Review article

CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Clin Exp Vaccine Res 2019;8:4-26
https://doi.org/10.7774/cevr.2019.8.1.4
pISSN 2287-3651 • eISSN 2287-366X

Rhoptry antigens as *Toxoplasma gondii* vaccine target

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Received: January 25, 2018
Revised: July 10, 2018
Accepted: January 14, 2019

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No potential conflict of interest relevant to this article was reported.

The authors would like to thank all staff of Department of Parasitology of Tarbiat Modares University, Iran.

Toxoplasmosis is a cosmopolitan zoonotic infection, caused by a unicellular protozoan parasite known as *Toxoplasma gondii* that belongs to the phylum Apicomplexa. It is estimated that over one-third of the world's population has been exposed and are latently infected with the parasite. In humans, toxoplasmosis is predominantly asymptomatic in immunocompetent persons, while among immunocompromised individuals may be cause severe and progressive complications with poor prognosis. Moreover, seronegative pregnant mothers are other risk groups for acquiring the infection. The life cycle of *T. gondii* is very complex, indicating the presence of a plurality of antigenic epitopes. Despite of great advances, recognize and construct novel vaccines for prevent and control of toxoplasmosis in both humans and animals is still remains a great challenge for researchers to select potential protein sequences as the ideal antigens. Notably, in several past years, constant efforts of researchers have made considerable advances to elucidate the different aspects of the cell and molecular biology of *T. gondii* mainly on microneme antigens, dense granule antigens, surface antigens, and rhoptry proteins (ROP). These attempts thereby provided great impetus to the present focus on vaccine development, according to the defined subcellular components of the parasite. Although, currently there is no commercial vaccine for use in humans. Among the main identified *T. gondii* antigens, ROPs appear as a putative vaccine candidate that are vital for invasion procedure as well as survival within host cells. Overall, it is estimated that they occupy about 1%-30% of the total parasite cell volume. In this review, we have summarized the recent progress of ROP-based vaccine development through various strategies from DNA vaccines, epitope or multi epitope-based vaccines, recombinant protein vaccines to vaccines based on live-attenuated vectors and prime-boost strategies in different mouse models.

Keywords: *Toxoplasma gondii*, Mice, Vaccines, Immunization, Adjuvant

Introduction

Toxoplasmosis is a cosmopolitan zoonotic infection, caused by a unicellular protozoan parasite known as *Toxoplasma gondii* that belongs to the phylum Apicomplexa [1,2]. This obligate intracellular parasite can infect a wide spectrum of warm-blooded vertebrate species such as men, birds, livestocks, marine mammals, etc. [1,3]. Recently, it has been shown that this parasite can infect snakes [4]. Role of rodents and birds in the spread of infection usually ignored [1,5]. Probably the global warming dilemma would play a substantial role in the epidemiological distribution of disease [5]. The infection is predominantly transmitted via consumption of raw/undercooked meats

contaminated with tissue cysts, drinking water or ingestion of raw/unwashed vegetables contaminated by mature oocysts, and vertically from mother to the fetus [1,3,6-9]. However, transmission via blood transfusion and organ transplantation occurs rarely [10-13]. The presence of *Toxoplasma* DNA in meat and meat products indicates the potential risk of food-transmitted toxoplasmosis [14,15].

Upon maternal infection, fetus is probably to be exposed with transplacental transmission. Toxoplasmosis may cause the miscarriage in those pregnant mothers that acquired the infection during pregnancy. Based on the gestational age, the consequent complications will be different including deafness, impaired mental development, retardation, microcephaly, hydrocephalus, brain focal lesions, etc. [16]. The infection may result in fetal death, neonatal loss and abortion in some animals, particularly amongst goats and sheep resulting severe economic losses in the industry of veterinary medicine and animal husbandry. Besides, they serve as a source of transmission to people [3].

Need for Vaccine

The present common primary control measures for men and animals *T. gondii* infection depends on chemotherapy. Since antiparasitic drugs are unable to prevent from the *T. gondii* infection in both men and animals and also have no effect on the encysted parasites within infected hosts [17], accordingly, discover and development of an effective vaccine urgently needed to prevent and control toxoplasmosis.

Now, the live-attenuated tachyzoites of *T. gondii* S48 strain, Toxovax is the only commercially licensed vaccine for use in the veterinary industry in some countries that decrease the incidence of abortion in sheep from congenital toxoplasmosis, however, it is inadequate and expensive [18]. Since this vaccine contains the live-attenuated tachyzoites, it is not appropriate for human use, especially in immunocompromised individuals. Besides, there is ascending concerns of safety for use in food-producing animals as well. On the other hands, *T. gondii* S48 strain may revert to the wild-type virulence, as previously the accidental infections have been reported in farmers [19-21].

As we all know, in regard to the major transmission routes of parasite and toxoplasmosis manifestations in the high risk groups, the main targets for vaccination strategy would cover [22].

- Vaccination to prevent acute parasitemia and protect against congenital toxoplasmosis
- Prevent or reduce tissue cysts in food animals to interrupt the transmission route to humans
- Prevention or reduction of oocyst shedding in cats to confine environmental contamination as well as minimize the risk of toxoplasmosis for all intermediate hosts

Rhoptry Proteins

Toxoplasma contains three apical secretory organelles known as the rhoptries, dense granules, and micronemes. Among them the rhoptries are club-shaped and has two distinct portions, including the anterior duct (neck) and the posterior bulb. This unique apical secretory organelle is shared merely in the all apicomplexan parasites [23,24]. Rhoptries are contributes in the active penetration of the parasite into the host cell and involved in the biogenesis of parasitophorous vacuole as a peculiar intracellular compartment, in which the parasite multiplies vigorously within it and avoid the intracellular elimination [24]. Rhoptry proteins (ROPs) are contributed in the multiple stages of the invasion of parasites and are also critical for survival within host cells [23-26]. In general, the rhoptries constitutes approximately 1%-30% of the total *Toxoplasma* cell volume [27]. The researchers were employed the proteomic and genomic approaches to recognize the contents of the rhoptries in *T. gondii* and other apicomplexan parasites [28-30]. They identified 38 ROPs, in which twenty of them were localized to the rhoptry organelle (eleven and nine for the rhoptry bulb and the rhoptry neck, respectively) [29-31]. Rhoptry family genes encode many substantial and pivotal proteins that contribute in the virulence and pathogenicity of *T. gondii*. Due to the key biological role of these proteins, ROPs have lately become popular and focused as hoping vaccine candidates against some parasitic disease such as toxoplasmosis [24-26,32-34]. These proteins are released by rhoptries which are the apical secretory organelles of parasite and mainly involved in the *T. gondii* invasion into the cytoplasm of host cells [25]. Some of these ROPs (such as ROP5, ROP16, and ROP18) so-called ROP kinases, act as serine-threonine kinases and play a pivotal role in the virulence and pathogenicity of parasite as well as host cell modulation [25,26,35].

In this review, we have summarized the recent developments via various strategies for ROP-based vaccines in different mouse models. The specific features and main functions of some ROPs have been summarized in Table 1.

In this field, great advances in the development of *T. gondii*

Table 1. The main features and functions of some ROPs

Antigens	Features or major effects on host	Reference
ROP1	Expressed in tachyzoite, bradyzoite, and sporozoite stages	[36-38]
	Has a key role in cell invasion	
	Enhance the invasion process of parasite *in vitro*	
	Related to the *T. gondii* penetrating enhancing factor	
ROP2	A member of the ROP2-protein family	[26,39-44]
	Expressed in sporozoites, tachyzoites, and bradyzoites stages	
	Containing T-cell and B-cell epitopes	
	Participates in the formation of PV and PVM	
	Molecular link between cell mitochondria and PVM	
	Pivotal for invasion and replication of parasites	
	Critical for parasite-host cell interaction	
	As the target of mucosal immune mechanisms	
	As a vaccine or diagnostic candidate Ag	
	Contributed in the uptake of iron from the infected host (serve as ligands for human hololactoferrin)	
ROP4	A member of the ROP2-protein family	[26,43,45]
	Expressed in sporozoites, tachyzoites, and bradyzoites stages	
	Contains a predicted serine/threonine PK domain in the C terminus	
	Participates in the formation of PV	
	Interaction with the mitochondrial import machinery	
	Release from the parasite during or shortly after invasion	
	Secreted ROP4 is linked to the PVM	
	Involvement in vacuole membrane function	
	Contributed in the uptake of iron from the infected host (serve as ligands for human hololactoferrin)	
ROP5	A secretory protein of the ROP2 family	[26,32,46-48]
	Responsible for the major virulence of parasite	
	Contribute to the intracellular proliferation of parasite	
	Major virulence determinant of immune evasion by inhibiting the accumulation of IRGs on PVM	
	Contains a tandem cluster of polymorphic alleles that differ in expression levels among different virulent strains, however, is confirmed to be responsible for the virulence of all types I strains	
	As a key component with a key role during the invasion process into the host cell	
	Dedicate to the formation of a PV and then becomes associated with the PVM	
	Act as essential cofactors for ROP18	
	A potential stimulators for both of humoral and cellular immune responses	
ROP7	A member of the ROP2 family	[46,49]
	After synthesis and maturation, it is localized in the rhoptries at the apical end of the permeabilized tachyzoites and colocalizes with ROP1	
	Translocated into the PV upon invasion	
ROP8	Has a conserved serine/threonine kinase domain	[50,51]
	One of the most abundant proteins belonging to the ROP2 family	
	An important protein in the pathogenesis of parasite	
	Containing T-cell and B-cell epitopes	
ROP9	A soluble rhoptry protein	[52,53]
	Only expressed in tachyzoite stage	
	Might be involved in the early stages of invasion	
	Contains putative B-cell epitopes	
	Induces an exclusive $CD4^+$ T cell response	
ROP13	A soluble protein that is proteolytically processed en route to the rhoptries and can be injected into the host cell cytoplasm	[29,54,55]
	ROP13 shows no homology to any known protein and lacks any identifiable domains.	

(*Continued to the next page*)

Table 1. Continued

Antigens	Features or major effects on host	Reference
ROP16	Contains a NLS, which injected during the invasion process into the host cell cytoplasm and then translocated rapidly into the nucleus A key virulence factor of *T. gondii* As a ROPK Key virulence determinant and regulator of host-cell transcription Activate both STAT3 and STAT6 signaling pathways IL-12 downregulation (ROP16 knockout parasites induce higher value of IL-12) Arginase 1 induction Containing T-cell epitopes	[56-59]
ROP17	Containing a key ATP-binding domain and conserved residues in its catalytic triad region Verified as a ROPKs	[26,60-62]
ROP18	A member of the ROP2-protein family A key virulence factor in *T. gondii* A highly polymorphic serine-threonine kinase (as a ROPK) ROP18 protein is secreted into the host cell cytoplasm during the infection and localizes to PVM Inhibiting accumulation of the IRGs on the PVM Contribution in controlling the intracellular proliferation of *T. gondii*, in which overexpression of ROP18 increases the replication of the parasite Downregulate $CD8^+$ T cell-mediated type I adaptive immune responses Inactivation of host innate and adaptive immune responses	[33,34,63-65]
ROP19	Has a key role in the PVM As an active kinase located in the PV Containing T-cell and B-cell epitopes	[30,66]
ROP38	It is predicted to be an active ROPK Has an inhibitory effect on host cell transcription by suppression of MAPK signaling Involved in the regulation of host transcription factor expression and cell proliferation Due to the low sequence variation in ROP38 gene among different *Toxoplasma* strains, this gene proposed may be suitable for vaccine candidate against toxoplasmosis	[30,67,68]
ROP54	A rhoptry pseudokinase effector involved in *T. gondii* invasion Modulate the innate immunity of the host cell	[69,70]

ROP, rhoptry protein or rhoptry antigens; *T. gondii*, *Toxoplasma gondii*; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; Ag, antigen; PK, protein kinase; IRGs, immunity-related GTPases; NLS, nuclear localization sequence; ROPK, rhoptry protein kinase; IL, interleukin; MAPK, mitogen activated protein kinase.

vaccination has been done such as the following:

- Several vaccine candidates have been evaluated from ROPs, particularly ROP1, ROP2, ROP5, ROP16, and ROP18.
- Novel genetic adjuvants and traditional adjuvants have been surveyed to induce a stronger cellular immunity.
- An increasing number of papers have examined the nanoparticles and microparticles as delivery system approaches to enhance a long-lasting protective immunity.

Mouse Models

Mice are being used frequently in experimental vaccine studies against toxoplasmosis as the main biological models to evaluate the outcome of acute and chronic infection before and post challenge with *T. gondii* strains. These little animals are also used for acute parasitemia and congenital toxoplasmosis or acquired infection. The reason is that toxoplasmosis in mice is histologically alike to that of men. On the other hand, ease of manipulating and cheap maintenance of them compared with larger animals such as livestock and domestic animals are other reasons for widely use of different mouse models [21,71]. Furthermore, the immunology of mice is well characterized [72]. It has been shown nearly 99% homology between mouse genome and the human genome [73]. However, some differences exist between the immune systems and immune responses of men and mice [72,74].

Type I strains of *T. gondii* is highly virulent in mouse models, while types II and III are extremely less virulent. The experimental mouse models have different major histocompatibility complex (MHC) haplotypes and different susceptibility

to the parasite. For example, BALB/c (H-2^d) mice are defined genetically with a low susceptibility to toxoplasmosis [75]. Also, C3H/HeJ (H-2^k) and C57BL/6 (H-2^b) mouse models with different genetic backgrounds are considered as intermediate and high susceptibility to *T. gondii* infection, respectively [76,77].

DNA Vaccines

The DNA vaccination is a novel method that recruits the plasmid vector in order to transfer and expression of the target gene [78,79]. These vaccines can be delivered through various routes, including intramuscular, subcutaneous, or mucosal. It is well known that DNA vaccination is a powerful strategy to provoke and elicit both specific cellular and humoral immune responses [80]. DNA vaccination as a robust method have become a major focus and they have many advantages in comparison to traditional vaccines in several parameters as follows [22,80,81]:

- Design (more rapid design as well as can be rapidly isolated and cloned).
- Versatility (ease in adapting or improving plasmid sequence, capability to deliver multi-antigen vaccines into a host only with a single dose, ease in formulation with different adjuvants).
- Production (cost effective, ease of production, capable of large-scale production, appropriate protein folding for correct epitope expression).
- Transport (stability at room temperature and no need to cold chain).
- Safety (cannot revert to the pathogenic form, safer than live or attenuated vaccines).
- Immune responses (able to induce a long-lasting immunity, elicit efficient and specific humoral and cellular immune responses).

Following the injection, the naked DNA plasmid enter to the cell cytoplasm and express encoded proteins within the host cells [19,80,82,83]. Production of specific-IgG antibodies as one of the robust protective immune responses can prevent and inhibit from the attachment of *T. gondii* to its host cell receptors. Besides, it helps macrophages (MQs) to kill and eliminate the parasite and preventing reactivation [84]. Specific and strong cellular and humoral immune responses are enhanced during the course of *T. gondii* infection. Generally, the secretion of interferon γ (IFN-γ) from T cells as the adaptive cellular immunity has a crucial role in the controlling and restricting growth of the parasite in both acute and chronic infection stages. Also, this important cytokine, inhibit the reactivation of bradyzoites within dormant tissue cysts [85,86]. It is well established that protection against toxoplasmosis generally is developed through both types of $CD4^+$ and $CD8^+$ T cells as cellular immunity arms. However, the role of $CD8^+$ T cells and IFN-γ are evident to be more substantial to limit infection [19,82]. It has been reported that DNA vaccination with ROP5, ROP9, ROP16, ROP17, ROP18, and ROP38 strongly increased the percentages of $CD4^+$ T and $CD8^+$ cells in immunized mice [47,52,62,67,87,88].

DNA vaccination against *T. gondii* infection would induce a strong Th1 type immune response (predominance of IgG2a over IgG1) with increased secretion of IFN-γ and interleukin (IL)-2 inflammatory cytokines to confine toxoplasmosis [85, 86]. Noteworthy, previous studies have shown that DNA vaccination against toxoplasmosis with Th1-type immune response and significant values of IL-2 and IFN-γ (compared with controls), does not guarantee the desirable outcome in mice. It can be concluded that the immunogenicity can not necessarily predictive of brain cyst load and survival rates in mice post challenge [19,46,52,82,83,89-91]. However, it should be mentioned DNA vaccinations generally trigger the activation and proliferation of both $CD4^+$ and $CD8^+$ T lymphocytes, along with increasing in essential specific antibodies for limit the infection [19,36,47,67,82,83,87,88]. Chen et al. (2014) [52] showed that pVAX-ROP9 induce a mixed Th1/Th2 response with the predominance of IgG2a levels (as an indicator of Th1-type response) than IgG1 (as an indicator of Th2-type response). In this regard, in the mice immunized with pVAX-ROP9 the production of IFN-γ, IL-2, IL-4, and IL10 were significantly increased, compared with those groups that injected pVAX1 or phosphate buffered saline ($p<0.05$). Moreover, pVAX-ROP9 considerably enhanced the percentages of $CD4^+$ and $CD8^+$ T cells and prolonged the survival time in Kunming mice post intraperitoneally challenge with 1×10^3 tachyzoites of the highly virulent RH strain (12.9±2.9 days, $p<0.05$). The authors noted that TgROP9 plasmid could be considered as a potent promising vaccine candidate for acute toxoplasmosis [52]. ROP18 as a main virulence factor of *T. gondii*, involved in the controlling of intracellular proliferation of parasite [34, 65]. For the first time, this protein has been shown to be a promising vaccine candidate by Yuan et al. (2011) [88]. Briefly, intramuscular vaccination of Kunming mice with the plasmid construct pVAX-ROP18 enhanced both humoral and cellular Th1-biased (predominance of IgG2a levels over IgG1) immune responses and increased activation of $CD4^+$ and $CD8^+$

 https://doi.org/10.7774/cevr.2019.8.1.4

T cells. Also, pVAX-ROP18 vaccination lead to higher survival time (27.9±15.1 days, $p<0.05$) than those mice in control groups when challenged with 1×10^3 tachyzoites of RH strain [88]. More examples of immunization experiments with DNA vaccines (single antigens) against *T. gondii* in different mouse models has been listed in Supplementary Table 1.

Accumulating evidence has been shown that multiantigenic DNA vaccinations could overcome the single antigen limitation and enhance the protective immunity against toxoplasmosis either survival duration time and/or brain cyst load [36,46,64,87,90,92-94]. More details can be found in Supplementary Table 2. For example, Chen et al. (2015) [47] evaluated the ROP5 and GRA15 antigens alone or in combination. The Kunming mice vaccinated intramuscularly with pVAX-ROP5 or pVAX-GRA15 and then two weeks after the third inoculation, were challenged with 1×10^3 tachyzoites of RH strain (type I) intraperitoneally for acute infection and 10 tissue cysts PRU strain (type II) orally for chronic infection. The immunized mice with alone antigen showed dramatically IgG2a titers in sera, high production of IL-12 p40, IL-12 p70, IFN-γ, and IL-2 production as Th1-type immune responses, augmentation of cell-mediated cytotoxic activity with high frequencies of IFN-γ secreting $CD8^+$ T cells, increased survival time (19.4±4.9 and 17.8±3.8 days for pVAX-ROP5 and pVAX-GRA15, respectively) and reduction of brain cysts (57.4% and 65.9% for pVAX-ROP5 and pVAX-GRA15, respectively), compared with control groups. As it was predictable, co-administration of these antigens, boosted the protective efficiency and elicited the cellular and humoral immune responses, compared to single antigen vaccines. Furthermore, significantly prolonged the survival time (22.7±7.2 days) and reduced the number of brain cyst load (79%) [47].

In regard to some delivery problems for DNA vaccines, their immunogenicity is occasionally confined. Several factors are affecting in the immune efficacy of DNA vaccines such as dosage and delivery routes. Albeit, 10 to 100 μg are routinely inoculated intramuscularly in mice [57,80]. For instance, the intramuscular injection of DNA vaccines is poorly distributed, inefficiently expressed and quickly degraded, thereby evoke relatively moderate humoral and cell mediated immune responses [95,96]. Besides, the degradation of plasmid DNA by lysosomes and DNAses may reduce the expression of plasmid DNA in small experimental animals as well as may affect DNA expression in men [95]. There are several strategies for adjuvanting plasmid DNA to augment their immunogenicity. An adjuvant could boost DNA delivery as well as enhance either the magnitude or time of DNA expression [96,97]. Moreover, by recruiting the immune cells to the site of injection, increases the immunostimulatory properties of plasmid. It is speculated, those adjuvants that enhance the magnitude/duration of plasmid DNA expression might help the uptake of DNA into host cells or increase taken up by professional antigen-presenting cells (APCs) as well as protect plasmid against degradation by DNAses [80,81,96,97]. Since the immunogenicity of DNA vaccines to stimulate the specific immune responses are often weak, recruiting cytokines and costimulatory molecules as genetic adjuvants or alum as non-genetic adjuvants as well as liposomes as vehicle adjuvants would be enhanced and modulated these immune responses [36,78,80,81,87,93,97-99]. Meanwhile, during recent years to enhance the immunogenicity of DNA vaccines, various strategies have been employed to conquer the present drawbacks and obstacles such as progress and improvement in plasmid design, antigen codon optimization to increase the expression of proteins, utilization of molecular and/or traditional adjuvants, co-expression of molecular adjuvants, electroporation, and prime-boost or combination immunization strategies [80,81]. Some examples of adjuvants in vaccination experiments against *T. gondii* infection in mouse models have been summarized in Table 2 [100-126].

Liposomes as carriers are composed of a diverse cholesterol and phospholipids, can encapsulate or bind plasmid DNA as well as they act as a suitable vehicle adjuvant [96,97]. Liposomes also help the entrace of DNA into cells through interaction with the lipid bilayer of the cell membrane and protect DNA from extracellular degradation by serum proteins [126]. Notably, following the formulation of DNA vaccine into liposomes, specific humoral and cellular immune responses considerably could elicit [81,97,126]. In this context, Chen et al. [125] highlighted that the approach of vaccination with a liposome-encapsulated DNA encoding ROP1-SAG1 of *T. gondii*, has potential capability to augment the humoral and cell mediated immune responses in BALB/c mice.

Genetic adjuvants have become recently as attractive tools to augment the protective efficacy of DNA vaccine [78]. In general, they are expression plasmid vectors that encoded biologically active molecules such as cytokines (IFN-γ, IL-12, IL18, etc.), chemokines (regulated upon activation normal T-cell expressed and secreted [RANTES]), co-stimulatory molecules (B7-1, B7-2, etc.), etc. (A_2/B subunits of cholera toxin [$CTXA_2$/B]) [19,78,82]. It should be noted, genetic adjuvants can be either encoded on a separate vector, or expressed on

Table 2. Examples of adjuvants in vaccination experiments against *Toxoplasma gondii* infection in mouse models

Category	Adjuvant	Function	Summary of results	Reference
Genetic adjuvants	pIFN-γ	Cytokine One of the important Th1 cytokines Play a main role in protective immunity against toxoplasmosis	Co-delivery of pIFN-γ with pcROP1 elicited the cellular immune responses with high production of IFN-γ and IL-2 cytokines [100].	[81,100]
	pIL-12	Cytokine Expressing the p35 and p40 subunits of murine IL-12 Enhancement of Th1 cellular immune responses Promotes NK cell activity Enhances CTLs response Stimulates the secretion of IFN-γ Lead to decrease the plasmid dose required for immune response stimulation and improve the immunogenicity of the vaccines Essential for the development of innate and adaptive immunity to limit toxoplasmosis	Co-administration of pIL-12 with pGRA7-ROP1 enhanced the levels of IgG titers, elicited Th1-biased responses with predominance of the IgG2a over IgG1, evoked higher secretion of IL-10, IFN-γ and TNF-α, prolonged survival time (50% survival rate 4 weeks post challenge) and decreased the percentage of brain cysts loads [36]. Co-administration of pIL-12 with pcSAG1+pcROP2 increased the survival time, compared with the controls ($p<0.05$). Enhancement of IgG antibodies and IFN-γ production also was observed [93,98]. The group co-administered pIL-12 plus pSAG1-ROP2 or pSAG1-ROP2-GRA2 elicited stronger humoral and Th1-type cellular immune responses as well as higher survival times [99,101]. Multiantigenic DNA vaccine (pSAG1-ROP2-SAG2) with pIL-12 co-delivery is a very effective approach in the protection against *T. gondii* [94]. DNA immunization of CBA/J mice with pROP18 induced specific humoral and cellular immune responses and co-administration of pIL12 did not enhance these responses [102].	[36,93,94,98,99,101-103]
	pIL-18	Cytokine Activates NK cells Enhances Th1-type immunity Induces IFN-γ Synergizes with IL-12	Coimmunization of pVAX-ROP13 with pVAX-IL-18 dramatically enhanced the survival duration, reduced the brain cysts load and provoked the IFN-γ, IL-2, IL-4, and IL-10 production, compared with pVAX-ROP13 alone [54].	[54,104-106]
	pB7-2	Co-stimulatory molecule Play a key role in providing co-stimulatory signals required for the generation and maintenance of antigen-specific immune response Has a key role to stimulate the T-cell differentiation toward Th1 pathway Play a central role in the antigen-specific induction of $CD8^+$ CTL response	The co-inoculation of pB7-2 with pROP16-GRA7 or single-gene vaccines considerably augmented humoral and cellular immune responses as well as the survival duration of time [87].	[87,107,108]
	CpG-ODN	As the TLR-9 ligand Increase antigen-specific immunity Boost the immunogenicity of DNA vaccines Strong enhancers of Th1-biased immune Activate the DCs	Coimmunization of CpG-ODN with rROP2 and rROP2+rGRA4 dramatically reduced the percentage of brain cysts (63% and 66%, respectively) [109].	[80,96,109]
	$pCTXA_2/B$	A powerful mucosal adjuvant Composed of five non-toxic B subunits and one A subunit. Subunit A contains A_1 and A_2, of which A_1 is the major toxin and is not essential for its adjuvant effect. Induce mucosal immune response	In the group co-administered $pCTXA_2/B$ with pSAG1-ROP2, there was no obvious enhancement of immunity in terms of humoral and Th1-type cellular immune responses as well as survival time [99]. The use of $pCTXA_2/B$ with a multi-epitope DNA vaccine lead to boost both humoral and cellular immune responses in BALB/c mice [110].	[99,110-112]

(*Continued to the next page*)

Table 2. Continued

Category	Adjuvant	Function	Summary of results	Reference
Conventional adjuvants	Alum	Leads to increased vaccine uptake Enhanced stability at the site of injection Promotes antigen phagocytosis by APCs Activates MQs Increases MHC II expression and antigen presentation	The mice immunized by pcROP1 with or without alum produced higher Th1 immune response compared with control groups. It should be noted, the levels of IFN-γ in mice immunized with PBS, pcROP1, and pcROP1+alum were 46.61 ± 1.79, 1,161.00 ± 76.10, and 433.00 ± 51 pg/mL, respectively. Also, higher levels of IgG2a was observed in pcROP1+alum group. All mice in both groups that received pcROP1 and pcROP1+alum were died within seven days and no significant difference was seen between experimental and control groups (the controls were died within 6 days) [89]. Co-administration of alum with pcSAG1+pcROP2 increased the survival time, compared with the controls ($p<0.05$). However, no significant difference was seen between groups that received adjuvants or no adjuvant. Enhancement of IgG antibodies and IFN-γ production also was observed [98]. rGRA4-rROP2-alum immunized mice from both strains of C57BL/6 (H-2^b) and C3H (H-2^k) with ME49 cysts resulted in fewer brain cysts than the controls ($p<0.01$), whereas vaccination with rROP2-alum, only conferred protection to C3H mice ($p<0.01$) [113].	[89,97,98,113]
	PLG/PLGA	Microparticle/nanoparticle As a safe delivery system and a potent adjuvant Extended antigen release Reduce protein degradation Generate a long-lasting immune response Facilitate Ag uptake via APCs	rROP18+PLGA administered intranasally enhanced the specific IgA and IgG2a levels in comparison to the group that immunized subcutaneously with rROP18-montanide adjuvant ($p<0.05$) [114]. rROP18 and/or rROP38 encapsulated into PLG, induced a long-term humoral and cellular immune response with dramatic reduction in the brain cyst formation (81.3% reduction in PLG+rROP38+rROP18 vaccinated mice) [115]. rROP18 and/or rCDPK6 encapsulated into PLG microparticles, induced a long-term humoral and cellular immune response with dramatic reduction in the brain cyst formation (ranged from 47.7% to 73.6%) [116].	[82,114-117]
	Quil-A	Classified as saponins Obtained from the bark of a tree *Quillaja saponaria* Used as veterinary adjuvant Has the following advantages: low cost, easily formulated and generally safe	rROP2-Quil-A adjuvant enhanced humoral and cellular responses in the immunized BALB/c mice [118].	[97,118]
	Re	Ginseng, the root of *Panax ginseng* C.A. Meyer-root Ginseng saponins, i.e., ginsenosides, are believed to be one of the active fractions in the root Ginsenosides have adjuvant properties and the adjuvant activity Elicited the antibody response against viral and bacterial antigens Safe and relatively low cost	Co-administration of rROP18 with Re induced humoral and cellular immune responses [119].	[119-124]
	Liposomes	As vesicles composed of phospholipids and cholesterol Proper for Ag or plasmid delivery Protect DNA from degradation by serum proteins Enhanced cellular and humoral immunity	Immunization with a liposome-encapsulated DNA encoding ROP1-SAG1, increased both humoral and cellular immune responses [125].	[81,97,126]
	Montanide	-	Significantly higher levels of IgG (with the predominance of IgG1 over IgG2a) were observed in mice vaccinated with rROP18-Montanide adjuvant, compared with rROP18 group ($p<0.05$) [114].	[114]

IFN-γ, interferon-γ; Th, T helper; ROP, rhoptry protein or rhoptry antigens; IL, interleukin; GRA, dense granule antigens; NK cells, natural killer cells; CTLs, cytotoxic T lymphocytes; TNF-α, tumor necrosis factor α; CpG ODN, oligodeoxynucleotides contained CG motifs; TLR, Toll-like receptor; DCs, dendritic cells; $CTXA_2$/B, A_2/B subunits of cholera toxin; APCs, antigen-presenting cells; MQs, macrophages; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PLGA, polylactide-co-glycolide acid; Ag, antigen; PLG, polylactide-co-glycolide; Re, Ginsenoside Re; SAG, surface antigens.

the same vector as the antigen and thereafter co-administrated with the vaccine [78,80,81,97,99]. As noted, one of the best approaches to augment the immunogenicity of DNA vaccines is the co-inoculation of plasmids encoding cytokines that has some advantages such as simplicity, ease of cloning procedure, and cost benefits. Since the cytokine is expressed and acts at the site of antigen expression, thus the risk of toxicity of systemically administered cytokines will be minimized [81]. In 2001, Guo et al. [100] evaluated the efficacy of pcIFN-γ as genetic adjuvant in the outcome of a DNA vaccine encoding ROP1 with or without adjuvant. They reported that the proliferation response of spleen T cells, natural killer cells (NK cells) killing activity, the serum concentrations of IFN-γ, IL-2, and nitric oxide in BALB/c mice vaccinated with pcROP1+pcIFN-γ regimen were markedly higher than in those immunized with pcROP1 alone, however, no obvious difference was shown in terms of IgG antibody values between the two groups. The authors concluded that pcIFN-γ is able to considerably elicit the cellular immune responses [100]. The pivotal role of IL-2 for the growth, multiplication, and differentiation of T lymphocytes is well known, as well as contributed in the prevention of parasitic invasion process combined with IFN-γ [85,115].

IL-12 is a pivotal proinflammatory cytokine for control and restriction of acute and chronic toxoplasmosis [127]. This critical cytokine is secreted by MQs and dendritic cells (DCs) during antigen stimulation and has a crucial role in the activation of NK cells and development of a Th1-biased immune responses and IFN-γ production which is indispensable for limit to *T. gondii* [85,93,98,127]. IL-12 has been frequently investigated as a proper genetic adjuvant against toxoplasmosis [36,93,94,98,99,101,102]. For instance, Quan et al. (2012) [36] reported that co-administration of IL-12 plus pGRA7-ROP1 in BALB/c vaccinated mice leads to increase survival time, in which 28 days post challenge with the lethal dose, 50% of them were survived. Moreover, the number of tissue cysts in the brain significantly reduced [36]. At another study, Khosroshahi et al. (2012) [98] compared the effect of IL-12 and alum as genetic and non-genetic adjuvants, receptively, on the efficiency of cocktail DNA vaccine that encoded psSAG1+psROP2. They found that co-administration of IL-12 and alum with psSAG1+psROP2 enhanced the survival time of experimental groups than controls ($p<0.05$). Moreover, increased levels of IgG antibody, high levels of IFN-γ and low levels of IL-4 were also observed, compared with control groups. Noteworthy, no significant difference was found between IL-12 and alum adjuvants to induce immune responses [98]. The same results also was found by Zhang et al. (2007) [93] on a multi-antigenic DNA vaccine encoding pSAG1-ROP2 combined with pIL-12.

IL-18 is an important cytokine with diverse functional roles. This cytokine potentially enhances the NK cell activity and also synergize with IL-12 to stimulate NK cell production of IFN-γ by T cells [104,105]. Alike to IL-12 cytokine, the predominant role of IL-18 is the boost of immune response toward Th1-type responses. For the mentioned reason, it can confer as a candidate adjuvant to be involved in the *Toxoplasma* resistance [104-106]. For this purpose, Wang et al. (2012) [54] designed an investigation to evaluate the immunogenicity and immunoprotection of TgROP13 by constructing pVAX-ROP13 alone or with IL-18 as genetic adjuvant. They reported that co-immunization of pVAX-ROP13 with pIL-18 in Kunming mice dramatically ($p<0.05$) enhanced the survival duration, compared with pVAX-ROP13 alone (32.3±2.7 and 24.9±2.3 days, respectively) against highly virulent RH strain (type I). The rate of reduction of brain cyst load in the mice immunized with pVAX-ROP13 and pVAX-ROP13+pVAX-IL-18 was 39.82% and 66.03%, respectively ($p<0.05$), after challenge with PRU strain (type II). Besides, co-delivery of pVAX-IL-18 plus pVAX-ROP13 considerably provoked the secretion of IFN-γ, IL-2, IL-4, and IL-10 cytokines, compared with the group that injected pVAX-ROP13 alone. The above-mentioned valuable findings suggest that ROP13 triggered strong humoral and cellular responses against the parasite and can be considered as a potentially efficient vaccine candidate. Also, the use of pVAX-IL-18 as a genetic adjuvant successfully boosted the protective immunity in terms of humoral and cellular responses, prolonged the survival time as well as with fewer brain cysts [54]. Thus, it seems that IL-18 cytokine could be extensively effective as Th1 adjuvant for future studies on other ROPs.

As noted, careful selection of a suitable genetic adjuvant would enhance the protective immunity of DNA vaccines and perhaps is a pivotal strategy to induce an appropriate response [78,80,81,97]. B7 costimulatory molecules such as B7-1 (CD_{80}) and B7-2 (CD_{86}) have an important role in providing costimulatory signals which are extremely necessary for the generation and maintenance of antigen-specific immune response. Hence, if a plasmid which expressing B7-1 or B7-2 as costimulatory molecules co-injected with a plasmid DNA vaccine, it is anticipated that can highly stimulate vaccine-elicited specific antibodies and $CD8^+$ responses [87,107,108]. It has been shown that co-administration of plasmid encod-

 https://doi.org/10.7774/cevr.2019.8.1.4

ing B7-2 was more efficient than that encoding B7-1 for the generation of antigen-specific cytotoxic T lymphocytes (CTLs) response [128,129]. In this regard, Liu et al. (2014) [87] constructed a multiantigenic DNA vaccine expressing the ROP16 and GRA7 (pROP16, pGRA7, and pROP16-GRA7) antigens and a plasmid encoding murine costimulatory molecule B7-2 (pB7-2) as a genetic adjuvant and evaluated the protective efficiency of these antigens with or without pB7-2 in Kunming mice against acute toxoplasmosis. They showed immunization with pROP16-GRA7 produced higher levels of IgG titers (predominance of IgG2a over IgG1), increased the secretion of IFN-γ, enhanced the percentage of $CD8^+$ T cells and median survival times, compared with those of mice received pROP16 or pGRA7 and those in control groups after lethal challenge with 1×10^3 tachyzoites of RH strain. Noteworthy, the formulation of pB7-2 with multiantigenic DNA vaccine (pROP16-GRA7) or single-gene vaccine (pROP16 or pGRA7), significantly boosted humoral and cellular immune responses as well as the survival duration of time. The authors proposed that B7-2 would be a feasible and promising genetic adjuvant to increase protective immunity, however, further studies are required in future [87].

Recombinant Protein Vaccines

In several past years, constant efforts of investigators have made considerable advances to elucidate the different aspects of the cell and molecular biology of *T. gondii* [25,26,29,32,33,45,49,55,56,70,130]. These attempts thereby provided great impetus to the present focus on vaccine development, according to the defined subcellular components of the parasite. Among the main identified *T. gondii* antigens, the ROPs appear as putative vaccine candidates that are vital for invasion procedure as well as survival within host cells [24,82]. Recombinant subunit vaccines are one of the current approaches that may offer an alternative way for the development of vaccine candidates against toxoplasmosis in humans and animals. These vaccines are able to elicit systemic humoral and cell mediated responses as well as being apt for large-scale production [82].

ROP5 is responsible for the major virulence of parasite and involved in the intracellular proliferation. This protein significantly decreases the accumulation of immunity-related GTPases in parasitophorous vacuole membrane (PVM), thereby maintains the PVM integrity [26,32]. To evaluate the protective efficacy of recombinant form of ROP5 (rROP5), Zheng et al. (2013) [48] performed a study to understand this issue. They vaccinated the BALB/c mice subcutaneously with 100 μg protein+Freund's complete adjuvant in first immunization and 100 μg protein+Freund's incomplete adjuvant (FIA) in second and third immunization. Enhanced IgG titers ($p<0.01$), mixed Th1/Th2 responses with the predominance of IgG2a over IgG1 ($p<0.05$), high production of IFN-γ, IL-2, IL-4, and IL-10 cytokines ($p<0.05$) as well as prolonged survival time ($p<0.05$) was observed as the outcome of vaccination, compared with control groups. Besides, co-injection of rSAG1 with rROP5 boosted the protective efficiency. As it is clear, immunization with compound polyvalent vaccine has better efficacy than a single antigen. The authors proposed evaluation of the brain cyst burden in both immunized and control groups using low virulence strains of parasites can helpful in future studies [48]. Noteworthy, IL-2 in combination with IFN-γ play a key role in preventing parasitic invasion [85,116].

The use of traditional and molecular adjuvants has become attractive recently because of their potential ability in eliciting specific and long-lasting protective immunity [97]. Also, admirable attempts have been made to introduce novel delivery systems to boost protective efficiency [82,97]. Aluminum salt-based adjuvants (alum) have been utilized as vaccine adjuvant since 1926 and is the most common used vaccine adjuvant in men. They are widely used in various vaccine formulations with some advantages such as enhance stability and immunogenicity of antigen following the adsorption to the alum particles [97]. It is well known that alum increases the expression of MHC II and adhesion or costimulatory molecules, including intercellular adhesion molecule 1, lymphocyte function-associated antigen 3, and CD40. Also, it has been reported that alum absorption enhances antigen uptake at the site of injection and augment the antigen phagocytosis by professional APCs such as DCs, MQs, and B cells as well as induce the production of some chemokines including the chemokine (C-C motif) ligand 2, the chemokine (C-X-C motif) ligand 1 and CCL11 in mice [97,131]. Noteworthy, alum is unable to induce a strong cell mediated response (Th1 or CTL) that are critical to limit and elimination of intracellular parasites [97]. In 2004, a paper by Martin et al. [113] was published that were evaluated the efficacy of alum adjuvant on the immunogenicity of rROP2 and rGRA4 in C57BL/6 (H-2^b) and C3H (H-2^k) mouse models. These experimental models have different MHC haplotypes and different susceptibility to the parasite. Vaccination with rROP2-rGRA4-alum regimen resulted significantly reduction ($p<0.01$) in brain cyst

load after oral challenge with 20 (sublethal dose) ME49 tissue cysts. While, immunization with rROP2-alum, only was considerably reduced the number of brain cysts in C3H (H-2^k) mice ($p<0.01$). They concluded that use of alum adjuvant could be used in vaccination against *T. gondii* infection [113].

Oligodeoxynucleotides contained CG motifs (CpG-ODN) were illustrated that could increase antigen-specific immunity to protein vaccines in a variety of hosts ranging from mouse models, humans and various veterinary species [132]. These adjuvants are strong enhancers of Th1-biased immune responses via activation of TLR-9 dependent cascades [80]. Nevertheless, the formulation of CpG-ODN adjuvant as a vaccination approach against *T. gondii* has been rarely investigated [109,110,133-136] and investigations, according to CpG-ODN+ROPs of *T. gondii* has very limited explored [109]. Lately, were shown co-inoculation of rROP2+ CpG-ODN elicit a strong humoral and Th1-biased immune responses with the predominance of IgG2a over IgG1 and enhanced the production of IFN-γ and IL-10 and negligible values of IL-4 cytokines. The brain cyst burden significantly reduced in the C3H/HeN (H-2^k) mice vaccinated with rROP2+ CpG-ODN (63%, $p<0.001$) and rROP2+rGRA4+CpG-ODN (66%, $p<0.001$) following a non-lethal challenge with 20 tissue cysts of Me49 (type II) strain orally. The authors have declared CpG-ODN is a potential adjuvant which can induce strong Th1-type immune responses, however, they proposed more studies are needed because of the different pathogenicity of *T. gondii* strains [109].

Due to easily proteolytically degradation of recombinant proteins that entails, the more frequent immunizations, a favorable delivery system to protect from degradation would be indispensable [137]. More recently, some studies have focused on polylactide-co-glycolide (PLG), a biodegradable and biocompatible polymer as a safe delivery system for antigens. PLG can extend the protein releasing period to induce a long-lasting immune response and reduce the protein degradation, thereby, increase the uptake of antigen and its presentation by APCs [115,117]. PLG also considered as a potent vaccine adjuvant as well as encapsulate the recombinant subunit vaccines and can maintain their antigenicity to elicit an efficient protection [82,114-117]. More recently, rROP18 and rROP38 were encapsulated into PLG microparticles to prolong the antigenicity. Vaccination of Kunming mice with rROP18 and rROP38 entrapped into PLG enhanced significantly humoral and cellular immune responses in terms of total IgG titers ($p<0.01$), IgG2a subclass ($p<0.01$), IFN-γ cytokine ($p<0.01$), and mixed Th1/Th2 immunity responses (but bias to Th1) as well as reduction of brain tissue cysts ($p<0.01$). The use of mixed antigen (rROP38+rROP18+PLG) boosted the protective immunity. For example, the percentage of $CD4^+$ and $CD8^+$ T cells were enhanced with the brain tissue cyst reduction of 81.3%. The authors have declared that PLG polymer microparticles with preserving the protein immunogenicity for extended duration, could be a promising novel adjuvant, however, further studies are required [115].

As we all know, *T. gondii* infects a wide spectrum of hosts, mainly via the mucosal surfaces of the digestive tracts. Also, this opportunistic agent can attack all nucleus cells and easily spread throughout the body. Hence, the development of potential vaccine candidate that capable to augment systemic and mucosal immunity has of great priority [138,139]. The nasal route of vaccination as a non-invasive and needle-free strategy has high priority in immunization investigations [140]. The i.n route is superior than the oral route because of requires fewer antigen and less proteolytic activity in the nasal cavity. Besides, i.n route could induce both systemic and mucosal protective immunity to recombinant protein antigen [112, 141]. Increased stimulation of IgG and IgA antibodies have been frequently demonstrated in papers following nasal administration [102,112,118,138,140]. The specific IgG antibodies have a key role in restriction of *T. gondii* infection and involved in the activation and promotion of the classical complement pathway, MQs phagocytosis and block invasion, other vital roles against intracellular parasites [84]. Many evidence showed specific secretory immunoglobulin A (SIgA) has a key role in mucosal surfaces and acts as the first line defense against several infectious agents that colonize mucosal tissues such as *Toxoplasma* [138,139]. In this context, previously, rROP2+Quil-A adjuvant and rROP2-GRA5-GRA7+cholera toxin (CT) adjuvant have been tested as immunogens to vaccinate BALB/c mice intranasally and shown acceptable responses including increased IgG and IgA titers. Moreover, rROP2+Quil-A elicited a significant lymphocyte proliferation response and rROP2-GRA5-GRA7+CT lead to 58.3% protection against brain cyst formation (after oral challenge with 50 cysts from VEG strain), compared with the control group ($p<0.05$) [112,118]. More recently, Wang et al. (2014) [138] were reported that nasal immunization of BALB/c mice, elicited IgG antibody production ($p<0.01$), promoted mixed Th1/Th2 immune response ($p<0.05$) with the predominance of IgG2a over IgG1, increased production of IFN-γ ($p<0.01$), IL-2 ($p<0.01$), and IL-4 cytokines ($p<0.05$), enhanced SIgA antibody titers in the nasal, vaginal and intestinal washes of rROP17-vacci-

 https://doi.org/10.7774/cevr.2019.8.1.4

nated mice ($p<0.01$), increased survival rate (75% protection 30 days post challenge with 4×10^4 tachyzoites of RH strain orally, $p<0.01$) and reduced the liver and brain parasite burden ($p<0.05$), compared with the control group. The authors remarked that intranasal vaccination of rROP17 can strongly provoke both systemic and mucosal immunity and would be considered as a potential vaccine candidate against *T. gondii* infection [138]. The examples of vaccination with protein vaccines against *T. gondii* in different mouse models have been summarized in Supplementary Tables 3 and 4.

Epitope Mapping and Epitope-Based Vaccines

Recognize and construct novel vaccines for prevent and control of toxoplasmosis in both humans and animals is still remains a great challenge for researchers to select highly potential protein sequences as the ideal antigens [19,82]. Bioinformatics is an interdisciplinary science that analyzes the biological data by using defined technologies and algorithms from mathematics, statistics, computer sciences, physics, medicine and biology [142]. Bioinformatics had many advantages than the traditional methods including: affordable and required low-cost, effective, satisfactory precision and accuracy and required lower times [142,143]. Recently, this novel science became popular and widely employed for various purposes such as predict protein structures, functions, biological characteristics, and epitopes as well as the design of new vaccines [51,130,142,143]. Particularly, prediction of epitopes is an indispensable tool in the immunogenicity design and reverse Vaccinology [66,143]. Several papers were found through database searching about ROP-based vaccines that analyzed the potential B and T cells epitopes using bioinformatics online servers to introduce novel vaccine candidates [27,39,51, 59,66,110,144-147]. However, for some of them there is a lack of confirmation of the protective efficacy in mouse models. Since the immunogenicity of the predicted sequences should be approved in suitable animal models, therefore, both *in silico* and *in vivo* approaches are required to evaluate the potency of protein as vaccine candidates [82,130].

The life cycle of *T. gondii* is very complex, indicating the presence of a plurality of antigenic epitopes. It has been proven that vaccination with stage-specific antigens only lead to stage-limited protection [130,148]. Accordingly, immune responses against *T. gondii* antigens that are express in various stages of parasite life cycles, are presumably more efficient and such vaccines likely confer increased survival time and lower brain cyst load than control groups. Thus, vaccination with compound polyvalent vaccines probably to be more efficient over a single antigen. Bioinformatics method helps researchers to predict the highly potential B and T cell epitopes [59,82,110,145,147]. Recently, the use of multi-epitope vaccines has become popular as a novel strategy in vaccine design against the opportunistic agent of toxoplasmosis, *T. gondii*. An ideal epitope-based vaccine should contain both B and T-cell epitopes that are vital for eliciting antibody responses and induce CTL responses, respectively [130]. In this context, for the first time, in 2008 Cong et al. [110], constructed a DNA vaccine encoding multi-epitope gene (MEG) including several putative immunodominant T-cell and B-cell epitopes of *T. gondii* SAG1 (59-67), SAG1 (246-256), GRA1 (176-186), ROP2 (199-216), and GRA4 (235-245) and CpG motif, with or without $CTXA_2$/B as a genetic adjuvant and then tested in BALB/c mice. After immunization, increased levels of IgG antibody in the mice immunized with pVAX1-MEG ($p=0.009$) and pVAX1-MEG-$CTXA_2$/B group ($p=0.006$) were recorded, compared than negative controls. Furthermore, in subsets of IgG, the predominance of IgG2a over IgG1 (especially in mice immunized with pVAX1-MEG-$CTXA_2$/B) was observed. In addition, IgG2a levels in the group vaccinated with pVAX1-MEG-$CTXA_2$/B were markedly higher compared with a pVAX1-MEG immunization regimen ($p<0.001$), whereas similar concentrations of IgG1 titers existed between these groups ($p=0.834$). CTL activity was enhanced, mainly in mice immunized with pVAX1-MEG-$CTXA_2$/B. After cytokine assay, the results showed pVAX1-MEG-$CTXA_2$/B immunized mice had higher amounts of IFN-γ and IL-2 than pVAX1-MEG group ($p=0.009$). Eventually, these results lead to prolonged survival time ($p<0.05$ and $p<0.001$ in mice immunized with pVAX1-MEG and pVAX1-MEG-CTXA2/B, respectively) following the challenge of mice with 1×10^3 tachyzoites of highly virulent RH strain, compared with three control groups. pVAX1-MEG-$CTXA_2$/B immunization regimen resulted 20% survival rate in this group, while all mice in other groups succumbed. As evident, all of the above-mentioned findings suggests that the formulation of CpG motif and $CTXA_2$/B as adjuvants in combination of this DNA vaccine, considerably boosted the protective efficacy against acute infection of *T. gondii* [110]. Until now, toll-like receptor (TLR-1–TLR-13) genes have been discovered in men that have critical roles in the innate immune system [80,149]. Oligodeoxynucleotides contained CG motifs (CpG ODN) as the TLR-9 ligand, was shown to be ef-

fective to boost the immunogenicity of DNA vaccines [80,96]. CpG motifs are also able to activate the DCs and stimulate the production of some cytokines from them such as type I IFN from $CD11c^{+}B220^{+}$ plasmacytoid DCs or IFN-γ and IL-12p70 from $CD11c^{+}CD8^{+}B220^{-}$ DCs [96]. Examples of vaccination experiments with epitope-based vaccines against *T. gondii* in mouse models have been embedded in Supplementary Table 5.

Previously, the antigenic characteristics of ROP19 and SAG1 were analyzed and compared together using bioinformatics databases. For this purpose, the Immune Epitope Database (IEDB) online service was employed to predict the T-cell epitopes and linear B-cell epitopes of the antigens. The DNASTAR software showed that ROP19 is superior to SAG1 in terms of antigenic index and surface probability. The authors claimed that ROP19 had good linear B-cell epitopes compared to SAG1. Additionally the 50% inhibitory concentration (IC_{50}) values of peptides binding to the MHC class II molecules of ROP19 were also predicted and lower IC_{50} values (low percentile rank=high level binding) were estimated for ROP19 indicating that ROP19 has viable T-cell epitopes [66]. These researchers, were performed a similar study with same design on ROP54 and SAG1. The linear-B cell epitopes analysis showed the superiority of ROP54 than SAG1 in terms of antigenic index and significant surface probability. Besides, Th-cell epitopes on ROP54 also were analyzed by the bioinformatics methods to predict the capability of binding to MHC class II molecules. Briefly, the minimum percentile ranks for 4 different MHC II alleles were chosen and listed on SAG1 and ROP54. Overall, the IC_{50} values for ROP54 were estimated lower than SAG1 (low percentile=high binding) which indicates better Th-cell epitopes. The authors concluded that the bioinformatics prediction of ROP54 sequence on linear-B cell epitopes and Th-cell epitopes revealed positive results with high potentiality to become an excellent vaccine candidate for toxoplasmosis [144]. ROP54 as a novel rhoptry protein pseudokinase is associated with the PVM after being injected into the host cell [70,144].

In 2016 Zhou et al. [27], for the first time performed a new survey on ROP48 with multiple bioinformatics approaches to predict some characteristics of the protein sequence in terms of physical and chemical features, epitope, and topological structure. They demonstrated that ROP48 was mainly located in the membrane. Moreover, several positive B- and T-cell epitopes with favorable flexibility and surface probability also were identified, which indicated positive antigenicity, suggesting this protein could be a potential DNA vaccine candidate against toxoplasmosis for future studies [27]. Camejo and colleagues reported that deletion of ROP48 in a type II strain (Δrop48 parasites) did not show significant affect on the *in vitro* growth or virulence in female C57BL/6 J mice [23].

Vaccines Based on Live-Attenuated Vectors

Since *Toxoplasma* is an obligatory intracellular protozoa, the use of live, attenuated vectors (bacteria or viruses) as vehicles to deliver and express the parasite antigen, can mimic the intracellular niche of *T. gondii* [19,83]. It has been shown that this immunization strategy against *T. gondii* infection capable to provoke a strong humoral and cell mediated immune responses lead to high protection or complete protection in some studies, because of its intrinsic adjuvant properties and/or mimicry of a natural infection [147,150-154]. These vaccine types can also be administered intramuscularly, intraoral, intranasal, subcutaneous, and intravenous and induce effective immune responses and protection in terms of enhancing the survival time and/or reduce the brain cyst burden [146,147].

Bacille Calmette–Guerin (BCG), an attenuated strain derived from *Mycobacterium bovis*, has been employed widely during recent decades as a live vaccine against tuberculosis and has peculiar intrinsic adjuvant properties thereby develop the cellular type responses within host [155,156]. Moreover, some advantages are cost effectiveness, ease of production, relatively thermostable as well as is unable to revert toward virulent phenotype [153,157]. Both *M. bovis* BCG and *T. gondii* are obligatory intracellular microbes, thus, the use of recombinant BCG as a foreign antigen delivery system would be very suitable for the vaccine development against toxoplasmosis [153,156]. Immunization with recombinant *M. bovis* BCG expressing TgROP2 (BCG/pMV262-ROP2) was found to elicit both humoral and cellular immune responses and increase survival rate post challenge intraperitoneally with 5×10^{2} tachyzoites of RH strain in BALB/c mice ($p<0.05$). The authors concluded that *M. bovis* BCG is an adequate vector to express TgROP2 antigen. Nevertheless, they proposed further studies are required to evaluate the protective efficiency of BCG/pMV262-ROP2 in other mouse models such as C57BL/6 and C3H mice [153].

Vaccinia virus as a prominent vehicle increase the antigen presentation to the immune system of the host and is a powerful immunostimulant against those antigens that normally not identified by the immune system [154,158]. A study

 https://doi.org/10.7774/cevr.2019.8.1.4

showed that a new recombinant modified vaccinia virus Ankara (MVA) expressing TgROP2 (MVA ROP2) induced mixed Th1/Th2 immune response (predominance of IgG2a over IgG1) and prolonged the survival duration (11 days vs. 8-9 days, $p=0.04$) post challenge with 300 tachyzoites of RH strain in female Swiss mice. However, the brain cyst load do not differ between experimental and control mice. Interestingly, all mice vaccinated with ts-4 strain of *T. gondii* survived following challenge. The authors remarked that MVA ROP2 generated an effective immune response which lead to delaying the mortality time [154].

It is well known that the use of recombinant viral vectors has the excellent ability to elicit effectual expression of the foreign antigens, thereby, help the presentation and stimulation of humoral and cellular immune responses [159]. For example, it has been reported that adenoviral vectors are safe and efficient for transgene expression *in vivo* as well as having intrinsic adjuvant properties which lead to activation the innate immune response through TLRs and nod-like receptors [160,161]. Adenoviruses (Ad) are considered as popular vaccine vectors and have been used extensively, because of their powerful capability to provokation the T-cell mediated immunity [151,152]. Since, there is frequently pre-existing immunity against the classically human adenovirus type 5 (AdHu5), alternatively canine adenovirus type 2 (CAV-2) has been suggested as vectors for human gene transfer [162]. Besides, the use of CAV-2 has the following advantages: well-characterized biology of CAV-2, ease of genetic manipulation, suitable for gene transfer into the central nervous system, able to induce strong protective immunity of humoral and cellular immune responses [152,163]. Thus, currently CAV-2 is appointed as one of the most applicable non-human adenoviruses for vaccine vector purposes [151,152]. In this case, Li et al. (2016) [151] constructed a novel recombinant CAV-2 carrying TgROP16 (CAV-2-ROP16) and then evaluated the immune response and survival status of BALB/c mice. CAV-2-ROP16 was able to elicit significantly both humoral and cell mediated responses (mixed IgG1 and IgG2a levels with the predominance of IgG2a titers, $p<0.05$) and increased production of IFN-γ, IL-2, and IL-4 ($p<0.05$). Furthermore, the enhanced survival rate (25% protection 80 days after challenging with 1×10^3 tachyzoites of RH strain) was observed, compared with control mice that died within 7 days ($p<0.05$). They showed this system could markedly enhance the protection with eliciting humoral and cell-type immune responses [151]. The same authors were demonstrated that CAV-2-ROP18 has also been potentially capable to induce the same immune response with CAV-2-ROP16. Briefly, CAV-2-ROP18 immunization elicited a strong IgG antibody response ($p<0.05$), increased levels of a mixed IgG1 and IgG2a ($p<0.05$) with the predominance of IgG2a production, enhanced splenocyte proliferation (about 21-fold than control groups, $p<0.05$), enhanced production of IFN-γ, IL-2, and IL-4 cytokines ($p<0.05$), increased CTL activity in Kunming mice ($p<0.05$), and elevated numbers of $CD4^+$ T and $CD8^+$ T cells ($p<0.05$). The immunized mice with CAV-2-ROP18 showed 40% protection (60-day post infection intraperitoneally with 1×10^3 tachyzoites of RH strain), while all control mice died within seven days ($p<0.05$). Besides, 57.3% reduction of brain tissue cyst burden was recorded following challenge intragastrically via oral gavage with 5 cysts of PRU strain (genotype II) ($p<0.05$). The authors declared that the potential utilization of a CAV vector carrying the TgROP18 gene in the development of a useful vaccine against chronic and acute toxoplasmosis in future investigations [152]. It should be noted in addition to cell-type immune responses, humoral immunity with high production of antigen-specific IgG antibodies are vital to limit the *T. gondii* invasion by preventing the attachment of parasites to host cell receptors [84]. Furthermore, $CD8^+$ T cells, especially in synergy with $CD4^+$ T cells are very critical for the control of toxoplasmosis [152,164]. Noteworthy, the safety concerns and hazards was outlined regarding the use of live or attenuated vectors and need the more consideration during their development in the future that must be further investigated [165]. Supplementary Table 6 listed the examples of immunization with live-attenuated vectors expressing *T. gondii* antigens in mouse models.

Prime-Boost Strategies

The prime-boost approaches such as DNA prime/viral vector boost (i.e. using Adenovirus, fowlpox, vaccinia virus, etc.), DNA prime/protein boost, and protein prime/DNA boost have been shown extensively to be an efficient strategy to induce both cellular and humoral immune responses against some micro-organisms like human immunodeficiency virus (HIV), *Plasmodium, Leishmania*, *T. gondii*, etc., which would further provide a foundation for the development of appropriate vaccine candidates [81,145,166-170]. Homologous prime-boost approach involves the similar formulation employed in both the prime and boost regimens, while heterologous prime-boost strategies contains different formulations used in more

than one immunization [81,169]. Notably, the interval between prime and boost is extremely important for vaccine response and excellent efficacy. In addition, the arrangement of vaccination may influence the outcome of prime–boost strategies [80,171]. Some papers suggested that heterologous prime-boost regimens are more effectual than the homologous prime-boost [169,172]. Heterologous prime-boost strategy predominantly uses a DNA or a viral vector for priming and a protein-based vaccine for boosting [172].

It is well established that different vaccination strategies lead to different immune response. For instance, as noted, heterologous prime-boost regimens strongly elicit both of humoral and cell-mediated immunity against an antigen using each delivery system individually. In the other hand, subunit vaccines often provoke a predominant humoral immune response, whereas DNA vaccines or recombinant live vector-based vaccines mainly elicit an efficient cellular immunity [169]. Li et al. (2011) [168] reported that a DNA prime/protein boost immunization based on ROP2 and SAG1 (pcROP2-SAG1/rROP2-SAG1+FIA), pcROP2-SAG1 and rROP2-SAG1 formulations could elicit similar humoral and cellular immunity against toxoplasmosis. However, the BALB/c mice immunized with the rROP2-SAG1 enhanced humoral response (IgG specific antibodies with the predominance of IgG1 over IgG2a), slightly increased IFN-γ production and more vigorous specific lymphoproliferative responses, compared with other antigen formulations [168]. In another study, Yin et al. (2015) [145] designed an excellent and comprehensive investigation to evaluate the efficacy of a *T. gondii* vaccine encoding all stages of the parasite antigens. At first, main antigens present in sporozoite, tachyzoites, and bradyzoite stages were predicted for $CD8^+$ T cell epitopes conserved regions based on their binding affinity to human leukocyte antigen (HLA-A*02, HLA-A*03, and HLA-B*07) and H2 (H2-Ld, H2-Dd, and H2-Kd) supertype molecules using bioinformatic algorithms from IEDB online service. Then protein fragments of $SAG3_{101–144}$, $ROP18_{347–396}$, $MIC6_{288–347}$, $GRA7_{182–224}$, $MAG1_{58–125}$, $BAG1_{156–211}$, and $SPA_{142–200}$ were selected and ubiquitin-conjugated multi-stage antigen segments (UMAS) plasmid DNA were constructed. They reported that among different formulations of prime/boost regimens (DNA/DNA, Ad/Ad, DNA/Ad, and Ad/DNA), priming with DNA and boosting with Ad-UMAS elicited higher values of specific IgG (predominance of IgG2a over IgG1) and higher production of IFN-γ (1,691±35.18 pg/mL) and IL-2 (561±19.68 pg/mL) cytokines were achieved, compared with p-UMAS or Ad-UMAS injection alone ($p<0.05$). Also p-UMAS prime/Ad-UMAS boost regimen significantly increased survival rate (67%, 28 days post challenge intraperitoneally with 1×10^3 tachyzoites of RH strain) compared than controls which died within 8-10 days and reduced the brain cyst load ($p<0.01$) [145]. Ubiquitin is a 76-amino-acid peptide which documented increase DNA vaccine responses against targeted antigen in the adjuvant setting [145]. Conjugating ubiquitin to a DNA construct was determined to increase the proteasome dependent degradation of endogenously synthesized antigens, thereby elicit cellular immune responses toward the conjugated antigen in animal models [145,146,173,174]. Heterologous vaccination oftentimes shows a powerful synergistic effect in comparison to homologous regimens [80]. Some studies on vaccines against hepatitis B virus [175], hepatitis C virus [176], HIV [177], and *T. gondii* [145] have been emphasized that the best effective prime-boost approaches recruit priming with a DNA vaccine and then followed by recombinant adenovirus vaccine as boosting. The examples of heterologous prime-boost vaccination against *T. gondii* in mouse models have been inserted in Supplementary Table 7.

Conclusion

T. gondii can infect a wide spectrum of warm-blooded vertebrate species. Toxoplasmosis is almost asymptomatic in immunocompetent individuals, however, in immunocompromised persons may be cause severe complications or even may result in death if not treated. Since, current common drugs are unable to prevent from *T. gondii* infection in both humans and animals and also have no effect on the encysted parasites within infected hosts, thus, the development of an effective vaccine urgently needed to prevent and control toxoplasmosis. During the two past decades, the different vaccine types with various strategies have been tested experimentally worldwide. However, currently there is a lack of a licensed commercial vaccine for human applications. The vaccination with stage-specific antigens only lead to stage-limited protection. Accordingly, recently the use of epitope mapping for design of multi-epitope vaccines has become popular as a novel approach against toxoplasmosis. Moreover, these vaccine types remove undesirable factors which often lead to improve the highly specific responses and better protection. The use of live-attenuated vectors as vehicles to deliver and express the antigen are another strategy for vaccine development that demonstrated excellent protection up to 60% for ROP-based antigens. Also, heterologous prime-boost regimens appear

 https://doi.org/10.7774/cevr.2019.8.1.4

very effective that showed up to 67% survival rate. Notably, frequently was shown that the use of traditional and molecular adjuvants as well as delivery systems has become attractive recently because of their potential ability in eliciting specific and long-lasting protective immunity. Collectively, the results are widely diverse, but extremely valuable findings have been obtained, so that they gave promising perspectives for future investigations. It should be mentioned that several limitations might influence the outcomes of experimental vaccine studies because of the following reasons: unsuitable immunization protocol, inadequate evaluation criterion, the vaccine construct, the strain of *T. gondii*, dosage of inoculum, the delivery route, the various mouse models, etc. The future investigations should be addressed all these facets to minimize the faults. Also optimize immunization protocol and use of different types of delivery systems, genetic and/or non-genetic adjuvants surely would affect the findings.

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Supplementary Material

Supplementary materials are available at Clinical and Experimental Vaccine Research website (http://www.ecevr.org).

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Supplementary Table 1. Baseline characteristics of included studies based on immunization experiments with *T. gondii* DNA-encoding ROPs in mouse models (single antigens)

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP1	-	Plasmid, i.m	BALB/c	NR	Elicited cellular and humoral immune responses ↑ Proliferative activity of spleen T lymphocytes ($p<0.01$) ↑ Percentages of $CD8^+$ T cells ($p<0.05$) The numbers of $CD4^+$ T cells showed no obvious increase compared with the control group ↑ NK cell activity ($p<0.05$)	NR	NR	pcROP1 could elicit both cellular and humoral immune responses in vaccinated mice.	[1]
	pcIFN-γ	Plasmid, i.m	BALB/c	NR	↑ Levels of IgG antibody in sera of pcROP1 and pcROP1+pcIFN-γ groups, compared with the control group ($p<0.01$) ↑ NK cell activity, especially in pcROP1+pcIFN-γ group ($p<0.05$) ↑ proliferative activity of spleen T lymphocytes in mice immunized with pcROP1 and pcROP1+pcIFN-γ (especially in the latter ($p<0.01$) The levels of IFN-γ and NO in the pcROP1+pcIFN-γ group was significantly higher than that with pcROP1 alone ($p<0.05$) ↑ IL-2 in vaccinated mice with pcROP1+pcIFN-γ, compared with the controls ($p<0.05$) The levels of IL-2 in the pcROP1+pcIFN-γ group was significantly higher than that with pcROP1 alone ($p<0.05$)	NR	NR	The genetic adjuvant pcIFN-γ could enhance the cellular immune response induced by DNA vaccine of pcROP1 in mice against *T. gondii* infection.	[2]
	pIL-12	Plasmid, i.m	BALB/c	6 Mice were challenged with a lethal dose of Me49 strains (1,500 cysts per mouse) 5 Mice per group were challenged with a nonlethal dose of strain Me49 (20 cysts per mouse), orally	↑ IgG titers Predominance of the levels of IgG2a over IgG1 (especially in mice immunized with pROP1+pIL-12) ↑ IL-10, IFN-γ, and TNF-α ($p<0.05$) ↑ splenocyte proliferation ($p<0.05$)	Reduced ($p<0.01$)	Increased survival rate ($p<0.01$)	The study showed that a DNA vaccine expressing the *T. gondii* ROP1 Ag induced specific humoral and cellular immune responses and immunization with vaccine combined with pIL12 induces greater Th1-type immune responses and protective efficacy against *T. gondii* infection.	[3]
	Alum	Plasmid, i.m	BALB/c	5×10^5 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ IgG2a ($p<0.05$) ↑ IFN-γ (1,161.00±76.10 pg/mL and 433.00±51 pg/mL for pcROP1 and pcROP1+alum groups, respectively, $p<0.05$)	NR	Increased survival time (7 days compared with 6 days in control)	The study showed that ROP1 DNA vaccine can induce partial protective response against toxoplasmosis.	[4]
	-	Plasmid, i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced humoral immune response ↑ SI (2.04±0.12, $p<0.001$) ↑ IFN-γ (712±28.1 pg/mL, $p<0.001$) and IL-4 (94±14.5 pg/mL, $p<0.01$)	NR	Increased survival rate (50%, 23-day post challenge, $p<0.05$) All control mice died within 9 days.	These findings proposed that the ROP1 Ag is a potential candidate for the development of vaccine against toxoplasmosis. Complete protection may be achieved by combining ROP1 with other immunogenic rhoptry antigens.	[5]

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https://doi.org/10.7774/cevr.2019.8.1.4 http://www.ecevr.org/

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Supplementary Table 1. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP2	-	Plasmid, i.m	C57BL/6 (H-2^b), BALB/c (H-2^d) and CBA/J (H-2^k)	6×10^3 Tachyzoites, RH strain, s.c	↑ IgG in mice of all three strains (especially in BALB/c mice) ↑ IgG1 in mice of all three strains (especially in BALB/c and C57BL/6 mice) ↑ IgG2a (BALB/c: homogeneous response; CBA/J: heterogeneous response) ↑ IgG2c in C57BL/6 mice Induced mixed Th1/Th2 response	NR	All DNA-immunized mice of the three strains died after the challenge. Nevertheless, in BALB/c mice a slight delay (2 days) was observed, compared with control (p=0.04).	These results suggest that plasmid immunization using the ROP2 gene generates a mixed Th1/Th2 response against ROP2.	[6]
	-	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	↑ IgG antibodies ($p<0.05$) ↑ Splenocyte proliferation ↑ IFN-γ (335.00 ± 9.7982 pg/mL, $p<0.05$), IL-2 (200.82 ± 8.7593 pg/mL, $p<0.05$), and TNF-α (198.91 ± 9.2450 pg/mL, $p<0.05$)	NR	Increased survival time (7 days compared with 5 days in control)	-	[7]
	-	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	↑ Ratio IgG2a to IgG1 ↑ IFN-γ (651 ± 120 pg/mL) and IL-12 (430 ± 36 pg/mL)	NR	Increased survival time (10 days compared with 7-8 days in control)	-	[8]
	-	Plasmid, i.m	C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H (H-2^k)	Acute: C57BL/6: 10 cysts of strain IPB-G (a zymodeme II type strain) BALB/c: either 50 or 200 cysts of strain IPB-G, orally C3H: 50 cysts of strain IPB-G or 76K, orally Chronic: BALB/c and C3H: 25 cysts of strain IPB-G, orally C57BL/6: 10 cysts of strain 76K, i.p	Induced a strong antibody response ↑ Splenocyte proliferation in BALB/c ($p<0.05$) and C3H ($p<0.01$) mice ↑ IFN-γ in BALB/c ($p<0.01$) and C3H ($p<0.01$) mice	C3H: Reduced ($p<0.05$)	C3H (challenged with 50 cysts of strain IPB-G): 90% survival during 20 days ($p<0.001$) C3H (challenged with 50 cysts of strain 76K): 100% survival during 20 days ($p<0.02$) C57BL/6 (challenged with 10 cysts of strain IPB-G): 20% survival during 20 days BALB/c (challenged with 200 cysts of strain IPB-G): 20% survival during 20 days BALB/c (challenged with 50 cysts of strain IPB-G): 90% survival during 20 days	In this study, we show that DNA immunization with potentially protective *T. gondii* Ag induces both humoral and cellular immune responses in mice of three different genetic backgrounds. In addition, we show that in one mouse strain, DNA vaccination not only reduces the mortality associated with the acute phase of infection, but also limits the parasite load during the chronic phase of the disease.	[9]

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Supplementary Table 1. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP5	-	Plasmid, i.m	Kunming	Acute: 1×10^3 tachyzoites, RH strain (type I), i.p Chronic: 10 tissue cysts PRU strain type II), orally	Induced a strong IgG antibody response ($p<0.05$) Predominance of IgG2a over IgG1 (IgG2a/IgG1 ratio: 1.51 ± 0.03) ↑ Splenocyte proliferation (SI: 3.32 ± 0.05, $p<0.05$) ↑ Levels of the Th1 cytokines IFN-γ, IL-2, IL-12p70, and IL-12p40 ($p<0.05$) ↑ Levels of IL-4 and IL-10 ($p<0.05$) ↑ Percentages of $CD4^+$ T and $CD8^+$ cells ($p<0.05$) Cell-mediated cytotoxic activity with increased frequencies of IFN-γ secreting $CD8^+$ T cells ($p<0.05$)	Reduction in brain tissue cysts load (57.4%, $p<0.05$)	Increased survival time (19.4 ± 4.9 days, $p<0.05$) All control mice died within 9 days.	Results demonstrated that a potential DNA vaccine expressing the *T. gondii* ROP5 elicit Th1-biased responses, as well as $CD8^+$ cell-mediated cytotoxic T cell response, against acute or chronic *T. gondii* infection in mice.	[10]
	-	Plasmid, i.m	BALB/c	Acute: 1×10^4 tachyzoites, RH strain, i.p Chronic: 20 cysts PRU strain type, i.g	↑ IgG antibodies ($p<0.05$) The predominance of IgG2a over IgG1 ↑ IFN-γ (672.6 ± 43.17 pg/mL) and IL-2 (256.89 ± 11.81 pg/mL) ($p<0.05$)	Reduced ($p<0.05$)	Increased survival time ($p<0.05$)	Our results showed that a DNA vaccine encoding ROP5 significantly enhanced protection against *T. gondii* challenge.	[11]
ROP7	-	Plasmid, i.m	BALB/c	Acute: 1×10^4 tachyzoites, RH strain, i.p Chronic: 20 cysts PRU strain, i.g	↑ IgG antibodies ($p<0.05$) The predominance of IgG2a over IgG1 ↑ IFN-γ (662.76 ± 42.42 pg/mL) and IL-2 (264.42 ± 18.31 pg/mL) ($p<0.05$)	Reduced ($p<0.05$)	Increased survival time ($p<0.05$)	Our results showed that a DNA vaccine encoding ROP7 significantly enhanced protection against *T. gondii* challenge.	[11]
ROP8	-	Plasmid, i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ production (816 ± 26.3 pg/mL, $p<0.05$) and IL-4 (148 ± 18.3 pg/mL, $p<0.05$)	NR	Increased survival rate (50%, 29- day post challenge, $p<0.05$) Control mice died within 9 days.	Results presented in this study suggest that ROP8 DNA is a promising and otential vaccine candidate against toxoplasmosis.	[12]
ROP9	-	Plasmid, i.m	Kunming	1×10^3 Tachyzoites, RH strain, i.p	↑ IgG antibodies ($p<0.05$) High ratio (1.69) of IgG2a to IgG1 demonstrating that immunization of pVAX-ROP9 primarily induced a Th1 type response. ↑ Splenocyte proliferation ($p<0.05$) ↑ Percentages of $CD4^+$ and $CD8^+$ T cells ($p<0.05$) ↑ IFN-γ (466.62 ± 12.72 pg/mL, $p<0.05$), IL-2 (305.88 ± 15.02 pg/mL, $p<0.05$), IL-4 (231.72 ± 9.89 pg/mL, $p<0.05$), and IL-10 (212.00 ± 16.23 pg/mL, $p<0.05$)	NR	Prolonged survival time in mice (12.9 ± 2.9 days; $p<0.05$) Control mice were died within 6 days.	The results of the present study showed that immunization with a TgROP9 plasmid induced strong humoral and cellular Th1-type immune responses, and prolonged survival time against lethal challenge. Although TgROP9 elicited only partial protection against acute toxoplasmosis, it could be used as a potential vaccine candidate in further studies of multi-component. *T. gondii* vaccines against toxoplasmosis in the mice model.	[13]

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Supplementary Table 1. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP13	pIL-18	Plasmid, i.m	Kunming	Acute: 1×10^3 tachyzoites, RH strain (type I), i.p Chronic: 10 cysts PRU strain (type II), orally	↑ Level of IgG antibodies in the sera of mice immunized with pVAX-ROP13 and pVAX-ROP13 plus pVAX-IL-18 ($p<0.05$) ↑ Lymphocyte response compared with the control ($p<0.05$) ↑ IFN-γ in mice immunized with pVAX-ROP13 plus pVAX-IL-18 (1,107.28±26.74 pg/mL, $p<0.05$) and pVAX-ROP13 (826.75±18.91 pg/mL, $p<0.05$) ↑ IL-2 in mice immunized with pVAX-ROP13 plus pVAX-IL-18 (934.52±13.03 pg/ml, $p<0.05$) and pVAX-ROP13 (793.07±22.09 pg/mL, $p<0.05$) ↑ IL-4 in mice immunized with pVAX-ROP13 plus pVAX-IL-18 (257.54±4.17 pg/mL, $p<0.05$) and pVAX-ROP13 (163.23±6.05 pg/mL, $p<0.05$) ↑ IL-10 in mice immunized with pVAX-ROP13 plus pVAX-IL-18 (246.02±10.61 pg/mL, $p<0.05$) and pVAX-ROP13 (160.49±3.14 pg/mL, $p<0.05$)	pVAX-ROP13 plus pVAX-IL-18: Reduction in brain tissue cyst load (66.03%, $p<0.05$) pVAX-ROP13: Reduction in brain tissue cysts load (39.82%, $p<0.05$)	Increased survival time pVAX-ROP13: 24.9±2.3 days, $p<0.05$ pVAX-ROP13 plus pVAX-IL-18: 32.3±2.7 days, $p<0.05$ Control mice were died within 10 days.	The results suggest that ROP13 DNA vaccine induced strong protective humoral and cellular responses against *T. gondii*, indicating that it has the potential to be a vaccine candidate worthy of further development. The use of an IL-18-encoding plasmid as an adjuvant successfully enhanced the immune protection and survival time of immunized mice. Further studies are warranted to evaluate the immune efficacy of this DNA vaccine construct in other animal host species against toxoplasmosis.	[14]
ROP16	-	Plasmid, i.m	Kunming	1×10^3 Tachyzoites, RH strain, i.p	↑ Specific anti-ROP16 IgG ($p<0.05$) ↑ Th1 immune responses ↑ Splenocyte proliferation (~8-fold higher than control, $p<0.05$) ↑ IFN-γ (918±12.77 pg/mL, $p<0.05$), IL-2 (887.33±24.94 pg/mL, $p<0.05$), IL-4 (172.67±7.51 pg/mL, $p<0.05$), and IL-10 (168±19.52 pg/mL, $p<0.05$)	NR	Increased survival time (21.6±9.9 days), compared with that of control mice, which died within 7 days after challenge ($p<0.05$).	*T. gondii* ROP16 should provide a promising vaccine candidate against toxoplasmosis, worth further evaluation and development using other animal species.	[15]
	pB7-2	Plasmid, i.m	Kunming	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) The predominance of IgG2a over IgG1 ($p<0.05$) ↑ Percentage of $CD8^+$ T cells ($p<0.05$) ↑ IFN-γ ($p<0.05$)	NR	Increased survival time ($p<0.01$)	The formulation of pB7-2 with pROP16, resulted in dramatically enhanced antibody titers, both Thl nd $CD8^+$ T cell mediated immune responses.	[16]
ROP17	-	Plasmid, i.m	BALB/c	1×10^3 Tchyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.01$) Elicited both Th1- and Th2-specific responses (IgG2a/IgG1 ratio >1) ↑ IFN-γ (186.17±11.47 pg/mL) and IL-2 (158.41±11.38 pg/mL), $p<0.05$ Increased number of $CD8^+$ T cells ($p<0.05$)	NR	Prolonged survival time (15.6±5.4 days, $p<0.05$) Control mice were died within 4 to 8 days.	Despite the partial protective efficacy of the DNA vaccine, ROP17 appears to be a potential candidate for the development of vaccines against toxoplasmosis.	[17]

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Supplementary Table 1. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP18	pIL-12	Plasmid, i.m	CBA/J ($H\text{-}2^k$)	60 Cts of the 76 K strain, orally	↑ Secific IgG antibody Mixed Th1/Th2 response The predominance of IgG2a over IgG1 in mice immunized with pROP18 and pROP18-pIL-12 ↑ IFN-γ and IL-2 in mice immunized with pROP18 and pROP18-pIL-12, compared to controls ($p<0.05$) Similar percentage of $CD8^+$ T cells between vaccinated and control group ($p>0.05$)	None significant	NR	These results suggest that ROP18 could be component of a subunit vaccine against toxoplasmosis and could lead to more encouraging results.	[18]
	-	Plasmid, i.m	Kunming	1×10^3 Tchyzoites, RH strain, i.p	↑ Secific IgG antibody ($p<0.05$) ↑ Ratio IgG2a to IgG1 ↑ $CD4^+$ and $CD8^+$ T cells in the spleen ($p<0.05$) ↑ IFN-γ (1,008.67 ± 32.47 pg/mL, $p<0.05$), IL-2 (980 ± 54.84 pg/mL, $p<0.05$), IL-4 (149 ± 12.49 pg/mL, $p<0.05$), and IL-10 (143 ± 9.64 pg/mL, $p<0.05$)	NR	Increased survival time (27.9 ± 15.1 days) Control mice were died within 7 days.	For the first time was shown that a ROP18 vaccine construct can enhance the *T. gondii*-specific CTL. Th1 responses and increased survival suggested that ROP18 is a promising vaccine candidate against infection with *T. gondii*.	[19]
	-	Plasmid, i.m	ICR	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response with predominance of IgG2a over IgG1 ($p<0.05$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ (427 ± 40 pg/mL, $p<0.05$) and IL-4 (56 ± 9 pg/mL, $p<0.05$)	NR	Increased survival time (16 days compared with 7 days in control, $p<0.05$)	The study indicates that the introduction of multiantigenic DNA vaccine is more powerful and efficient than single-gene vaccine.	[20]
ROP19	-	Plasmid, i.m	BALB/c	20 Cysts PRU strain, i.g	↑ Levels of IgG antibodies ($p<0.05$) ↑ IFN-γ (485.04 ± 64.559 pg/mL, <0.05)	Reduced ($p<0.05$)	NR	The results suggest that the DNA vaccine encoding ROP19 induced a significant immune response and provided protection against a challenge with *T. gondii* strain PRU cysts.	[21]
ROP38	-	Plasmid, i.m	Kunming	Acute: 1×10^3 tachyzoites, RH strain (type I), i.p Chronic: 10 cysts PRU strain (type II), orally	↑ Level of IgG antibody ($p<0.01$) The predominance of IgG2a over IgG1 Proliferation SI measured at $OD_{570\,nm}$ in mice vaccinated with pVAX-ROP38 (0.90 ± 0.02) was similar to that immunized with PBS (0.91 ± 0.01), pVAX I (0.89 ± 0.07), and blank control (0.97 ± 0.01) ($p>0.05$) ↑ Percentages of $CD4^+$ and $CD8^+$ T cells than control ($p<0.01$) No significant differences in the ratio of $CD8^+/CD4^+$ between mice immunized with pVAX-ROP38 and in controls ($p>0.05$) ↑ IFN-γ (575.2 ± 123.0, $p<0.01$) and IL-2 (195.3 ± 28.4, $p<0.05$) ↓ IL-10 ($p<0.01$)	Reduction in brain tissue cyst burden (76.6%, $p<0.01$)	Increased survival time (10 days compared with 8 days in control, $p>0.05$)	The present study revealed that the pVAX-ROP38 vaccine could elicit strong humoral and cellular immunity response against chronic *T. gondii* infection in mice, resulting in the reduction of the brain cyst formation effectively, which suggests that TgROP38 is a desirable vaccine candidate against chronic *T. gondii* infection.	[22]

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Supplementary Table 1. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP54	-	Plasmid, i.m	Kunming	Acute: 1×10^3 tachyzoites, RH strain, i.p Chronic: 10 cysts of the PRU strain, orally	Induced a strong IgG antibody response ($p<0.05$) Mixed IgG1/IgG2a response with the predominance of IgG2a over IgG1 ↑ Proliferation SI (1.90 ± 0.02, $p<0.05$) ↑ IFN-γ (986.9 ± 14.74 pg/mL, $p<0.05$), IL-2 (360.98 ± 20.45 pg/mL, $p<0.05$), IL-12 p-70 (310.4 ± 21.57 pg/mL, $p<0.05$), IL-4 (90.59 ± 8.45 pg/mL, $p<0.05$), and IL-10 (131.71 ± 15.22 pg/mL, $p<0.05$)	Reduced (35.9%, $p<0.05$)	Increased survival time (13.0 ± 1.15 days, $p<0.05$) All control mice died within 8 days	These results indicate that the recombinant ROP54 plasmid can provide partial protection and might be a potential vaccine candidate against acute and chronic toxoplasmosis.	[23]

↑, increase; Ag, antigen; CTLs, cytotoxic T lymphocytes; i.g, intragastrically; i.m, intramuscular; i.p, intraperitoneally; IFN-γ, interferon-γ; IL, interleukin; NK cells, natural killer cells; NO, nitric oxide; NR, not reported; PBS, phosphate buffered saline; ROP, rhoptry protein or rhoptry antigens; s.c, subcutaneous; SI, stimulation index; *T. gondii*, *Toxoplasma gondii*; Th, T helper; TNF-α, tumor necrosis factor α.

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Supplementary Table 2. Baseline characteristics of included studies based on immunization experiments with *T. gondii* DNA-encoding ROPs in mouse models (mixed antigens)

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP1+SAG1	Liposome	Plasmid, i.m	BALB/c	NR	Induced a strong IgG antibody response ($p<0.05$) ↑ IL-2 (163 ± 25 pg/mL) and IFN-γ (1,950 ± 110 pg/mL) significantly	NR	NR	Immunization with a liposome-encapsulated DNA construct encoding the *T. gondii* SAG1-ROP1 can induce humoral and cell-mediated immune responses.	[24]
ROP1+GRA7	pIL-12	Plasmid, i.m	BALB/c	6 Mice were challenged with a lethal dose of Me49 strains (1,500 cysts per mouse) 5 Mice per group were challenged with a nonlethal dose of strain Me49 (20 cysts per mouse), orally	↑ IgG titers ($p<0.01$) The predominance of the levels of IgG2a over IgG1 ↑ IL-10, IFN-γ, and TNF-α ($p<0.05$) ↑ splenocyte proliferation ($p< 0.05$)	Reduced ($p<0.01$)	Increased survival rate ($p<0.01$) pROP1-GRA7: 33.3% survival rate 4 weeks after infection pROP1-GRA7+pIL-12: 50% survival rate 4 weeks after infection All control mice were died within 18 days.	The study suggest that the multiantigenic DNA antigen pGRA7-ROP1 was very effective in stimulating host protective immune responses than separately injected single antigens, and that IL-12 serves as a good DNA adjuvant.	[3]
ROP2+SAG1	-	Plasmid, i.m	BALB/c	1×10^5 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response Th1-type response with the predominance of the levels of IgG2a over IgG1 ↑ Splenocyte proliferation significantly ↑ IFN-γ, compared with the controls ($p<0.05$) IL-4 was undetectable in splenocyte supernatants from all experimental and control animals	NR	Increased survival time ($p<0.01$)	The results demonstrated that DNA cocktail immunization might be an important approach to achieve a multi-component vaccine against *T. gondii*, particularly with respect to generating an efficient, long-lasting protective immune response.	[25]
	-	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) Predominance of the levels of IgG2a over IgG1 ↑ Splenocyte proliferation ↑ IFN-γ (687 ± 136 pg/mL, $p<0.05$) and IL-12 (456 ± 48 pg/mL, $p<0.05$)	NR	Increased survival time (12 days compared with 8 days in control)	The current study showed that multi-antigenic DNA produced potent, effective and long-term protection against *T. gondii* challenge.	[26]
	pIL-12 and Alum	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	↑ IgG antibodies ($p<0.05$) than control groups (especially in the group immunized with pcSAG1+pcROP2+alum) ↑ IFN-γ ($p<0.05$) ↓ IL-4 in the group immunized with pcROP2+pcSAG1 ($p<0.05$)	NR	Increased survival time (10 days in the group immunized with pcROP2+pcSAG1 compared with 5 days in control, $p<0.05$)	The results of the study showed that use of adjuvants (IL-12 and alum) coincident with DNA cocktail leads to significant change in the survival time of the experiment groups in comparison with control groups. Also, there is no significant difference between adjuvants to induce immune responses.	[27]

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Supplementary Table 2. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
	-	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ IFN-γ (902 ± 155.8 pg/mL, $p<0.05$) and IL-4 (52.0 ± 17 pg/mL, $p<0.05$) The predominance of IgG2a over IgG1	NR	Increased survival time (11 days compared with 6 days in control)	The cocktail DNA containing the recombinant plasmids can be an appropriate candidate for immunization against toxoplasmosis.	[28]
	pIL-12	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) The predominance of IgG2a over IgG1 IgG2a values in the pSAG1-ROP2 plus pIL-12 immunized group were significantly higher than the pSAG1-ROP2 immunized group ($p<0.001$). ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ and IL-12 significantly ↓ IL-4 significantly	NR	Increased survival time compared to controls, but not complete protection (death within 11 to 16 days) The protection induced by pSAG1-ROP2 was markedly enhanced by pIL-12 co-administration (death within 16 to 22 days)	The study indicates that the introduction of multiantigenic DNA vaccine is more powerful and efficient than the single gene vaccine, and the co-delivery of pIL-12 further enhanced the potency of multiantigenic DNA vaccine.	[8]
	$pCTXA_2$/B and pIL-12	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	Higher levels of IgG antibodies in the sera of mice immunized with pSAG1-ROP2 combined with pIL-12 ($p<0.01$). The predominance of the levels of IgG2a over IgG1 in all three groups (especially in mice immunized with pcDNA3.1-SAG1-ROP2+pIL-12) ↑ Splenocyte proliferation (especially in mice immunized with pcDNA3.1-SAG1-ROP2+pIL-12, $p<0.001$) ↑ IFN-γ and IL-12 (especially in mice immunized with pcDNA3.1-SAG1-ROP2+pIL-12, $p<0.05$) ↓ IL-4 in the group (especially in mice immunized with pcDNA3.1-SAG1-ROP2+pIL-12, $p<0.05$)	NR	Increased survival time (pcDNA3.1-SAG1-ROP2+pIL-12, $p<0.05$)	The results show that the IL-12 is superior to $CTXA_2$/B as vaccine adjuvant of anti-*T. gondii* by i.m challenge. It may be potentially a better choice as a vaccine adjuvant, which provides a basis for further research on cytokine adjuvants and the relationship between the properties of adjuvants and the route of immunization.	[29]

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Supplementary Table 2. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP2+SAG1+SAG2	pIL-12	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	↑ Levels of IgG (especially in mice immunized with pROP2-SAG1-SAG2+pIL-12, $p<0.05$) The predominance of the levels of IgG2a over IgG1 in all groups (especially in mice immunized with pROP2-SAG1-SAG2+pIL-12) ↑ Splenocyte proliferation (pIL-12 augmented splenocytes proliferation over 4 fold more than the group immunized without the co-administration of pIL-12, $p<0.01$). ↑ IFN-γ and IL-12 (especially in mice immunized with pROP2-SAG1-SAG2+pIL-12) ↓ IL-4 in mice immunized with pROP2-SAG1-SAG2+pIL-12 ($p<0.05$)	NR	Increased survival time (pROP2-SAG1-SAG2 and pROP2-SAG1-SAG2+pIL-12: 25 days and 31-32 days respectively, compared with 8 days in control)	Multiantigenic DNA vaccine expressing SAG1, ROP2 and SAG2 is more potent than single gene vaccine and double gene vaccine. Simultaneous murine IL-12 expression plasmid vaccination could enhance the potency of a multiantigenic DNA vaccine. These results will contribute to the development of an efficient and long-term protective immunity against *T. gondii*.	[30]
ROP2+SAG1+GRA2	pIL-12	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) The predominance of the levels of IgG2a over IgG1 ↑ Splenocyte proliferation ($p<0.05$) IL-12 augmented splenocyte proliferation about 2.5 fold more than the group immunized with pSAG1–ROP2–GRA2 ($p<0.01$) ↑ IFN-γ and IL-12 significantly ↓ IL-4 significantly	NR	Increased survival time pROP2-SAG1-GRA2: death within 18 days pROP2-SAG1-GRA2+pIL-12: death within 23 days Control mice were died within 8 days.	The use of IL-12 encoding plasmid as an adjuvant, successfully enhanced the level of protection induced by the multiple antigens encoding plasmid alone, and would be a promising immunization protocol. Thus, this immunization regimen may represent an effective vaccine strategy for generating an efficient long-term protective immunity against *T. gondii* infection.	[26]
ROP2+GRA5	-	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ IFN-γ (892 ± 196 pg/mL, $p<0.05$) and IL-4 (68 ± 28.9 pg/mL, $p<0.05$) The predominance of IgG2a over IgG1	NR	Increased survival time (12 days compared with 6 days in control)	The cocktail DNA containing the recombinant plasmids can be an appropriate candidate for immunization against toxoplasmosis.	[28]
ROP2+GRA5+SAG1	-	Plasmid, i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ IFN-γ ($1,278\pm136$ pg/mL, $p<0.05$) and IL-4 (120 ± 48 pg/mL, $p<0.05$) The predominance of IgG2a over IgG1	NR	Increased survival time (12 days compared with 6 days in control)	The cocktail DNA containing the recombinant plasmids can be an appropriate candidate for immunization against toxoplasmosis.	[28]

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Supplementary Table 2. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP2+GRA1+ GRA7	-	Plasmid, i.m	C3H/HeN ($H\text{-}2^k$)	Acute: 100 *T. gondii* 76K cysts, orally Chronic: a sublethal dose of 20 brain cysts of *T. gondii* 76K, orally	↑ Antibody titers against the antigens from two gene DNA vaccine cocktails, but lower titers when immunized with the three-gene cocktail. ↑ IFN-γ	Reduced in the three-gene cocktail DNA vaccinated group (81%, $p<0.01$) Reduced in the pGRA7+pROP2 vaccinated group (79%, $p<0.05$) Reduced in the pGRA1+pROP2 vaccinated group (57%, none-significant) Reduced in the pROP2 vaccinated group (43%, none-significant)	Increased survival time after lethal challenge experiment in mice vaccinated with the three-gene DNA vaccine cocktail (complete protection, $p<0.01$), whereas 56% of control vaccinated mice succumbed to acute toxoplasmosis with a median survival time of 12 days.	The presence of GRA7 in the DNA vaccine formulation was important for optimal protection and this was correlated with GRA7-specific IFN-γ production. We propose GRA7 as a main component in cocktail DNA vaccines for vaccination against *T. gondii*.	[31]
ROP5+ROP7	-	Plasmid, i.m	BALB/c	Acute: 1×10^4 tachyzoites, RH strain, i.p Chronic: 20 cysts PRU strain, i.g	Induced a strong IgG antibody response High level of Th1 type immune response (predominance of IgG2a over IgG1) ↑ IFN-γ (1,109.52±129.66 pg/mL, $p<0.05$) and IL-2 (511.59±70.14 pg/mL, $p<0.05$)	Reduced ($p<0.05$)	Mice vaccinated with pROP5/ROP7 showed a longer survival time than single-gene-immunized mice ($p<0.05$) or control mice ($p<0.05$)	The results suggest that the multiple-gene vaccine had the ability to partly protect mice against virulent and low-virulent *T. gondii* strains.	[11]
ROP5+GRA15	-	Plasmid, i.m	Kunming	Acute: 1×10^3 tachyzoites, RH strain type I), i.p Chronic: 10 tissue cysts PRU strain type II), orally	Induced a strong IgG antibody response ($p<0.05$) The predominance of IgG2a over IgG1 (IgG2a/IgG1 ratio: 1.72±0.03) ↑ Splenocyte proliferation (SI: 4.45±0.05, $p<0.05$) ↑ Levels of the Th1 cytokines IFN-γ, IL-2, IL-12p70, and IL-12p40 ($p<0.05$) ↑ Levels of IL-4 and IL-10 ($p<0.05$) ↑ Percentages of $CD4^+$ T and $CD8^+$ cells ($p<0.05$) Cell-mediated cytotoxic activity with increased frequencies of IFN- γ secreting $CD8^+$ T cells ($p<0.05$)	Reduction in brain tissue cysts load (79%, $p<0.05$)	Increased survival time (22.7±7.2 days, $p<0.05$) All control mice died within 9 days	Co-immunization of pVAX-ROP5 and pVAX-GRA15 boosted the immune responses and increased protective efficacy against *T. gondii* infection compared to single antigen vaccines.	[10]

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Supplementary Table 2. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP16+GRA7	pB7-2	Plasmid, i.m	Kunming	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.01$) The predominance of IgG2a over IgG1 (especially for the pROP16-GRA7 co-delivery with pB7-2 group) ↑ Percentage of $CD4^+$ and $CD8^+$ T cells ($p<0.05$) ↑ IFN-γ especially for the pROP16-GRA7 co-delivery with pB7-2 group ($p<0.05$)	NR	Increased survival time ($p<0.01$)	The formulation of pB7-2 with either a multiantigenic DNA vaccine (pROP16-GRA7) or a single-gene vaccine (pROP16 or pGRA7), all resulted in dramatically enhanced antibody titers, both Thl and $CD8^+$ T cell mediated immune responses, therefore, it might be a feasible method of boosting protective immunity induced by a recombinant DNA vaccine.	[16]
ROP18+MIC3	-	Plasmid, i.m	ICR	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) The predominance of IgG2a over IgG1 (IgG2a values in the pROP18–MIC3 immunized group were significantly higher than the single-gene immunized group ($p<0.05$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ (849±86 pg/mL, $p<0.05$) and IL-4 (66±14 pg/mL, $p<0.05$)	NR	Increased survival time (19 days compared with 7 days in control, $p<0.05$)	Our study indicates that the introduction of multi-antigenic DNA vaccine is more powerful and efficient than single-gene vaccine. These results suggested that multiantigenic DNA immunization might be an important approach to achieve an effective vaccine against *T. gondii*.	[20]

↑, increase; ↓, decrease; Ag, antigen; $CTXA_2/B$, A_2/B subunits of cholera toxin; GRA, dense granule antigens; i.g, intragastrically; i.m, intramuscular; i.p, intraperitoneally; IFN-γ, interferon-γ; IL, interleukin; MIC, microneme antigens or microneme proteins; NR, not reported; ROP, rhoptry protein or rhoptry antigens; SAG, surface antigens; SI, stimulation index; *T. gondii*, *Toxoplasma gondii*; Th, T helper; TNF-α, tumor necrosis factor α.

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Supplementary Table 3. Baseline characteristics of included studies based on immunization experiments with protein vaccines against *T. gondii* in mouse models (single antigens)

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP1	FCA and FIA	Protein (10 μg)+FCA for prime injection+FIA for boosters, s.c	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced humoral immune response ↑ SI (3.04±0.21, p<0.001) ↑ IFN-γ (1,457±31.19, p<0.001) and IL-4 (186±14.17, p<0.01)	NR	Increased survival rate (mean survival of 29 days, p<0.05)	These findings proposed that the ROP1 Ag is a potential candidate for the development of vaccine against toxoplasmosis. Complete protection may be achieved by combining ROP1 with other immunogenic rhoptry antigens.	[5]
ROP2	Alum	Protein (10 μg), i.m	C57BL/6 (H-2^b) and C3H (H-2^k).	20 (sublethal dose) or 100 (lethal dose) ME49 tissue cysts, orally	-	20 (sublethal dose) ME49 tissue cysts, orally C57BL/6: reduced (none significant) C3H: reduced (p<0.01)	100 (lethal dose) ME49 tissue cysts, orally There were no significant differences in the survival rates from both strains of immunized mice compared to the control groups	The results reinforce the value of alum as a possible adjuvant to be used in immunization against *T. gondii*, allowing the development of a vaccine for wide application for either humans or animals. We consider that combinations with other effective antigens that generate immunity by different strategies should also be taken into account in the future.	[32]
	CpG-ODN	Protein (10 μg)+CpG (10 μg), i.m	C3H/HeN (H-2^k)	20 (sublethal dose) tissue cysts, Me49 (Type II) strain, orally	Induced a strong humoral Th1-biased response High IgG2a to IgG1 antibody ratio ↑ IFN-γ and IL-10	Reduced (63%, p<0.001)	NR	Our results indicate that CpG-ODN is an important candidate adjuvant for use in potential vaccines against this pathogen.	[33]
	Quil-A	Protein (10 μg)+10 μg Quil-A, i.n	BALB/c	NR	↑ IgG (in 5/10 mice, 50%) and IgA (in 2/10 mice, 20%) antibodies in sera of mice at day 62 of the experiment Elicited significant lymphocyte proliferation response	NR	NR	These results indicate that intranasal immunization with recombinant protein ROP2 plus Quil-A can elicit both cellular and humoral immune responses in BALB/c mice.	[34]
ROP5	FCA and FIA	Protein (100 μg)+ FCA+2 boosters in FIA, s.c	BALB/c	1×10^2 Tachyzoites, RH strain, i.p	↑ Level of IgG antibodies (p<0.01) Induced mixed Th1/Th2 immune responses with the predominance of IgG2a over IgG1 ↑ IFN-γ, IL-2, IL-4, and IL-10 (p<0.05) ↑ Splenocyte proliferation (p<0.05)	NR	Prolonged survival time (p<0.05)	This study demonstrated the novel finding that ROP5 induced a strong protective humoral and cellular response against *T. gondii* infection, which indicated that it is a potential vaccine candidate against toxoplasmosis.	[35]

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Supplementary Table 3. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP17	-	Proteins (15, 25, 35 or 45 μg of rTgROP17), i.n	BALB/c	1×10^4 and 4×10^4 tachyzoites of RH strain for chronic and acute assay, respectively, orally	↑ IgG antibody production of the mice immunized with 25, 35, and 45 μg rTgROP17 ($p<0.05$) ↑ IgG1 and IgG2a in the sera of all the mice immunized with rTgROP17, particularly in those immunized with 35 and 45 μg rTgROP17 ($p<0.05$) Mixed Th1/Th2 immune response (predominance of IgG2a over IgG1) ↑ Splenocyte proliferation ($p<0.01$) ↑ IFN-γ ($p<0.01$), IL-2 (p 0.01), IL-4 ($p<0.05$) ↑ Mucosal immune responses (SIgA antibody titers) in the nasal, vaginal and intestinal washes of rTgROP17-immunized mice ($p<0.01$)	↓ Liver and brain parasite burdens	Increased survival rate 30 days post challenge (75% protection, $p<0.01$)	The study suggests that intranasal immunization of mice with rTgROP17 can induce both systemic and local immune responses to provide protection against lethal *T. gondii* infection through reduction of the tachyzoite burdens in the host tissues and increases of the animal survivals. We conclude that ROP17 is a promising vaccine candidate against infection with *T. gondii*.	[36]
ROP18 Groups: ROP18S2+ Montanide™ ISA 71+poly I:C (s.c-M-P-ROP18), s.c ROP18S2+CT (i.n-CT-ROP18), i.n Control groups	Montanide ISA 71, poly (I:C) and CT	s.c and i.n	CBA/J ($H\text{-}2^k$)	60 Cysts of the 76 K strain, orally	Significantly higher specific IgG antibody in s.c-M-P-ROP18 group, compared with i.n-CT-ROP18 group ↑ Specific IgG antibody, compared to controls (especially in the s.c-M-P-ROP18 group) The predominance of IgG1 over the IgG2a ↑ Intestinal IgA response ↑ IFN-γ, IL-2, and IL-5 responses in immunized mice compared to the control groups ($p<0.05$) Mixed Th1/Th2 immune response with the predominance of Th1 Similar percentage of $CD8^+$ T cells between vaccinated and control groups ($p>0.05$)	Reduced for i.n-CT-ROP18 group (50%, $p<0.001$)	NR	These results suggest that ROP18 could be a component of a subunit vaccine against toxoplasmosis and that strategies designed to enhance mucosal protective immune responses could lead to more encouraging results.	[18]
ROP18	Re	Proteins (100 μg rROP18)+different dosages of Re (10, 50 or 100 μg), s.c	ICR	5×10^2 Tachyzoites, RH strain, i.p	↑ IgG antibody than control ($p<0.05$). Co-administration of rROP18 with Re (50 μg and 100 μg) induced numerically higher specific antibody level than that with10 μg Re ($p<0.05$) Mixed IgG1/IgG2a response, with the predominance of IgG1 production ↑ Splenocyte proliferation in mice immunized with rROP18+Re (50 μg and 100 μg, $p<0.05$) ↑ IFN-γ and IL-4 ($p<0.05$)	NR	Increased survival time (15 days in mice immunized with rROP18+Re, compared with 6 days in control, $p<0.05$)	The data demonstrate that by the addition of ginsenoside Re, the rROP18 triggered a stronger humoral and cellular response against *T. gondii*, and that Re is a promising vaccine adjuvant against toxoplasmosis, deserves further evaluation and development.	[37]

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Supplementary Table 3. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP18 Groups: G1. Montanide adjuvant only, i.p G2. 10 μg rROP18, i.p G3. 10 μg rROP18+ Montanide, i.p G4. PLGA only, i.n G5. rROP18-PLGA, i.n	Montanide and PLGA	Proteins (10 μg)+ PLGA, i.p and i.n	Swiss-Webster mice	NR	Significantly higher levels of IgG in mice immunized with rROP18-adjuvant, compared with rROP18 group ($p<0.05$) Significantly higher levels of IgG in mice immunized with rROP18-PLGA, compared with PLGA group ($p<0.05$) IgA levels in rROP18-PLGA were significant ($p<0.05$) as compared to PLGA The predominance of IgG2a over IgG1 in mice vaccinated with rROP18-PLGA ($p<0.05$) The predominance of IgG1 over IgG2a in mice vaccinated with rROP18-adjuvant ($p<0.05$)	NR	NR	It was concluded that nanospheres of ROP18 would be a non-invasive approach to develop vaccines against *T. gondii*. Further experiments are needed to determine the cellular response to these nanospheres in a mouse model for chronic toxoplasmosis.	[38]

↑, increase; Ag, antigen; CpG ODN, oligodeoxynucleotides contained CG motifs; CT, cholera toxin; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; i.m, intramuscular; i.n, intranasal; i.p, intraperitoneally; IFN-γ, interferon-γ; IL, interleukin; NR, not reported; PLGA, polylactide-co-glycolide acid; poly (I:C), polyinosinic–polycytidylic acid; Re, Ginsenoside Re; ROP, rhoptry protein or rhoptry antigens; s.c, subcutaneous; SI, stimulation index; SIgA, secretory immunoglobulin A; *T. gondii*, *Toxoplasma gondii*; Th, T helper.

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Supplementary Table 4. Baseline characteristics of included studies based on immunization experiments with protein vaccines against *T. gondii* in mouse models (mixed antigens)

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP2+GRA4	Alum	Protein (10 μg each), i.m	C57BL/6 (H-2^b) and C3H (H-2^k)	20 (sublethal dose) or 100 (lethal dose) ME49 tissue cysts, orally	-	20 (sublethal dose) ME49 tissue cysts, orally C57BL/6: reduced ($p<0.01$) C3H: reduced ($p<0.01$)	100 (lethal dose) ME49 tissue cysts, orally There were no significant differences in the survival rates from both strains of immunized mice compared to the control groups	The results reinforce the value of alum as a possible adjuvant to be used in immunization against *T. gondii*, allowing the development of a vaccine for wide application for either humans or animals. We consider that combinations with other effective antigens that generate immunity by different strategies should also be taken into account in the future.	[32]
	CpG-ODN	Protein (10 μg each)+CpG (10 μg), i.m	C3H/HeN (H-2^k)	20 (sublethal dose) tissue cysts, Me49 (type II) strain, orally	Induced a strong humoral Th1-biased response High IgG2a to IgG1 antibody ratio ↑ IFN-γ and IL-10	Reduced (66%, $p<0.001$)	NR	These results indicate that CpG-ODN is an important candidate adjuvant for use in potential multicomponent anti-*T. gondii* vaccines for animals and humans.	[33]
ROP2-LiHsp83 Groups: ROP2 LiHsp83 ROP2+LiHsp83 (mixture) ROP2-LiHsp83 (fused) PBS	*L. infantum* Hsp83	Footpad injections	BALB/c, C57BL/6 and C3H	Acute: 1×10^5 tachyzoites, RH strain, i.p Chronic: 20 cysts of the ME49 strain, orally	Mice immunized with fusion ROP2-LiHsp83 elicited a stronger humoral and cellular response in comparison to mice immunized with ROP2 alone, or a mix of LiHsp83 and ROP2. ↑ IFN-γ secretion and Th1 type response, with predominance of specific IgG2a/IgG2c isotype in mice immunized with fusion protein ROP2 alone or mixed with LiHsp83 induced a Th1/Th2 mixed response (predominance of IgG1 response) ↑ IFN-γ in ROP2-LiHsp83 immunized compared with other groups ($p<0.01$) ↑ IL-4 in mice immunized with ROP2 alone or mixed with LiHsp83, compared with ROP2-LiHsp83 or PBS ($p<0.05$) ↑ Splenocyte proliferation in mice immunized with ROP2-LiHsp83 compared with other groups ($p<0.01$), highest in BALB/c strain	C57BL/6 (reduced in all groups, especially in fusion ROP2-LiHsp83 group, $p<0.05$) C3H (reduced in all groups, especially in fusion ROP2-LiHsp83 group, $p<0.01$)	Increased survival time in C57BL/6 and BALB/c mice immunized with fusion ROP2-LiHsp83 Increased survival time in BALB/c mice immunized with ROP2 alone	In conclusion, here we demonstrate that a member of heat shock protein 90 family, LiHsp83, is a good candidate to carry antigens and develop an adjuvant-free vaccine. This carrier based vaccine system has the capability to produce an immunoresponse that activates antibody secretion, cytokine production and stimulates cellular immune response, all positive features to control parasite infection.	[39]

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Supplementary Table 4. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP2+GRA4+ SAG1	FIA	Proteins (total amount of each recombinant antigen: 10 μg)+FIA, s.c	C3H/HeJ (H-2^k), C57BL/6 (H-2^b), and BALB/c (H-2^d)	5 tissue cysts, DX strain (type II), i.p	Induced cellular and humoral immune responses	C57BL/6: Reduced (55%, p=0.021) C3H/HeJ: No significant reduction in brain tissue cysts, compared with control BALB/c: Reduced (46%, p<0.001)	NR	This study revealed that immunization with a mixture of recombinant antigens could be a very promising tool in immunoprophylaxis of toxoplasmosis	[40,41]
ROP2+ROP4	FCA and FIA	Protein (10 μg each)+FCA+2 boosters in FIA, s.c	C3H/HeJ (H-2^k)	5 tissue cysts, DX strain (type II), i.p	Predominance of IgG1 over IgG2a Both antigens generated a strong systemic mixed Th1/Th2 response polarized towards IgG1 antibody isotype ↑ Splenocyte proliferation significantly ↑ IFN-γ and IL-2 (p=0.008)	Immunization with rROP2 or rROP2 alone was not sufficient to reduce the brain cysts ROP2+ROP4: Reduced (46%, p=0.003)	NR	Results suggest that, similar to ROP2, ROP4 could be a very good candidate for future anti-*T. gondii* multi-component vaccine based on the recombinant forms of different parasite proteins.	[42]
ROP2+ROP4+GRA4	FIA	Proteins (total amount of each recombinant antigen: 10 μg)+FIA, s.c	C3H/HeJ (H-2^k), C57BL/6 (H-2^b), and BALB/c (H-2^d)	5 tissue cysts, DX strain (type II), i.p	Induced cellular and humoral immune responses	C57BL/6: Reduced (41%, p=0.042) C3H/HeJ: Reduced (59%, p=0.021) BALB/c: Reduced (84%, p<0.001)	NR	This study revealed that immunization with a mixture of recombinant antigens could be a very promising tool in immunoprophylaxis of toxoplasmosis	[40,41]
ROP2+ROP4+SAG1	FIA	Proteins (total amount of each recombinant antigen: 10 μg)+ FIA, s.c	C3H/HeJ (H-2^k), C57BL/6 (H-2^b) and BALB/c (H-2^d)	5 tissue cysts, DX strain (type II), i.p	Induced cellular and humoral immune responses	C57BL/6: Reduced (90%, p<0.001) C3H/HeJ: Reduced (71%, p<0.001) BALB/c: Reduced (77%, p<0.001)	NR	This study revealed that immunization with a mixture of recombinant antigens could be a very promising tool in immunoprophylaxis of toxoplasmosis.	[40,41]
ROP2+GRA5+GRA7	CT	Protein (12.5 μg each)+0.5 μg CT, i.n	BALB/c	50 Cysts from VEG strain, orally	↑ IgG antibody titers ↑ IgA antibody titers in intestinal washes, feces and sera	Reduced (p<0.05)	NR	These results indicate that i.n immunization in BALB/c mice with recombinant proteins rROP2, rGRA5 and rGRA7 associated with CT induced partial protection against tissue cyst formation after oral infection with tissue cysts from *T. gondii*.	[43]

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Supplementary Table 4. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP5+SAG1	FCA and FIA	Proteins (100 μg each)+FCA+2 boosters in FIA, s.c	BALB/c	1×10^2 Tachyzoites, RH strain, i.p	↑ Level of IgG antibodies ($p<0.01$) The predominance of IgG2a over IgG1 ↑ IFN-γ, IL-2, IL-4, and IL-10 ($p<0.05$) Mixed Th1/Th2 immune response. ↑ Splenocyte proliferation ($p<0.01$)	NR	Prolonged survival time (12.1 ± 3.4 days; $p<0.05$) compared to the control or single-Ag vaccinated groups.	The strategy of using ROP5 protein combined with other antigens appears to be a promising approach to develop a new subunit multi-component vaccine against toxoplasmosis by generating partial, but significant, pro-tective immune responses.	[35]
ROP5+ROP18 ROP5 (full-length ROP5) 1–549 (549 aa) ROP5-C (C-terminal ROP5) 278–549 (272 aa) ROP18 (full-length ROP18) 1–554 (554 aa) ROP18-C (C-terminal ROP18) 316–554 (239 aa) Groups: Blank control Adjuvant control ROP5 ROP18 ROP5+ROP18	poly I:C	Proteins, s.c	BALB/c and C3H/HeOuJ	Acute: 1×10^3 tachyzoites, RH strain (type I), i.p Chronic: 5 tissue cysts, DX strain (type II), i.p	Induced a significant IgG1 and IgG2a production in BALB/c and C3H/HeOuJ mice Induced a mixed type (Th1/Th2) immune response In most cases, the determined titres of Ag-specific antibodies were significantly higher in C3H/HeOuJ mice compared to those in BALB/c mice ($0.00101 \leq p \leq 0.0101$)	BALB/c: Reduced (only in the ROP18-immunized BALB/c mice were significant) C3H/HeOuJ: Reduced (only in ROP5+ROP18 immunized mice were significant)	Increased survival time 25% survival rate in the ROP18-immunized BALB/c mice	The results demonstrated that immunization with ROP5 and ROP18 proteins leads to the activation of both humoral and cellular immune mechanisms, resulting in the partial protection against highly virulent and cysts-forming strains of *T. gondii*. Although the outcomes of the experiments might not be fully satisfactory, these results provide additional evidence that ROP5 and ROP18 proteins may be valuable components of a multi-antigen vaccine against *T. gondii*.	[44]

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Supplementary Table 4. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP18+ROP38 Groups: Blank control PBS PLG rTgROP38 rTgROP18 rTgROP38+rTgROP18 PLG+rROP38 PLG+rROP18 PLG+rROP38+rROP18	PLG	Proteins+PLG, s.c	Kunming	10 cysts of the PRU strain (genotype II), orally	↑ Specific IgG antibody responses in the mice immunized with various proteins, compared with the control groups ($p<0.01$) ↑ Levels of IgG1 and IgG2a in the mice immunized with various proteins, compared with the control groups ($p<0.01$) Mixed Th1/Th2 immune response Lymphocyte proliferation indexes were similar between the vaccinated and control groups ($p>0.05$) ↑ $CD4^+$ T cells in the mice immunized with different protein vaccines (highest in PLG-rROP38-rROP18 group), compared with the control groups ($p<0.01$) ↑ $CD8^+$ T cells only in the mice immunized with PLG-rROP18 ($p<0.05$) or PLG-rROP38-rROP18 ($p<0.01$) ↑ IFN-γ in the mice immunized with different protein vaccines, compared with the control groups ($p<0.01$) ↑ IL-2 in the mice immunized with different protein vaccines, compared with the control groups (non-significant) ↓ IL-4 and IL-10 in mice immunized with protein vaccines, compared with the control groups ($p<0.05$)	Reduced in the mice immunized with various proteins, compared with the control groups ($p<0.01$) The best was PLG-rROP38-rROP18 (with a cyst reduction of 81.3%)	NR	The findings of the present study indicated that recombinant rhoptry antigens encapsulated in PLG could maintain the protein immunogenicity in an extended period and elicit effective protection against chronic *T. gondii* infection, which has implications for the development of long-lasting vaccines against chronic toxoplasmosis in animals.	[45]

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Supplementary Table 4. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP18+CDPK6 Main groups: PBS rROP18 rROP18+rCDPK6 rROP18+206 rROP18+rCDPK6+206 rROP18+PLG rROP18+rCDPK6+PLG	206 and PLG	Proteins (10 μg of each), s.c	Kunming	Acute: 1×10^3 tachyzoites, RH strain (type I), ip Chronic: 10 cysts of the PRU strain (type II), orally	↑ Specific IgG antibody responses in the mice immunized with rROP18+PLG or rCDPK6+rROP18+PLG ↑ Splenocyte proliferation significantly (highest in mice immunized with rCDPK6+rROP18+PLG, $p<0.001$), compared with the control groups ↑ $CD4^+$ and $CD8^+$ T cells in mice immunized with the various protein vaccines ↑ Levels of $CD4^+$ ($p<0.001$) and $D8^+$ T lymphocytes ($p<0.01$) in mice immunized with protein–PLG microparticles, compared with controls ↑ Percentages of $CD4^+$ cells in mice immunized with rROP18+206 and rROP18+PLG, compared with the controls ($p<0.05$) ↑ IFN-γ and IL-2 significantly in the mice immunized with various proteins, compared with the control groups ($p<0.05$) ↓ IL-4 and IL-10, compared with the control groups ($p<0.05$) Similar levels of IL-12 between vaccinated and control groups ($p>0.05$) Induced Th1-biased immune responses	Reduced (varied from 47.7% to 73.6%, $p<0.001$) The average survival time of the mice immunized with the various protein vaccines (8.56 days) was slightly longer than that in the controls (8 days) rROP18+PLG (10.9 ± 2.58 days compared with the controls, $p<0.05$) rROP18 (10.1 ± 1.52 days compared with the controls, $p<0.05$)	Increased survival time	These findings suggest that the two recombinant *T. gondii* proteins encapsulated in PLG conferred immunity to *T. gondii* for an extended period, providing the foundation for the further development of a commercial vaccine against toxoplasmosis.	[46]

↑, increase; ↓, decrease; 206, Montanide™ ISA 206 VG; Ag, antigen; CDPK, calcium dependent protein kinase; CpG ODN, oligodeoxynucleotides contained CG motifs; CT, cholera toxin; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; GRA, dense granule antigens; HSPs, heat shock proteins; i.m, intramuscular; i.n, intranasal; i.p, intraperitoneally; IFN-γ, interferon-γ; IL, interleukin; *L. infantum, Leishmania infantum*; LiHsp83, *Leishmania infantum* heat shock protein 83; NR, not reported; PBS, phosphate-buffered saline; PLG, polylactide-co-glycolide; poly (I:C), polyinosinic–polycytidylic acid; ROP, rhoptry protein or rhoptry antigens; s.c, subcutaneous; SAG, surface antigens; *T. gondii, Toxoplasma gondii*; Th, T helper.

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Supplementary Table 5. Examples of immunization experiments with epitope-based vaccines against *T. gondii* in mouse models

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MEG, containing T- and B-cell epitopes SAG1 (59-67), P1 SAG1 (246-256), P2 GRA1 (176-186), P3 ROP2 (199-216), P4 GRA4 (235-245), P5 Groups: pVAX1-MEG pVAX1-MEG-CTXA$_2$/B pVAX1-CTXA$_2$/B pVAX1 Saline	CpG motif and CTXA$_2$/B	Plasmid, i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	↑ Antibody response (IgG) in the mice immunized with pVAX1-MEG (p= 0.009) and pVAX1-MEG-CTXA$_2$/B (p=0.006) group, compared than negative controls ↑ Serum IgA response in the mice immunized with pVAX1-MEG-CTXA$_2$/B (p<0.05, compared to other immunized group) The predominances of the levels of IgG2a over IgG1 (especially in mice immunized with pVAX1-MEG-CTXA$_2$/B) Higher IgG2a values in mice immunized with pVAX1-MEG-CTXA$_2$/B, compared with pVAX1-MEG (p<0.001) and similar values of IgG1 between these groups (p=0.834) ↑ Splenocyte proliferation significantly (especially in mice immunized with pVAX1-MEG-CTXA$_2$/B) ↑ CTL activity (especially in mice immunized with pVAX1-MEG-CTXA$_2$/B) ↑ IFN-γ and IL-2 (p<0.05)	NR	Increased survival time (p<0.05 and p<0.001) in mice immunized with pVAX1-MEG and pVAX1-MEG-CTXA2/B, respectively, compared with three control groups 20% Survival in mice immunized with pVAX1-MEG-CTXA2/B All mice in control groups died within 7 days.	This study is the first report of a multi-epitope DNA construct strategy as a potential DNA vaccine against toxoplasmosis. Furthermore, we have also demonstrated that the use of a combination of this DNA vaccine component with CpG motif and CTXA$_2$/B as genetic adjuvant enhanced both the magnitude and breadth of immune responses accompanied by significant increasing of survival rate in vaccinated mice.	[47]
MEG, containing T- and B-cell epitopes SAG1 (40-50 aa) – (236-247 aa) – (181-192 aa) GRA2 (153-169 aa) – (113-125 aa) GRA7 (162-175 aa)– (221-235 aa) – (153–162 aa) ROP16 (240-253 aa) – (364-372 aa) – (470-483 aa) – (541-549 aa) Groups: pTgMEG pRANTES pTgMEG plus pRANTES pEGFP-C1 PBS	pRANTES	Plasmid, i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	↑ Levels of IgG in mice vaccinated with pTgMEG and pTgMEG+pRANTES (especially in the latter group), compared with controls (p<0.05) The predominance of IgG2a over IgG1 (especially in the pTgMEG+pRANTES group) IgG1 titers among all groups did not differ significantly ↑ IFN-γ in the experimental groups, compared with the control groups (p<0.05) Higher IFN-γ secretion in immunized mice with pTgMEG+pRANTES compared with pTgMEG group (p<0.05) IL-4 or IL-10 production did not differ among all groups. ↑ Percentages of $CD4^+$ and $CD8^+$ T cells in pTgMEG and pTgMEG+pRANTES (especially in latter group)	NR	Increased survival time (p<0.05) pTgMEG: 13 days pTgMEG+pRANTES: 17 days All control mice died within 6-7 days.	The DNA vaccine and the genetic adjuvant revealed in this study might be new candidates for further vaccine development against *T. gondii* infection, although developing an effective vaccine against *T. gondii* is not only a tedious mission but, also adifficult a challenge.	[48]

↑, increase; Ag, antigen; CTLs, cytotoxic T lymphocytes; CTXA$_2$/B, A$_2$/B subunits of cholera toxin; GRA, dense granule antigens; i.m, intramuscular; i.p, intraperitoneally; IFN-γ, interferon-γ; IL, interleukin; MEG, multi-epitope genes; NR, not reported; PBS, phosphate-buffered saline; RANTES, regulated upon activation normal T-cell expressed and secreted; ROP, rhoptry protein or rhoptry antigens; SAG, surface antigens; *T. gondii*, *Toxoplasma gondii*.

Supplementary Table 6. Examples of immunization with live-attenuated vectors expressing *T. gondii* antigens in mouse models

Antigen	Adjuvant/ Carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Refer-ence
ROP2 Groups: MVA and MVA-ROP2 viruses (10^6,10^7, and 10^8 pfu) PBS	None/MVA	i.m	Female Swiss mice	Acute: 3×10^2 tachyzoites, RH strain, i.p Chronic: 20 cysts of the ME49 strain, orally	↑ Secific IgG antibodies against the ROP2 protein Mixed Th1/Th2 immune response (predominance of IgG2a over IgG1)	Animals injected either with MVA, MVA-ROP2 or PBS showed the same results (none-significant)	Increased survival time significantly (11 days vs. 8-9 days, p=0.04)	We conclude that MVA ROP2 recombinant vaccinia virus can possibly generate an immune response, which could be useful in protection against toxoplasmosis.	[49]
ROP2 Groups: BCG/pMV262-ROP2 BCG/pMV262 PBS	None/*M. bovis* BCG	s.c	BALB/c	5×10^2 Tachyzoites, RH strain, i.p	↑ Specific immune responses against ROP2 protein in mice immunized with BCG/pMV262-ROP2 ↑ IFN-γ and IL-2 in both groups that received a single inoculation and a boost inoculation of BCG/pMV262-ROP2 ↑ Percentages of $CD4^+$ T ($p<0.01$) and $CD8^+$ cells (non-significant)	NR	Increased survival rate ($p<0.05$) Control mice were died within 8-9 days	These results indicated that *M. bovis* BCG is an adequate vector to express and present antigens of *T. gondii*, and it may be used to further study the induction of protective immunity in other animals.	[50]
MEG SAG1-$I_{59–67}$ SAG1-$II_{246–255}$ $GRA1_{176–186}$ $ROP2_{200–215}$ $GRA4_{235–243}$ $SAG2C_{36–44}$ $SAG2X_{215–223}$	$CTXA_2$/B/ *S. typhimurium* strain BRD509 $aroA^-$ and $aroD^-$ mutant	i.o, i.n and i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Higher levels of IgG antibody in mice vaccinated with pVAX1-MEG-$CTXA_2$/B DNA plasmid via i.m route, compared with mice immunized with BRD509/pVAX1-MEG-CTXA2/B orally and intranasally ($p<0.05$) Higher levels of IgA antibody in mice immunized with BRD509/pVAX1-MEG-$CTXA_2$/B via i.o and i.n routes, in comparison to mice immunized intramuscularly with pVAX1-MEG-$CTXA_2$/B plasmid ($p<0.05$) ↑ Percentages of $CD8^+$ T cells in the three immunization routes, compared to the controls (the highest percentages were seen in mice vaccinated i.o with BRD509/pVAX1-MEG-$CTXA_2$/B) ↑ IFN-γ and IL-2 in mice vaccinated with pVAX1-MEG- $CTXA_2$/B, compared with the control groups ($p<0.05$) Significantly higher secretion of IFN-γ and IL-2 in the mice via i.n and i.o vaccinated with BRD509/pVAX1-MEG-$CTXA_2$/B, compared with i.m vaccination route (p=0.02) Similar values of IL-4 and IL-5 between vaccinated groups and control groups ($p>0.05$) Higher Ag specific lymphocyte proliferation activity in BRD509/pVAX1-MEG-$CTXA_2$/B i.n and i.o immunization groups, compared with i.m vaccination route with pVAX1-MEG$CTXA_2$/B ($p<0.05$)	NR	Increased survival rate i.m: 20% survival rate 10 days after challenge (pVAX1-MEG-$CTXA_2$/B) Control mice were died within 4-5 days (saline and pVAX1) i.n: 40% survival rate 10 days after challenge (BRD509/ pVAX1-MEG-$CTXA_2$/ B) Control mice were died within 5-7 days (BRD509 and BRD509/pVAX1) i.o: 60% survival rate 10 days after challenge (BRD509/ pVAX1-MEG-$CTXA_2$/ B) Control mice were died within 4-8 days (BRD509 and BRD509/pVAX1)	The results from this study indicate that a DNA vaccine encoding multi-epitopes of *T. gondii* delivered by attenuated *Salmonella* is promising.	[51]

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Supplementary Table 6. Continued

Antigen	Adjuvant/ Carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP16 Groups: CAV-2-ROP16 CAV-2 PBS Blank control	None/CAV-2	i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response in the recombinant virus CAV-2-ROP16 group, compared to other groups ($p<0.05$) ↑ Levels of a mixed IgG1 and IgG2a ($p<0.05$) with the predominance of IgG2a production Predominant Th1-type response had developed ↑ IFN-γ (791.13 ± 42.76 pg/mL, $p<0.05$), IL-2 (418.94 ± 34.43 pg/mL, $p<0.05$), and IL-4 (173.27 ± 18.93 pg/mL, $p<0.05$) ↑ IFN-γ and TNF-α production induced by $CD4^+$ and $CD8^+$ T cells ($p<0.05$) ↑ Splenocyte proliferation in mice immunized with CAV-2-ROP16 ↑ Percentages of $CD4^+$ T and $CD8^+$ cells in CVA-2-ROP16 immunized group	NR	Increased survival rate (25% protection until 80 days after challenge, $p<0.05$) Control mice were died within 7 days	This study presents the successful use of recombinant virus CAV-2-ROP16 in vaccination protocols to protect against i.p challenge with the virulent RH strain of *T. gondii*. This system was shown to be extremely efficient in eliciting humoral and cellular immune responses that led to a significant improvement in survival time in mice.	[52]
ROP18 Groups: CAV-2-ROP18 CAV-2 PBS Blank control	None/CAV-2	i.m	Kunming	Acute: 1×10^3 tachyzoites, RH strain (genotype I), i.p Chronic: 5 cysts PRU strain (genotype II), i.g via oral gavage	Induced a strong IgG antibody response in the recombinant virus CAV-2-ROP18 group, compared to other groups ($p<0.05$) ↑ Levels of a mixed IgG1 and IgG2a ($p<0.05$) with the predominance of IgG2a production ↑ Splenocyte proliferation in mice immunized with CAV-2-ROP18 (approximately ~21-fold higher than other groups, $p<0.05$) ↑ CTL activity in mice immunized with CAV-2-ROP18 ($p<0.05$) ↑ Percentages of $CD4^+$ T and $CD8^+$ cells in CAV-2-ROP18 immunized group ↑ IFN-γ (914.26 ± 36.56 pg/mL, $p<0.05$), IL-2 (431.07 ± 28.94 pg/mL, $p<0.05$), and IL-4 (197.29 ± 29.98 pg/mL, $p<0.05$) ↑ IFN-γ and TNF-α production in both $CD4^+$ and $CD8^+$ T cell compartments	Reduced (57.3%, $p<0.05$)	Increased survival rate (40% protection until 60 days after challenge, $p<0.05$) Control mice were died within 7 days	These results demonstrate the potential use of a CAV vector harboring the ROP18 gene in the development of a vaccine against acute and chronic toxoplasmosis.	[53]

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Supplementary Table 6. Continued

Antigen	Adjuvant/ Carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Refer-ence
Encoding MAS and UMAS $ROP18_{347–396}$, $SAG3_{101–144}$, $MIC6_{288–347}$, $GRA7_{182–224}$, $MAG1_{58–125}$, $BAG1_{156–211}$, and $SPA_{142–200}$ DNA vaccine or/and Ad vaccine	Ubiquitin/ Ad	DNA vaccines (p-MAS or p-UMAS plasmid, 100 μg each), i.m or recombinantAd vaccine (Ad-UMAS virus, 3×10^8 PFU each), i.m or the combination of DNA vaccine (p-UMAS, 100 μg each) and recombinant Ad vaccine (Ad-UMAS virus, 3×10^8 PFU each).	BALB/c	Acute: 1×10^3 tachyzoites, RH strain (genotype I), i.p Chronic: 20 cysts PRU strain (genotype II), i.g via oral gavage	Induced a strong IgG antibody response in both p-MAS and p-UMAS immunized mice (especially in the p-UMAS group), compared to control groups ↑ Splenocyte proliferation in both p-MAS and p-UMAS immunized mice (a further 30% increase in latter group) ↑ IFN-γ and IL-2 secretion in both p-MAS and p-UMAS immunized mice (especially in the p-UMAS group), compared to control groups ↑ Levels of an IgG1 and IgG2a in p-MAS and p-UMAS immunized mice (predominance of IgG2a over IgG1), compared to control groups ↑ Percentages of $CD4^+$ T and $CD8^+$ cells in p-MAS and p-UMAS groups Significantly higher levels of IFN-γ and IL-2 secretion and increased splenocyte proliferation in Ad-UMAS immunized mice compared with p-UMAS group ($p<0.05$) ↑ Percentages of $CD8^+$ T cells in immunized with Ad-UMAS compared with p-UMAS group ($p<0.05$)	Reduced ($p<0.01$) The brain cyst burden was 50% lower in p-MAS group (833 ± 116), compared with the control groups p-UMAS (570 ± 98) Ad-UMAS (469 ± 103)	Increased survival rate p-MAS: 33% survival 28 days after challenge p-UMAS: 50% survival 28 days after challenge Control mice were died within 8-10 days	Distinct humoral and cellular immunity induced by immunization with DNA vaccine and recombinant Ad vaccine encoding ubiquitin conjugated multistage Ag of *T. gondii*. The DNA vaccine had the advantage of inducing a stronger humoral response, whereas the Ad-vectored vaccine improved the cellular immune response.	[54]

(*Continued to the next page*)

http://www.ecevr.org/ https://doi.org/10.7774/cevr.2019.8.1.4

Supplementary Table 6. Continued

Antigen	Adjuvant/ Carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
ROP18 Encoding Ad-UMAS ROP18$_{347–396}$, SAG3$_{101–144}$, MIC6$_{288–347}$, GRA7$_{182–224}$, MAG1$_{58–125}$, BAG1$_{156–211}$, and SPA$_{142–200}$	Ubiquitin/ Ad	i.m, i.n, s.c, i.o, i.v	BALB/c	Acute: 1×10^3 tachyzoites, RH strain (type I), i.p Chronic: 20 cysts PRU strain (type II), i.g via oral gavage	↑ Levels of *T. gondii*-specific IgG antibodies in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$) Highest titer of IgG antibody was observed by i.m route and followed by s.c, i.n, i.o and i.v ↑ IgG subtypes in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$) Significantly higher values of IgG2a in i.m and s.c vaccination groups, compared with other vaccination routes Significantly higher values of IgA in i.n and i.o vaccination groups, compared with other vaccination routes ↑ Percentages of $CD4^+$ and $CD8^+$ T cells in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$) Significantly higher percentages of $CD4^+$ and $CD8^+$ T cells in i.n and i.o vaccination groups, compared with other vaccination routes ↑ IFN-γ and IL-2 in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$) Significantly higher secretion of IFN-γ and IL-2 in i.n and i.o vaccination groups, compared with other vaccination routes ↑ Lymphocyte proliferation ability in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$) Significantly higher lymphocyte proliferation ability in i.n and i.o vaccination groups, compared with other vaccination routes	Reduced ($p<0.05$)	Increased survival rate i.m, i.o, and i.n vaccinated groups: 50% survival rate 28 days after challenge i.v and s.c vaccinated groups: 40% survival rate 28 days after challenge All the control mice died within 8 days	Ad-UMAS could be an effective and safe mucosal candidate vaccine to protect animals and humans against *T. gondii* infection	[55]

↑, increase; Ad-UMAS, adenovirus expressing ubiquitin-conjugated multistage antigen segments; Ad, adenovirus; Ag, antigen; BCG, Bacillus Calmette-Guerin; CAV-2, canine adenovirus type-2; CTLs, cytotoxic T lymphocytes; $CTXA_2$/B, A_2/B subunits of cholera toxin; GRA, dense granule antigens; i.g, intragastrically; i.m, intramuscular; i.n, intranasal; i.o, intraoral; i.p, intraperitoneally; i.v, intravenous; IFN-γ, interferon-γ; IL, interleukin; *M. bovis, Mycobacterium bovis*; MAS, multi-stage antigen segments; MEG, multi-epitope genes; MIC, microneme antigens or microneme proteins; MVA, modified vaccinia virus Ankara; NR, not reported; PBS, phosphate-buffered saline; PFU, plaque-forming unit; ROP, rhoptry protein or rhoptry antigens; s.c, subcutaneous; *S. typhimurium, Salmonella typhimurium*; SAG, surface antigens; *T. gondii, Toxoplasma gondii*; Th, T helper; TNF-α, tumor necrosis factor α; UMAS, ubiquitin-conjugated multistage antigen segments.

Supplementary Table 7. Examples of heterologous prime-boost immunization against *T. gondii* in mouse models

Antigen/Adjuvant	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
rROP2-SAG1/FCA pcROP2-SAG1 pcROP2-SAG1 boost rROP2-SAG1/FIA	s.c i.m	BALB/c	NR	↑ IgG antibody (especially in mice immunized with rROP2-SAG1 compared with pcROP2-SAG1 and pcROP2-SAG1+rROP2-SAG1 groups after 70 days of the first immunization, $p<0.05$) Predominance of IgG1 over IgG2a (significant for mice immunized with rROP2-SAG1 and pcROP2-SAG1+rROP2-SAG1 groups, $p<0.05$) More vigorous specific lymphoproliferative responses in mice of group rROP2-SAG1 ↑ IFN-γ in groups rROP2-SAG1, or pcROP2-SAG1, or pcROP2-SAG1 boosted with rROP2-SAG1 (non-significant between these groups)	NR	NR	The results indicate that fusion proteins ROP2-SAG1 exhibit immunogenicity by three immunization procedures, using a recombinant protein vaccine, or DNA vaccine, or DNA boosted with protein. Immune effects based on the recombinant protein are stronger than that of the DNA vaccine.	[56]
ROP18 Encoding MAS and UMAS $ROP18_{347–396}$, $SAG3_{101–144}$, $MIC6_{288–347}$, $GRA7_{182–224}$, $MAG1_{58–125}$, $BAG1_{156–211}$, and $SPA_{142–200}$ DNA vaccine or/and adenovirus vaccine Prime/bopst: DNA/DNA (p-UMAS/p-UMAS) Ad/Ad (Ad-UMAS/Ad-UMAS) DNA/Ad (p-UMAS/Ad-UMAS) Ad/DNA (Ad-UMAS/p-UMAS)	The combination of DNA vaccine (p-UMAS, 100 μg each) and recombinant adenovirus vaccine (Ad-UMAS virus, 3×10^8 PFU each), i.m	BALB/c	Acute: 1×10^3 tachyzoites, RH strain (genotype I), i.p Chronic: 20 cysts PRU strain (genotype II), i.g via oral gavage	Highest levels of humoral antibodies and cellular immune responses were achieved in mice immunization priming with the DNA vaccine and boosting with the Ad-UMAS vaccine Compared with p-UMAS or Ad-UMAS immunization alone, higher levels of a specific IgG (predominance of IgG2a) and higher levels of cytokines (IFN-γ and IL-2) were obtained by priming with p-UMAS and boosting with Ad-UMAS ($p<0.05$) Priming with p-UMAS and boosting with Ad-UMAS demonstrated higher proliferation activity, compared with the other immunization strategy ($p<0.05$)	Reduced ($p<0.01$) The most significant reduction of brain cyst burden was observed by the DNA prime-Ad boost approach.	Increased survival rate 67% Survival in mice vaccinated with p-UMAS prime and Ad-UMAS boost 28 days after challenge Control mice were died within 8-10 days	Priming vaccination with DNA vaccine and boosting with the recombinant Ad vaccine encoding ubiquitin conjugated multi-stage antigens of *T. gondii* was proved to be a potential strategy against the infection of type I and type II parasite.	[54]

↑, increase; Ad-UMAS, adenovirus expressing ubiquitin-conjugated multistage antigen segments; Ad, adenovirus; Ag, antigen; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; GRA, dense granule antigens; i.g, intragastrically; i.m, intramuscular; i.p, intraperitoneally; IFN-γ, interferon-γ; IL, interleukin; MAS, multi-stage antigen segments; MIC, microneme antigens or microneme proteins; NR, not reported; ROP, rhoptry protein or rhoptry antigens; s.c, subcutaneous; SAG, surface antigens; *T. gondii, Toxoplasma gondii*; UMAS, ubiquitin-conjugated multistage antigen segments.

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