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REVIEW OF DNA VACCINE APPROACHES AGAINST THE PARASITE *TOXOPLASMA GONDII*

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KEY WORDS

Toxoplasma gondii
DNA vaccines
Adjuvants
Antigens

ABSTRACT

Toxoplasma gondii is an apicomplexan parasite that affects both humans and livestock. Transmitted to humans through ingestion, it is the second-leading cause of foodborne illness-related death. Currently, there exists no approved vaccine for humans or most livestock against the parasite. DNA vaccines, a type of subunit vaccine which uses segments of the pathogen's DNA to generate immunity, have shown varying degrees of experimental efficacy against infection caused by the parasite. This review compiles DNA vaccine efforts against *Toxoplasma gondii*, segmenting the analysis by parasite antigen, as well as a review of concomitant adjuvant usage. No single antigenic group was consistently more effective within in vivo trials relative to others.

Toxoplasma gondii is an obligate intracellular parasite of the Apicomplexan phylum that can infect any nucleated mammalian cell and is carried by at least one-quarter of the world's human population. While chronic infection of healthy individuals is generally benign, transient cervical lymphadenopathy may occur during the initial acute phase of infection. However, infection of immunocompromised patients can cause pneumonia, encephalitis, and even death (Demar et al., 2007). Acute infection of pregnant women during or shortly before gestation may also cause neonatal death or fetal abnormalities due to transmission to the fetus (Assir et al., 2014). Once establishing a chronic infection, the parasite can be found in various solid organs, particularly muscle tissue and the brain (Dunay et al., 2018). Overall in the United States, it is estimated that *T. gondii* is the second leading cause of death due to foodborne illness and has an economic impact of over $3 billion each year (Scallan et al., 2011; Hoffmann et al., 2012).

Toxoplasma gondii has 3 stages to its life cycle: oocysts, tachyzoites, and bradyzoites. The oocyst stage is the only portion of the life cycle that is produced by sexual reproduction and originates in the feline intestine. *Toxoplasma* can be acquired by humans in several ways. Exposure to cat feces in food or water allows for the ingestion of oocysts, and eating undercooked mammalian flesh allows for the acquisition of *Toxoplasma* tissue cysts. Once inside the host, ingested parasites differentiate into tachyzoites, which define the acute stage of infection, leading to rapid replication in host tissue. Pressure from the host's immune system ultimately forces the parasites into the latent bradyzoite form, which results in intracellular cyst formation and characterizes chronic infection (Black and Boothroyd, 2000). The bradyzoite stage can be reactivated (usually through immune suppression) and will redifferentiate into tachyzoites, thus restarting the cycle of intracellular spread (McConkey et al., 2013).

Control of *Toxoplasma* in humans is limited due to several factors. *Toxoplasma gondii* can cross the blood-brain barrier to establish persistent infection in the central nervous system, making it a challenge for drug therapy capable of clearing infection due to the general chemical inaccessibility of this compartment (Alday and Doggett, 2017). Treatment for symptomatic disease (often in the form of reactivated toxoplasmosis) usually consists of a combination of 2 antimicrobials. Currently, the most common regimens include a combination of pyrimethamine and sulfadiazine, or pyrimethamine coadministered with other drugs such as clindamycin, atovaquone, clarithromycin, or azithromycin (Neville et al., 2015). Currently, all drugs used for clinical applications are only effective against the acute tachyzoite stage of infection and are associated with side effects, which has driven impressive efforts in search of additional antiparasitic compounds (McFarland et al., 2016).

Due to the limited applicability of these treatments, control may be more fully obtained via preventative measures such as that offered by vaccination. Ideally, a *Toxoplasma* vaccine would be able to offer protection for humans and agricultural animals, the latter leading to reduced transmission to humans (Innes et al., 2019). Currently, there is 1 vaccine on the market against *Toxoplasma*, called *Toxovax*, which is a live attenuated vaccine specifically designed to reduce fetal abortion in sheep. It is not approved for use in humans, or in the United States, and offers limited protection and duration of efficacy (Liu et al., 2012). More recently, *Toxovax* has been shown to reduce tissue cysts in pigs (Burrells et al., 2015).

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There are several different methods for creating protective vaccines including live attenuated vaccines, inactivated or killed vaccines, toxoid vaccines, and subunit vaccines. Live attenuated vaccines are created by weakening or altering the pathogen in a way that it is non-disease causing. The immunogenicity of these vaccines varies based on attenuation, and thus their efficacy is variable. Additionally, fears of the parasite reverting to a pathogenic strain make this type of vaccine approach less appealing. Killed or inactivated vaccines function by altering the pathogen by heating or using chemicals such as formalin to destroy the pathogen's ability to replicate, and thus the pathogen cannot revert to its more virulent state, making them safer than live attenuated vaccines. Due to the intracellular nature of infection of *T. gondii*, a toxoid vaccine, a vaccine made with an altered form of pathogen-essential toxin, would be less viable. Finally, subunit vaccines aim to expose the host immune system to a limited, often defined, portion of antigens derived from the pathogen. Subunit vaccines are effective against some diseases but often suffer from relatively low immunogenicity (Liu et al., 2012).

Attempts to develop experimental *T. gondii* vaccines for the last 30 yr have met limited success (Wang et al., 2019). Live-attenuated vaccines have been based on mutant strains such as strain S48 used in *Toxovax*. Other strains such as T-263 are used in live vaccine attempts in felines to suppress oocyst shedding, but live T-263 is difficult to produce and deliver large-scale (Verma and Khanna, 2013; Innes et al., 2019). Recent developments in genetic technology allow for new live vaccines based on gene editing, or the deletion of certain genes, which may produce a higher immune response in hosts. For example, there has been 1 live vaccine made using a CRISPR/Cas9 approach to delete a gene in an attempt to stop the fertilization of oocysts in felines that has proved to be completely effective (Ramakrishnan et al., 2019). However, a broader safety profile and multi-species efficacy still need to be established. Inactivated or killed vaccines have been attempted but with less efficacy than live vaccines, though they are considered safer because they cannot revert to a pathogenic strain (Innes et al., 2019). Subunit vaccines have been investigated in over 200 studies for a *Toxoplasma* vaccine with emphasis on antigens that affect *T. gondii* attachment to host cells and evasion of host immune response, mostly recombinant protein antigens. The main method of delivery in the past has usually involved live, nonpathogenic bacteria, viruses, and other microbes (Wang et al., 2019). Notably, using a major histocompatibility complex (MHC)-matched parasite peptide antigen (HF10), Blanchard et al. (2008) achieved complete protection from lethal infection by using antigen-pulsed bone marrow-derived dendritic cells. Recently, other delivery methods involving nanoparticles and exosomes have been explored for their ability to protect the antigen and thus produce a higher immune response and lower number of cysts (Beauvillain et al., 2009; Assolini et al., 2017). Major challenges to all vaccination attempts include the genetic diversity amongst different parasite strains and hosts, the multistage life cycle, the lack of knowledge about the transition from the acute stage to latent stage, and the absence of standard vaccination procedures across attempts (Wang et al., 2019).

DNA vaccines are an emerging type of subunit vaccines in which a portion of the pathogen's DNA is inserted into a bacterial vehicle vector and delivered to the host. Once the DNA has been injected into tissue, some cells will uptake the vector, which is then translocated to the nucleus. Injection of DNA vaccines may be done intramuscularly, intradermally, or subcutaneously. Next, the pathogen's DNA is transcribed and translated as if it were from the host. Following translation, the protein is presented to the immune system where it is recognized as foreign (Hobernik and Bros, 2018).

The use of DNA vaccines provides several advantages (Smith et al., 2020; Yu et al., 2020). DNA vaccines are easily adaptable to new pathogens, as there is little work required to change the specific antigens of the vaccine. Additionally, plasmid vectors are extremely malleable and can be readily modified. Because of the ability to select the specific antigens present in the vaccine, it can be specifically engineered to produce a tailored immune response, especially those suited for efficacy against intracellular pathogens such as *T. gondii*. The multiple stages in a parasite's complex life cycle have different proteins that evoke an immune response, thus the ability to tailor the immune response with specific antigens allows for more efficacy across stages (Ghaffarifar, 2015). Another distinct advantage is the ease of production and distribution. Propagation of the vaccine is very rapid and the cost to do so is low, even on a large scale. DNA vaccines also do not require cold-chain storage for stability, which allows for easy distribution. Finally, DNA vaccines have been considered safer than other vaccine options, as they usually require low dosage and are unable to revert to their virulent state (Shedlock and Weiner, 2000).

The efficacy of DNA vaccines is continuing to be explored in other parasites besides *T. gondii*. In *Trypanosoma cruzi*, which causes Chagas disease in humans, clinical trials that explored administration of a DNA vaccine to mice during an ongoing infection have shown that after just 2 doses there was a limitation of disease progression (Dumonteil, 2007). A DNA vaccine is also currently being developed for *Leishmania major*, the parasite associated with cutaneous leishmaniasis. The vaccine, named LEISHDNAVAX, contains several *Leishmania* protein antigens that have been shown to elicit T-cell responses in people of different genetic backgrounds and may move forward into clinical trials (Sacks et al., 2016). With these promising results demonstrated in other parasites, a DNA vaccine against *T. gondii* could also be efficacious.

Our aim in this review is to summarize recent *Toxoplasma* DNA vaccine attempts and provide a concise collection of outcomes aligned by antigen or adjuvant which could promote future vaccine investigations that lead to increased potency and efficacy against *Toxoplasma gondii*.

MATERIALS AND METHODS

Manuscripts were selected from PubMed and Toxoblog.com using the search term "*Toxoplasma gondii* DNA vaccine," with papers selected that were dated 2010 and after. In total 95 unique papers were reviewed to create a matrix that summarized methods used in *T. gondii* DNA vaccine experiments (Tables I–III). Papers were searched for genes used (and if they were used in conjunction with other genes), and the sequence of the genes and which parasite strain they were from, as well as the type of plasmid backbone and the inclusion of adjuvants. Papers excluded in this review matrix included papers that did not perform a parasite challenge in a mouse model, papers that did not use a plasmid delivery system (such an adenovirus) (Li et al., 2015a, 2016; Yin et

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al., 2015), papers that used a DNA prime/boost method to enhance response (Rashid et al., 2011, 2017; Min et al., 2012; Meng et al., 2013), and papers that were missing too many of the key variables such as challenge method and dosage. Further, publications before 2010 are only briefly summarized and not included in the tables. ToxoDB.org datasets were used to evaluate the levels of transcriptional expression of antigens listed in Table I.

RESULTS

As of 2018, there were more than 500 clinical trials that focused on DNA vaccines, with most targeting viral infections and cancer; however, due to the safety, stability, and cost-effectiveness of DNA vaccines, they are a valuable focus for other areas including bacterial infection, autoimmune diseases, and parasites. Some DNA vaccines have been approved by the Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) for veterinary use, including vaccines against West Nile Virus in horses and canine melanoma. To create a potent DNA vaccine, the design should include 2 well thought out elements: antigens (to target the immune response) and adjuvants (to enhance the immune response) (Hobernik and Bros, 2018).

Before the year 2000, there was minimal research into DNA vaccines against *T. gondii*. However, between 2000 and 2010, the investigation of DNA vaccines as possible protection against *T. gondii* began to take off, almost exclusively with work being conducted in mice. Early DNA vaccine work on *T. gondii* focused on antigens such as GRA1, GRA7, and ROP2 to determine whether DNA vaccines incorporating these antigens could elicit an immune response and whether they proved effective against *T. gondii* infection (Leyva et al., 2001; Bivas-Benita et al., 2003; Scorza et al., 2003; Martin et al., 2004; Roque-Reséndiz et al., 2004; Wei et al., 2006; Hiszczyńska-Sawicka et al., 2010). Several of these studies noted the efficacy of ROP2-base DNA vaccines to delay the death of mice given a lethal parasite challenge. GRA7 was also used in combination with other antigens, leading to increased survival rates (Jongert et al., 2007, 2008a; Rosenberg et al., 2009). Further studies investigated SAG1, MIC3, and GRA4 as antigens (Chen et al., 2002, 2009; Couper et al., 2003; Ismael et al., 2003, 2009; Martin et al., 2004; Yang et al., 2005; Zhang et al., 2007a; Liu et al., 2008; Fang et al., 2009, 2010; Shang et al., 2009). Many of these studies showed promising results at high doses or against non-fetal acquired parasites (Couper et al., 2003; Fachado et al., 2003). Additionally, some studies investigated the efficacy of bradyzoite antigens such as BAG1 and MAG1 (Nielsen et al., 2006; Dautu et al., 2007; Zimmermann et al., 2008). Studies also used different combinations of these antigens (Fachado et al., 2003; Martin et al., 2004; Cong et al., 2005; Mévélec et al., 2005; Yang et al., 2005; Jongert et al., 2007, 2008b; Zhang et al., 2007b; Cui et al., 2008; Xue et al., 2008a, 2008b; Liu et al., 2009; Qu et al., 2009; Rosenberg et al., 2009; Wang et al., 2009). Common adjuvants tested in these early DNA vaccines included CTxA2/b, GMCSF, CPG-ODN, IL-2, and IL-12 (Chen et al., 2002; Mévélec et al., 2005; Cong et al., 2008; Cui et al., 2008; Xue et al., 2008b; Zimmermann et al., 2008; Ismael et al., 2009).

Antigens

The biological function of the parasite antigen produced by a DNA vaccine, as well as its cellular localization, can play an important role in antigenicity. Figure 1 outlines several subcellular locations from which antigens are selected for vaccine experiments. Surface or secreted proteins allow for easier access by the immune system as opposed to intracellular proteins. As *T. gondii* is a complex organism with three different infectious stages, stage specificity and the variety of invasion pathways must be taken into consideration, as the acute and latent forms of toxoplasmosis are induced by very distinct forms of the pathogen. In addition, there are different strains of *Toxoplasma*, so the use of an antigen (portion) that is heavily conserved among strains makes the vaccine more versatile (Liu et al., 2012). Since immunity to *Toxoplasma* has been correlated to a Th1 immune response, most vaccines attempt to achieve a higher Th1 response (Ghaffarifar, 2015). Size is also an important consideration when choosing an antigen: a large plasmid will have a lower transfection rate when entering the cell, and also a large protein may have more difficulty in translation. While peptide-encoding DNA vaccines are less immunogenic than their full-length gene counterparts, they benefit from smaller size—however, fewer attempts with antigen protein regions have been conducted. It is also vital that the chosen antigen is not similar to those produced by humans, as these may be identified by the adaptive immune response as self-antigens and thereby ignored by the immune system (Dumonteil, 2007).

Codon optimization, or the accommodation of codon bias of the host organism, is also valuable when creating a DNA vaccine, as it can strongly enhance antigen expression in the host, thus providing greater amounts of antigen to be recognized by the immune response (Dumonteil, 2007). If foreign antigen production is sufficiently high, DNA vaccines have been shown to elicit antibody responses in vaccination trials. Notably, this is not always expected, as many DNA vaccine antigens are produced and remain in the cytoplasm where humoral immunity is considered inferior to cell-mediated immune responses (Shedlock and Weiner, 2000).

Given the nature of *T. gondii* virulence, antigens used in vaccines are often surface antigens (SAGs), and apically situated rhoptry antigens (ROPs), microneme antigens (MICs), and dense granule antigens (GRAs), as these are the main genes encoding surface proteins or secreted proteins necessary for successful host cell invasion, respectively (Fig. 2B). Another functional class of genes explored more recently as vaccine antigens are the essential parasite genes, those which encode proteins that are necessary for the parasite to replicate and survive. The majority of DNA vaccines also encode for most or all of the coding regions of antigen genes to ensure providing the highest number of epitopes.

Microneme proteins (MICs): Microneme proteins (MICs) are released onto the parasite's surface and interact with host cell receptors to aid in gliding motility machinery and successful host cell invasion (Yuan et al., 2013). This critical role makes MIC proteins attractive targets for DNA vaccine studies, and 7 different MIC genes were observed in *T. gondii* DNA vaccine studies reviewed herein.

The *T. gondii* MIC1/MIC4/MIC6 complex is involved in host cell attachment. Thus, each of these genes has been tested as possible antigens against both acute and chronically infected

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Table I. Summary of challenge results from single antigens DNA vaccines against *Toxoplasma gondii*. A summary of results from publications since 2010 employing a single parasite antigen (generally full-length) delivered via DNA vaccination followed by challenge, organized by antigen class. Tz = tachyzoites.

Gene	Dosage (per injection)	Acute challenge	Acute result*	Chronic challenge	Chronic result†	Prominent stage expression	Reference
Microneme Proteins (MICs)							
MIC1	10 μg	80 ME49 cysts	40–50% survival	40 ME49 cysts	52% reduced	Bz§, Tz, Sp	Pinzan et al., 2015
MIC3	100 μg	10,000 RH Tz	+8 days	100 RH Tz	unknown	Bz, Tz§, Sp	Gong et al., 2016
MIC3‡	100 μg	10,000 RH Tz	20% survival	None	N.D.	Bz, Tz§, Sp	Ghaffarifar et al., 2019
MIC3	100 μg	1000 RH Tz	+7 days	None	N.D.	Bz, Tz§, Sp	Qu et al., 2013
MIC4	10 μg	80 ME49 cysts	40–50% survival	40 ME49 cysts	47% reduced	Bz, Tz§, Sp	Pinzan et al., 2015
MIC6	10 μg	80 ME49 cysts	40–50% survival	40 ME49 cysts	27% reduced	Bz§, Tz, Sp	Pinzan et al., 2015
MIC6	100 μg	80 Pru cysts	+20 days	20 Pru cysts	40% reduced	Bz§, Tz, Sp	Yan et al., 2012
MIC6	100 μg	1,000 RH Tz	+10 days	20 Pru cysts	38% reduced	Bz§, Tz, Sp	Xu et al., 2019
MIC8‡	100 μg	1,000 RH Tz	+6 days	20 Pru cysts	25% reduced	Bz§, Tz, Sp	Li et al., 2014
MIC8‡	100 μg	80 Pru cysts	+11 days	None	N.D.	Bz§, Tz, Sp	Li et al., 2014
MIC8	100 μg	1,000 RH Tz	+6 days	None	N.D.	Bz§, Tz, Sp	Liu et al., 2010a
MIC11	100 μg	1,000 RH Tz	17% survival	None	N.D.	Bz, Tz§, Sp	Tao et al., 2013
MIC13	100 μg	1,000 RH Tz	+21 days	10 Pru cysts	57% reduced	Bz, Tz, Sp§	Yuan et al., 2013
Surface Antigens (SAGs)							
SAG1‡	50 μg	50 RH Tz	40% survival	None	N.D.	Bz, Tz§, Sp	Liu et al., 2010b
SAG1	100 μg	1,000 RH Tz	+7 days	None	N.D.	Bz, Tz§, Sp	Mavi et al., 2019
SAG1‡	100 μg	1,000 RH Tz	+2 days	None	N.D.	Bz, Tz§, Sp	Maraghi et al., 2019
SAG1	50 μg per plasmid (total of 100 μg)	10,000 RH Tz	+4 days	None	N.D.	Bz, Tz§, Sp	Hoseinian Khosroshahi et al., 2011
SAG1	100 μg	10,000 RH Tz	+6 days	None	N.D.	Bz, Tz§, Sp	Meng et al., 2012
SAG1	100 μg	10,000 RH Tz	+7 days	20 Pru cysts	37% reduced	Bz, Tz§, Sp	Han et al., 2017b
SAG1	100 μg	10,000 RH Tz	+6 days	None	N.D.	Bz, Tz§, Sp	Zhou et al., 2012
SAG1	100 μg	100,000 RH Tz	+3 days	None	N.D.	Bz, Tz§, Sp	Wu et al., 2012
SAG2C	100 μg	None	N.D.	20 Pru cysts	72% reduced	Bz	Zhang et al., 2013a
SAG2D	100 μg	None	N.D.	20 Pru cysts	23% reduced	Bz	Zhang et al., 2013a
SAG2X	100 μg	None	N.D.	20 Pru cysts	70% reduced	Bz	Zhang et al., 2013a
SAG4	100 μg	100,000 RH Tz	+7 days	20 Pru cysts	66% reduction	Bz§, Tz	Zhou and Wang, 2017
SAG5a	100 μg	None	N.D.	20 Pru cysts	35% reduced	Low expression	Lu et al., 2015
SAG5b	100 μg	10,000 RH Tz	+7 days	20 Pru cysts	44% reduced	Low expression	Lu et al., 2017
SAG5c	100 μg	10,000 RH Tz	+6 days	20 Pru cysts	41% reduced	Tz	Lu et al., 2017
SAG5d‡	100 μg	10,000 RH Tz	+7 days	None	N.D.	Tz	Lu et al., 2014
Dense Granule Proteins (GRAs)							
GRA1	100 μg	10,000 RH Tz	+6 days	100 RH Tz	unknown	Bz, Tz§, Sp	Gong et al., 2016
GRA1	100 μg	100,000 RH Tz	Not significant	None	N.D.	Bz, Tz§, Sp	Wu et al., 2012
GRA2	100 μg	1,000 RH Tz	+3 days	None	N.D.	Bz§, Tz, Sp	Ching et al., 2017
GRA2	100 μg	10,000 RH Tz	+4 days	None	N.D.	Bz§, Tz, Sp	Zhou et al., 2012
GRA5	100 μg	1,000 RH Tz	+3 days	None	N.D.	Bz, Tz§, Sp	Ching et al., 2017
GRA6‡	100 μg	1,000 RH Tz	40% survival	None	N.D.	Bz, Tz§, Sp	Sun et al., 2011
GRA7‡	50 μg per plasmid (total of 100 μg)	1,500 ME49 cysts	+12 days	20 ME49 cysts	55% reduced	Bz§, Tz, Sp	Quan et al., 2012
GRA7	100 μg	1,000 RH Tz	+5 days	None	N.D.	Bz§, Tz, Sp	Mavi et al., 2019
GRA7‡	100 μg	1,000 RH Tz	+12 days	None	N.D.	Bz§, Tz, Sp	Liu et al., 2014
GRA8	100 μg	1,000 RH Tz	+4 days	None	N.D.	Bz, Tz§, Sp	Chu et al., 2018
GRA14‡	100 μg	100,000 RH Tz	+5 days	None	N.D.	Bz§, Tz, Sp	Ahmadpour et al., 2017b
GRA14‡	100 μg	100,000 RH Tz	+5 days	None	N.D.	Bz§, Tz, Sp	Ahmadpour et al., 2017a
GRA16	100 μg	1,000 RH Tz	+2 days	10 Pru cysts	44% reduced	Bz, Tz§	Hu et al., 2017
GRA17	100 μg	1,000 RH Tz	+2 days	None	N.D.	Bz, Tz§	Zhu et al., 2017
GRA23	100 μg	1,000 RH Tz	+4 days	None	N.D.	Bz, Tz§	Zhu et al., 2017
GRA24	100 μg	100 RH Tz	+25 days	None	N.D.	Bz§, Tz, Sp	Zheng et al., 2019b
GRA24	100 μg	1,000 RH Tz	+5 days	20 Pru cysts	29% reduced	Bz§, Tz, Sp	Xu et al., 2019
GRA25	100 μg	1,000 RH Tz	+7 days	20 Pru cysts	41% reduced	Bz, Tz§, Sp	Xu et al., 2019

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Table I. Continued.

Gene	Dosage (per injection)	Acute challenge	Acute result*	Chronic challenge	Chronic result†	Prominent stage expression	Reference
Rhoptry Proteins (ROPs)							
ROP1‡	50 μg per plasmid (total of 100 μg)	1,500 ME49 cysts	20% survival	20 ME49 cysts	55% reduced	Bz, Tz§, Sp	Quan et al., 2012
ROP1	100 μg	1,000 RH Tz	+12 days	None	N.D.	Bz, Tz§, Sp	Sonaimuthu et al., 2016
ROP2	50 μg per plasmid (total of 100 μg)	10,000 RH Tz	+2 days	None	N.D.	Low expression	Hoseinian Khosroshahi et al., 2011
ROP5	100 μg	10,000 RH Tz	+4 days	20 Pru cysts	58% reduced	Bz, Tz§, Sp	Wang et al., 2016b
ROP5	100 μg	1,000 RH Tz	+14 days	6 Pru cysts	52% reduced	Bz, Tz§, Sp	Zhu et al., 2020
ROP7	100 μg	10,000 RH Tz	+5 days	20 Pru cysts	58% reduced	Bz, Tz§, Sp	Wang et al., 2016b
ROP8	100 μg	1,000 RH Tz	50% survival	None	N.D.	Bz, Tz§, Sp	Parthasarathy et al., 2013
ROP8	100 μg	2,000 RH Tz	+2 days	None	N.D.	Bz, Tz§, Sp	Foroutan et al., 2020b
ROP8‡	100 μg	2,000 RH Tz	+2 days	None	N.D.	Bz, Tz§, Sp	Foroutan et al., 2020b
ROP9	100 μg	1,000 RH Tz	+12 days	None	N.D.	Bz§, Tz, Sp	Chen et al., 2014b
ROP13‡	100 μg	1,000 RH Tz	+49 days	10 Pru cysts	40% reduced	Bz§, Tz, Sp	Wang et al., 2012
ROP13	100 μg	10,000 RH Tz	N.D.	N.D.	N.D.	Bz§, Tz, Sp	Alizadeh et al., 2019
ROP16‡	100 μg	1,000 RH Tz	+13 days	None	N.D.	Bz§, Tz, Sp	Liu et al., 2014a
ROP16	100 μg	1,000 RH Tz	+24 days	None	N.D.	Bz§, Tz, Sp	Yuan et al., 2011a
ROP17	100 μg	1,000 RH Tz	+14 days	None	N.D.	Bz§, Tz, Sp	Wang et al., 2016a
ROP18	100 μg	80 Pru cysts	+12 days	20 Pru cysts	30% reduced	Bz, Tz§, Sp	Chen et al., 2018
ROP18	100 μg	1,000 RH Tz	+17 days	6 Pru cysts	47% reduced	Bz, Tz§, Sp	Zhu et al., 2020
ROP18	100 μg	1,000 RH Tz	+9 days	None	N.D.	Bz, Tz§, Sp	Qu et al., 2013
ROP18	100 μg	1,000 RH Tz	+48 days	None	N.D.	Bz, Tz§, Sp	Yuan et al., 2011b
ROP19	100 μg	None	N.D.	20 Pru cysts	57% reduced	Bz, Tz§	Zhou et al., 2016
ROP21	100 μg	1,000 RH Tz	+7 days	10 Pru cysts	37% reduced	Bz§, Tz	Zhang et al., 2018d
ROP22	100 μg	1,000 RH Tz	+8 days	10 Pru cysts	40% reduced	Low expression	Zhang et al., 2019
ROP29‡	100 μg	None	N.D.	20 Pru cysts	53% reduced	Low expression	Lu et al., 2018
ROP35	100 μg	1,000 RH Tz	+8 days	10 Pru cysts	38% reduced	Bz, Tz§, Sp	Zhang et al., 2018c
ROP38	unknown	1,000 RH Tz	+2 days	10 Pru cysts	77% reduced	Tz§	Xu et al., 2014
ROP54	100 μg	1,000 RH Tz	+8 days	10 Pru cysts	36% reduced	Bz, Tz§, Sp	Yang et al., 2017
Other Antigens							
PLP1	100 μg	80 Pru cysts	+20 days	20 Pru cysts	44% reduced	Bz§, Tz, Sp	Yan et al., 2012
PLP1	100 μg	80 Pru cysts	+12 days	20 Pru cysts	30% reduced	Bz§, Tz, Sp	Chen et al., 2018
PLP1‡	100 μg	1,000 RH Tz	+8 days	None	N.D.	Bz§, Tz, Sp	Yan et al., 2011
ADF	100 μg	1,000 RH Tz	+2 days	None	N.D.	Bz, Tz§, Sp	Li et al., 2011
ROM4‡	100 μg	1,000 RH Tz	+6 days	None	N.D.	Bz, Tz§, Sp	Rahimi et al., 2017
ROM4	100 μg	10,000 RH Tz	+6 days	20 Pru cysts	41% reduced	Bz, Tz§, Sp	Han et al., 2017b
ROM4 (peptide)	100 μg	10,000 RH Tz	+5 days	20 Pru cysts	36% reduced	Bz, Tz§, Sp	Han et al., 2017b
ROM4 (full gene + peptide)	100 μg	10,000 RH Tz	+12 days	20 Pru cysts	57% reduced	Bz, Tz§, Sp	Han et al., 2017b
ROM4	unknown	1,000 RH Tz	13.3% survival	10 Pru cysts	44% reduced	Bz, Tz§, Sp	Zhang et al., 2015
ROM5	unknown	1,000 RH Tz	20% survival	10 Pru cysts	72% reduced	Bz§, Tz, Sp	Zhang et al., 2015
RON5	100 μg	1,000 RH Tz	+8 days	10 Pru cysts	26% reduced	Bz§, Tz	Zhao et al., 2016
ESA10	100 μg	10,000 RH Tz	+10 days	None	N.D.	Bz§, Tz, Sp	Wang et al., 2015c
MDH	100 μg	20,000 RH Tz	+9 days	None	N.D.	Bz, Tz§	Hassan et al., 2014c
DPA	100 μg	20,000 RH Tz	+12 days	None	N.D.	Bz	Hassan et al., 2014a
CDPK1‡	100 μg	1,000 RH Tz	+17 days	20 Pru cysts	46% reduced	Bz§, Tz, Sp	Chen et al., 2014a
CDPK1‡	100 μg	1,000 RH Tz	+14 days	10 Pru cysts	46% reduced	Bz§, Tz, Sp	Chen et al., 2016
CDPK1	100 μg	1,000 RH Tz	+10 days	None	N.D.	Bz§, Tz, Sp	Huang et al., 2019
CPDK2	100 μg	1,000 RH Tz	+11 days	None	N.D.	Bz§, Tz, Sp	Chen et al., 2017
CDPK3	100 μg	1,000 RH Tz	+17 days	10 Pru cysts	54% reduced	Bz, Tz, Sp§	Zhang et al., 2013b
CDPK5	100 μg	1,000 RH Tz	+4 days	10 Pru cysts	39% reduced	Bz§, Tz	Zhang et al., 2014
IF-2a	100 μg	1,000 RH Tz	+16 days	20 Pru cysts	44% reduced	Bz, Tz§, Sp	Chen et al., 2013b
IF-4a	100 μg	1,000 RH Tz	+29 days	None	N.D.	Bz, Tz§, Sp	Chen et al., 2013a
EF-1a	100 μg	10,000 RH Tz	+9 days	None	N.D.	Bz, Tz§	Wang et al., 2015a
CP-B	100 μg	10,000 RH Tz	+5 days	None	N.D.	Bz, Tz, Sp§	Zhao et al., 2013a

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Table I. Continued.

Gene	Dosage (per injection)	Acute challenge	Acute result*	Chronic challenge	Chronic result†	Prominent stage expression	Reference
CP-C1‡	100 μg	10,000 RH Tz	+6 days	None	N.D.	Bz, Tz§, Sp	Han et al., 2017a
CP-L	100 μg	10,000 RH Tz	+4 days	None	N.D.	Bz, Tz, Sp§	Zhao et al., 2013a
IMP1	100 μg	500 RH Tz	+22 days	None	N.D.	Bz, Tz§, Sp	Cui et al., 2012
ADF	100 μg	1,000 RH Tz	+2 days	None	N.D.	Bz, Tz§, Sp	Li et al., 2011
DOC2C	100 μg	1,000 RH Tz	+10 days	10 Pru cysts	48% reduced	Bz§, Tz, Sp	Zhang et al., 2018b
HSP40	100 μg	1,000 RH Tz	+3 days	10 Pru cysts	62% reduced	Bz§, Tz, Sp	Li et al., 2018b
HSP60	100 μg	1,000 RH Tz	+19 days	10 Pru cysts	48% reduced	Bz, Tz§	Li et al., 2018a
HSP70‡	10 μg	10 ME49 cysts	N.D.	N.D.	N.D.	Bz§, Tz, Sp	Czarnewski et al., 2017
ASP1	100 μg	10,000 RH Tz	+9 days	None	N.D.	Bz, Tz, Sp§	Zhao et al., 2013b
Profilin‡	100 μg	None	N.D.	10 Pru cysts	39% reduced	Bz, Tz, Sp§	Gao et al., 2018
Profilin	100 μg	None	N.D.	10 Pru Cysts	49% reduced	Bz, Tz, Sp§	Zhang et al., 2018a
MYR1	100 μg	100 RH Tz	+29 days	None	N.D.	Bz, Tz§, Sp	Zheng et al., 2019a
SOD	100 μg	1,000 ME49 Tz	+10 days	None	N.D.	Bz, Tz§	Liu et al., 2017
NTPase	5 μg	2,000 RH(Δku80)	+10 days	20 Pru cysts	78% reduced	Bz, Tz§	Zheng et al., 2017b
p14-3-3	100 μg	10,000 RH Tz	+5 days	None	N.D.	Bz, Tz§, Sp	Meng et al., 2012
Toxofilin‡	50 μg	10,000 RH Tz	+9 days	20 Pru cysts	33% reduced	Bz§, Tz, Sp	Song et al., 2017
CyP	100 μg	500 RH Tz	38% survival	None	N.D.	Bz, Tz§, Sp	Gong et al., 2013
PP2C	100 μg	10,000 RH Tz	+11 days	None	N.D.	Sp	Song et al., 2020
GST	100 μg	10,000 RH Tz	+6 days	None	N.D.		Wang et al., 2015b
SPATR	100 μg	100 RH Tz	+10 days	None	N.D.	Bz§, Tz, Sp	Zheng et al., 2017a
GR	100 μg	20,000 RH Tz	+7 days	None	N.D.	Bz, Tz§	Hassan et al., 2014b
ERK7	100 μg	1,000 GT1 Tz	+1 day	None	N.D.	Bz§, Tz, Sp	Guo et al., 2019
ERK7	100 μg	20 Pru Cysts	+2 days	None	N.D.	Bz§, Tz, Sp	Guo et al., 2019

* Acute result: n % survival indicates the percentage of vaccinated mice surviving parasite challenge, typically via administration of parasite tachyzoites (Tz); or.+n days indicates the number of days vaccination permitted survival beyond the control group.
† Chronic result: indicates reduction in brain cyst numbers compared to the unvaccinated group. N.D. indicates not determined.
‡ Indicates antigen + adjuvant was also tested and included in Table III.
§ Indicates the parasite stage with the highest transcriptional expression according to ToxoDB.org. Bz: bradyzoite, Sp: sporozoite.

mice. Parasite MIC1 and MIC4 both act as adhesins and will bind to host cell receptors (Reiss et al., 2001). DNA vaccination incorporating MIC1 administered to mice showed a 40–50% survival rate against the acute challenge of the parasite, and chronically infected mice showed a 52% cyst burden reduction. Similarly, MIC4 showed a 40–50% acute infection survival rate and 47% cyst burden reduction in chronically infected mice. DNA vaccination with MIC6, which acts as an escort for both MIC1 and MIC4, permitted a 40–50% survival rate amongst acutely infected mice and in all other studies showed an increase in survival ranging from 10 to 20 days post-treatment (Meissner et al., 2002). In preventing the establishment of the chronic stage of the parasite, MIC6-based DNA vaccines reduced cyst burden by 27–40%.

MIC3 is expressed in all 3 infectious stages of *T. gondii* and is a potent adhesion protein of the parasite. It has been shown to elicit an early and powerful immune response in vivo (Fang et al., 2009; Yuan et al., 2013). Incorporating MIC3 into a DNA vaccination led to 20% overall survival or an increase in survival by 7–8 days in acutely infected mice.

MIC8 and MIC13 have been described as involved in *Toxoplasma* host cell adhesion (Friedrich et al., 2010; Li et al., 2014). Treatment with a MIC8-based DNA vaccine led to an increased survival of acutely infected mice by 6–11 days and a 25% cyst burden reduction in chronically infected mice. MIC13-based DNA vaccination led to an increase in survival of 21 days against acutely infected mice and led to a 57% cyst burden reduction in chronically infected mice. MIC 11 has been described as involved in the organization of other MICs during adhesion (Tao et al., 2013). MIC11-based DNA vaccination led to a 17% survival of acutely infected mice.

A full summary of the use of micronemes in *Toxoplasma* DNA vaccine papers and their results can be found in Table I. In summary, DNA vaccines encoding MIC antigens reduced chronic parasite loads from 30–60%, while extending survival from acute infection by approximately 10 days (median).

Surface antigens (SAGs): The surface of *T. gondii* is covered in often heavily modified DNA-encoded protein surface antigens (SAGs) that play various roles in the pathogenesis of the parasite, as they are the first portion of *T. gondii* to make contact with the host cell and are thus an attractive target for vaccine development. There are 5 proteins in the superfamily of surface antigens, SAG1–SAG5. SAG1 is the most abundant of the surface antigens, making up about 3–5% of all *T. gondii* tachyzoite proteins, and it plays a major role in tachyzoite adhesion and invasion of the host cell (Roozbehani et al., 2018). SAG1-based DNA vaccines led to an increase in survival ranging from 2 to 7 days against an acute challenge and a 37% chronic cyst burden reduction.

Unlike SAG1, the SAG2CDXY cluster, made of SAG2C, SAG2D, SAG2X, and SAGY, as well as SAG4, are detectable on the surface of bradyzoites, which contribute to chronic *T. gondii* infection (Zhang et al., 2013a; Zhou and Wang, 2017). SAG2C-based, SAG2D-based, and SAG2X-based vaccines led to 72, 23,

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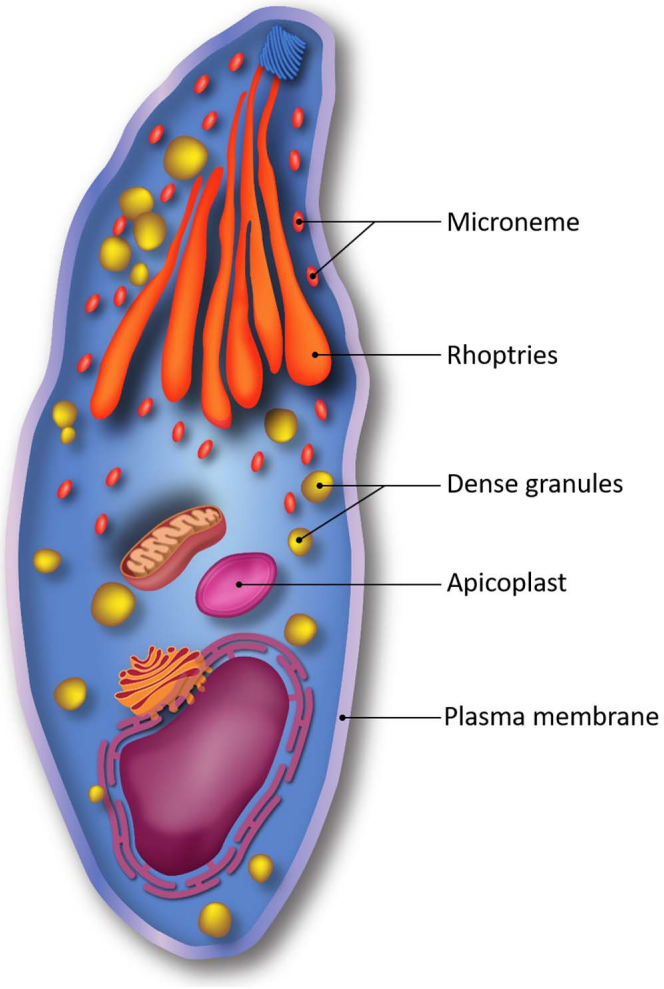

Figure 1. A graphical illustration of common subcellular sources of antigens from *Toxoplasma gondii* for the development of vaccines. Color version available online.

and 70% reduction of chronic cyst burden, respectively. Studies that investigated SAG4 found increased survival of 7 days from the acute infection. Additionally, a 66% reduction in cyst burden was observed when tested against the chronic infection in mice. SAG5 is broken up into 5 subtypes (SAG5a–SAG5e). SAG5a, SAG5b, and SAG5c are all found on both tachyzoites and bradyzoites and specifically play a role in the persistence of cysts in the host (Lu et al., 2015). Administration of a SAG5a-based DNA vaccine in mice showed a 35% cyst burden reduction. DNA vaccines incorporating SAG5b and SAG5c showed an increased survival of 7 and 6 days, respectively. Additionally, chronically infected mice showed a 44% cyst burden reduction and 41% cyst burden reduction for SAG5b-based and SAG5c-based DNA vaccines, respectively. SAG5d, which is only expressed in tachyzoites, has been shown to have immunogenicity similar to SAG1 (Lu et al., 2014). Mice that were acutely infected with *T. gondii*, when administered with a SAG5d-based DNA vaccine, had an increased survival of 7 days in comparison to untreated mice.

An additional summary of the SAG proteins found in *T. gondii* DNA vaccine papers and their effectiveness against the parasite can be found in Table I. The non-SAG1 surface antigens, as a group, generally outperformed MIC DNA-encoded proteins in reducing cyst loads (37–72% reduction) associated with the chronic infection, while acutely challenged mice often experienced a reduced duration of survival (2–7 days beyond control death) among the SAG-vaccinated groups.

Dense granule proteins (GRAs): Dense granule proteins (GRAs) play a vital role in host cell invasion by forming a parasitophorous vacuole (PV) from the membrane of the host cell. The PV is a wrapping that contains invading parasites and is derived from both host membrane and parasite secretions. The PV permits the parasites to grow within a self-contained vacuole and resists the leakage of antigens into the host cell, thereby limiting the activation of the immune response. Their secretory nature, as well as their role in host cell invasion function, makes GRAs an appealing target for DNA vaccines. Because of their role in host invasion, GRA proteins showed little efficacy against chronic infection, and many studies do not evaluate DNA vaccine constructs incorporating GRA antigens against chronic infection.

GRA1 is a calcium-binding protein that is secreted by both tachyzoites and bradyzoites and has been shown to illicit heavy T-cell proliferation (Vercammen et al., 2000). Against the acute stage of infection, DNA vaccines containing GRA1 as an antigen showed an increase in survival of 6 days. GRA2 is involved in the formation of the intravacuolar network of the PV (Ching et al., 2017). DNA vaccines incorporating GRA2 showed an increase in survival of 3 days against acute infection. GRA5 protects the parasite during cell invasion by inhibiting apoptosis of infected cells (Min et al., 2012). Against acute infection, a GRA5-based DNA vaccine showed an increased survival of 3 days.

Mice vaccinated using GRA6 as an antigen showed a 40% survival of the acute infection. A specific carboxyl-terminal portion of GRA6, known as HF10, has been known to have MHC-restricted immunodominance and protective capability against lethal infection (Sun et al., 2011). GRA7 plays an integral role in host cell invasion and, once inside the host cell, works in conjunction with other *T. gondii* proteins. GRA7-treated mice showed a 5–12 day increase against acute infection and a 55% chronic cyst burden reduction. GRA8 is commonly used to identify and diagnose acute infection (Ybañez et al., 2020). When tested as a DNA vaccine antigen, it led to a 4-day survival increase against the acute infection. GRA14, expressed in all stages of the *Toxoplasma* life cycle and during infection, is secreted into the PV where it can be transferred between the PV membrane and the intravacuolar network (Pagheh et al., 2019). Vaccination with a GRA14-based DNA vaccine showed increased survival of 5 days against an acute challenge.

GRA16 and GRA 24 are exported from the PV and mediate host gene expression. GRA16 regulates the production of p53, a protein involved in inflammatory host response and the cell cycle (Hu et al., 2017). DNA vaccines incorporating GRA16 as an antigen led to a 2-day survival increase of the acute stage and a 44% cyst burden reduction against the chronic stage. GRA24 binds with a mitogen-activated protein kinase known as p38α and causes transcriptional changes across the cell (Bougdour et al., 2014). GRA24-based DNA vaccines showed an increase in survival against the acute infection of 3–26 days. When chronically challenged, these vaccines led to a 29% cyst burden reduction. Similar to GRA16 and GRA24, GRA25 may act as an inhibitor of chemokines CCL2 and CXCL1, which are involved in

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host immune response. However, the mechanism by which GRA25 works is still unknown (Xu et al., 2019). When tested as a DNA vaccine antigen, GRA25 led to an increase in acute infection survival of 7 days and a 41% cyst burden reduction in chronic infection.

GRA17 and GRA23 are involved in PV membrane permeability and nutrient transportation (Gold et al., 2015). DNA vaccines containing these antigens were tested against acute infection and showed an increased survival of 2 and 4 days, respectively.

In comparison to SAG and MIC antigens, vaccines using GRA antigens showed variable success against both the acute and chronic stages of the parasite. GRA-based vaccines led to a 5-day (median) acute survival increase, which is similar to that of SAG-based DNA vaccines. Against the chronic stage, these vaccines showed a 29–55% cyst burden reduction, which is less than that of both SAG and MIC-based DNA vaccines. DNA vaccines that used GRA6 antigens showed the highest efficacy against the acute stage of the parasite, leading to 40% overall survival. This is comparable to DNA vaccines incorporating MIC4, MIC6, and SAG1. A full summary of GRA antigens explored in *T. gondii* DNA vaccine papers can be found in Table I.

Rhoptry proteins (ROPs): Rhoptry proteins (ROPs) are apical secretory organelles that are known to be involved in parasites' penetration of the host cell, specifically in the formation of the PV. ROP proteins have a variety of different functions and contribute heavily to the virulence of the parasite. Some play a role in early, acute virulence, such as ROP1 and ROP9 (Chen et al., 2014b; Sonaimuthu et al., 2016). DNA vaccines incorporating ROP1 as an antigen led to 20% survival or a 12-day survival increase against acute infection and a 55% cyst burden reduction against chronic infection. ROP9-based DNA vaccines led to increased mouse survival of 12 days against acute infection.

Another family of ROP proteins exists that contributes to the virulence of the parasite and is involved in the membrane of the PV. Included in this family are ROP2, ROP7, ROP8 (Hajj et al., 2007; Labesse et al., 2009; Li et al., 2015b). Several studies have used these ROP proteins as antigens in DNA vaccine candidates. Against the acute stage of the parasite in mice, DNA vaccines using ROP2 as an antigen led to increased survival of 2 days. ROP7-based DNA vaccine-treated mice had an increased acute infection survival of 5 days and a 58% cyst burden reduction during the chronic stage. Against an acute challenge, DNA vaccines incorporating ROP8 showed 50% survival and increased survival of 2 days.

ROP5 is an essential virulence factor of *T. gondii* and leads to the inactivation of IFN-γ-inducible immunity-related GTPases (IRGs) (Wang et al., 2016b). These IRG proteins lead to an immunity from the parasite within mice by accumulating on the PV and killing the parasite (Khaminets et al., 2010). Studies that investigated ROP5 as a DNA vaccine antigen found that it led to a 4–14 day survival increase against the acute stage of the parasite and a 52–58% reduction in chronic infection cyst burden. Additionally, ROP5 complexes with ROP17 and ROP18, which are both ROP kinases, to aid in the mediation of the IRG pathway (Etheridge et al., 2014). ROP17 is also a member of the ROP2 family, and a ROP17-based DNA vaccine led to a 14-day survival increase against acute infection. ROP18, a polymorphic serine-threonine kinase that is secreted during the invasion process and can inhibit the host's innate and adaptive immune responses, has been studied more frequently against both acute and chronic infection. In acutely infected mice, those that had been administered a ROP18-based DNA vaccine led to 9–48-day survival increases in others. Against the chronic infection, 30–47% cyst burden reduction was found in mice administered this DNA vaccine.

Another category of these proteins is ROP kinases (ROPK), which include ROP17 and ROP 18 as mentioned previously, as well as ROP16, ROP19, ROP21, ROP22, ROP29, and ROP38 (Liu et al., 2014; Xu et al., 2014; Zhou et al., 2016; Lu et al., 2018; Zhang et al., 2019). Additionally, ROP54 is a pseudokinase (Yang et al., 2017). ROP16 is considered a key virulence factor in the pathogenesis of *Toxoplasma*, as it can subvert STAT3/6 signaling and target the host nucleus directly (Liu et al., 2014). Against acute infection, ROP16-based DNA vaccines led to an increase in survival of 13–27 days. ROP19 is another ROP kinase present in the PV membrane and is considered to be a target for less virulent strains of *T. gondii* (Zhou et al., 2016). An ROP19-based DNA vaccine was tested against the chronic stage of the parasite and led to a 57% cyst burden reduction. A DNA vaccine using ROP21 as an antigen showed a 7-day survival increase against acute infection and a 37% chronic cyst burden reduction. A ROP22-based vaccine led to an 8-day survival increase against acute infection and a 40% cyst burden reduction against the chronic stage. ROP29 was tested as a DNA vaccine antigen in mice against the chronic infection and showed a 53% cyst reduction. Mice administered a ROP38-based DNA vaccine showed a 2-day survival increase against the acute infection and a 77% cyst burden reduction against the chronic infection. A study that investigated ROP54 as a potential DNA vaccine antigen observed an 8-day survival increase against the acute infection and a 36% cyst reduction in chronically infected mice.

ROP13 has an effector function that is involved in the host cell response to *T. gondii* and is not similar to any other known protein. Studies have found it present in evacuoles, or rhoptry-derived secretory vesicles, and suggest that it might be toxic to the host (Håkansson et al., 2001; Turetzky et al., 2010). Studies testing a DNA vaccine incorporating ROP13 found a 49-day increased survival against the acute stage of the parasite and a 40% cyst burden reduction when chronically challenged.

ROP35 is a protein found within the PV membrane, though its direct function is unclear (Zhang et al., 2018c). Against acute infection, a ROP35-based DNA vaccine led to an 8-day survival increase. This DNA vaccine was tested against the chronic infection as well and a 38% cyst burden reduction was observed in mice that had the vaccine administered.

A full summary of ROP antigens that were examined in DNA vaccine papers against *T. gondii* can be found in Table I. Noticeably, ROP38 DNA vaccination stands out as producing an impressive 77% reduction in cyst load, compared to the other evaluated ROP proteins which averaged 44% reduction. ROP13-based and ROP18-based DNA vaccines also show great efficacy, leading to a 49- and 48-day survival increase against acute infection, respectively.

Other antigens: Many other genes have been employed as DNA vaccine antigens against *T. gondii*, although with less regularity. Perforin-like proteins (PLPs) are pore-forming proteins, and the PLP1 protein has been shown to affect the permeability of the PV membrane in *T. gondii* (Kafsack et al., 2009). When explored as a potential DNA vaccine antigen, it showed an increase in acute infection survival by 8–20 days and a 30–44% cyst burden

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reduction against the chronic infection. Rhomboid proteases (ROMs) are used in *T. gondii* to cleave adhesive proteins to facilitate the entry into the host cells and the separation of parasites from receptors (Foroutan et al., 2019). Two ROMs have been explored as DNA vaccine candidates within the last decade, ROM4 and ROM5. DNA Vaccines incorporating ROM4 showed a 13% survival, or a 5–12-day survival, increase and generated a 36–57% cyst burden reduction against the chronic infection. ROM5 provided a 20% survival in acutely infected mice and produced a 72% cyst reduction. *Toxoplasma gondii* secreted protein with an altered thrombospondin repeat (SPATR) has been described as part of the microneme family and involved in virulence and invasion of the parasite (Zheng et al., 2017a). SPATR-based DNA vaccination led to a 10-day survival increase against an acute challenge.

Rhoptry neck (RON) proteins combine with ROP proteins mentioned earlier to create a larger moving junction complex and have only recently been discovered. RON5 is present in the moving junction as well and acts as an escort for other RON proteins (Zhao et al., 2016). Acutely infected mice that had been administered a RON5-based DNA vaccine survived 8 days longer than untreated control and produced a 26% cyst reduction in chronically infected mice.

Some studies have taken interest in molecules that function in the parasite's metabolism as possible DNA vaccine antigens. *Toxoplasma gondii* requires a lot of energy during the invasion, and the production of malate dehydrogenase (MDH) has been shown to contribute to virulence (Hassan et al., 2014c). MDH treatment led to 9 days of increased survival against the acute infection. Deoxyribose phosphate aldolase (DPA) is functional in the parasite's glycolysis cycle and functions to connect the MIC2 protein to the host cell surface (Hassan et al., 2014a). When mice that had been administered a DPA-based DNA vaccine were challenged with the acute parasite infection, they survived 12 days longer than untreated mice. *Superoxide dismutase* (SOD) is an enzyme that protects cells from oxidative stress and is involved in the process of metabolizing oxygen. In *T. gondii*, it is involved in the growth of both the acute and chronic stages of the parasite (Liu et al., 2017). Treatment with a DNA vaccine incorporating SOD as an antigen showed a 10-day survival increase against an acute challenge. Nucleoside triphosphate hydrolase (NTPase) promotes host invasion in *T. gondii* by breaking down ATP and ADP to AMP, forcing the parasite to acquire purines from host cells (Zheng et al., 2017b). DNA vaccines incorporating NTPase showed a 10-day survival increase against the acute infection and led to a 78% cyst burden reduction against a chronic challenge.

Calcium-dependent protein kinases (CDPKs) are kinases present in many apicomplexans and play a role in biological function. In *T. gondii*, CDPK1 is involved in the parasite's life during infection and in the secretion of microneme proteins (Chen et al., 2014a). In DNA vaccines using CDPK1 as an antigen, a 10–17 day survival increase against the acute form of the parasite and a 46% chronic stage cyst burden reduction were observed. CPDK2, which plays a role in amylopectin metabolism, was also tested as a DNA vaccine antigen and led to a survival increase of 11 days against the acute infection (Chen et al., 2017). Another kinase, CDPK3, has a similar function to other CDPKs and is located at the apical end of the parasite (Zhang et al., 2013b). A CDPK3-based DNA vaccine led to a 17-day survival increase in acutely infected mice and a 54% cyst burden reduction against the chronic infection. CPDK5 was also tested as a DNA vaccine antigen but showed less efficacy. The DOC2C protein acts downstream of CDPK proteins in *T. gondii* and facilitates secretory vesicle and plasma membrane fusion (Zhang et al., 2018b). A DNA vaccine incorporating DOC2C showed a 10-day survival increase in acutely infected mice.

Heat shock proteins (HSPs) assist the cell with post-translational processes such as stabilization, folding, and translocation (Li et al., 2018b). HSPs tested as DNA vaccine candidates include HSP40 and HSP60. HSP40 showed promise against the chronic stage of the parasite, which led to a 62% chronic cyst burden reduction. When tested against the acute infection, an HSP60-based DNA vaccine showed a 19-day survival increase and a 48% cyst burden reduction against the chronic stage.

Other molecules that displayed positive results as a DNA vaccine antigen include Initiation factor 2a, Initiation factor 4a, Immune mapped protein 1, Myc regulation 1, Phosphatase 2C, and Cyclophilin. *Toxoplasma gondii* eukaryotic initiation-factor 2a (IF-2a) is vital to the viability of the parasite when phosphorylated (Chen et al., 2013b). IF-2a antigen treatment led to a 16-day survival increase against the acute infection and a 44% reduction in cyst burden against a chronic challenge. *Toxoplasma gondii* eukaryotic translation initiation factor 4a (IF-4a) aids in beginning translation and unwrapping inhibiting mRNA (Chen et al., 2013a). Against an acute challenge, treatment with IF-4a led to a 29-day survival increase. Immune mapped protein 1 (IMP1) has shown immunogenicity and protection against *Eimeria maxima*. This protein is also present in the RH strain of *T. gondii* and, thus, shows significant interest as a vaccine candidate (Cui et al., 2012). IMP1 treatment showed increased survival against acute infection by 22 days. Myc regulation 1 (MYR1) is a recently discovered virulence factor in *T. gondii* present in the PV to help alter host cell transcription to promote host–parasite interactions. Additionally, it also serves to regulate several tachyzoite pathways (Zheng et al., 2019a). Against an acute challenge, treatment with the MYR1 antigen led to a 29-day survival increase. Phosphatase 2C (PP2C) is a protein described as a rhoptry secretory protein and interferes with host gene expression during infection (Song et al., 2020). Against an acute challenge, a PP2C-based DNA vaccine led to a survival increase of 11 days. Cyclophilin (CyP) has been described as a protein family responsible for protein folding in many prokaryotes and eukaryotes (Gong et al., 2013). CyP-based DNA vaccine treatment led to a 38% survival against an acute challenge.

Several other antigens were tested but did not display results as positive as those mentioned above. These include actin-depolymerizing factor (ADF), cathepsin proteases B, C1, and L (CP-B, CP-C1, CP-L), ESA10, Aspartic protease 1 (ASP1), elongation factor 1-alpha (EF-1α), Profilin (PF), p14-3-3, Toxofilin, Glutathione reductase (GR), Glutathione-S-Transferase (GST), and ERK7.

Many of these genes chosen as candidate vaccine antigens play a role in parasite invasion and metabolism, which is, perhaps, why they showed promising results as a DNA vaccine candidate. These other genes showed a survival increase of 9 days (median) and an average cyst burden reduction range of 26–78%. A full list of other genes that were tested in *T. gondii* DNA vaccines and their results can be found in Table I, and summarized in Figure 2.

Antigen considerations: Two final important factors when designing the antigen contents of a DNA vaccine include the

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Table II. Summary of challenge results from DNA vaccines containing multiple *Toxoplasma gondii* antigens. A summary of results from publications since 2010 employing two or more generally full-length (or peptide portions, where indicated) parasite antigens delivered via DNA vaccination followed by challenge, organized by the number of antigens co-administered. Bz = bradyzoite, Tz = tachyzoites, Sp = sporozoite.

Gene	Acute challenge	Acute result*	Chronic challenge	Chronic result†	Reference
Dual Genes					
MIC1 + MIC4	80 ME49 cysts	70% survival	40 ME49 cysts	59% reduced	Pinzan et al., 2015
MIC3 + GRA1	10,000 RH Tz	10% survival	1,000 RH Tz	unknown	Gong et al., 2016
MIC3 + ROP18	1,000 RH Tz	+12 days	None	N.D.	Qu et al., 2013
MIC6 + PLP1‡	80 Pru cysts	+25 days	20 Pru cysts	62% reduced	Yan et al., 2012
SAG1 + GRA1	100,000 RH Tz	30% survival	None	N.D.	Wu et al., 2012
SAG1 + GRA2‡	10,000 RH Tz	+9 days	None	N.D.	Zhou et al., 2012
SAG1 + GRA5	10,000 RH Tz	+4 days	None	N.D.	Naserifar et al., 2015
SAG1 + GRA7‡	1,000RH Tz	+8 days	None	N.D.	Mavi et al., 2019
SAG1 + ROP2	1,000 RH Tz	+3 days	None	N.D.	Sun et al., 2020
SAG1 + ROP2	10,000 RH Tz	+5 days	None	N.D.	Naserifar et al., 2015
SAG1 + ROP2	10,000 RH Tz	+5 days	None	N.D.	Hoseinian Khosroshahi et al., 2011
SAG1 + p14-3-3	10,000 RH Tz	+11 days	None	N.D.	Meng et al., 2012
SAG5B + SAG5C	10,000 RH Tz	+12 days	20 Pru cysts	70% reduced	Lu et al., 2017
GRA5 + ROP2	10,000 RH Tz	+6 days	None	N.D.	Naserifar et al., 2015
GRA7 + ROP1‡	1,500 ME49 cysts	33% survival	20 ME49 cysts	62% reduced	Quan et al., 2012
GRA7 + ROP16‡	1,000 RH Tz	+16 days	None	N.D.	Liu et al., 2014
GRA14 + ROM4‡	1,000 RH Tz	+8 days	None	N.D.	Rahimi et al., 2017
GRA17 + GRA23	1,000 RH Tz	+10 days	None	N.D.	Zhu et al., 2017
ROP5 + ROP18‡	1,000 RH Tz	+23 days	6 Pru cysts	64% reduced	Zhu et al., 2020
ROP5 + ROP7	1,000 RH Tz	+9 days	20 Pru cysts	75% reduced	Wang et al., 2016b
ROP18 + PLP1‡	80 Pru cysts	+28 days	20 Pru cysts	66% reduced	Chen et al., 2018
CPB + CPL	10,000 RH Tz	+10 days	None	N.D.	Zhao et al., 2013a
Tri-Gene					
MIC1 + MIC4 + MIC6	80 ME49 cysts	80% survival	40 ME49 cysts	68% reduced	Pinzan et al., 2015
SAG1 + GRA5 + ROP2	10,000 RH Tz	+6 days	None	N.D.	Naserifar et al., 2015
SAG2C + SAG2D + SAG2X	None	N.D.	20 Pru cysts	77% reduced	Zhang et al., 2013a
Multiple DNA-encoded Peptides					
SAG1/AMA1/ROP2/GRA4‡	10,000 RH Tz	+7 days	None	N.D.	Roozbehani et al., 2018
SAG1/GRA2/GRA7/ROP16‡	1,000 RH Tz	+7 days	None	N.D.	Cao et al., 2015
ROP16/ROP18/MIC6/CDPK3	None	N.D.	10 Pru cysts	38% reduced	Zhang et al., 2018a
PF/ROP16/ROP18/MIC6/CDPK3	None	N.D.	10 Pru cysts	80% reduced	Zhang et al., 2018a
SAG1/ROP2/GRA1/GRA4/SAG2C/SAG2X	1,000 RH Tz	20% survival	None	N.D.	Cong et al., 2014

* Acute result: n % survival indicates the percentage of vaccinated mice surviving parasite challenge, typically via administration of parasite tachyzoites (TzTz); or.+n days indicates the number of days vaccination permitted survival beyond the control group.
† Chronic result: indicates reduction in brain cyst numbers compared to the unvaccinated group. N.D. indicates not determined.
‡ Indicates antigen + adjuvant was also tested and included in Table III.

decision between using a full-length gene or a peptide sequence, as well as using one or multiple genes.

DNA-encoded peptide sequences have some advantages, such as their relative ease of production and storage, but they are less stable in vivo than the full-length gene (Roozbehani et al., 2018). In this *T. gondii* DNA vaccine review, 3 of 95 papers used peptide sequences in their vaccines. Two papers used various short amino acid sequences from multiple antigens, which led to protection for 7 days more than the control in the acute challenge in both cases: one tested SAG1, AMA1, ROP2, GRA4 while the other used SAG1, GRA2, GRA7, and ROP16 (Cao et al., 2015; Roozbehani et al., 2018).

It has also been shown that using multiple genes in a vaccine can offer a more wide-range immune response, especially for a pathogen such as *Toxoplasma* that has multiple stages in its life cycle during which not all genes are present. Of the papers reviewed, 29 of the 149 DNA vaccine candidates incorporated at least 2 separate antigens in their vaccine design (Figure 2A). A full summary of multiple genes and multiple peptide sequences explored in *T. gondii* DNA vaccine papers can be found in Table II.

Dual antigens with the longest increase in days of protection included MIC6 + PLP1 and ROP18 + PLP1, which led to 25 and 28 days of increased survival, respectively (Yan et al., 2012; Chen et al., 2018). However, a vaccine using MIC1 + MIC4 resulted in 70–80% survival against the acute challenge (Lourenço et al., 2006; Pinzan et al., 2015). The most effective dual antigens vaccines against the chronic challenge were the combination of SAG5b + SAG5c with a 70% cyst reduction (Lu et al., 2017) and ROP5 + ROP7 with a 75% cyst reduction (Wang et al., 2016b). Multiple sources used vaccines with SAG1 in union with various other antigens that demonstrated between 4–8 days of increased survival for the acute challenge (Hoseinian Khosroshahi et al.,

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2011; Naserifar et al., 2015; Mavi et al., 2019). A DNA vaccine incorporating SAG1 with GRA1 led to 30% survival against the acute challenge (Wu et al., 2012). Three papers used a tri-gene attempt in creating a vaccine—the most effective being the combination of 3 MIC genes (MIC1, MIC4, MIC6), which led to an 80% survival against the acute challenge (Pinzan et al., 2015). Another of the 3 studies use a combination of 3 SAG antigens (SAG2C, SAG2D, and SAG2X), which resulted in a 77% reduction in cysts after a chronic challenge (Zhang et al., 2013a).

In summary, a vaccine containing multiple antigens had the potential to produce higher levels of protection depending on the antigens used. Against the acute challenge, results vary from 4 days of increased survival to 80% survival. Against the chronic challenges, results vary between 32–80% cyst reduction.

Ruminant models of vaccine efficacy

Few *T. gondii* DNA vaccine studies have been conducted in ruminant animals, despite human *Toxoplasma* infection often originating from consumption of cysts found in meat and the large economic losses that result from congenital abnormalities caused by toxoplasmosis in livestock. In sheep, it was found that the use of ROP1 in combination with CpG elicited a higher interferon-gamma response than did other vaccine candidates; however, no humoral response was observed against tachyzoite lysate (Li et al., 2010). Another study reported that the fusion of ROP1 with ovine CD154 could induce a mixed Th1/Th2 response, while ROP1 alone only produced a Th1 response (Hiszczyńska-Sawicka et al., 2011a). Another study in sheep found that a significant humoral and cellular response could be elicited in response to MIC3 (Hiszczyńska-Sawicka et al., 2012). A more antigen-diverse study in sheep found that of GRA1, GRA4, GRA6, or GRA7 DNA vaccine antigens, all constructs evoked some amount of humoral and cellular response, with GRA7 producing the most robust response. (Hiszczyńska-Sawicka et al., 2011b). DNA vaccine efficacy has been evaluated in pigs, including GRA1 and GRA7 in a DNA cocktail vaccine that produced strong interferon-gamma and antibody response to recombinant protein (Jongert et al., 2008a). Overall, the understanding of the efficacy of *T. gondii* DNA vaccines in rudiment animals is still in its infancy. In addition to exploring the humoral and cellular responses to DNA vaccines in these animals, it would be prudent to focus on effects against cyst formation and congenital defects.

Adjuvants

DNA vaccines evoke T-cells and potentially humoral responses; however, the efficacy of vaccination is related to dosage, antigenicity, and potentially the size of the recipient (Babiuk et al., 2003). Therefore, adjuvants have been considered for use in DNA vaccines to enhance immune responsiveness. Despite the potential of an adjuvant to enhance the immunogenicity, and therefore protective effect, of the vaccine, only 26 reviewed papers included an adjuvant. The adjuvants used generally fall into 2 categories: host-derived biomolecules or chemical elements. A summary of adjuvants explored in *T. gondii* DNA vaccine papers can be found in Table III.

Host proteins as immunostimulants: Using host immune molecules to produce a stronger immune response, often Th1-directed, is a potent option for DNA vaccination (Ghaffarifar, 2015; Ghaffarifar et al., 2019). The most common host genes used as adjuvants were interleukins capable of directing the host to a Th1 response, considered most desirable for an intracellular infection caused by *T. gondii* (Quan et al., 2012; Ahmadpour et al., 2017a; Rashid et al., 2017; Ghaffarifar et al., 2019). IL-12 has been the most extensively studied adjuvant due to its role in activating Th1 responses and natural killer cells and has shown varying efficacy (Ghaffarifar, 2015). In some experiments, it led to a decreased survival, such as when used with GRA14, but it has also led to increased survival by 17% when co-administered with both GRA7 and GRA7 + ROP1 (Quan et al., 2012; Ahmadpour et al., 2017a; Ghaffarifar et al., 2019). In chronic challenges, the addition of IL-12 increased the cyst reduction by an additional 5% with GRA7 + ROP1 (Quan et al., 2012). Another interleukin, IL-15, provided an additional 2% cyst reduction beyond profilin antigen alone (Gao et al., 2018). IL-18 enhanced survival by 20% when used with SAG1 (Liu et al., 2010b) and reduced cyst burden by a further 26% compared to ROP13 alone (Wang et al., 2012). Lastly, recently IL-33 was co-administered with ROP5 + ROP18, leading to a 7-day increase in survival for the acute challenge and a further 16% reduction in cysts for the chronic challenge (Zhu et al., 2020). Combinations of interleukins have also been attempted, leading to similar enhancements (Table III).

Other host molecules serving as adjuvants included RANTES, which is secreted from many cell types and is associated with chronic inflammation and wound repair (Cao et al., 2015). As an adjuvant, it functions by increasing the immune response of CD4+ and CD8+ T-cells. RANTES was used with the DNA vaccine encoding peptides from SAG1/GRA2/GRA7/ROP16 and contributed to increased survival by 4 days (Cao et al., 2015).

Other adjuvants: Non-host derived adjuvants have also been explored to enhance *T. gondii*-focused DNA vaccines. For example, the flagellin FliC gene from *Salmonella*, which acts as a TLR5 agonist, increased acute survival by 3 days (Maraghi et al., 2019). CPG-ODN, a synthetic oligodeoxynucleotide containing unmethylated CpG motifs that can activate TLR9, coadministered with SAG1+GRA7 resulted in an additional 11 days of survival from an acute challenge (Mavi et al., 2019). The adjuvant α-GalCer, which stimulates natural killer T-cells, increased acute survival by 4–6 days (Han et al., 2017b).

Non-nucleic acid-based molecules have also been used as coadministered adjuvants for DNA vaccines against *T. gondii*. The R848 molecule that activates both TLR7 and TLR8 modestly reduced cyst loads (Lu et al., 2018) (Table III).

A protein from the HBV genome, HbsAg, encodes for a B- and T-cell epitope and has also been tested as a possible DNA vaccine adjuvant against *T. gondii* (Sun et al., 2020). When coadministered with a multiple-antigen DNA vaccine encoding SAG1 + ROP2, HbsAg allowed for a 20% survival against acute challenge.

Aluminum hydroxide gel (alum), when used with the DNA vaccine encoding Toxofilin, led to an increase in survival by 13 days (Song et al., 2017). However, when used with a DNA vaccine encoding peptides from SAG1/AMA1/ROP2/GRA4, the survival against an acute challenge was decreased by 5 days (Roozbehani et al., 2018). However, levamisole, a chemical used to treat parasitic worm infections and thought to foster a Th2 immune response, modestly increased survival by 13% when co-administered with GRA6 (Sun et al., 2011).

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Table III. Summary of adjuvant effect on DNA vaccinations against *Toxoplasma gondii*. A summary of results from publications since 2010 employing adjuvants co-administered with or by DNA vaccination followed by challenge, organized by adjuvant class. Tz = tachyzoites.

Adjuvant	Antigen gene(s)	Acute challenge	Acute result*	Chronic challenge	Chronic result†	Reference
Host Proteins						
IL-12	MIC3	10,000 RH Tz	+1 day	None	N.D.	Ghaffarifar et al., 2019
IL-12	GRA7	1,500 ME49 cysts	+17% survival	20 ME49 cysts	+5% reduction	Quan et al., 2012
IL-12	GRA14	100,000 RH Tz	−3 days	None	N.D.	Ahmadpour et al., 2017a
IL-12	ROP1	1,500 ME49 cysts	Not significant	20 ME49 cysts	+5% reduction	Quan et al., 2012
IL-12	ROP8	2,000 RH Tz	+1 day	None	N.D.	Foroutan et al., 2020a
IL-12	GRA7 + ROP1	1,500 ME49 cysts	+17% survival	20 ME49 cysts	+5% reduction	Quan et al., 2012
IL-15	Profilin	None	N.D.	10 Pru cysts	+2% reduction	Gao et al., 2018
IL-18	SAG1	50 RH Tz	+20% survival	None	N.D.	Liu et al., 2010b
IL-18	ROP13	1,000 RH Tz	Not significant	10 Pru cysts	+26% reduction	Wang et al., 2012
IL-18	PLP1	1,000 RH Tz	+1 day	None	N.D.	Yan et al., 2011
IL-18	MIC6 + PLP1	80 Pru cysts	+4 days	20 Pru cysts	+3% reduction	Yan et al., 2012
IL-18	ROP18 + PLP1	80 Pru cysts	+3 days	20 Pru cysts	+5% reduction	Chen et al., 2018
IL-33	ROP5 + ROP18	1,000 RH Tz	+7 days	6 Pru cysts	+16% reduction	Zhu et al., 2020
IL-7 + IL-15	CDPK1	1,000 RH Tz	+5 days	10 Pru cysts	+28% reduction	Chen et al., 2016
IL-15 + IL-21	MIC8	1,000 RH Tz	+7 days	20 Pru cysts	+39% reduction	Li et al., 2014
IL-15 + IL-21	MIC8	80 Pru cysts	+16 days	None	N.D.	Li et al., 2014
IL-15 + IL-21	CDPK1	1,000 RH Tz	+3 days	20 Pru cysts	+27% reduction	Chen et al., 2014a
RANTES	SAG1/GRA2/ GRA7/ROP16	1,000 RH Tz	+4 days	None	N.D.	Cao et al., 2015
B7-2	GRA7	1,000 RH Tz	+5 days	None	N.D.	Liu et al., 2014
B7-2	ROP16	1,000 RH Tz	+6 days	None	N.D.	Liu et al., 2014
B7-2	GRA7 + ROP16	1,000 RH Tz	+6 days	None	N.D.	Liu et al., 2014
Other						
FliC gene	SAG1	1,000 RH Tz	+3 days	None	N.D.	Maraghi et al., 2019
CpG-ODN	SAG1 + GRA7	1,000 RH Tz	+11 days	None	N.D.	Mavi et al., 2019
a-GalCer	SAG5d	10,000 RH Tz	+4 days	None	N.D.	Lu et al., 2014
a-GalCer	CP-C1	10,000 RH Tz	+6 days	None	N.D.	Han et al., 2017b
R848	ROP29	None	N.D.	20 Pru cysts	+27% reduction	Lu et al., 2018
Aluminum Hydroxide	HSP70	10 ME49 cysts	N.D.	N.D.	N.D.	Czarnewski et al., 2017
Aluminum Hydroxide	SAG1/AMA1/ ROP2/GRA4	10,000 RH Tz	−5 days	None	N.D.	Roozbehani et al., 2018
Aluminum Hydroxide	SAG1	1,000 RH Tz	+0 days	None	N.D.	Maraghi et al., 2019
Aluminum Hydroxide	Toxofilin	10,000 RH Tz	+13 days	20 Pru Cysts	+9% reduction	Song et al., 2017
MPLA	Toxofilin	10,000 RH Tz	+14 days	20 Pru Cysts	+16% reduction	Song et al., 2017
Alum + MPLA	Toxofilin	10,000 RH Tz	+23 days	20 Pru cysts	+33% reduction	Song et al., 2017
Calcium Phosphate Nanoparticles	GRA14	100,000 RH Tz	+0 days	None	N.D.	Ahmadpour et al., 2017b
Calcium Phosphate Nanoparticles	ROM4	1,000 RH Tz	+1 day	None	N.D.	Rahimi et al., 2017
Calcium Phosphate Nanoparticles	ROM4 + GRA14	1,000 RH Tz	+1 day	None	N.D.	Rahimi et al., 2017
Levamisole	GRA6	1,000 RH Tz	+13% survival	None	N.D.	Sun et al., 2011
pSPreS2	SAG1 + GRA2	10,000 RH Tz	+1 day	None	N.D.	Zhou et al., 2012
Saponin	SAG1	1,000 RH Tz	+0 days	None	N.D.	Maraghi et al., 2019
HbsAg	SAG1 + ROP2	1,000 RH Tz	20% survival	None	N.D.	Sun et al., 2020

* Acute result: n % survival indicates the percentage of vaccinated mice surviving parasite challenge; or +n days indicates the number of days vaccination permitted survival beyond antigen alone group.
† Chronic result: indicates reduction in brain cyst numbers beyond antigen alone group. N.D. indicates not determined.

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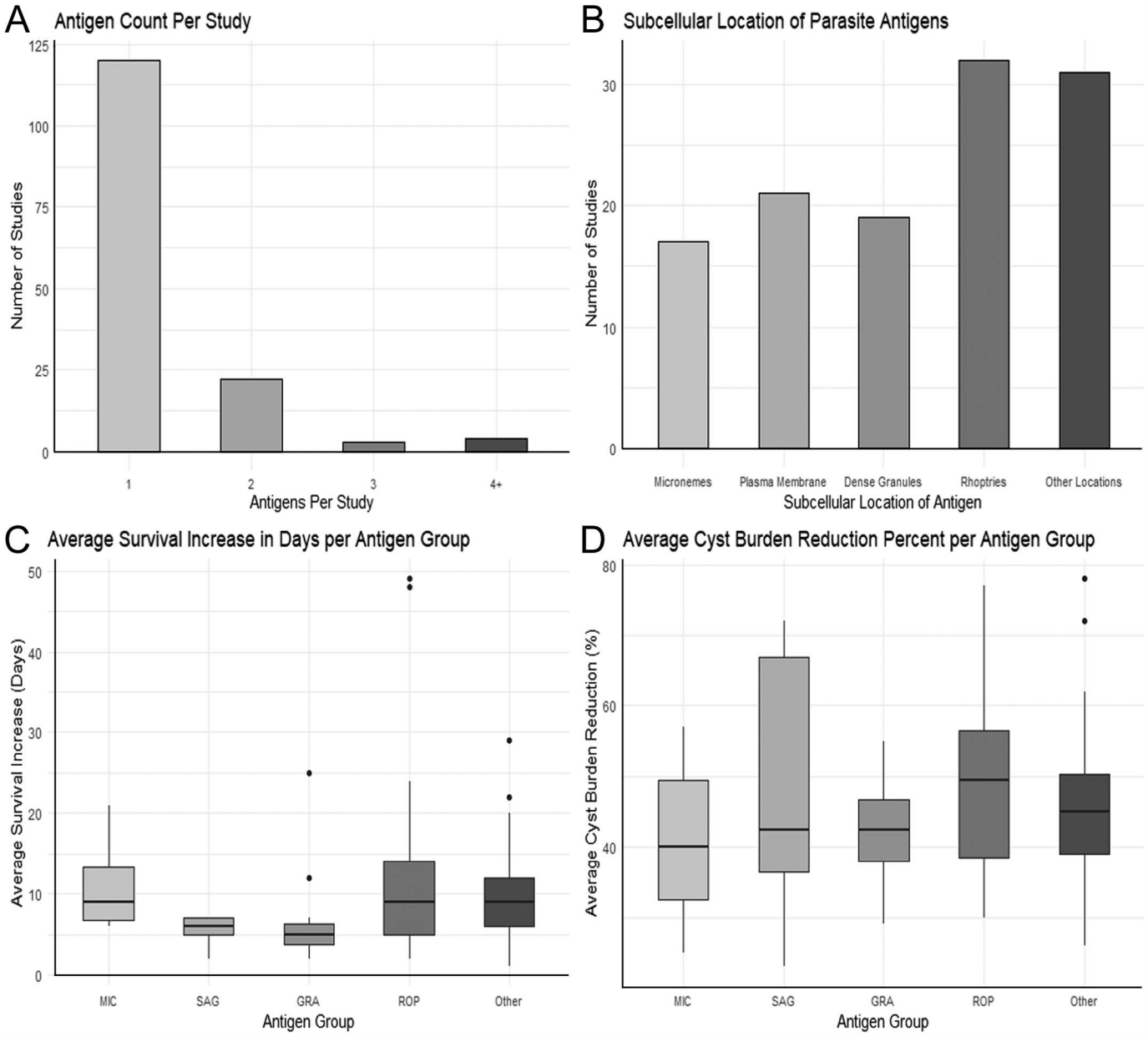

Figure 2. Metrics of published experimental *Toxoplasma gondii* DNA vaccine designs by antigen number and subcellular location. (**A**) Reviewed studies evaluated varying numbers of investigated DNA antigens, with few investigating multiple antigens (i.e., antigen cocktails). (**B**) Reviewed studies evaluated antigens found in various parasite subcellular locations. (**C, D**) Antigens found in various subcellular locations demonstrate overlapping levels of efficacy in vivo. MIC = microneme-associated proteins; SAG = surface antigen-associated proteins; GRA = dense granule-associated proteins; ROP = rhoptry-associated proteins.

In summary, interleukins demonstrated varying degrees of efficacy in acute and chronic conditions: between a decrease in survival by 3 days to an increase in survival rates by 20% when added to antigens for acute challenges, and between 0–39% additional cyst reduction when added to antigens for chronic challenges. Other adjuvants also showed increases in the immune response: an increase in survival of 4–23 days when added to antigens for an acute challenge, and 0–27% additional cyst reduction when added to antigens for chronic challenges. Efficacy is likely a product of the combination of both antigen efficacy and adjuvant activity. A full summary of additional adjuvants can be found in Table III.

CONCLUSION

DNA vaccines are an attractive approach for controlling morbidity and mortality associated with *T. gondii* infection in humans as well as in susceptible animal species. While many studies have been undertaken, no single vaccine antigen or adjuvants stands out as the single most promising lead for a DNA

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vaccine candidate, e.g., one capable of 100% cyst reduction and complete protection against death from acute challenge in model outbred species. However, the comparison of antigen efficacy between studies is complicated by the fact that there is no standardization regarding immunization protocol and evaluation criteria, including dose and route of inoculation, or parasite strain and infection number (Liu et al., 2012). Chronic infection efficacy studies are somewhat more standardized in measuring reduction in cyst loads, but variation in mouse and parasite strain is common. Further, it is known to be difficult to measure a significant change in cyst load due to wide variations between individual mice (Watts et al., 2015).

This review attempts to join together varying approaches to provide a basis for comparison, particularly by antigens and adjuvants. In almost all cases, the use of an adjuvant increased the survival time or rate as well as decreased the cyst burden in mice, although the increase is rarely impressive (no more than 20%) (Liu et al., 2010b).

The DNA vaccines with the greatest efficacy against acute infection measured by percent survival were the vaccines using microneme proteins MIC1 + MIC4 + MIC6 and MIC1 + MIC4, which led to 80% and 70% survival over 30 days against an acute challenge of 80 ME49 cysts, respectively (Pinzan et al., 2015; Foroutan et al., 2018).

However, the two most effective DNA vaccines against acute infection, as measured by the number of days surviving beyond the untreated group, were ROP18 + PLP1 with the adjuvant IL-18 and ROP5 + ROP18 with the adjuvant IL-33, which led to an increase in survival by 31 and 30 days, respectively (Chen et al., 2018; Zhu et al., 2020).

The most impressive DNA vaccine attempt against the chronic challenge was the antigen ROP29 with adjuvant R848. Using 20 PRU cysts, the ROP29 and R848 vaccine demonstrated an 80% reduction in cysts (Lu et al., 2018). Another DNA vaccine that showed of similar efficacy was a multi-antigen vaccine encoding Profilin, ROP16, ROP18, MIC6, and CDPK3, which led to an 80% reduction in cyst burden (Zhang et al., 2018a). Two other antigens with impressive cyst reduction include NTPase with a 78% cyst reduction and ROP38 with a 77% cyst reduction (Xu et al., 2014; Zheng et al., 2017b). The adjuvants which contributed to the greatest reduction in cysts compared to an antigen-only vaccine was the combination of IL-15 + IL-21, which led to a 39% further cyst reduction than the antigen MIC8 when challenged with 20 PRU cysts (Li et al., 2014).

The combination of multiple genes, especially the microneme proteins and rhoptry genes, demonstrated more efficacy as antigens in DNA vaccines and may warrant further investigation. For adjuvants, the use of interleukins, especially the combination of IL-15 + IL-21, produced relatively effective results and thus may be of interest to be tested in conjunction with other antigens.

Taken together, the field of *T. gondii* DNA vaccines has demonstrated that the more potent DNA vaccine candidates have the following features: (1) multiple antigens, (2) antigens either found on the surface of, or is secreted by, the parasite, and (3) inclusion of an adjuvant. Perhaps in the future, some combination of these factors can be combined to produce a more potent outcome than is currently available.

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