100 µL pcDNA 3.1, PBS: Receiving 100 µL PBS

Challenge and brain cyst formation

Brain cyst counts varied significantly among groups (Fig. 5). The DNA vaccine + IL-12 induced the lowest cyst numbers ($P < 0.001$), followed by the DNA vaccine alone ($P < 0.001$). TLA caused a modest reduction ($P < 0.05$), while IL-12 alone, pcDNA, and controls showed the highest cyst burdens.

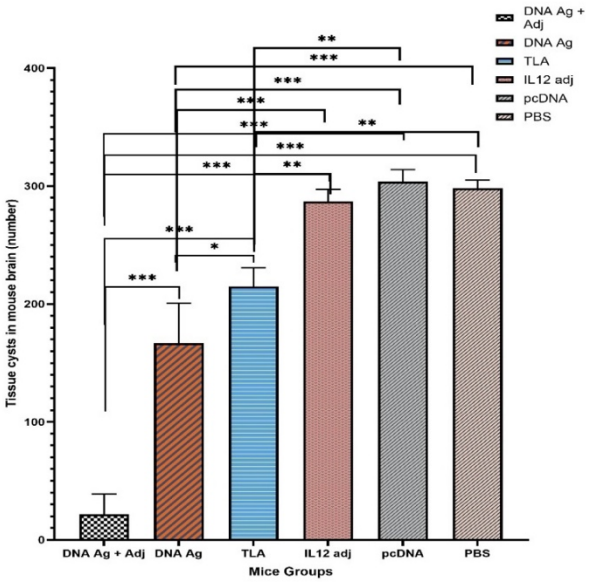

Fig. 5: Brain cyst number after challenge with avirulent dose of *T. gondii* Tehran strain DNA Ag + Adj: Receiving 50 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag + 50 µL IL-12, DNA Ag: Receiving 100 µL CDPK3–ROP22–MIC8 multi-epitope DNA Ag, TLA: Receiving 100 µL TLA, Adj IL-12: Receiving 100 µL IL-12, pcDNA: Receiving 100 µL pcDNA 3.1, PBS: Receiving 100 µL PBS

Discussion

Efforts to develop a vaccine for the *T. gondii* parasite have focused on utilizing appropriate antigens such as inactive forms, crude or purified antigens, recombinant antigens, and DNA vaccines, combined with adjuvants that promote Th1 immune responses, given the parasite's intracellular nature. Adjuvants under development include emulsions, liposomes, virosomes, alum, immune-stimulating complexes, cytokines, and toll-like receptor (TLR) agonists, several of which have been evaluated in *T. gondii* vaccines, such as alum, naltrexone, CpG, and propranolol (34).

In the present study, to enhance both cellular and humoral immunity, we employed a multi-epitope DNA vaccine. Compared with conventional vaccines, DNA vaccines provide enhanced immunogenicity by enabling in vivo expression of the encoded antigens and were therefore combined with a genetic adjuvant. Incorporating unmethylated CpG motifs within the plasmid or vaccine formula further enhances Th1 responses, elevating IL-12, IL-6, and IFN-γ levels, effectively making DNA a self-adjuvant (35). Guided by previous evidence, MIC8, ROP22, and CDPK3 antigens, together with IL-12, were selected for multi-epitope DNA vaccine construction. According to our results, DNA vaccine combined with IL-12, significantly enhanced antigen-specific splenocyte proliferation, whereas TLA induced weaker cellular responses, supporting the superior immunostimulatory capacity of DNA-based antigen delivery. Concordantly, IFN-γ levels were highest in the DNA + IL-12 group, reflecting Th1 polarization, macrophage activation, and intracellular parasite control. Humoral immunity contributed to protection too, as IL-12-adjuvanted DNA vaccination increased TLA-specific total IgG, reflecting effective B-cell activation. IL-12 indirectly augmented antibody production through robust T-helper support and promoted IgG2a dominance consistent with Th1/IFN-γ–mediated switching. TLA-only and control groups showed minimal antibodies, confirming antigen-specific vaccine overall immunogenicity. These immune responses translated into robust in vivo protection, as DNA + IL-12 immunization significantly improved survival following lethal *T. gondii*, IFN-γ production, and challenge, likely through synergistic $CD4^+/CD8^+$ T-cell macrophage effector function. While DNA vaccination alone delayed mortality, its activation protective effect was limited without strong immunostimulation. Brain cyst quantification confirmed that co-delivery of IL-12 effectively reduced chronic parasite burden, highlighting the importance of sustained Th1 responses for long-term control. Overall, these results demonstrate that the CDPK3–ROP22–MIC8 multi-epitope DNA vaccine, particularly when adjuvanted with IL-12, induces potent Th1-biased cellular responses, balanced humoral immunity, improved survival, and reduced cyst burden, supporting its potential as a promising candidate against both acute and chronic toxoplasmosis.

Our findings align with Xue et al., reported enhanced humoral and Th1 cellular responses and prolonged survival in the IL-12–adjuvanted group, demonstrating potent, long-lasting protection against *T. gondii* challenge (36). Our findings are also in agreement with Mavi et al, who showed that a dual-promoter DNA vaccine encoding SAG1 and GRA7 enhanced both humoral and cellular immunity in BALB/c mice. They observed increased total IgG, particularly IgG2a, elevated IFN-γ, and Th1-dominant responses. CpG-ODN adjuvant further improved lymphocyte proliferation and immune responses, prolonging survival after RH strain challenge, although complete protection was not achieved (17).

Despite the promising immunological and protective outcomes, several limitations warrant consideration. First, experiments were conducted exclusively in BALB/c mice, which are Th2-biased, potentially affecting the mag-

nitude and polarization of vaccine-induced immunity and limiting generalizability to other genetic backgrounds such as the Th1-prone C57BL/6 model. Second, although increased IFN-γ production and lymphocyte proliferation indicate robust cellular immunity, specific T-cell subsets, including $CD8^+$ cytotoxic and memory T cells, were not directly characterized. In particular, intracellular cytokine staining (ICS) and flow cytometric analysis were not performed to determine the relative contribution of $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells to the observed IFN-γ response. Therefore, the precise cellular sources and functional profiles of vaccine induced T-cell responses remain to be elucidated. Moreover, a direct cytotoxic T lymphocyte (CTL) functional assay was not conducted. While increased IFN-γ production indirectly supports activation of cytotoxic responses, confirmation through chromium-release assays, LDH-based cytotoxicity assays, or flow cytometry-based killing assays would provide stronger mechanistic evidence of $CD8^+$ T cell mediated effector function. Another limitation of this study is that the duration of protective immunity beyond the study period was not assessed, leaving long-term vaccine efficacy undetermined. Although a 18 days post infection duration was sufficient to demonstrate significant differences in acute protection, long term monitoring, particularly using less virulent strains would allow more comprehensive evaluation of durability of protection and sustained immune control. Also, a larger sample size in challenge experiments (more than 5 mice per group) would enhance statistical power and strengthen generalizability. Furthermore, although IL-12 significantly enhanced immunogenicity, its dose-dependent toxicity and safety were not evaluated, which is critical for translational applications. Additionally, the vaccine was not compared with alternative adjuvants or delivery platforms, highlighting the need for future studies to optimize and refine this strategy. Taken together, discrepancies in previous studies indicate that robust immunogenicity does not always ensure effective protection against *T. gondii*. While some multi-gene DNA vaccines elicited strong Th1 responses without full survival under lethal challenge(17), our findings underscore the importance of rational antigen selection and immune modulation. The combined targeting of CDPK3, MIC8, and ROP22, together with IL-12 as a genetic adjuvant, resulted in a balanced and functionally effective immune response characterized by enhanced cellular immunity, improved survival, and reduced cyst burden. These results support the potential of this multi-epitope DNA vaccine strategy as a promising approach for controlling both acute and chronic toxoplasmosis.

Conclusion

The multi-antigen DNA vaccine encoding MIC8, ROP22, and CDPK3 can induce both humoral and cellular immunity and increase survival in BALB/c mice. Co-administration of IL-12 further enhanced immunogenicity and protection, as evidenced by prolonged survival and reduced brain cyst burden compared with TLA and control groups. However, these results are limited to a single animal model and a single parasite strain. Therefore, IL-12–adjuvanted multi-epitope DNA vaccines may have potential utility for protection against toxoplasmosis and provide a foundation for future studies exploring additional antigens, alternative animal models, diverse parasite strains, and optimized immunization and challenge protocols.

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Artificial intelligence tools were used solely for language editing to enhance clarity and readability. The authors assume full responsibility for the content of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical Consideration

This study was approved by the Research Ethical Committee of Tehran University of Medical Sciences with the code number of IR. TUMS.AEC. 1401.085.

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