Review

# A systematic review on the role of GRA proteins of *Toxoplasma gondii* in host immunization

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ABSTRACT

*Toxoplasma gondii* is a widespread obligatory intracellular parasite infecting humans and most of all other warm-blooded animals. Currently there is no any accepted vaccine for prevention of *T. gondii* infection. Many studies are focused on using of various excretory secretory antigens (ESA); and among them dense granule antigens (GRAs) being involved in parasite survival, virulence and replication processes, are considered as one of the predominant vaccine candidates. The aim of this systematic review is to prepare more comprehensive understanding of these antigens to reduce *T. gondii* infection in humans and animals. English databases, including PubMed, Science Direct, Google Scholar, Scopus, ISI Web of Science were systematically searched and papers evaluating GRA antigens published until June 2019 were selected. Evaluation of selected publications revealed that GRA4 and GRA7 substantially increased survival time of the experimental animals. It is noticeable that the maximum reduction in cyst burden was observed in BALB/c mice vaccinated with combination of GRA3, GRA7 and M2AP antigens (93.5%). GRA6 and GRA10 have shown high immunogenicity and GRA1 and 2 are important for virulence and induction of immune responses. This review will be helpful for researchers to conduct more effective studies in the field of immunization against *T. gondii* infection.

## 1. Introduction

*Toxoplasma gondii* (*T. gondii*) belonging to the phylum Apicomplexa, is a widespread obligatory intracellular parasite, that infects humans and many vertebrate animal hosts (Meng et al., 2013). This ubiquitous parasite is estimated to infect approximately one-third of the world's population. *T. gondii* infection has economic importance in veterinary medicine due to neonatal losses and abortion in domestic animals and threats to public health via food-borne outbreaks (Liu et al., 2012). Toxoplasmosis can lead to serious complications such as encephalitis and chorioretinitis in immunocompromised hosts. It also results chorioretinitis in congenital disease in fetus if pregnant women get infected for the first time during pregnancy. Generally, toxoplasmosis is asymptomatic in the immunocompetent individuals. But there is an evidence indicating that ocular toxoplasmosis can occur in adults with

acquired infections (Roberts and McLeod, 1999). Additionally some authors believe that toxoplasmosis can cause mental disorders such as schizophrenia due to presence of cysts in the brain (Dubremetz and Lebrun, 2012). So *T. gondii* infection has great importance from medical and veterinary aspects.

*T. gondii* has a complex life cycle, consisting of the sexual stage in felines and the asexual stage in humans and other intermediate hosts. Sporozoites in oocysts, tachyzoites as rapidly multiplying form and bradyzoites in tissue cysts are three pathogenic forms of *T. gondii*. During infection, tachyzoites are responsible for clinical symptoms and acute phase. Bradyzoites remain in the form of tissue cysts, which are not eliminated by the host's cellular immune responses and are responsible for chronic phase of the disease. *T. gondii* is transmitted through the placenta, thus humans can be infected by ingesting tissue cysts from undercooked meat or materials contaminated with

Toxoplasma cysts, and consuming food or water contaminated with oocysts, likewise *T. gondii* infection can occur following the accidental ingestion of oocysts present in cat feces.

In the recent years, our knowledge about *T. gondii* biology increases, yet toxoplasmosis treatment is limited to control the proliferative tachyzoite stage of the life cycle and dose not eliminate the cyst stages during chronic infection. Immunization against *T. gondii* infection is another way to control the disease. Therefore, development of a well-tolerated, safe and effective vaccine would be highly valuable in preventing and possibly treating toxoplasmosis (Ahn et al., 2006; Jongert et al., 2008).

At present, there is no licensed vaccine available for humans. Live attenuated S48 strain "Toxovax" is the only accepted vaccine against toxoplasmosis that is licensed for use in sheep (Liu et al., 2012). This vaccine is not suitable for human use because it may be changed into a pathogenic strain. To prevent toxoplasmosis, vaccine can induce strong protective immunity against toxoplasmosis, or passive immunization in cases of recrudescence (Zhang et al., 2013). Several trials using DNA or protein based vaccines, live attenuated vaccines, and heterologous vaccines have been performed. Recently, many studies started to focus on *T. gondii* antigens belonging to surface antigens (SAGs), and excretory - secretory antigens (ESA) such as micronemal proteins, rhoptry proteins (ROPs) and dense granule proteins (GRAs) that have been shown to play a major role in stimulating protective immunity (Dlugonska, 2008; Jongert et al., 2008; Weilhammer and Rasley, 2011).

Among them, GRA antigens are major proteins of the excretory secretory antigens, stored within *T. gondii* dense granules (a secretory vesicular organelles), which expresses proteins in tachyzoite, bradyzoite, and sporozoite stages of *T. gondii*. They participate in the modification of the parasitophorous vacuole (PV) and PV membrane for the maintenance of intracellular parasitism in host cells and are involved in parasite survival, virulence and replication. These proteins can be protective antigens, secreted in abundance and are major components of the vacuole surrounding tachyzoite and the cyst wall surrounding bradyzoite (Meng et al., 2013; Min et al., 2012). GRA proteins are capable of strongly stimulating the host immune responses. These molecules are potent antigens that trigger strong T and B cell responses upon infection (Scorza et al., 2003; Yin et al., 2015; Zhang et al., 2007).

The aim of this review is to retrieve published studies related to evaluation of GRA antigens as target antigen to prevent and control toxoplasmosis.

## 2. Materials and methods

### 2.1. Search process and selection criteria

This review was performed and prepared in accordance with the specified process as the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) statement (Moher et al., 2009).

English databases, including PubMed, Science Direct, Google Scholar, Scopus, ISI Web of Science were systematically searched for papers evaluated GRA antigens for vaccination and immunization against *T. gondii* infection, published from January 1999 to June 2019.

The keywords and medical subject headings (MeSH) terms have included: "*Toxoplasma gondii*"; "GRA antigens"; "DNA-Vaccine"; "Protective"; "Recombinant" and "Immune responses". To avoid missing of any article, all references of selected papers were meticulously hand-searched. Abstracts of papers published in congresses' proceedings were not included.

Studies matching the following criteria were included: (1) papers published in English language; (2) researches using of each form of DNA or recombinant vaccine defined against *T. gondii* either as a single vaccine, cocktail vaccine or in combination with adjuvant or carrier; (3) studying mice challenged by tachyzoites or cysts of *T. gondii*; and (4) having a comparison group.

Papers were excluded for at least one of the following criteria: (1)

Abstracts with insufficient information (2) papers that had not the minimum score of the criteria, (3) investigations without animal as experimental model.

All identified titles, abstracts, methods and results were carefully examined by two independent reviewers (AD and FR). The full texts of papers were independently examined by the same two reviewers. Each disagreement was resolved by discussion and the involvement of two other authors (AP and SD).

### 2.2. Data extraction, quality evaluation and statistical analysis

Information was extracted from each study including, first author, publication year, used gene/ antigen, adjuvant, route of vaccine, animal model, number of injected parasites, inoculated vaccine dose, immunology, challenge route, survival time, and parasite burden. Three reviewers (AD, FR and AP) have independently extracted the data after a discussion on the controversial literatures by using the standardized form of data collection.

The quality of all selected papers was evaluated by using the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) risk of bias tool that was adapted from the Cochrane Risk of Bias Tool to assess the methodological quality using criteria specific to animal studies (Table 1). These criteria include assessments for selection bias, performance bias, detection bias, attrition bias and reporting bias. One scoring approach was used for grading each parameter for type of bias, and at least 6 scores assigned to each study.

All experimental studies that carried out evaluation of survival time in mice, immunology survey and determination of parasite burden in immunized mice were included. Analysis and results of all included papers were carried out by two reviewers, and reported systematically (AD, FR).

This systematic review protocol is format by SYRCLE (www. SYRCLE.NL), version 2.0 (December 2014) and available in http://syrf.org.uk/protocols/.

## 3. Results

### 3.1. Number of the included studies

In total, 39 articles published from January 1999 to June 2019 (DNA vaccine and Protein vaccine), were included in the study. In Fig. 1, the PRISMA flow diagram (Moher et al., 2009) describes completely the search process in this study.

### 3.2. DNA and protein vaccine candidates

Several antigens have been evaluated as vaccine candidates of dense granule organelles including GRA1, GRA2, GRA3, GRA4, GRA5, GRA6, GRA7, GRA8,GRA14, GRA15, GRA24 and GRA41. Most of studies were focused on GRA7 (11 studies) (Fatoohi et al., 2002; Hiszczyńska-Sawicka et al., 2010; Hiszczyńska-Sawicka et al., 2011; Jongert et al., 2008; Liu et al., 2014; Min et al., 2012; Quan et al., 2012; Rosenberg et al., 2009; Vazini et al., 2018; Vercammen et al., 2000; Yin et al., 2015), GRA4 (9 studies) (Desolme et al., 2000; Hiszczyńska-Sawicka et al., 2011; Li et al., 2007; Martin et al., 2004; Meng et al., 2013; Mévélec et al., 2005; Picchio et al., 2018; Yácono et al., 2012; Zhang et al., 2007), GRA2 (8 studies) (Allahyari et al., 2016; Babaie et al., 2018; Ching et al., 2016; Golkar et al., 2007; Golkar et al., 2005; Xue et al., 2008; Zhou et al., 2007; Zhou et al., 2012) and GRA1 (7 studies) (Bivas-Benita et al., 2003; Fatoohi et al., 2002; Hiszczyńska-Sawicka et al., 2011; Jongert et al., 2008; Scorza et al., 2003; Supply et al., 1999; Vercammen et al., 2000). These antigens were tested either alone or in combination (18 as single and 21 as cocktail) for evaluation of potential effects against *T. gondii*. Based on findings, vaccines comprising more than one antigen were generally more effective than single forms. SAG1 and ROP2 as effective antigens were further applied than other antigens

**Table 1**
SYRCLE tool for assessing risk of bias.

| Item | Type of bias | Domain | Description of domain | Review authors' judgment |
|---|---|---|---|---|
| 1 | Selection bias | Sequence generation | Describe the methods used, if any, to generate the allocation sequence in sufficient detail to allow an assessment whether it should produce comparable groups. | Was the allocation sequence adequately generated and applied?[a] |
| 2 | Selection bias | Baseline characteristics | Describe all the possible prognostic factors or animal characteristics, if any, that are compared in order to judge whether or not intervention and control groups were similar at the start of the experiment. | Were the groups similar at baseline or were they adjusted for confounders in the analysis? |
| 3 | Selection bias | Allocation concealment | Describe the method used to conceal the allocation sequence in sufficient detail to determine whether intervention allocations could have been foreseen before or during enrolment. | Was the allocation adequately concealed?[a] |
| 4 | Performance bias | Random housing | Describe all measures used, if any, to house the animals randomly within the animal room. | Were the animals randomly housed during the experiment? |
| 5 | Performance bias | Blinding | Describe all measures used, if any, to blind trial caregivers and researchers from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective. | Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment? |
| 6 | Detection bias | Random outcome assessment | Describe whether or not animals were selected at random for outcome assessment, and which methods to select the animals, if any, were used. | Were animals selected at random for outcome assessment? |
| 7 | Detection bias | Blinding | Describe all measures used, if any, to blind outcome assessors from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective. | Was the outcome assessor blinded? |
| 8 | Attrition bias | Incomplete outcome data | Describe the completeness of outcome data for each main outcome, including attrition and exclusions from the analysis. State whether attrition and exclusions were reported, the numbers in each intervention group (compared with total randomized animals), reasons for attrition or exclusions, and any re-inclusions in analyses for the review. | Were incomplete outcome data adequately addressed?[a] |
| 9 | Reporting bias | Selective outcome reporting | State how selective outcome reporting was examined and what was found. | Are reports of the study free of selective outcome reporting?[a] |
| 10 | Other | Other sources of bias | State any important concerns about bias not covered by other domains in the tool. | Was the study apparently free of other problems that could result in high risk of bias?[a] |

[a] Items in agreement with the items in the Cochrane Risk of Bias tool. Hooijmans et al. BMC Medical Research Methodology 2014 14:4 doi:https://doi.org/10.1186/1471-22
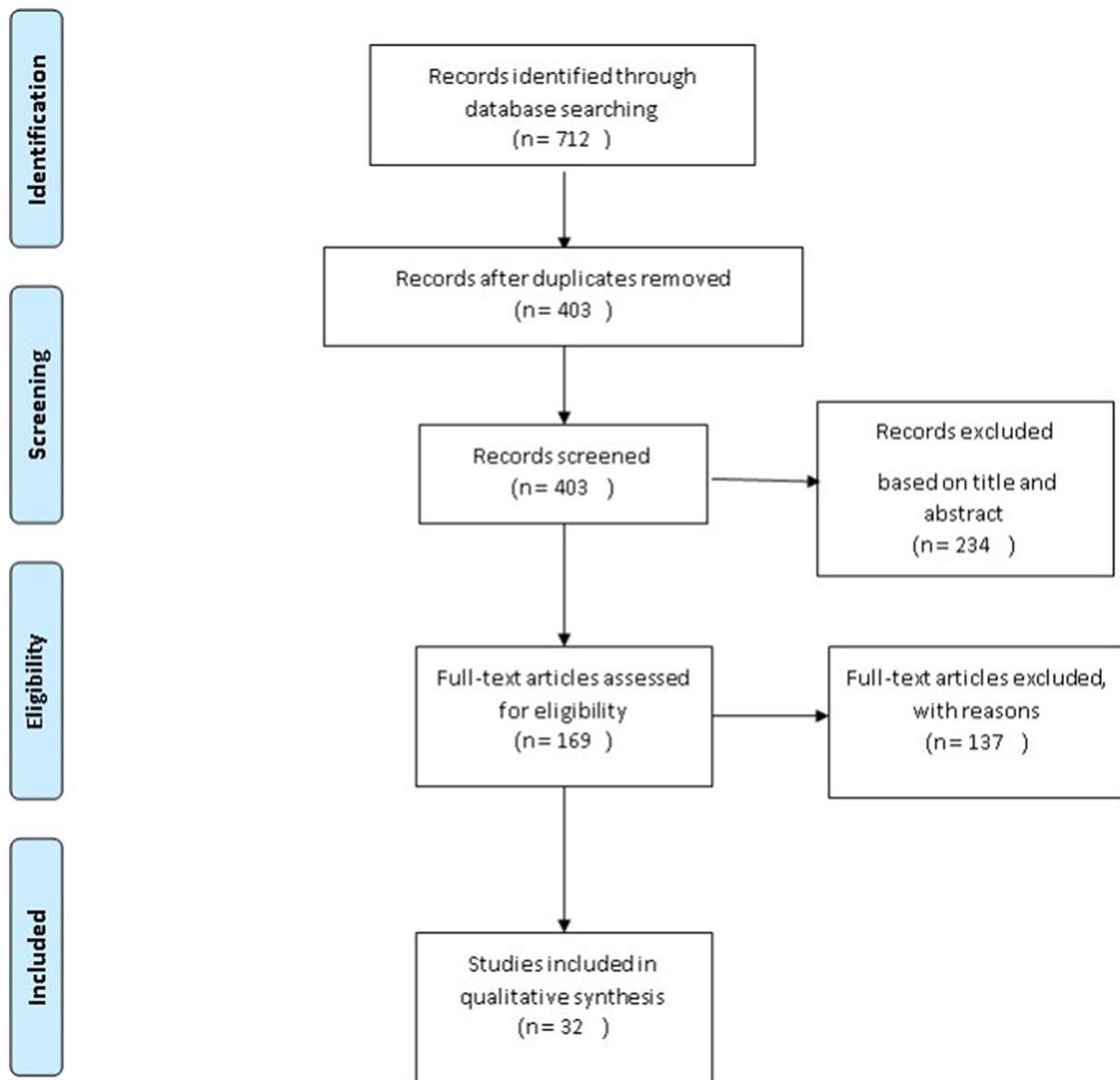
**Fig. 1.** The PRISMA flow diagram describing the study design process.

(Allahyari et al., 2016; Fatoohi et al., 2002; Martin et al., 2004; Mévélec et al., 2005; Naserifar et al., 2015; Picchio et al., 2018; Rosenberg et al., 2009; Vazini et al., 2018; Vercammen et al., 2000; Xue et al., 2008; Zhou et al., 2007; Zhou et al., 2012). Characterization of the studies containing GRAs of *T. gondii* for immunization are presented in Tables 2.

### 3.3. Adjuvants

Several adjuvants have been used in different studies to improve efficiency of vaccines. Among them, five studies have used Freund's adjuvant (Ching et al., 2016; Min et al., 2012; Supply et al., 1999; Zhang et al., 2007; Zhou et al., 2007), three -IL-12 (Desolme et al., 2000; Quan et al., 2012; Xue et al., 2008), and two other studies used Liposome (Hiszczyńska-Sawicka et al., 2010; Hiszczyńska-Sawicka et al., 2011) MPL (Babaie et al., 2018; Golkar et al., 2007) and adjuvants such as Emulsigen (Hiszczyńska-Sawicka et al., 2010), CPG (Rosenberg et al., 2009), GM-CSF (Mévélec et al., 2005), Chitosan (Bivas-Benita et al., 2003), PreS2 (Zhou et al., 2012), BCG (Li et al., 2007), CpG motifs (Jongert et al., 2008), Alum (Martin et al., 2004), and B7–2 (Liu et al., 2014) were used only once.

### 3.4. Animal models

In several studies, animal species such as mouse, sheep, pig and ewe were used for a range of experiments including evaluation of cellular and humoral responses. Mice were the most widely used as animal models. The three murine models utilized most frequently for vaccination included -BALB/c, C57BL/6 and C3H. However, a few studies used also OF1, Kumming, and CBA/J strain of mice as animal models.

### 3.5. Route of inoculation

In different studies different routes of administration in animal models were used including intramuscular, subcutaneous, intraperitoneal and oral. However, intramuscular administration was the most frequently used (25 studies) (Chen et al., 2015; Chu et al., 2018; Desolme et al., 2000; Ghaffarifar et al., 2014; Hiszczyńska-Sawicka et al., 2010; Hiszczyńska-Sawicka et al., 2011; Li et al., 2007; Liu et al., 2014; Martin et al., 2004; Meng et al., 2013; Mévélec et al., 2005; Naserifar et al., 2012; Naserifar et al., 2015; Pagheh et al., 2019; Quan et al., 2012; Rosenberg et al., 2009; Scorza et al., 2003; Sun et al., 2011; Vazini et al., 2018; Vercammen et al., 2000; Xue et al., 2008; Yin et al.,

**Table 2**
The detailed information about the included studies.

| Antigene(s) | Adjuvant(s) | Antigen delivery/Animal model (s) | Challenge Strain/route | Results — Immune responses/ Survival | Results — Cyst reduction | Score | Authors/year |
|---|---|---|---|---|---|---|---|
| GRA1 | Incomplete Freund's adjuvant | i.p. Mice (12.5 mg) s.c.Sheep (25 mg) OF1 mice sheep | 1000 Cysts Mice and 2000 oocysts Sheep of 76 K (oral) | Humoral immune response with developed IgG and Increased IFN-γ 13 day | … | 8 | (Supply et al., 1999) |
| GRA1 | – | i.m. (100 μg) C3H | Type II IPB-G and IPB-M | High level of IgG, CD8+ and CD4+ T cells | | 6 | (Scorza et al., 2003) |
| GRA 1 | Chitosan nanoparticle | Intragastric. (50 μg) C3H/ HeN | RH (i.p) | High levels IgG2a and IgG1 | | 6 | (Bivas-Benita et al., 2003) |
| GRA 2 | | s.c. (100 μg) CBA/J | RH (i.p) | Humoral immune response with developed IgG | | 6 | (Golkar et al., 2005) |
| GRA 2 | MPL | i.p. (20 brain cysts) C57BL/6 | RH (i.p) | Strong humoral response, predominantly of IgG1 subclass and cellular response | GRA2 decreased brain cysts production by 37.1%, compared to the MPL group (p < .01; by 44% as compared to the PBS group | 9 | (Babaie et al., 2018) |
| GRA 4 | IL-12 | i.m. (100 μg) C57BL/6 | 76 K (oral) | Increased IFN-γ and IL-10 and IgG 62% With IL12 25% | | 9 | (Desolme et al., 2000) |
| GRA4 | Freund's complete adjuvant | i.p. (100 μg) C57BL/6 | 20,000 of RH and PLK/GFP (i.p) | Th2-dominant immunity with a higher level of IgG1 than IgG2a, high level of IFN-γ 45 days | The cyst numbers were 400 and 600, respective ly | 9 | (Zhang et al., 2007) |
| GRA4 | … | oral. (1 μg) C57BL/6 | 20 cysts of Me49 (oral) | Humoral immune response with developed IgG | 59% | 7 | (Yácono et al., 2012) |
| GRA 4 | | i.m. (100 μg) BALB/c | $10^3$ of RH (i.p) | High levels of total IgG, IgG2a isotype and (IFN- γ), which suggested a specific Th1 immunity was activated 11.8 ± 4.8 days | … | 7 | (Meng et al., 2013) |
| GRA 5 | | i.m. (75μg) BALB/c | RH (i.p) | High levels of Il-4, IFN-γ and IgG, IgG2a, IgG subtypes 9 day | | 7 | (Naserifar et al., 2012) |
| GRA 6 | LMS | i.m. (100 μg) BALB/c | 1000 of RH (i.p) | Increased IgG 53.3% | | 7 | (Sun et al., 2011) |
| GRA 7 | Liposomes, Emulsigen P and Emulsigen D | i.m. (1 mg two injections 4 weeks apart to maximum of 2 ml per injection) Ewes | RH (i.p) | Liposomes formulated GRA7 vaccine induced a Th1 type of immune response, whilst formulation with Emulsigen D elicited a significant antibody response | | 6 | (Hiszczyńska-Sawicka et al., 2010) |
| GRA 7 | Complete Freund's | s.C and i.m. (100 μg) BALB/c | RH (i.p) | High levels of IgG and IgG2a IFN- γ | 60% | 7 | (Min et al., 2012) |
| GRA 8 | | i.m. (100 μg) BALB/c mice | $10^3$ of RH (i.p.) | higher IgG antibody titers with predominant IgG2a production; increased levels of IL-10, IL-12 (p70), IFN-γ, TNF-α 9 days | | 8 | (Chu et al., 2018) |
| GRA 14 | Calcium phosphate (CaPNs) or Aluminum hydroxide (Alum) nano-adjuvants | i.m. (100 μg) BALB/c mice | $10^4$ of RH (i.p.) | Increased IgG1 and IgG2a high level of IFN-γ and IL4 | 50% | 9 | (Pagheh et al., 2019) |
| GRA 24 | | i.m. (100 μg) BALB/c mice | $10^2$ of RH (i.p.) | high levels of a mixed Th1/Th2 cytokines Humoral immune response with developed IgG and Increased IFN-γ, IL-4, IL-10, IL-12 24 days | | 8 | (Zheng et al., 2019) |
| GRA 41 | | i.p. (100 μg) BALB/c mice | RH (i.p) and cysts of PRU | higher IgG antibody titers with predominant IgG2a production and Increased IFN-γ, IL-4, IL-10 | 59.34% | 9 | (Zhou et al., 2019) |

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**Table 2** (*continued*)

| Antigene(s) | Adjuvant(s) | Antigen delivery/Animal model (s) | Challenge Strain/route | Results — Immune responses/ Survival | Cyst reduction | Score | Authors/year |
|---|---|---|---|---|---|---|---|
| GRA1 GRA7 | CpG | i.p. (250 µg) bred pigs | $4 \times 10^3$ of RH tachyzoites (i.p)$2 \times 10^3$ brain cysts of IPB-G (oral) | Strong humoral and Type 1 cellular immune response characterized by the production of IFN-γ | | 6 | (Jongert et al., 2008) |
| GRA2 GRA6 | Monophospho ryl lipid A (MPL) | s.c. (20 µg single.10 µg cocktail) CBA/J mice | 20 cysts of Pru-gal (i.p.) | Humoral immune response with developed IgG and Increased IFN-γ | rGRA2: 69.8% rGRA6: failed | 8 | (Golkar et al., 2007) |
| GRA2 SAG1 | Freund's complete adjuvant | s.c. (100 µg) BALB/c | $10^4$ of RH (i.p.) | Strong immune response with high level of IgG and Increased IFN-γ 15 days | … | 8 | (Zhou et al., 2007) |
| GRA2 SAG1 | Hepatitis B virus surface antigen, PreS2 | i.m. (100 µg) BALB/c mice | $10^4$ of RH (i.p.) | Humoral immune response with developed IgG and Increased IFN-γ, IL-4, IL-10 SAG1: 13 day GRA2:11 day SAG1 + GRA2: 16 day | | 8 | (Zhou et al., 2012) |
| GRA2 SAG5 | Complete /incomplete Freud's adjuvant | s.c. (10 µg) BALB/c | $10^2$ of RH (i.p.) | Increased of IgG, IgG1, IgG2a and IFN-γ 16 day | … | 7 | (Ching et al., 2016) |
| GRA2 SAG1 | … | s.c. (10 µg rGRA2, Rsag1, 20 µg rGRA2, rSAG1) BALB/c | $10^3$ of RH (i.p.) | Enhanced IgG and high level of IL-4, IL-10 and IFN-γ 21 day | … | 7 | (Allahyari et al., 2016) |
| GRA4 ROP2 | Alum | i.m. (10 µg rROP2, rGRA4 100 µg rGRA4) C57BL/6 C3H mice | 20 cysts forsublethal dose, 100 cysts for lethal dose of ME49 (oral) | Enhanced IgG and IFN-γ and splenocyte proliferation … | sublethal dose: rR + rG > rGRA4 > Rrop2 > rGRA4 lethal dose: rROP2 > rR + rG > rGRA4 | 8 | (Martin et al., 2004) |
| GRA 4 SAG 1 | GM-CSF | i.m. (100 µg pSAG1mut or 100 µg pGRA4 or 50 µg pSAG1mut plus 50 µg pGRA4 or 50 µg pSAG1mut plus 50 µg pGRA4 with 50 µg, pGM-CSF) C57BL/6 | 40 cysts of 76K (oral) | High level of IL-4, IL-10 and IFN-γ 75% With GM-CSF 87% survival | | 8 | (Mévélec et al., 2005) |
| GRA4 SAG2 | BCG | i.m. (100 µg) BALB/c | RH (i.p.) | Enhanced IgG, IgG1, IgG2a and IFN-γ, Lymphocyte proliferation 8 day | … | 7 | (Li et al., 2007) |
| GRA 5 MIC 3 | | i.m. (100 µg) BALB/c | $10^4$ of RH (i.p.) | Increased IgG and IgG2a high level of IFN-γ and low level of IL4 The mean days of survival is 4, 4.6, 7.8, 7,8 and 8.6 for PBS, pc GNA3, GRA4, MIC3 and GRA4 + MIC3 respectively | | 7 | (Ghaffarifar et al., 2014) |
| GRA 7 ROP 1 | IL12 | i.m. (100 µg) BALB/c | RH (i.p) and 20 cysts of Me49 (oral) | Increased IgG2a, (IFN- γ), IL-10, and (TNF-); Tcell proliferation 27 day | 80% | 9 | (Quan et al., 2012) |
| GRA 7 ROP 2 | | i.m. (100 µg) BALB/c | $5 \times 10^4$ of RH (i.p.) | Increased IgG1 and IgG2a high level of IFN-γ and low level of IL4 | | 7 | (Vazini et al., 2018) |
| ROP 16 GRA 7 | B7–2 | i.m. (500 µg) Kunming | 1000 of RH (i.p) | High level of IgG and IgG2a subclass titers, IFN-γ, cD8+ T cells IL-4 and IL-10 27 day | | 8 | (Liu et al., 2014) |
| ROP 5 GRA 15 | | i.m. (100 µg) Kunming | $10^3$ of RH (i.p), 10 tissue cysts of PRU (oral) | High level of IgG2a, IFN-g, IL-2, IL12 p40 and IL-12 p70; CD8+ T cells 19.4 ± 4.9 days for ROP5; 17.8 ± 3.8 days for GRA15 | 57.4% for ROP5; 65.9% for GRA15 | 8 | (Chen et al., 2015) |

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**Table 2** (*continued*)

| Antigene(s) | Adjuvant(s) | Antigen delivery/Animal model (s) | Challenge Strain/route | Results — Immune responses/ Survival | Cyst reduction | Score | Authors/year |
|---|---|---|---|---|---|---|---|
| GRA1, GRA7, ROP2 | | i.m. (100 µg) C57BL/6, C3H, and BALB/c | 50 cysts of IPB-G in C3H (perorally) either 50 or 200 cysts of 76 K in BALB/c (perorally), and 10 cysts of IPB-G in C57BL/6 (perorally) | Increased IgG1, IgG2a and IFN-γ  Survival rates increased from 10% in controls to at least 70% after vaccination in one case and from 50% to at least 90% in the other | GRA1, 266 ± 137 GRA7, 152 ± 45 ROP2, 134 ± 54 | 8 | (Vercammen et al., 2000) |
| SAG 1 ROP 2 GRA 2 | IL-12 | i.m. (100 µg) BALB/c | 10⁴ of RH (i.p.) | Increased of IFN-γ, IL-4 and IL-12, IgG2a, IgG1 23 day | | 8 | (Xue et al., 2008) |
| GRA4 ROP2 TgPI-1 | | intradermal and intranasal  C3H/HeN | Me49 (oral) | High level of IgA, IgG, IgG1, IgG2a (The high titer of IgG2a to IgG1), IFN-γ and IL-12 | 50% | | (Picchio et al., 2018) |
| GRA 5 SAG 1 ROP 2 | | i.m. (75 µg) BALB/c | 10⁴ of RH (i.p.) | Strong production of IgG1 and IgG2a, IFN-γ, 12 day | | 7 | (Naserifar et al., 2015) |
| GRA1 GRA6 GRA7 SAG1 | ... | 10 µg/ml Whole-blood cultures from pregnant women | Three tachyzoites/cell of RH (i.p.) | High level of IgA, IgG, IgG1, IgG2a (The high titer of IgG2a to IgG1), IFN-γ and IL-12 ... | ... | 6 | (Fatoohi et al., 2002) |
| GRA1 GRA 4 GRA6 GRA 7 | liposomes | i.m. (1 mg) Sheep | RH (i.p.) | Strong production of IgG2 antibody and IFN-γ, Th-1 and IgG1 | | 6 | (Hiszczyńska-Sawicka et al., 2011) |
| pcEC3 (GRA3-GRA7-M2AP) pcEC2 (MIC2-MIC3-SAG1 | CpG motifs | i.m. (100 µg) BALB/c | 20 Prugniaud isolate type II, 10 Trousseau isolate type III (oral) | Humoral and cellular responses with increased level of IgG, IFN-γ, and IL-10 16 day | pcEC2: 84.7% pcEC3: 93.5% pcEC2 + pcEC3: 100% | 9 | (Rosenberg et al., 2009) |
| SAG3ROP18MIC6 GRA7MAG1 BAG1 SPA | | i.m. (100 µg) BALB/c | 10³ of RH (i.p) 20 cysts of PRU (gastric route) | Induced a more robust Th1-prone immunity confirmed by the higher level of IgG2a subtype compared with IgG1. Higher Th1 cytokine levels of IL-2 and IFN-γ, and enhanced CD8+ Tcells 67% | (296 ± 92) | 8 | (Yin et al., 2015) |

2015; Zheng et al., 2019; Zhou et al., 2012; Zhou et al., 2019).

### 3.6. Challenge route and immune response

The high virulent strain RH and the low virulent strains such as PRU and ME49 were predominantly used in both DNA and protein vaccines for evaluation of survival rates and reduction in brain cyst burden. Regularly, the mice were challenged 2–4 weeks after the immunization with $10^2$–$10^4$ tachyzoites or 20–80 cysts from different parasite strains by injection intraperitoneally (31 studies) (Allahyari et al., 2016; Babaie et al., 2018; Bivas-Benita et al., 2003; Chen et al., 2015; Ching et al., 2016; Chu et al., 2018; Fatoohi et al., 2002; Ghaffarifar et al., 2014; Golkar et al., 2007; Golkar et al., 2005; Hiszczyńska-Sawicka et al., 2010; Hiszczyńska-Sawicka et al., 2011; Jongert et al., 2008; Li et al., 2007; Li et al., 2015; Liu et al., 2014; Meng et al., 2013; Min et al., 2012; Naserifar et al., 2012; Naserifar et al., 2015; Pagheh et al., 2019; Quan et al., 2012; Sun et al., 2011; Supply et al., 1999; Vazini et al., 2018; Xue et al., 2008; Yin et al., 2015; Zheng et al., 2019; Zhou et al., 2007; Zhou et al., 2012; Zhou et al., 2019) or orally (12 studies) (Chen et al., 2015; Desolme et al., 2000; Jongert et al., 2008; Martin et al., 2004; Mévélec et al., 2005; Picchio et al., 2018; Quan et al., 2012; Rosenberg et al., 2009; Supply et al., 1999; Vercammen et al., 2000; Yácono et al., 2012; Yin et al., 2015).

Table 2 indicates immunity response in mice vaccinated with GRAs, after the vaccination. The concentration of inoculation for immunization of mice was between 50 and 500 µg, while many studies utilized 100 µg injected in a volume of 1 ul antigen. The survival rate in immunized mice challenged with different amount of inoculated *T. gondii* virulent strain was between 9 and 45 days or 25–90%. Single vaccine forms of GRA4 and GRA7 have substantially extended mice survival time compared to other antigens. In addition, combination vaccines with SAG1 or ROP2 demonstrated better results for survival time, but none of the antigens could completely protect against acute toxoplasmosis after challenge with *T. gondii* RH. The maximum amount of reduction of cyst burden was up to 93.5% in BALB/c mice with a mixture of GRA3, GRA7 and M2AP antigens.

## 4. Discussion

A lot of vaccination trials against *T. gondii* have been carried out to evaluate their immunological potentials in animal models, including inactivated and attenuated vaccine, DNA vaccine, subunit vaccine, and genetically engineered vaccine.

GRA proteins, that are secreted in abundance, are the major components of ESA expressing by both the vacuole-surrounding tachyzoites and the cyst-wall-surrounding bradyzoites (Yin et al., 2015).

According to current systematic review, among GRA proteins, GRA7 was the most tested candidate for vaccine development. TgGRA7 has been found in all infectious stages of parasite. Intermediate hosts, can be infected with *T. gondii* via oocyst ingestion released from cat feces, tissue-cyst ingestion of infected animals and congenital route (Jongert et al., 2008). Thus, immunization with GRA7 can destroy sporozoites (before releasing tachyzoites) and bradyzoites (before replace of disease). Importantly, GRA7 elicits strong humoral and cellular immunity against *T. gondii*. GRA7 recently has been recognized as a 29-kDa protein (Hiszczyńska-Sawicka et al., 2010) and it also reacts with sera of humans infected with acute and chronic forms (Golkar et al., 2005). Therefore, it can be considered as a good candidate for vaccine development.

Recent reports have demonstrated that vaccination with combination of antigens boosts the immune responses efficacy compared to single antigens. For example, cocktail vaccines GRA15-ROP5 (Chen et al., 2015), GRA 5- MIC3 (Ghaffarifar et al., 2014), GRA1- GRA7- ROP 2 (Vercammen et al., 2000) and GRA2- SAG1 (Allahyari et al., 2016) have indicated stronger increased protection against *T. gondii* infection than single antigen vaccines (Meng et al., 2013). However,

heterologous prime-boost strategy has been introduced as a potent vaccination approach for inducing both very strong humoral and cellular immune responses, which utilize two different vaccine types expressing the same antigen, injected at different intervals (Sun et al., 2011). The results indicated that DNA prime-protein boost (GRA7) (Chen et al., 2015) and DNA prime-peptide boost (GRA4) (Vercammen et al., 2000) vaccination generated significant protective immune response against *T. gondii*.

Moreover, multi-epitope-based vaccines are presently a popular subject for research, and they have obtained important results, since they are able to remove unfavorable factors and also induce immune protection that is highly specific (Ghaffarifar et al., 2014; Min et al., 2012). surveyed epitopes of several antigens derived from tachyzoite, bradyzoite and sporozoite of *T. gondii*, including SAG3 (101–144), ROP18 (347–396), MIC6 (288–347), GRA7 (182–224), MAG (158–125), BAG1 (156–211) and SPA (142–200), by Prime-DNA and boost-recombinant protocol and this immunization proved to be a potential way to protect mice challenged with *T. gondii* (Naserifar et al., 2012).

In our systematic review we compared vaccines using different factors such as adjuvant, the animal model species, route of injection, parasite strains and doses for challenge, strain and stage of the parasite utilized.

The aim of vaccination is the generation of a high immune response and long-term protection against infection. To achieve this objective, the addition of an adjuvant is often required. Adjuvants are substances that enhance the specific immune response of the vaccine antigens. These can be used for various purposes, such as reduce the amount of antigen or the number of immunizations needed for immunity; boost the immunogenicity of purified or recombinant antigens or improve the efficacy of vaccines in immunocompromised persons, elderly or newborns.

Several adjuvants have been used as part of the vaccine formulation such as IL-12, freund's complete adjuvant (FCA), freund's incomplete adjuvant (FIA), liposome, emulsigen, CPG, Granulocyte-macrophage colony-stimulating factor, GM-CSF, Chitosan, PreS2, BCG, CpG motifs, Alum, monophosphoryl lipid A (MPL) and B7–2 that improve a vaccine's efficacy. Ideally, adjuvants should promote an appropriate immune response, not induce immune responses against themselves, be stable with long shelf life and cheap to produce. In these reviewed studies, the most used adjuvants were Freund's (complete, incomplete or both), that enhance the immune response strongly and increase the survival of mice challenged with *T.gondii*. Despite, FCA being the gold standard adjuvant, FCA causes severe local reactions and is considered toxic for human use. But Freund's incomplete adjuvant (FIA) is less toxic and has been used in human vaccine formulations (Petrovsky and Aguilar, 2004).

According to studies, mice BALB/c (Allahyari et al., 2016; Ching et al., 2016; Chu et al., 2018; Ghaffarifar et al., 2014; Li et al., 2007; Meng et al., 2013; Min et al., 2012; Naserifar et al., 2012; Naserifar et al., 2015; Pagheh et al., 2019; Quan et al., 2012; Rosenberg et al., 2009; Sun et al., 2011; Vazini et al., 2018; Vercammen et al., 2000; Xue et al., 2008; Yin et al., 2015; Zheng et al., 2019; Zhou et al., 2007; Zhou et al., 2012; Zhou et al., 2019), C57BL/6 (Babaie et al., 2018; Desolme et al., 2000; Golkar et al., 2007; Martin et al., 2004; Mévélec et al., 2005; Vercammen et al., 2000; Yácono et al., 2012; Zhang et al., 2007) and C3H (Bivas-Benita et al., 2003; Martin et al., 2004; Picchio et al., 2018; Scorza et al., 2003; Vercammen et al., 2000) mice were utilized more than other mice and animal models (sheep, pig and ewe) for surveying immunization. In a study, BALB/c and C3H mice DNA-vaccinated with any of the three antigens (GRA1, GRA7, and ROP2) revealed specific cellular immune responses (Vercammen et al., 2000) by proliferation of splenocyte and secretion of cytokine (IFN- γ). In contrast, C57BL/6 mice vaccinated with antigens couldn't significantly induce cellular immune responses (Hiszczyńska-Sawicka et al., 2011). Therefore, selection of a proper experimental model is necessary for

immunization study. BALB/C mice are excellent models for lethal challenge. With increasing of IFN-γ production and having higher survival time than other murine models, mice seem to be proper as animal model.

Routes of administration in animal models is another factor that can effect on success of vaccination. Unfortunately, it was not discussed in any of the included studies what was the reason for the use of a certain route of injection for immunization. It is observed that intramuscular route has been used more than other routes, which can be due to the presence of many blood vessels in the musculature that create rapid passage for inoculated vaccine(McCluskie et al., 1999).

Challenge methods are one of the effective factors in vaccination studies, and the parasite inoculation routes depend on target of vaccine. If the goal of vaccine study is acute phase infection or preventing congenital toxoplasmosis, usually challenge routes are performed as intraperitoneal or subcutaneous using tachyzoites. But if target of study is decreasing the parasite load or examination of parasite burden in brain cysts, oral route using of tissue cysts or oocysts is better than others (Chen et al., 2015).

According to findings, almost 81% of studies used strain RH for challenge experiments in animal models (Allahyari et al., 2016; Babaie et al., 2018; Bivas-Benita et al., 2003; Chen et al., 2015; Ching et al., 2016; Chu et al., 2018; Fatoohi et al., 2002; Ghaffarifar et al., 2014; Golkar et al., 2005; Hiszczyńska-Sawicka et al., 2010; Hiszczyńska-Sawicka et al., 2011; Jongert et al., 2008; Li et al., 2007; Liu et al., 2014; Meng et al., 2013; Min et al., 2012; Naserifar et al., 2012; Naserifar et al., 2015; Pagheh et al., 2019; Quan et al., 2012; Sun et al., 2011; Vazini et al., 2018; Xue et al., 2008; Yin et al., 2015; Zhang et al., 2007; Zhou et al., 2007; Zhou et al., 2012; Zhou et al., 2019). The RH strain, type I genome, is incomplete in tissue cyst formation in organs and was found to be fully virulent in mice and other animal models. Vaccination with strain RH, in addition to increased survival time during acute toxoplasmosis (Mévélec et al., 2005), provided a considerable protection against brain cyst formation and defective protection against congenital toxoplasmosis (Naserifar et al., 2015). Of course, in immunization studies, it is better to use both high and low pathogen strains to evaluate vaccine against acute and chronic toxoplasmosis.

*T. gondii* induces a strong cellular immune response. Tachyzoites stimulate macrophages to produce tumour necrosis factor-alpha (TNF-α) and interleukin-12 (IL-12) (Bivas-Benita et al., 2003; Xue et al., 2008). IL-12 acts on natural killer (NK) cells and T lymphocytes to produce IFN- γ, which is crucial for protection. IFN-γ and TNF-α kill synergistically tachyzoites by macrophages (Xue et al., 2008). Among the T lymphocytes, CD8+ T lymphocytes are the main effectors for protection against toxoplasmosis, whereas CD4+ T lymphocytes play a synergistic role. Other cytokines including IL-10 IL-4, IL-5 have a vital function in balancing immune responses. Experimental models immunized with vaccine candidates also induce humoral response (Liu et al., 2014).

Moreover, different studies related to vaccines against *T. gondii,* evaluate survival time of animal models and the number of brain cysts in them (Quan et al., 2012). The survival time of vaccinated mice against *T. gondii* infection is thought to be the most direct parameter for evaluating a vaccine candidate (Zhang et al., 2007). With regards to findings, survival time in different GRAs, was 9–45 days or 25–90%. The results and evidence of GRA-based vaccines presented in this systematic review of cellular and humoral production and the survival rate, suggest that the order of the immunization is very important and can be a valuable starting point to develop an effective and safe vaccine against *T. gondii* infection for animal and human use. Thus, the presenting of this systematic review article encourage and support future studies to select effective target antigens and then inoculate them to animal models via an optimal delivery strategy for stimulating appropriate protective immunity.

## 5. Conclusion

As *T. gondii* GRA$_S$ are involved in parasite survival, virulence and replication, these antigens are one of the predominant vaccine candidates. For successful vaccination procedure, previous studies emphasized that immune responses can be enhanced by DNA immunization with cocktails of more than single antigen and vaccinated animal models showed the lowest tissue cyst burden in the brain and the highest survival time. Among GRAs antigens, GRA4 and GRA7 prolonged substantially survival time in the experimental animals. It is noticeable that the maximum reduction in cyst burden was observed in BALB/c mice vaccinated with combination of GRA3, GRA7 and M2AP antigens. GRA6 and GRA10 have shown high immunogenicity and GRA1 and 2 are important for virulence and induce immune response. Therefore, these antigens could be effective candidates for immunization against toxoplasmosis. We do hope that this systematic review will help researchers to plan and conduct studies more effectively in the field of immunization against *T. gondii* infection.

## Declaration of Competing Interest

None.

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