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Review

Vaccination challenges and strategies against long-lived *Toxoplasma gondii*

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ABSTRACT

Since the discovery of *Toxoplasma gondii* in 1908, it is estimated that one-third of the global population has been exposed to this ubiquitous intracellular protozoan. The complex life cycle of *T. gondii* has enabled itself to overcome stress and transmit easily within a broad host range thus achieving a high seroprevalence worldwide. To date, toxoplasmosis remains one of the most prevalent HIV-associated opportunistic central nervous system infections. This review presents a comprehensive overview of different vaccination approaches ranging from traditional inactivated whole-*T. gondii* vaccines to the popular DNA vaccines. Extensive discussions are made to highlight the challenges in constructing these vaccines, selecting adjuvants as well as delivery methods, immunisation approaches and developing study models. Herein we also deliberate over the latest and promising enhancement strategies that can address the limitations in developing an effective *T. gondii* prophylactic vaccine.

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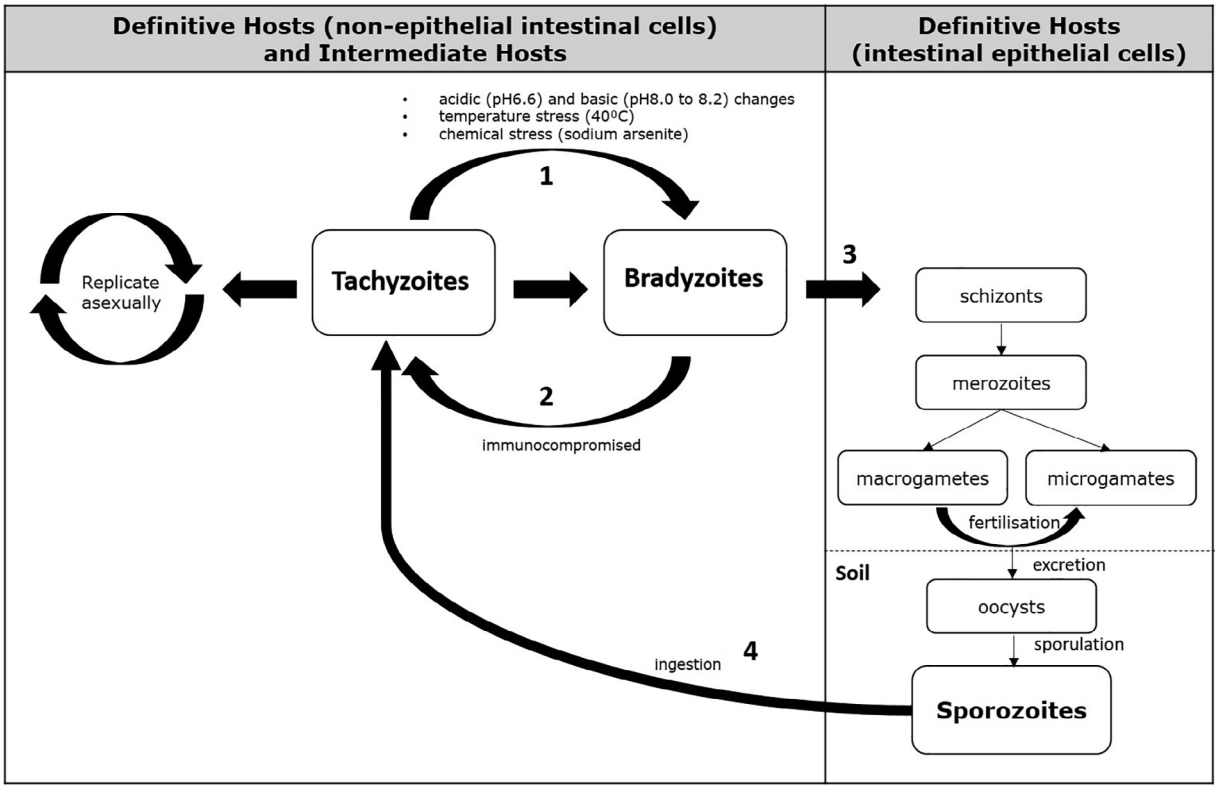

Fig.1. *T. gondii* interconverts between tachyzoite, bradyzoite and sporozoite stages under different conditions. It can replicate both sexually and asexually within definitive hosts, but can only divide asexually within the intermediate hosts. (1) Tachyzoites differentiate into bradyzoites in response to changes in pH [3], temperature stress and chemical stress [4]. (2) Bradyzoites convert into tachyzoites in immunocompromised hosts. (3) Bradyzoites have to complete the schizont stages to form merozoites prior to differentiation into macrogametes and microgametes. Fertilised gametes eventually form oocysts and divide into sporozoites in the soil. (4) Ingested sporozoites convert into active tachyzoites after ingestion by hosts such as poultry and humans.

1. Introduction

Toxoplasma gondii is an obligate intracellular Apicomplexan parasite that has successfully infected one-third of the global population. The parasite can infect almost all nucleated cells, hence, it has broad host range of warm-blooded mammals including humans. Strains are mainly isolated in North America and Europe and classified into three major clonal lineages, type I, II and III strains [1]. The genetic diversity of *T. gondii* strains is generally limited and uniform.

The cat family (Felidae) has been determined as *T. gondii*'s definitive host, where the parasite undergoes sexual reproduction [2]. *T. gondii* is also able to replicate asexually in both definitive hosts and intermediate hosts including almost all mammals. The life cycle of *T. gondii* can be divided into three main stages which are tachyzoite, bradyzoite and sporozoite. The fast-replicating tachyzoites could differentiate into dormant bradyzoites under certain conditions including acidic (pH 6.6) and basic (pH 8.0–8.2) changes [3], temperature stress (40 °C) and chemical stress (sodium arsenite) [4]. Bradyzoites multiply very slowly in a quiescent metabolic stage. They develop in cysts within host cells and persist in the human body for life and remain asymptomatic. Despite that, recrudescent infection usually occurs in an immunocompromised host, resulting in stage conversion back to the destructive tachyzoite [5]. Within 2 days in the feline intestine, bradyzoites progress through all five stages of schizonts and develop into merozoites, the first sexual stage. The merozoites then undergo doublings and differentiate into macrogametes (male) and microgametes (female) followed by fertilisation to form diploid oocysts. The oocysts, surrounded by a thick impermeable oocyst wall, are shed in the feces and then sporulated to form sporozoites [6]. The complex life cycle of *T. gondii* is summarised in Fig. 1.

The three principal transmission routes of *T. gondii* are: (1) foodborne transmission - ingestion of tissues cysts from undercooked meat or oocysts from contaminated food or water, (2) zoonotic transmission - accidental ingestion of oocysts when coming into contact with an infected host or contaminated environment, (3) congenital transmission - when tachyzoites cross the placenta during pregnancy [7]. Due to the ease of *T. gondii* transmission, a high seroprevalence rate of 90% has been reported in some European and South American countries. In the United States, it is estimated that 22.5% of the population aged 12 years and older have toxoplasmosis [8].

Most *T. gondii* infections are asymptomatic as the parasite is suppressed by effective immune control, however, tissue cysts can recrudesce in immunocompromised hosts and cause diverse complications [5]. For instance, AIDS/HIV patients have an increased frequency of toxoplasma encephalitis [9]. In pregnant women, *T. gondii* could transmit across the placenta or cause miscarriages. The infants with congenital infection may be diagnosed with a wide variety of manifestations including chorioretinitis, calcification and hydrocephalus [10]. Meta analyses have also deduced a positive correlation between *T. gondii* seropositivity and psychotic symptoms such as schizophrenia and bipolar disorder [11,12].

The combination of pyrimethamine and sulfadiazine (PS) has been the most established regimen to treat active toxoplasmosis in humans by inhibiting *T. gondii* folate metabolism and eventually blocking nucleic acids synthesis. However, it imposes severe side effects such as drop in platelet counts, neutropenia and hypersensitivity reactions [13]. Moreover, pyrimethamine could

be teratogenic, thus pregnant women are usually treated with spiramycin, which has minimal foetal toxicity. As spiramycin cannot pass through the placenta, it is only limited to prevent vertical transmission of *T. gondii* rather than treating foetal infection [14]. To date, all the drugs used in clinical practice are solely active against tachyzoite and not able to clear the cyst formed during chronic infection. This could be explained by the low level of DNA synthesis in the bradyzoite residing within tissue cysts, hence reducing the folate biosynthesis for the anti-folate drugs to act on [15]. Currently, there is only one live attenuated *T. gondii* vaccine, Toxovax, available commercially for the control of sheep and goats abortions due to toxoplasmosis. It is used in the UK, New Zealand, France, and Ireland [16]. Toxovax is not able to completely eradicate the parasite and cannot be used for humans as it carries potential risks of reversion to pathogenic form and provoke iatrogenic infection [17].

As noted above, *T. gondii* is a very successful parasite with the capability of interconversion in response to different stresses, leading to its flexibility to survive and propagate both sexually and asexually in a wide range of hosts globally. Hence, active immunisation serves as the ideal and long-term strategy to induce the host immune response against *T. gondii*. This review presents the progress of current *T. gondii* vaccine studies, highlighting some of the new techniques and approaches used. We also provide insights for the future development of *T. gondii* vaccines.

2. Current advances in toxoplasmosis vaccines

The search for effective *T. gondii* vaccines has shown progress with numerous preclinical studies reported. Four major approaches for developing *T. gondii* vaccines will be discussed in the following sections.

2.1. Nucleic acid vaccines

DNA vaccines have many advantages in terms of safety, rapid production, stable storage and effective stimulation of both humoral and cellular immune responses [18]. Over the last five years, more than 50 *T. gondii* DNA vaccine candidates have shown good protection in various challenge studies. Several methods were employed to enhance vaccine effectiveness: (i) the addition of adjuvant to enhance immunity, (ii) the employment of bacteria or viruses as carriers for improved antigen delivery, (iii) the incorporation of multiple antigens for cross-protection, (iv) the use of bioinformatics to analyse antigenic characteristics of candidate genes, and (v) the use of heterologous prime-boost regimen to induce humoral and cellular immunity via two vaccination approaches. Table 1 summarises the DNA candidate vaccines that extended host survival by at least 30 days against type I tachyzoites or more than 70% cyst reduction against type II cysts. Rhoptry proteins (ROPs) are the most highly explored DNA vaccine candidates. Their key role in host cell invasion could have contributed to the antigenic properties for inducing a strong immune response. As shown in Table 1, ROP16 and ROP18 have conferred the highest protection against Type I RH. Both antigens were delivered by canine adenovirus type 2 (CAV2) to elicit Th1 response characterised by high levels of proinflammatory cytokines IFN-γ and IL-2 [19,20]. As ROP18 is tachyzoite-specific antigen, ROP16 appears to be a more promising antigen to provide cross-protection against both tachyzoites and bradyzoites. Apart from using single antigen, Wang et al., 2017 constructed a DNA vaccine encoding multi-stage antigens (SAG3, ROP18, MIC6, GRA7, MAG1, BAG1, SPA) delivered as recombinant adenovirus, which resulted in the highest reduction of Type II PRU brain cysts (Table 1). The DNA construct was conjugated with ubiquitin to facilitate proteasome dependent degradation, thus enhancing the presentation of antigens to $CD8^+$ T cells [21].

RNA-based vaccines that require an efficient delivery system have also been explored for *T. gondii*. Chahal *et al.* (2016) has reported the first study to construct the modified dendrimer nanoparticle (MDNP)-delivered mRNA replicon encoding 6 *T. gondii* antigens. The MDNP delivery technology is a fully synthetic system that uses replicons, ionizable delivery materials and lipid anchored polyethylene glycol to protect the naked mRNA from degradation and enhance its delivery to the host. The novel platform showed promising results with complete protection against the Type II PRU strain with only a single vaccination. Additionally, vaccine production requires a relatively short time of about 1 week [22]. Nevertheless, protection against Type I virulent strains and reduction in cyst numbers for the Type II avirulent strains were not investigated. Following that, Luo *et al.* (2017) developed a lipid nanoparticle-delivered self-amplifying RNA vaccine encoding *T. gondii* nucleoside triphosphate hydrolase-II (NTPase-II). Significant protection against the Type II PRU strain with a 62.1% reduction in the number of brain cysts was observed in immunized mice [23]. In brief, these studies demonstrate the potential of developing efficient RNA vaccines against *T. gondii* [22,23].

2.2. Recombinant protein vaccines

Recombinant protein subunit vaccines are formulated using defined and highly purified antigens composed from epitopes of pathogens. These antigens could be less immunogenic as they contain fewer pathogen-associated molecular pattern (PAMPs) as compared to the attenuated or inactivated whole-pathogen vaccines [24]. Furthermore, proteolytic degradation of these antigens could happen in the host cells. Therefore, adjuvants are commonly included in recombinant protein vaccines to improve its immunogenicity and protect it from degradation [25]. Nevertheless, the number of *T. gondii* protein vaccines being studied has increased over the last 5 years. This is most likely due to the emergence of different categories of adjuvants to enhance the immune response. Table 2 highlights protein vaccines that have extended host survival at least 30 days against type I tachyzoites or more than 70% cyst reduction of type II cysts. Similar to DNA vaccines, the rhoptry protein ROP is the most frequently selected, either alone or in combination with other antigens to achieve high protection levels. Recombinant ROP18 has shown high efficiency in reducing Type II PRU brain cysts, when combined with recombinant ROP38 [26], or CDPK6 [27]. The immunisation of recombinant antigen combination alone was able to decrease more than 70% (for rROP18-rROP38) and 50% (for rROP18-rCDPK6) of brain cysts as compared to the control group [26,27]. As shown in Table 2, higher percentages – 81.3% and 73.6% respectively, were achieved after the antigens were encapsulated in poly(lactide-co-glycolide) (PLG), which protects the antigens from degradation and releases the antigens over a long period. On the contrary, some antigens could only exhibit high immunogenicity when they are combined with the adjuvants. For instance, the loading of *T. gondii* antigen lysates in porous nanoparticle adjuvant can reduce 70% of brain cysts, but the usage of the lysates or nanoparticle alone can only obtain 20% of cysts reduction [28].

2.3. Live attenuated vaccines

Live attenuated vaccines contain the target pathogen with reduced virulence but retaining its ability for limited replication within the host cell to induce an immune response. Since an attenuated *T. gondii* vaccine is able to express a wider range of antigens at specific life cycle stages, it is deemed ideal for combatting toxoplasmosis. As summarised in Table 3, most of the live attenuated

Table 1
Summary of DNA vaccine candidates achieved high protection against *T. gondii* published between 2014 and 2018.

No.	Types of Antigen	Antigen/Adjuvant/Delivery Approach	Immune Responses	Challenge Strain/ Amount	Protection	Mouse Strain	Immunisation Approach	Ref.
1	Rhoptry proteins (ROP)	ROP38	higher ratio of IgG2a to IgG1; higher levels of IFN-γ and IL-2	PRU – 10 cysts	76.6% reduction in brain cysts	Kunming	Intramuscular (i.m.)	[92]
		ROP18 Expressed by recombinant canine adenovirus type-2	higher ratio of IgG2a to IgG1; higher levels of IFN-γ, IL-2 and IL-4	RH – 1×10^3 tachyzoites	40% protection in 60 days	Kunming	i.m.	[19]
		ROP16 Expressed by recombinant canine adenovirus type-2	predominance of IgG2a; higher levels of IFN-γ, IL-2, and TNF-α	RH – 1×10^3 tachyzoites	25% survival in 80 days	BALB/c	i.m.	[20]
2	Enzymes	Calcium dependent protein kinase (CDPK)1 Adjuvant - IL-21 and IL-15	predominance of IgG2a over IgG1; higher levels of IFN-γ and IL-2	PRU – 20 cysts	72.7%, reduction in cysts	Kunming	i.m.	[93]
		CDPK1 Adjuvant - IL-7 and IL-15	higher IgG2a/IgG1 ratio; higher levels of IFN-γ and IL-2	PRU – 10 cysts	73.5% reduction in cysts	Kunming	i.m.	[94]
		Nucleoside triphosphate hydrolase-II (TgNTPase-II) Using DNA-based alphaviral RNA replicon vectors	predominance of IgG2a; higher levels of IFN-ϫ, IL-2, IL-4 and IL-10	PRU – 20 cysts	71% reduction in brain cysts	BALB/c	i.m., electroporation	[95]
3	Rhomboid (ROM) proteins	ROM5 Adjuvant - emulsified in Freund's complete adjuvant	higher IgG2a to IgG1 ratio; higher levels of IFN-γ, IL-2 and IL-12	RH – $1X10^3$ tachyzoites PRU – 10 cysts	20% survival in 35 days 72.04% reduction in brain cysts	Kunming	i.m.	[96]
4	Surface antigen (SAG)	SAG5B and SAG5C	predominance of IgG2a over IgG1; higher level of IFN-ϫ	PRU-20 cysts	75% reduction in brain cyst	BALB/c	i.m.	[97]
5	Combination of different types of antigens	SAG3, ROP18, microneme protein (MIC)6, dense granule antigen (GRA)7, matrix antigen (MAG)1, bradyzoite specific antigen (BAG)1, sporozoite surface antigen (SPA) Expressed by recombinant adenovirus vaccine Adjuvant – ubiquitin	higher level of IgA; higher level of IL-2, IFN-γ, low level of IL-10	PRU – 20 cysts	78.6% reduction in brain cyst	BALB/c	Intranasal (i.n.)	[21]
		Perforin-like Protein (PLP)1 and ROP18 Adjuvant- IL-18	higher IgG2a to IgG1 ratio; higher levels of IFN-γ, IL-2, IL-12, IL-4, and IL-10	PRU- 20 cysts	72.4% reduction in brain cysts	Kunming	i.m.	[59]

Table 2
Summary of protein vaccine candidates achieved high protection against *T. gondii* published between 2014 and 2018.

No.	Types of Antigen	Antigen/Adjuvant/Delivery Approach	Immune Responses	Challenge Strain/ Amount	Protection	Mouse Strain	Immunisation Approach	Ref.
1	Tachyzoite lysate antigen	*T. gondii* antigens Adjuvant - Loading of porous nanoparticles (DGNP)	mixed IgG1/IgG2a response; higher levels of IFN-ϫ, TNF-α, IL-2, IL-4, IL-6, and IL-17	76 K strain – 30 cysts	70% brain cyst reduction	CBA/J (H-2 k)	mucosal vaccine	[28]
	Toxoplasma lysate Adjuvant - chitosan nanospheres, Freund's incomplete adjuvant	increase in IgG and IgM; higher level of IFN-γ	RH – 2500 tachyzoites ME49 – 10 cysts	80 days survival 76.8% reduction in parasitic count	Swiss strain albino	Intraperitoneal (i.p.)	[63]
2	Rhoptry protein	ROP17 Fusion with glutathione S-transferase (GST)	greater IgG2a than IgG1, higher SIgA antibody; higher levels of IFN-γ, IL-2, and IL-4	RH – 4×10^4 tachyzoites	75% survival rate in 30 days	BALB/c	i.n.	[98]
	ROP38 and ROP18 Adjuvant – polylactide-co-glycolide (PLG) encapsulation	higher ratio of IgG2a to IgG1; higher level of IFN-γ and IL-2	PRU – 10 cysts	81.3% of cyst reduction	Kunming	Subcutaneous (s.c.)	[26]
3	Enzymes	Phosphoglycerate mutase (PGAM)2	higher IgG2a/IgG1 ratio, higher IgA antibody; Higher levels of IFN-γ, IL-2 and IL-4	RH- 4×10^4 tachyzoites	70% survival in 30 days	BALB/c	i.n.	[99]
4	Actin-binding protein	Profilin Adjuvant - encapsulated in oligomannose-coated liposomes	higher total IgG (higher IgG2c than IgG1); increase of IFN-γ	RH – 1×10^3 tachyzoites	66.7% survival in 30 days	C57BL/6	s.c.	[100]
5	Surface antigen	Actin depolymerizing factor (ADF)	higher sIgA and IgG; higher levels of IFN-γ and IL-2	RH – 4×10^4 tachyzoites	36.3% survival in 30 days	BALB/c	i.n.	[101]
	SAG1 fused with an antibody fragment scFv directed against DEC205 endocytic receptors Adjuvant- Poly I:C	predominance of IgG1 over IgG2a; higher levels of IL-12, TNF-α, and IL-6	76 K – 15 cysts	80% reduction in brain cyst number	CBA/J (H-2 k)	s.c., i.n.	[81]
6	Dense granule protein	GRA7 self-assembling polypeptide nanoparticles (SAPNs) Adjuvant- PADRE	increase of IFN-γ secretion	ME49 -2×10^3 tachyzoites	72% reduction in brain cysts	HLA-B*0702	s.c.	[102]
7	Scaffold protein	Receptor for activated C kinase (RACK)1	higher sIgA antibody titers, predominance of IgG2a; Higher levels of IFN-ϫ, IL-2, and IL-4	RH - 1×10^4 tachyzoites	45% survival in 30 days	BALB/c	i.n.	[103]
8	Inner membrane complex (IMC)	IMC sub-compartment proteins 3 (ISP3) Virus-Like Nanoparticle Vaccine	higher levels of IgA and IgG (higher IgG2a than IgG1); higher levels of IFN-γ, IL-6 and IL-11	ME49- 20 cysts	75% reduction in brain cysts	BALB/c	i.n.	[66]
9	Combination of different types of antigens	CDPK6 and ROP18 Adjuvant - encapsulated in PLG microspheres	higher level of IgG and IFN-γ	PRU – 10 cysts	73.6% reduction in brain cyst	Kunming	s.c.	[27]

Table 3
Summary of live attenuated vaccine achieved high protection against *T. gondii* published between 2014 and 2018.

No.	Vaccination Approach	Immune Responses	Challenge Strain/ Amount	Protection	Animal/Strain	Immunisation Approach	Ref.
1	Nonreverting, nonreplicating, live attenuated uracil auxotroph vaccine strains in the type II *KU80* knockout background		RH – 1×10^3 tachyzoites PRU *KU80* knockout strain – 2×10^7 tachyzoites	100% survival- 30 days 0 cyst	C57BL/6 mice	i.p.	[32]
2	RH strain with AMA1 knockout (AMA1KO) tachyzoites		RH *KU80* knockout strain BALB/C (10^5 tachyzoites) C57B/6J (10^3 tachyzoites) ME49 CD-1 (10^5 tachyzoites) BALB/C (10^3 tachyzoites)	100% survival until 60 days 100% survival until 45 days 100% survival until 45 days reduction in number of parasites	BALB/C, C57B/6J, CD-1 mice	i.p., s.c.	[34]
3	Lactate dehydrogenase knockout in PruΔKu80::hxgprt parental strain		RH – 1×10^4 tachyzoites	double knockout of LDH1/LDH2- 100% survival in 30 days reduction in brain cyst number	BALB/c mice	i.p.	[33]
4	GRA17 knockout in RH strain	predominance of IgG2a over IgG1; higher level of IFN-ϒ, IL-2, IL-12, and IL-10	RH – 1×10^3 tachyzoites PRU – 20 cysts	100% survival in 35 days reduction in brain cyst number	Kunming mice	i.p.	[35]
5	CDPK2 deficient tachyzoites of Pru strain	higher level of IgG; higher level of IFN-ϒ, IL-2, IL-12, and IL-10	RH – 1×10^3 tachyzoites PRU – 20 cysts	100% survival in 35 days reduction in brain cyst number	Kunming mice	i.p.	[36]

T. gondii vaccines were able to provide 100% survival over 30 days post-challenge. The availability of the complete *T. gondii* genome sequence allows the selective gene deletion or disruption for targeted attenuation. Despite this, the use of live attenuated vaccines is limited in light of the risk of reverting back to the pathogenic form to cause disease.

The enzymes in the *de novo* pyrimidine biosynthesis pathway are the major targets to produce attenuated *T. gondii* strains. Knockout of the key regulatory enzyme, carbamoyl phosphate synthetase II (CPSII), created an uracil auxotroph and completely avirulent *T. gondii* mutant strain, *cps1-1*. The strain has demonstrated long-term protective immunity against the type I RH strain and type II ME49 strain with significant reduction in the number of brain cysts [29,30].

To ensure efficient targeted gene replacement, Fox and colleagues deleted the *KU80* gene in *T. gondii* [31]. KU80 protein is a component of the non-homologous end-joining (NHEJ) DNA repair pathway which induces a high frequency of non-homologous recombination that hampers the gene targeting approach. Following that, the *KU80*-deleted *T. gondii* was utilised as a background strain in combination with a further knockout of the *ompdc* gene in the *de novo* pyrimidine biosynthesis pathway. The strain induced fully protective $CD8^+$T cell dependent immunity that prevented acute infection by type I and type II strains of *T. gondii*. Cyst formation by the type II strain was also reduced [32]. A lactate dehydrogenase mutant in the *KU80* knockout background also provided full protection against the *T. gondii* type I strain [33]. In addition, the deletion of *T. gondii* essential genes such as AMA1, GRA17, and CDPK2, have produced vaccine strains that provide strong protection against both type I and type II strains [34–36].

The *T. gondii* strain attenuation techniques have shifted from natural attenuation to genetic deletion. In 1988, the only licensed Toxovax S48 strain was attenuated by extensive passages in excess of 3000 times in the laboratory [37]. The spontaneous mutations causes the strain to carry a risk of regaining its pathogenic phenotype. In comparison, the current available knockout strains have defined deletions that could prevent pathogenicity reversion, thus they are relatively safe for human use. Furthermore, the use of attenuated vaccine containing two deletion sites, such as MIC1-MIC3 double knockout strain [38], could be safer as the *in vivo* reversion of double mutants would be negligible [39].

2.4. Inactivated whole-pathogen vaccines

The production of inactivated pathogens using chemical and physical treatments serves as one of the earliest methods in manufacturing safe and stable vaccines. Radiation sterilisation has been used as it eliminates chemical contaminants and efficiently penetrates the pathogens to destroy nucleic acids while preserving their surface antigens. Moreover, the biological features such as cellular structures, DNA and protein synthesis, and the invasion ability are still maintained in the irradiate strains [40]. Since they have lost their replication and infection capabilities, they require booster immunisations or adjuvants for sufficient immune stimulation. Gamma-irradiated or UV-attenuated *T. gondii* strains have been used for vaccination against toxoplasmosis (Table 4). This vaccination approach is getting less common due to concerns of the high risk of *T. gondii* strain reverting to its virulent form.

3. Vaccine immunity enhancement strategies in response to current limitations

3.1. Rationale of antigen selection through effective bioinformatics

The precise selection of antigens serves as the first crucial step in vaccine design. Some of the *T. gondii* vaccine studies selected

Table 4
Summary of inactivated vaccine achieved high protection against *T. gondii* published between 2014 and 2018.

No.	Vaccination Approach/ Adjuvant	Immune Responses	Challenge Strain/ Amount	Protection	Animal/ Strain	Immunisation Approach	Ref.
1	Irradiated, sterilised *T. gondii* tachyzoites Adjuvant - oral immunization- aluminium hydroxide	BALB/c/i.p.- higher total IgG, IgM BALB/c/oral- higher total IgA BALB/c/i.p.- higher levels of IL-10, TNF-α, IFN-ɤ, IL-4, IL-2, and IL-6	ME49 VEG 10 cysts	BALB/c/i.p.- higher reduction of brain cyst	BALB/c mice	i.p. or oral	[104]
2	UV-attenuated *T. gondii* Adjuvant - disodium cromoglycate (DSCG)	higher level of TNF-α, IFN-ɤ, IL-2, IL-4, IL-10, IL-17	RH – 100 tachyzoites	prolonged survival until 27 days, lower parasite burden in liver	Kunming mice	i.p.	[105]

candidate antigens based on their roles in invasion and infection. However, high infection capability does not always correspond to high immunogenicity. Nonetheless, predicting and localising the antigen epitopes through immunoinformatics assays are strongly advised. *T. gondii* contains many antigenic compositions with each antigen capable of inducing distinct immune responses in the host. The genome resource of *T. gondii* strains, such as ME49, GT1, VEG and RH, is accessible through the ToxoDB online database (http://ToxoDB.org). The availability of sequence data for *T. gondii* has led to the advent of reverse vaccinology to filter the vaccine candidates based on their immunogenic potential.

The immune protection against *T. gondii* infection is mediated by both cellular and humoral immunity. Hence, *T. gondii* vaccines should contain both T-cell epitopes to induce cytotoxic T lymphocyte (CTL) responses and B-cell epitopes to induce protective antibody responses. T-cell epitope studies generally involve cytotoxic T lymphocyte (CTL) prediction and T helper (Th) cell prediction. Immune Epitope Database (IEDB) hosts tools that are commonly used in *T. gondii* vaccine research to predict the half maximal inhibitory concentration (IC50) values of peptides binding to the MHC or HLA molecules [41]. Since T-cell epitopes are present in linear form to MHCs, the interface between ligands and T-cells can be modeled with accuracy. The prediction of B-cell epitopes could be more challenging as they do not contain physicochemical patterns in their amino acid sequences for in silico prediction [42]. When some B-cell epitopes interact with the cognate antibody's paratope, they could change their conformation, hence it increases the difficulty in 3-dimensional (3D) structure-based prediction [43]. Bioinformatics software such as SOPMA and I-TASSER have been used to predict the 3D models of proteins [44].

The selection of conserved antigens among different *T. gondii* strains is crucial for eliciting cross-protective immune responses. Epitope conservancy analysis tools have been developed to assist in the selection of epitopes having a desired level of conservation [45]. Interestingly, the usage of protein antigens that are conserved with phylogenetically closely related parasites such as *Neospora*, may lead to vaccines developed for both parasitic diseases. Preliminary studies reported that mice immunised with the *T. gondii* microneme protein-encoding genes *MIC1* and *MIC3* knockout strains displayed significant protection against both *T. gondii* and lethal heterologous *N. caninum* infection, with up to 80% survival rates [46]. Also, mice immunised with plasmid-expressed *T. gondii* MIC3 showed lower parasite burden following *T. gondii* or *N. caninum* challenge [47]. The potential of developing cross-reactive vaccine candidates among closely related apicomplexan parasites could be further explored.

3.2. Stable and cross-protection antigen expression

Optimal and stable antigen expression in vaccinated hosts is desired for inducing effective immunity. Most *T. gondii* DNA vaccines employed eukaryotic expression vectors driven by viral-derived promoters to achieve high-level constitutive gene expression *in vivo*. Cytomegalovirus (CMV) immediate early-enhancer or promoter activity has often been shown to direct the highest level of transgene expression, explaining its widespread usage in DNA vaccines [48]. Despite that, the IFN-ɤ and tumour necrosis factor-α (TNF-α) produced by innate and adaptive immunity were found to inhibit transgene expression from DNA vaccines with virus-derived promoters [49,50]. The inhibitory effect was also seen in the reporter gene expression directed by the CMV promoter in an adenoviral vector [51]. These studies suggested that anti-viral function of certain cytokines could affect the promoter function thus regulating the transgene expression. By correlating these findings with most *T. gondii* DNA vaccine studies that utilise constitutive promoters, it is possible that high level of IFN-ɤ induced by the overexpressed antigens might in turn limit the promoter activity, which subsequently interrupts the amount and stability of antigen expression after a certain period. The inconsistency in antigen expression and regulation might partly explain the partial protection achieved. Thus, the usage of eukaryotic promoters that are not downregulated by IFN-ɤ, such as MHC class II promoter, could be more appropriate to drive *T. gondii* antigen expression [49].

Besides stable antigen expression, the incorporation of multiple antigens is necessary for cross protection against *T. gondii* strains that has changeable forms and complex life stages. Previous studies had reported that vaccination with stage-specific antigens led to stage-limited protection [52,53]. Conversely, improvement of protection levels can be seen in *T. gondii* vaccine candidates with multiple antigens. These vaccines are featured with the flexibility to include more than one antigen candidate for stimulation of multi-dimensional immune responses. Multiple antigen vaccines could also mimic antigen processing and presentation during natural infection [54]. Additionally, as the antigen presentation capability varies among individuals, vaccines with a broader array of antigens could be more efficient. For instance, two vaccine candidates were reported with both utilising attenuated *Salmonella typhimurium* as live vector to deliver plasmid-encoding *T. gondii* antigens with the first containing SAG1 and SAG2 [55] while the second containing multi-epitopes (SAG1, GRA1, ROP2, GRA4, SAG2C, and SAG2X) [56]. Although both had shown comparable protection levels, the latter could elicit a broader protective immunity against *T. gondii* as it encodes epitopes present in both tachyzoites and bradyzoites.

3.3. Adjuvant as immunity booster

Adjuvants are required to aid most inactivated vaccines to compensate for the lack of immune triggers as a result of inactivation and/or removal of pathogenic features. In general, improvement in the immunogenicity and protection by vaccines against *T. gondii* has been observed when adjuvants are used. Aluminum salts is one of the most established adjuvants that has been used in numerous licensed vaccines. However, it might not be an ideal choice for a

T. gondii vaccine as it exerts little effect on eliciting the Th1 cell-mediated immunity which serves a major role in the control of both acute and chronic *T. gondii* infection [30]. Previous studies have reported the Th2 biased or mixed Th1 and Th2 immune responses induced in alum-adjuvanted *T. gondii* vaccine [57,58]. In contrast, the most often used adjuvants to direct a Th1-biased immune response against *T. gondii* include A2/B subunit of cholera toxin and cytokine adjuvants [59,60].

The Adjuvant System (AS), which combines classical adjuvants such as aluminium salts, oil-water emulsions, liposomes and immunostimulators, shows a great potential to be incorporated in *T. gondii* vaccine. AS01 (monophosphoryl lipid A (MPL), QS21, liposomes), AS02 (MPL, QS21, emulsion) and AS04 (MPL, aluminum salt) might suit the needs of intracellular *T. gondii* protection that requires induction of Th1-bias response. All three systems contain MPL, a chemically detoxified derivative of the parent lipopolysaccharide (LPS) from *Salmonella Minnesota R595* strain and a toll-like receptor (TLR)4 agonist [24]. Direct stimulation of TLR4 by MPL leads to the maturation of antigen presenting cells (APCs), followed by an increased production of pro-inflammatory cytokines such as IFN-ɣ and IL-2, leading to the induction of Th1 immune responses [61].

Versatile delivery vehicles have been used as adjuvant in some of the *T. gondii* recombinant protein vaccine studies. Biodegradable and biocompatible polymers of poly(lactide-co-glycolide) (PLG) which is endowed with higher immunogenic properties has attracted extensive interest in *T. gondii* vaccine development. PLG nanoparticles (NPs) could provide continuous *in vitro* release of entrapped antigens over a long period of time, at the same time enhance the antigens uptake by antigen presenting cells (APCs) and protect the antigens from hydrolytic and enzymatic degradation *in vivo* [62]. On the other hand, the natural polymer, chitosan nanosphere, used to encapsulate *T. gondii* lysate antigens increased the survival time and enhanced both the cellular and humoral immune responses in vaccinated mice [63].

3.4. Efficient display and delivery of Toxoplasma gondii antigens

Presentation of antigens on the surface of a carrier, such as live bacteria or virus, allows it to be better recognised by the host immune system. The antigens displayed on highly repetitive surface structures can induce APCs maturation by triggering Toll-like Receptors (TLRs) and cross-link B cell receptors to stimulate strong B and T cell-mediated immune responses [64]. This approach was demonstrated by Tang *et al.* (2016), who engineered a transgenic *Eimeria tenella* line (Et-TgSAG1) expressing *T. gondii* SAG1 antigen on the cell surface. As a result, TgSAG1-specific humoral and cellular immune responses were elicited in chickens and Th1-dominant immune responses in mice [65]. Besides that, the insect cells Sf9/Baculovirus (BV) recombinant technology platform has been used to produce virus like particles (VLPs) that display *T. gondii* antigens. Using this approach, Sf9 insect cells were co-infected with recombinant BV expressing influenza matrix M1 and a *T. gondii* antigen. The M1 then acts as a core protein to generate VLPs while displaying repeated structures of the *T. gondii* antigen on its surface. Both VLPs displaying *T. gondii* inner membrane complex (IMC) sub-compartment proteins 3 (ISP3) or MIC8 were found to be highly immunogenic with higher levels of systemic and mucosal antibody responses [66,67].

Apart from antigens displayed on cell surfaces, the efficient delivery of antigens into host cells is crucial to enhance their uptake and presentation. Live antigen delivery systems, which include *S.* Typhimurium bacterial vector, Bacille Calmette–Guerin (BCG) vector, adenoviral vector and baculovirus vector have been employed in *T. gondii* vaccine studies. However, pre-existing immunity of vectors could induce robust B and T memory responses against the vector, and as such, when a vaccine is delivered, it may cause early pathogen clearance, loss of prolonged gene expression and lower immunogenicity [68]. Therefore, novel vectors such as chimpanzee-specific adenoviruses, which exhibit low pre-existing anti-vector immunity in humans, can be the alternative tool to deliver *T. gondii* antigens [69].

The concept of applying probiotic therapeutic against parasites is emerging in the infancy stage. Until the present, there is no reported probiotic strains that are utilised as carrier of *T. gondii* antigens. Benson *et al.*, 2009 suggested that the gut commensal microflora provides indirect stimulation of DCs to protect against *T. gondii* [70]. Most of probiotic lactic acid bacteria are resistant to the harsh condition in stomach, while some are able to colonise gastrointestinal mucosa, thus allowing longer time for antigen presentation to the immune system [71]. Since the probiotic strains are well-adapted to colonise mucosal tissue and capable to stimulate DCs, it is worth to be explored as *T. gondii* vaccine carrier.

3.5. Effective immunisation strategies

Heterologous prime-boost regimen exploits a synergistic effect where two different vaccines to the same pathogen are combined to augment protective immunity. Mendes *et al.* (2013) demonstrated the efficacy of a heterologous prime-boost regime with adenovirus encoding *T. gondii* SAG1 (AdSAG1), followed by a modified Vaccinia Virus Ankara encoding the same antigen. *T. gondii* infected C57BL/6 mice that received the heterologous vaccination regime displayed a significantly lower number of brain cysts than those which received a homologous vaccination regime consisting of two doses of AdSAG1 [72].

Multiple heterologous prime-boost regimes have been tried and the two major forms that showed enhancing protection levels against *T. gondii* are DNA prime-protein boost and DNA prime-recombinant viral boost immunisation [41,73,74]. In the DNA prime-protein boost approach, DNA vaccines induced robust cellular immune responses and prime antigen-specific memory B-cells, whereas protein vaccines elicited antigen-specific antibodies more prominently. On the other hand, DNA prime/viral vector boost approaches focused on the induction of T-cells response. Previous study showed that the sequence of vector administration in the prime-boost regimen induced different levels of immune responses depending on the properties of the vectors [75]. For instance, mice that received a multi-epitope *T. gondii* vaccine with DNA prime/adenoviral boost elicited higher levels of Th1-type immune response with higher survival rates and lower brain cyst numbers compared to mice that received the reverse adenoviral prime/DNA boost [74].

Choosing appropriate modes of vaccine administration could be a part of inducing desired immunogenicity. Most *T. gondii* DNA vaccines employed intramuscular vaccination routes. Nonetheless, intramuscular injection of naked DNA is typically inefficient, only a limited amount of the delivered genetic material is taken up and expressed by the host cells [76]. Furthermore, muscle cells usually lack co-stimulatory molecules required for CTL responses necessary to reduce *T. gondii* cyst burden [77]. Therefore, alternative routes of *T. gondii* vaccine immunisation should be considered. Intradermal immunisation of *T. gondii* vaccines could be studied as human skin dermis and epidermis are rich in lymphocytes, APCs such as Langerhans and DCs, and the immunologically active keratinocytes [76].

Mucosal surface of the intestine is the natural site of infection for *T. gondii*, thus, stimulating mucosal defenses would resemble the natural protective immunity. Mucosal vaccination allows the delivery of a vaccine to the professional APCs residing in the lamina propria underlying the intestinal epithelium. The highly vascularised intestinal epithelium would then enhance the induction of both mucosal and systemic immunity [78]. Multiple studies have been conducted to compare the immunogenicity induced by

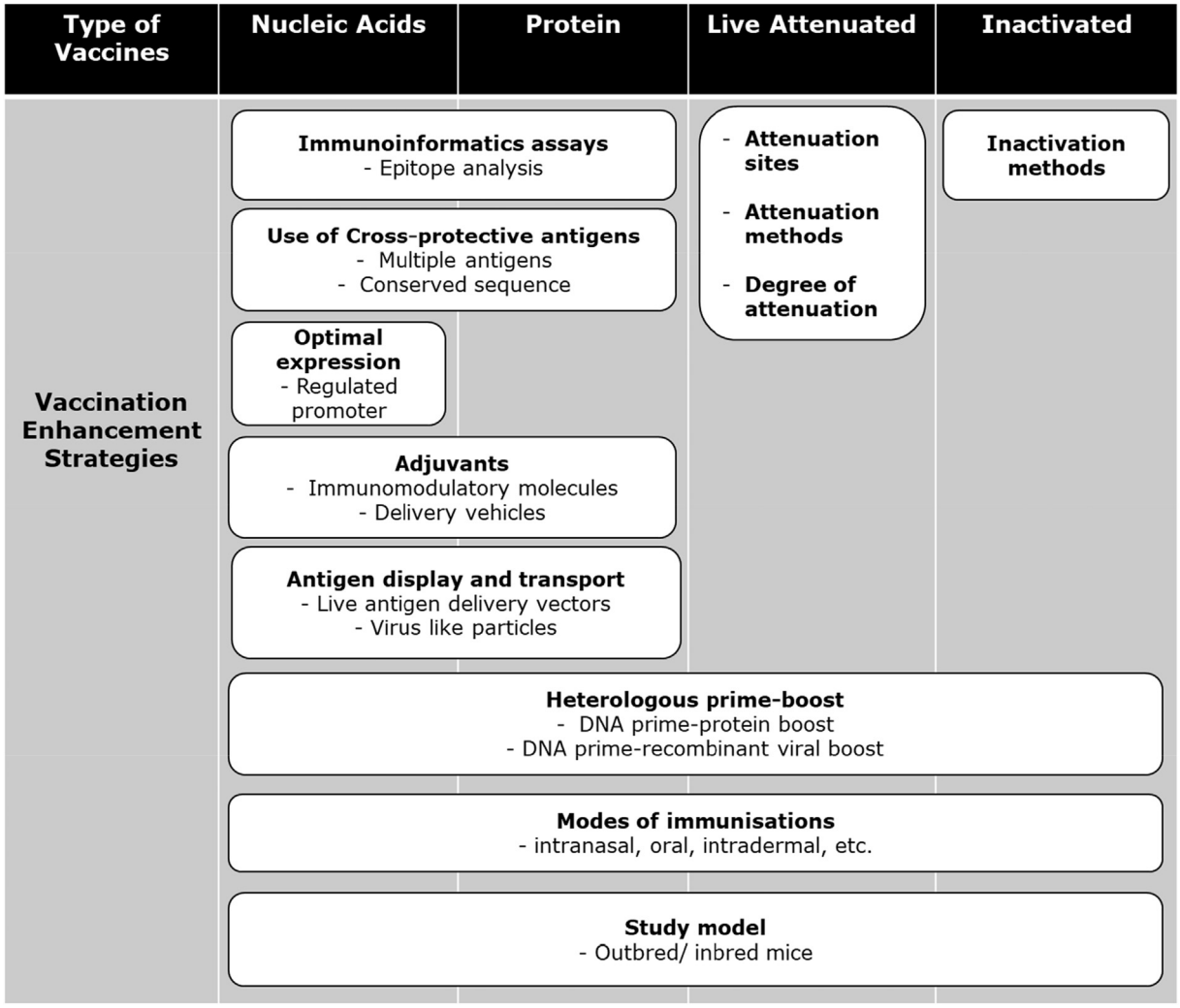

Fig. 2. An overview of strategies to improve the search for an efficacious vaccine against *T. gondii*. The enhancement strategies for vaccine design are varied among different approaches. In general, nucleic acids and protein vaccines require careful immunogenic antigens selection that could potentially provide cross protection against *T. gondii* strains that has changeable forms and complex life stages. Optimum antigen expression together with the inclusion of adjuvants and employment of antigen display or carrier are essential to enhance the immunogenicity. For live attenuated vaccines, maintaining optimal degree of attenuation with careful selection of the attenuation site and method are crucial. On the other hand, the efficacy of inactivated vaccine could be enhanced by choosing the optimal inactivation method. The combination of approaches via heterologous prime-boost regimen is also recommendable. The appropriate use of immunisations modes could further improve the protective immunogenicity. Lastly, the selection of a suitable study model is important to provide accurate examination of the vaccine efficacy.

the same antigen with different immunisation routes. Wang *et al.* (2017) reported higher levels of IgA antibodies in mice that received either intranasal or intraoral vaccination of recombinant adenovirus expressing ubiquitin-conjugated multi-stage *T. gondii* antigen, in comparison with those vaccinated via intramuscular, subcutaneous, or intravenous routes [21]. However, mucosal vaccines administered orally could be degraded by the host gastrointestinal tract natural defense and clearance mechanisms [79]. Hence, intranasal administration is more commonly used to induce mucosal immunity against *T. gondii.* Furthermore, intranasal vaccination is more efficient than intramuscular vaccination in stimulating early Th1 cellular immune responses against *T. gondii* [80]. The combination of delivery routes using the same antigen was also explored. The administration of SAG1 antigen using combined intranasal and subcutaneous routes has shown higher protection against chronic toxoplasmosis, as compared to the use of intranasal or subcutaneous route alone [81]. Similarly, the intradermal-prime and intranasal-boost vaccination of recombinant serine protease inhibitor 1 in mice showed stronger cellular and humoral immunity with lower brain cyst numbers, as compared to mice that received prime-boost via the same route [58]. Both studies suggest that a combination of immunisation approaches could enhance the protective effect of the vaccine [58,81]. As discussed in section 3.1, antigen selection is crucial in determining the vaccine immunogenicity, while the selection of immunisation routes could further enhance the type of immune responses required for protection. For instance, non-parenteral delivery routes (oral, intranasal) usually induce effective mucosal immunity characterised by higher IgA secretion and systemic immunity, while parenteral routes such as intramuscular and subcutaneous immunisation are generally lean towards systemic responses [81].

3.6. Selection of appropriate study model

As humans are outbred populations that have been exposed to microbes over millennia, using an outbred animal model for vaccine study could be more informative. Large animal models such as ewe, pig and chicken have been used for *T. gondii* vaccine challenge studies [38,65,82]. Nonetheless, due to high cost and lack of appropriate animal facilities, the usage of these animal models is limited. Therefore, the most commonly used animal model for *T. gondii* challenge is mouse, particularly the inbred mice strains

BALB/c and C57BL/6 and the outbred mouse strain, Kunming. The use of mice to model a human toxoplasma infection is mainly based on the common mammalian phylogeny between the two species. Nevertheless, both humans and mice are end-stage hosts not required for maintaining the parasite's life cycle, hence the study of *T. gondii* infection in a mouse model could mirror a physiologic host–parasite interaction as in nature [83].

The genetic background of the animal models could lead to a bias in terms of vaccine efficacy. In mice, proteins expressed by genes in the H-2 regions of the MHC complex have been associated with varies immune responses and susceptibilities against acute or chronic toxoplasmosis [84]. Mice with H-2^d haplotypes, such as outbred mice (e.g. NMRI strain) and inbred mice (e.g. BALB/c), developed less severe chronic toxoplasmosis. On the contrary, the H-2^b (e.g. C57BL/6) or H-2^k (e.g. C3H/HeOuJ) MHC haplotype are susceptible to chronic toxoplasmosis and may eventually succumb to the infection [85]. BALB/c mice (H-2^d) immunised with SAG1 protein showed 50% reduction in maternofetal transmission of *T. gondii*, whereas CBA/J mice (H-2^k) receiving the same vaccine showed 50% increase in the number of infected fetuses [86]. In another study, BALB/c mice (H-2^d) immunised with recombinant ROP5 and ROP18 showed higher reduction in brain cysts and higher survival rates as compared to immunised C3H/HeOuJ mice (H-2^k) [87]. Both studies suggest that variation in *T. gondii* vaccine efficacy is affected by the genetic background of mice. If inbred mouse strains are used, the same vaccine should be tested in a number of possible H-2 settings. Alternatively, the use of outbred strains could overcome immunological restrictions of the MHC [88].

3.7. Immunogenicity against Toxoplasma gondii: moving forward

Further exploration of the immune response (both innate and adaptive) and signaling resulting from a *T. gondii* infection should assist in the appropriate use of antigens, approaches and adjuvants to induce a protective immune response. Mice model has been used extensively to study immune response against *T. gondii*. It is one of the host species that use TLR11 and TLR12 to recognise *T. gondii* profilin, an actin-binding protein that is essential for parasite gliding motility. Subsequently, it initiates the downstream myeloid differentiation primary response 88 (MYD88) for cytokine IL-12 production. In human, TLR11 is a nonfunctional pseudogene and TLR12 is not present. Human innate cytokine response to *T. gondii* requires phagocytic uptake and endosomal processing of the live pathogen [89]. Therefore, it is believed that the phagocytosis-dependent mechanism could be masked by the profilin-driven pathway in mice. This finding has recently been extended with the demonstration of only live parasites, but not recombinant profilin, soluble tachyzoite antigen or heat-killed tachyzoites, are able to elicit IL-12 and TNF production in human monocytes and DCs. Moreover, tachyzoites that are genetically deficient in profilin can still produce cytokines [83]. These findings provide insights into utilising antigens that are able to enhance phagocytosis of live parasites for inducing better immune response in humans. Recently, B-cell lymphoma 3-encoded protein (Bcl-3), a member of the Iκβ family regulating NF-κβ complexes, has been shown to play a crucial role in combatting *T. gondii* infection. Bcl-3 expression in dendritic cells is needed to induce a protective Th1-type immune response and to prime protective T-cell-mediated immunity towards *T. gondii* [90]. Most probably, the use of genetic adjuvants together with Bcl-3 could further boost the desired immune response. Besides, Sardinha-Silva *et al.* (2017) showed that microneme proteins MIC1 and MIC4 in *T. gondii* can interact with the TLR2 and TLR4 N-glycans on APCs to induce early IL-12 response which is thought to contribute to acute control of *T. gondii* infection [91]. In brief, the development of *T. gondii* vaccines should be based on careful review of recent findings, especially the proteins and mechanisms involved in eliciting an immune response. This will help to improve the strategies for new vaccine design, formulations and delivery.

4. Conclusion

The major challenge in developing a new toxoplasmosis vaccine is to acquire both sterile protection and high safety standards within a vaccine construct. The reactogenicity of highly immunogenic live attenuated strains still remains a concern. Most studies have shown that the protection generated depends on the type of infection (acute or chronic), indicating the lack of strong cross protection against different *T. gondii* strains. As *T. gondii* has a complex life cycle, its antigenic specificity or makeup can change during different developmental stages, thus vaccination using stage-specific antigens is limited to stage-specific protection. Development of the first human vaccine against *T. gondii* shall be achievable through detailed considerations and employment of strategies at each stage of vaccine production as summarised in Fig. 2. In addition, the expansion of knowledge on toxoplasmosis immunity would provide more insights into designing an effective vaccine.

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Declaration of Competing Interest

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