Review article

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Rhoptry antigens as *Toxoplasma gondii* vaccine target

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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 $CD8^+$

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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-

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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-

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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

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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

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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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