Research Article

Resistance to Chronic *Toxoplasma gondii* Infection Induced by a DNA Vaccine Expressing GRA16

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Toxoplasma gondii can infect all warm-blooded animals including human beings. *T. gondii* dense granule protein 16 (TgGRA16) as a crucial virulence factor could modulate the host gene expression. Here, a DNA vaccine expressing TgGRA16 was constructed to explore the protective efficacy against *T. gondii* infection in Kunming mice. The immune responses induced by pVAX-GRA16 were also evaluated. Mice immunized with pVAX-GRA16 could elicit higher levels of specific IgG antibody and strong cellular response compared to those in controls. The DNA vaccination significantly increased the levels of cytokines (IFN-γ, IL-2, IL-4, and IL-10) and the percentages of CD4+ and CD8+ T cells in mice. After lethal challenge, mice immunized with pVAX-GRA16 (8.4 ± 0.78 days) did not show a significant longer survival time than that in controls (7.1 ± 0.30 days) ($p > 0.05$). However, in chronic toxoplasmosis model (administration of 10 brain cysts of PRU strain orally), numbers of tissue cysts in mice immunized with pVAX-GRA16 were significantly reduced compared to those in controls ($p < 0.05$) and the rate of reduction could reach 43.89%. The results indicated that the TgGRA16 would be a promising vaccine candidate for further development of effective epitope-based vaccines against chronic *T. gondii* infection in mice.

1. Introduction

Toxoplasma gondii, an apicomplexan parasite, can infect any nucleated cells of almost all warm-blood mammals including humans and birds [1–3]. Approximately one-third of the global human beings are considered *T. gondii* seropositive [2, 4]. Normally, *T. gondii* infections in immunocompetent people are asymptomatic. However, for people with immunodeficiency, such as AIDS patients and transplant recipients, *T. gondii* infection can lead to encephalitis, ophthalmopathy, or even death [4–6]. Pregnant women primarily infected with *Toxoplasma* could result in miscarriage, ocular complications, and severe neonatal malformations in the fetus [7, 8]. Toxoplasmosis occurring in animals can cause abortion and neonatal loss which can lead to significant economic losses to animal husbandry [9–11].

No drugs are available to eliminate *T. gondii* tissue cysts. Vaccination is considered one of effective options to control and prevent the parasite infection, but until now, no vaccine is suitable for use in humans [12, 13]. Over the last 20 years, encouraging efforts have been made in development of vaccines against *T. gondii* infection [14, 15]. A large number of vaccine candidates were evaluated using DNA vaccinal strategy and most of them were considered to induce effective humoral and cellular immune responses against the cellular pathogen invasion in animal models [16, 17]. However, none [18] of antigens from *T. gondii* could induce more than 80–90% protection against tissue cyst formation.

Dense granule (GRA) is an important secretory organelle in *T. gondii* cytoplasm, and many GRA proteins have been recognized as promising vaccine candidates against the parasite infection [18–21]. Among GRA proteins, *T. gondii* GRA16 (TgGRA16) can be exported beyond the parasitophorous vacuole (PV) to host cell nucleus to modulate the host genome expression [22]. TgGRA16 can bind to the host enzymes (the deubiquitinase HAUSP and PP2A phosphatase) to regulate the ability of p53 protein and the cell cycle in hosts. The p53 protein is a key molecule in control of the inflammation during glucocorticoid responses via regulation of the expression of NF-κB gene [23]. TgGRA16 is also considered a virulent factor for *T. gondii* genotype II strain functioning in the regulation of metabolism and cell cycle. However, it is yet to be known whether TgGRA16 could induce resistance to acute and chronic *T. gondii* infection in the mouse model.

The objectives of this study were to examine the immunogenicity of TgGRA16 using the eukaryotic plasmid pVAX-GRA16 and to estimate its potential protective effect against acute and chronic toxoplasmosis in Kunming mouse models.

2. Materials and Methods

2.1. Animals. Specific-pathogen-free (SPF) grade inbred Kunming mice (female) of 6–8 weeks of age were purchased from the Center of Laboratory Animals, Lanzhou Institute of Biological Products (Lanzhou, China). All mice were handled in strict accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China. The animal study was approved by the Animals Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science (Approval Number LVRIAEC2012-011).

2.2. Parasites. Two strains of *T. gondii* (RH and PRU) were used in the present study. Tachyzoites of the *T. gondii* RH strain (genotype I) were propagated by a serial intraperitoneal passage in Kunming mice. The tachyzoites were obtained from the peritoneal fluid of mice after centrifuging to remove the cellular debris and then were resuspended in sterile phosphate-buffered saline (PBS). The harvested tachyzoites were also used for the preparation of *Toxoplasma* lysate antigen (TLA) according to our previous description [15]. Tissue cysts of the PRU strain (type II) were maintained in the laboratory by the monthly passage of infective brain homogenate in Kunming mice.

2.3. Construction of DNA Vaccines. The TgGRA16 gene was amplified by polymerase chain reaction (PCR) from *T. gondii* genomic DNA with a pair of specific primers (forward primer: 5$'$-CGG GGTACC ATGTATCGAAACCACTCAG-3$'$ and reverse primer: 5$'$-CCG GAATTC TCACATCTG-ATCATTTTTCC-3$'$) designed according to the reference sequence from ToxoDB (Gene ID: TGME49_208830). The *Kpn* I and *Eco*R I restriction sites were introduced in the primers (underlined).

After sequencing, the PCR products were subcloned into the pVAX I (Invitrogen) through the two restriction sites using T4 DNA ligase and generated recombinant plasmid pVAX-GRA16. The recombinant plasmids pVAX-GRA16 were purified from transformed *E. coli* DH5α cells by anion exchange chromatography (EndoFree Plasmid Giga Kit, Qiagen Sciences, MD, USA) following the manufacturer's instructions. The concentration was determined by spectrophotometry at OD260 and OD280. Then the plasmids were dissolved in sterile phosphate-buffered saline (PBS) to a final concentration of 1 μg/μl and stored at $-20°$C.

2.4. Expression of pVAX-GRA16 Plasmids In Vitro. HEK293 cells grown on 6-well plates were transfected with recombinant plasmids pVAX-GRA16 using lipofectamine^{TM} 2000 reagent (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, the expression of pVAX-GRA16 *in vitro* was assayed by indirect immunofluorescence assay (IFA) as described previously [24]. The goat anti-*T. gondii* tachyzoites polyclonal antibody (1 : 50 dilution in PBST) and the fluorescein isothiocyanate- (FITC-) labeled rabbit anti-goat IgG antibody (1 : 2000, Sigma, USA) were used as the first antibody and the second antibody and were successively added to each well. As a negative control, the HEK293 cells were transfected with pVAX I.

2.5. Immunization and Challenge. A total of 4 groups were performed in the present study (28 mice per group). Mice in different groups were intramuscularly injected with pVAX-GRA16 plasmids, empty pVAX I, and PBS (100 μl/each) 3 times at a 2-week interval (Table 1). The mice that received nothing were used as negative control.

Two weeks after each immunization, blood samples from mice in all groups were collected from the mouse tail vein prior, and then the sera were separated and stored at $-20°$C. Preimmune serum samples were used as negative control. Two weeks after the third inoculation, 3 mice in each group were sacrificed, and their splenocytes were aseptically harvested for lymphocyte proliferation assay, flow cytometric analysis, and cytokine measurements.

For assessment of protection against acute toxoplasmosis, 10 mice in each group were intraperitoneally (IP) challenged with 10^3 tachyzoites of the virulent *T. gondii* RH strain 2 weeks after the last immunization. The survival time for each mouse and the percentages of mice survived were recorded until a fatal outcome for all animals. Meanwhile, 6 mice in all groups were inoculated orally with 10 tissue cysts as experimental chronic toxoplasmosis. One month after infection, brains of mice from each group were homogenized in 1 ml PBS. The

TABLE 1: Summary of treatments performed in mice.

Groups	Treatments	Route of administration
I	Nothing	—
II	PBS	Intramuscularly[c]
III	pVAX I[a]	Intramuscularly[c]
IV	pVAX-GRA16[b]	Intramuscularly[c]

[a]Each mouse, 100 μg pVAX I resuspended in 100 μl PBS. [b]Each mouse, 100 μg pVAX-GRA16 dissolved in 100 μl PBS. [c]Each mouse, individually injecting the leg muscle 3 times, at a 2-week interval.

number of cysts per brain was determined by three samples of 10 μl aliquots of each homogenized brain under an optical microscope.

2.6. *Determination of Antibodies.* The total IgG antibodies in serum samples were determined using SBA Clonotyping System-HRP Kit according to the manufacture's instruction (Southern Biotech Co., Ltd., Birmingham, USA). Briefly, ELISA plates were firstly coated with TLA (15 μg/ml). Twelve hours later, the mouse antisera were added to each well, subsequently with 100 μl of horseradish-peroxidase (HRP) conjugated anti-mouse IgG diluted in 1 : 250. Bound antibody was visualized by adding the substrate solution (PH 4.0) (1.05% citrate substrate buffer; 1.5% ABTS; 0.03% H_2O_2) for 20 min. All measurements were performed in triplicate.

2.7. *Lymphocyte Proliferation Assay by MTs.* The harvested splenocytes from 3 mice in each group were plated in triplicate at a density of 5×10^5 cells per well in complete medium (DMEM medium + 10% FCS + 100 U/ml penicillin/streptomycin). The spleen cells of mice from each group were stimulated with TLA (10 μg/ml) as positive controls. Wells that added medium (M) alone served as negative controls. The proliferative activity was measured using MTs method (Promega, USA) after 4 days. The stimulation index (SI) was calculated using the formula $OD_{570TLA} : OD_{570M}$.

2.8. *Flow Cytometry.* Analyses of CD4+ and CD8+ T lymphocytes were performed according to our previous study [25]. The percentages of CD4+ and CD8+ T lymphocytes were determined using flow cytometry with staining by the surface markers including phycoerythrin- (PE-) labeled anti-mouse CD3 (eBioscience), allophycocyanin- (APC-) labeled anti-mouse CD4 (eBioscience), and fluorescein isothiocyanate- (FITC-) labeled anti-mouse CD8 (eBioscience) antibodies. All the samples were analyzed regarding fluorescence profiles on a FACScan flow cytometer (BD Biosciences) by SYSTEM II software (Coulter).

2.9. *Cytokine Assays.* Splenocytes without RBC in 3 mice from each group were stimulated with 10 μg/ml TLA. Culture supernatants were harvested at 24 h for determination of IL-2 and IL-4, 72 h for IL-10, and 96 h for IFN-γ and IL-12 (p70) using commercial ELISA kits according to the manufacturer's instructions (Biolegend, USA). The analysis was performed with the data from three independent experiments.

2.10. *Statistical Analysis.* All statistical analyses were performed by SAS procedure (Statistical Analysis System, Version 9.1). The differences of all the data among the 4 groups were analyzed by one-way ANOVA. The results in comparisons between groups were considered different if $p < 0.05$.

3. Results

3.1. *Expression of the Plasmids in HEK293 Cells.* The expression of pVAX-GRA16 was identified using IFA. Specific green fluorescence was observed in HEK293 cells transfected with the eukaryotic recombinant plasmid pVAX-GRA16, but not
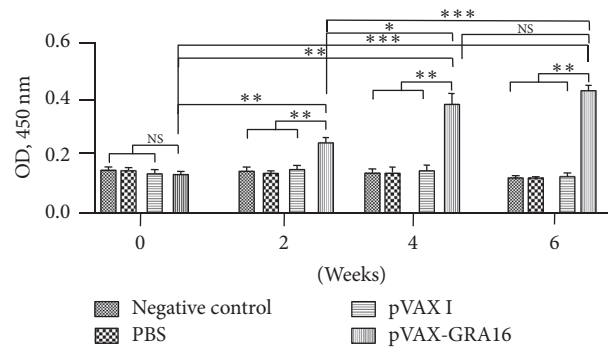

FIGURE 1: Determination of IgG antibody response induced by DNA immunization with pVAX-GRA16, pVAX I, PBS, and negative controls in the sera of Kunming mice at 0, 2, 4, and 6 weeks by ELISA. Each bar represents the mean OD ($\pm$SE, $n = 3$). $***p < 0.001$, $**p < 0.01$, and $*p < 0.05$; NS: not significant.

TABLE 2: Splenocyte proliferative responses and flow cytometry analysis of T cell subclasses in spleen cells between the immunization and the controls.

Groups	SI (mean $\pm$ SD)	CD3+CD4+CD8$-$ (%)	CD3+CD8+CD4$-$ (%)
Blank control	4.44 ± 0.78	4.17 ± 0.40	4.60 ± 0.95
PBS	3.50 ± 0.46	5.27 ± 1.82	3.60 ± 0.98
pVAX I	3.50 ± 0.51	3.43 ± 1.53	2.53 ± 1.74
pVAX-GRA16	$5.53 \pm 0.26^{**}$	$13.87 \pm 1.50^*$	$6.13 \pm 0.96^*$

SI stands for stimulation index; $^*p < 0.05$, $^{**}p < 0.01$.

in the controls transfected with the same amount of empty pVAX I.

3.2. *Evaluation of Antibody against TgGRA16.* Levels of IgG antibodies in mice immunized with pVAX-GRA16 were increased with successive DNA vaccination and reached the highest level at 2 weeks after the third booster. The antibody titers in sera collected at 6 weeks were significantly higher than that collected at 0 weeks ($p < 0.001$) and 2 weeks ($p < 0.001$) but showed no significant difference compared to that collected at 4 weeks ($p > 0.05$) (Figure 1). Mice injected with PBS and pVAX I did not generate specific antibodies, which revealed no significant differences compared to those in negative control group ($p > 0.05$).

3.3. *Evaluation of Splenocyte Proliferation.* After coculture of splenocytes with the stimulant, the proliferative response was examined by MTs and calculated as the ratio of the average OD_{570TLA} to the average OD_{490M}. The result showed that a vigorous splenocyte proliferation was detected in pVAX-GRA16 immunized mice ($p < 0.01$) compared with splenocytes from mice that received pVAX I, PBS, and nothing (Table 2).

3.4. *Percentages of CD4+ and CD8+ T Lymphocytes.* As shown in Table 2, the ratios of CD4+ T lymphocytes in mice

TABLE 3: Cytokine productions by splenocytes of immunized Kunming mice after stimulation by Toxoplasma lysate antigen (TLA)[a].

Groups	Cytokine production (pg/ml)			
	IFN-γ	IL-2	IL-4	IL-10
Blank control	169.80 ± 7.69	49.84 ± 3.59	<5	249.65 ± 13.15
PBS	172.40 ± 16.57	47.94 ± 2.94	<5	310.05 ± 37.59
pVAX I	171.36 ± 2.66	51.52 ± 6.07	<5	235.20 ± 36.73
pVAX-GRA16	1313.00 ± 48.33[***]	270.1 ± 6.20[***]	8.43 ± 1.58[*]	686.50 ± 87.36[***]

[a]Splenocytes from mice were obtained two weeks after the last immunization; [*]$p < 0.05$; [***]$p < 0.001$.

from pVAX-GRA16 immunized group (13.87 ± 1.50) were significantly higher than that in mice from pVAX I group (5.27 ± 1.82), PBS group (3.43 ± 1.53), and negative control (4.17 ± 0.40) ($p < 0.001$). CD8+ T cells in the spleen from mice immunized with pVAX-GRA16 were significantly increased (6.13 ± 0.96) compared to those in pVAX I group (3.60 ± 0.98), PBS group (2.53 ± 1.74), and negative control (4.60 ± 0.95) ($p < 0.05$). But there were no statistically significant differences in both the CD4+ and CD8+ T cells among the three control groups ($p > 0.05$).

3.5. Cytokine Production.

As shown in Table 3, the Th1-biased cytokines IFN-γ ($p < 0.001$) and IL-2 ($p < 0.001$) were significantly increased in spleen cells from mice immunized with pVAX-GRA16 compared to those in mice from pVAX I, PBS, and negative control groups. Meanwhile, splenocytes from pVAX-GRA16 immunized mice also produced higher levels of Th2-biased cytokines IL-4 ($p < 0.05$) and IL-10 ($p < 0.001$) compared to those in all the control groups (Table 3). Levels of various cytokines in spleen cells from mice in pVAX I, PBS, and negative control groups failed to be elicited after being cocultured with stimulants ($p > 0.05$).

3.6. Protection against T. gondii in Mice.

To evaluate whether pVAX-GRA16 can induce effective protection against acute toxoplasmosis, 10 mice from each group were challenged with lethal T. gondii tachyzoites of virulent RH strain two weeks after the last immunization. The average survival time of immunized mice (8.4 ± 0.78 days) showed an extension tendency compared to that of the control groups (7.1 ± 0.30 days), but the differences were not significant ($p > 0.05$) (Figure 2).

To determine the protection against T. gondii tissue cyst formation in the brain, mice were orally challenged with 10 cysts of the PRU strain. One month later, the brain cyst loadings were assessed. As shown in Table 4, compared to pVAX I, PBS, and negative control groups, immunization with pVAX-GRA16 significantly reduced brain cyst numbers in the immunized mice ($p < 0.001$), with a cyst reduction of 43.89%.

4. Discussion

Toxoplasmosis is a zoonotic disease with worldwide distribution [26]. However, no effective and safe medicines are available to control the parasite. It is thus particularly urgent to develop effective vaccines. In recent years, numerous studies have evaluated the protective efficacy of different
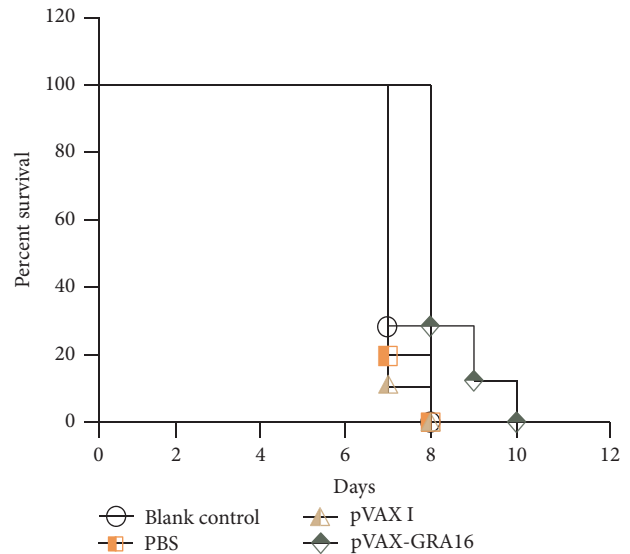

FIGURE 2: Survival times of mice immunized with pVAX-GRA16, pVAX I, PBS, and nothing followed by challenge with 1×10^3 tachyzoites 2 weeks after the last immunization.

TABLE 4: Tissue cyst loadings in the brains of mice after oral challenge with 10 cysts of the *Toxoplasma gondii* PRU strain.

Groups	Number of brain cysts (means $\pm$ SD)
Blank control	3366.67 ± 115.47
PBS	3463.33 ± 109.70
pVAX I	3150.00 ± 129.41
pVAX-GRA16	1866.67 ± 152.75[***]

[***]Statistically significant difference ($p < 0.001$).

antigens, such as MIC6 [27], ROP18 [28], NTPase II [29], TrxLp, and ENO2 [30]. Most of them showed partial protection against toxoplasmosis. It is reported that DNA vaccines can induce robust humoral and cellular immune responses [31, 32]. Herein, DNA immunization with TgGRA16 gene induced strong humoral and cellular immune responses, with a slightly longer survival time and a significant brain cyst reduction in mice compared to the controls, suggesting that TgGRA16 would be a potential vaccine candidate.

T cell-mediated immune responses are well known to be resistant against *T. gondii* infection [33]. Particularly, the activity of cytotoxic CD8+ T cells is considered crucial for the destruction of host cells where *T. gondii* niche by the

production of IFN-γ [34, 35]. Here, immunization of mice with pVAX-GRA16 induced significantly higher levels of CD4+ and CD8+ T lymphocytes, which contributed to a *Toxoplasma*-specific CTL response. The results of enhanced CD4+ and CD8+ T cells in mice immunized with pVAX-GRA16 were consistent with our previous study of DNA vaccination with TgCDPK5 [24].

Productions of IFN-γ and IL-2 cytokines could activate macrophage for killing *T. gondii* and also trigger multiple intracellular mechanisms to limit the parasite replication *in vivo* [31, 34]. Here, the significantly high levels of IFN-γ and IL-2 cytokines were detected in mice immunized with pVAX-GRA16, which contributed to the decrease of the brain cyst burden compared to that in controls. The pVAX-GRA16 also induced increasing levels of IL-4 and IL-10 in mice, which was consistent with the results of levels of IgG antibody. IL-4 and IL-10 are known as promotion of the proliferation and differentiation of activated B cells [31].

IgG antibodies can opsonize *T. gondii* for phagocytosis and inhibit the parasite reactivation through attaching to the host cell receptors [36]. Our results showed that pVAX-GRA16 could elicit a higher level of IgG antibodies in mice after the third immunization ($p < 0.001$) compared to that in controls, which would contribute to the mildly longer survival time after acute challenge infection.

To assess the protective immunity of the pVAX-GRA16 DNA vaccine, the Kunming mice were challenged intraperitoneally with lethal dosage of tachyzoites of the highly virulent *T. gondii* RH strain and orally with 10 cysts of PRU strain. The results showed that mice immunized with pVAX-GRA16 could slightly prolong the survival time (8.4 ± 0.78 days) and significantly decrease brain cysts (43.89%) compared to those in all the controls, suggesting that TgGRA16 would be a potential vaccine candidate. Further study should be focused on analysis of the epitopes of TgGRA16 by bioinformatics and evaluation of the protective effect of these antigen peptides derived from TgGRA16 against *T. gondii* infection.

In conclusion, the present study showed that DNA immunization with pVAX-GRA16 induced strong humoral and cellular immune responses and reduced the brain cyst formation in mice, indicating that TgGRA16 would be a potential candidate for further development of effective multiepitope vaccines against acute and chronic toxoplasmosis in the mouse model.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ling-Ying Hu and Nian-Zhang Zhang contributed equally to this work.

Acknowledgments

References

[1] J. P. Dubey, *Toxoplasmosis of Animals and Humans*, CRC Press, Boca Raton, Fla, USA, 2nd edition, 2010.

[2] D. E. Hill, S. Chirukandoth, and J. P. Dubey, "Biology and epidemiology of *Toxoplasma gondii* in man and animals," *Animal Health Research Reviews*, vol. 6, no. 1, pp. 41–61, 2005.

[3] J. P. Dubey, "The history of *Toxoplasma gondii*—the first 100 years," *The Journal of Eukaryotic Microbiology*, vol. 55, no. 6, pp. 467–475, 2008.

[4] P. Zhou, Z. Chen, H.-L. Li et al., "*Toxoplasma gondii* infection in humans in China," *Parasites & Vectors*, vol. 4, article 165, 2011.

[5] T. Štajner, Z. Vasiljević, D. Vujić et al., "Atypical strain of *Toxoplasma gondii* causing fatal reactivation after hematopoietic stem cell transplantion in a patient with an underlying immunological deficiency," *Journal of Clinical Microbiology*, vol. 51, no. 8, pp. 2686–2690, 2013.

[6] J. C. Sepúlveda-Arias, J. E. Gómez-Marin, B. Bobić, C. A. Naranjo-Galvis, and O. Djurković-Djaković, "Toxoplasmosis as a travel risk," *Travel Medicine and Infectious Disease*, vol. 12, no. 6, pp. 592–601, 2014.

[7] H. Andiappan, V. Nissapatorn, N. Sawangjaroen et al., "*Toxoplasma* infection in pregnant women: a current status in Songklanagarind hospital, southern Thailand," *Parasites & Vectors*, vol. 7, no. 1, article no. 239, 2014.

[8] M. Reich, M. Ruppenstein, M. D. Becker, and F. Mackensen, "Risk of recurrence of preexisting ocular toxoplasmosis during pregnancy," *Ocular Immunology and Inflammation*, vol. 16, pp. 1–6, 2014.

[9] A. Diakoua, E. Papadopoulos, N. Panousis et al., "*Toxoplasma gondii* and Neospora caninumseroprevalence in dairy sheep and goats mixed stock farming," *Vet Parasitol*, vol. 198, pp. 387–390, 2013.

[10] D. E. Hill and J. P. Dubey, "*Toxoplasma gondii* prevalence in farm animals in the United States," *International Journal for Parasitology*, vol. 43, no. 2, pp. 107–113, 2013.

[11] J. M. Unzaga, G. Moré, D. Bacigalupe et al., "*Toxoplasma gondii* and *Neospora caninum* infections in goat abortions from Argentina," *Parasitology International*, vol. 63, no. 6, pp. 865–867, 2014.

[12] E. A. Innes and A. N. Vermeulen, "Vaccination as a control strategy against the coccidial parasites *Eimeria, Toxoplasma* and *Neospora*," *Parasitology*, vol. 133, no. 2, pp. S145–S168, 2006.

[13] E. Hiszczyńska-Sawicka, J. M. Gatkowska, M. M. Grzybowski, and H. Długońska, "Veterinary vaccines against toxoplasmosis," *Parasitology*, vol. 141, no. 11, pp. 1365–1378, 2014.

[14] D. O. Villarreal, K. T. Talbott, D. K. Choo, D. J. Shedlock, and D. B. Weiner, "Synthetic DNA vaccine strategies against persistent viral infections," *Expert Review of Vaccines*, vol. 12, no. 5, pp. 537–554, 2013.

[15] N.-Z. Zhang, S.-Y. Huang, D.-H. Zhou et al., "Protective immunity against *Toxoplasma gondii* induced by DNA immunization with the gene encoding a novel vaccine candidate: calcium-dependent protein kinase 3," *BMC Infectious Diseases*, vol. 13, article 512, 2013.

[16] N.-Z. Zhang, M. Wang, Y. Xu, E. Petersen, and X.-Q. Zhu, "Recent advances in developing vaccines against *Toxoplasma gondii*: an update," *Expert Review of Vaccines*, vol. 14, no. 12, pp. 1609–1621, 2015.

[17] V. Feliu, V. Vasseur, H. S. Grover et al., "Location of the CD8 T Cell Epitope within the antigenic precursor determines immunogenicity and protection against the *Toxoplasma gondii* parasite," *PLoS Pathogens*, vol. 9, no. 6, article e1003449, 2013.

[18] N.-Z. Zhang, J. Chen, M. Wang, E. Petersen, and X.-Q. Zhu, "Vaccines against *Toxoplasma gondii*: new developments and perspectives," *Expert Review of Vaccines*, vol. 12, no. 11, pp. 1287–1299, 2013.

[19] J. Chen, Z.-Y. Li, E. Petersen, S.-Y. Huang, D.-H. Zhou, and X.-Q. Zhu, "DNA vaccination with genes encoding *Toxoplasma gondii* antigens ROP5 and GRA15 induces protective immunity against toxoplasmosis in Kunming mice," *Expert Review of Vaccines*, vol. 14, no. 4, pp. 617–624, 2015.

[20] M. Meng, A. Zhou, G. Lu et al., "DNA prime and peptide boost immunization protocol encoding the Toxoplasma gondii GRA4 induces strong protective immunity in BALB/c mice," *BMC Infectious Diseases*, vol. 13, no. 1, article 494, 2013.

[21] E. Hiszczyńska-Sawicka, G. Oledzka, L. Holec-Gasior et al., "Evaluation of immune responses in sheep induced by DNA immunization with genes encoding GRA1, GRA4, GRA6 and GRA7 antigens of *Toxoplasma gondii*," *Veterinary Parasitology*, vol. 177, no. 3-4, pp. 281–289, 2011.

[22] A. Bougdour, I. Tardieux, and M.-A. Hakimi, "*Toxoplasma* exports dense granule proteins beyond the vacuole to the host cell nucleus and rewires the host genome expression," *Cellular Microbiology*, vol. 16, no. 3, pp. 334–343, 2014.

[23] A. Bougdour, E. Durandau, M.-P. Brenier-Pinchart et al., "Host cell subversion by *Toxoplasma* GRA16, an exported dense granule protein that targets the host cell nucleus and alters gene expression," *Cell Host and Microbe*, vol. 13, no. 4, pp. 489–500, 2013.

[24] N.-Z. Zhang, S.-Y. Huang, Y. Xu et al., "Evaluation of immune responses in mice after DNA immunization with putative *Toxoplasma gondii* calcium-dependent protein kinase 5," *Clinical and Vaccine Immunology*, vol. 21, no. 7, pp. 924–929, 2014.

[25] Y. Xu, N. Z. Zhang, Q. D. Tan et al., "Evaluation of immunoefficacy of a novel DNA vaccine encoding *Toxoplasma gondii* rhoptry protein 38 (TgROP38) against chronic toxoplasmosis in a murine model," *BMC Infectious Diseases*, vol. 14, no. 1, article 525, 2014.

[26] F. Robert-Gangneux and M. Dardé, "Epidemiology of and diagnostic strategies for toxoplasmosis," *Clinical Microbiology Reviews*, vol. 25, no. 2, pp. 264–296, 2012.

[27] G.-H. Peng, Z.-G. Yuan, D.-H. Zhou et al., "*Toxoplasma gondii* microneme protein 6 (MIC6) is a potential vaccine candidate against toxoplasmosis in mice," *Vaccine*, vol. 27, no. 47, pp. 6570–6574, 2009.

[28] Z.-G. Yuan, X.-X. Zhang, R.-Q. Lin et al., "Protective effect against toxoplasmosis in mice induced by DNA immunization with gene encoding *Toxoplasma gondii* ROP18," *Vaccine*, vol. 29, no. 38, pp. 6614–6619, 2011.

[29] F. Tan, X. Hu, F.-J. Luo, C.-W. Pan, and X.-G. Chen, "Induction of protective Th1 immune responses in mice by vaccination with recombinant *Toxoplasma gondii* nucleoside triphosphate hydrolase-II," *Vaccine*, vol. 29, no. 15, pp. 2742–2748, 2011.

[30] M. Wang, X.-Y. Yang, N.-Z. Zhang, D.-L. Zhang, and X.-Q. Zhu, "Evaluation of protective immune responses induced by recombinant TrxLp and ENO2 proteins against *Toxoplasma gondii* infection in BALB/c mice," *BioMed Research International*, vol. 2016, Article ID 3571962, 8 pages, 2016.

[31] M.-H. Bessieres, B. Swierczynski, S. Cassaing et al., "Role of IFN-γ, TNF-α, IL4 and IL 10 in the regulation of experimental *Toxoplasma gondii* infection," *Journal of Eukaryotic Microbiology*, vol. 44, no. 6, p. 87s, 1997.

[32] R. Liang, J. V. van den Hurk, A. Landi et al., "DNA prime-protein boost strategies protect cattle from bovine viral diarrhea virus type 2 challenge," *Journal of General Virology*, vol. 89, no. 2, pp. 453–466, 2008.

[33] E. Y. Denkers and R. T. Gazzinelli, "Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection," *Clinical Microbiology Reviews*, vol. 11, no. 4, pp. 569–588, 1998.

[34] L. David Sibley, "Invasion and intracellular survival by protozoan parasites," *Immunological Reviews*, vol. 240, no. 1, pp. 72–91, 2011.

[35] R. T. Gazzinelli, F. T. Hakim, S. Hieny, G. M. Shearer, and A. Sher, "Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-γ production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine," *Journal of Immunology*, vol. 146, no. 1, pp. 286–292, 1991.

[36] P. C. Sayles, G. W. Gibson, and L. L. Johnson, "B cells are essential for vaccination-induced resistance to virulent *Toxoplasma gondii*," *Infection and Immunity*, vol. 68, no. 3, pp. 1026–1033, 2000.