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REVIEW

Current progress toward vaccines against *Toxoplasma gondii*

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João Luis Garcia[1]
Elisabeth A Innes[2]
Frank Katzer[2]

[1]Department of Preventative Veterinary Medicine, Center of Agricultural Science, State University of Londrina, Parana, Brazil; [2]Moredun Research Institute, Pentlands Science Park, Edinburgh, Scotland

Abstract: *Toxoplasma gondii* is an intracellular protozoan parasite that can infect many warm-blooded animal species and humans. Despite substantial knowledge of the biology, epidemiology, and host-pathogen interactions of *T. gondii*, there are still very few effective control strategies to prevent oocyst shedding in cats, tissue cysts in livestock for consumption, and infection and disease in humans. This article reviews current progress and targets for vaccination against *T. gondii*.

Keywords: toxoplasmosis, vaccination, livestock animals, cats, immune response, organelles

Introduction

Toxoplasma gondii is a protozoan parasite with a worldwide distribution,[1] and may be found in animals from the arctic,[2] rain forest,[3] and arid zones,[4] and even in marine mammals.[5] *T. gondii* normally causes a subclinical infection in most animal species; however, a primary infection during pregnancy can cause fetal pathology, as well as abortion in humans and some animal species.[6,7] When the human fetus is infected, it may present with hydrocephalus, chorioretinitis, deafness, and impaired mental development.[8] The incidence of congenital toxoplasmosis in humans is highly variable across different countries/regions.[9] The recorded incidence is usually lower in Europe and North America (six cases per 1,000 live births),[10,11] when compared with Brazil, where cases range from 0 to 77 per 1,000 live births depending on the level of poverty in the region studied.[7] A study in Brazil reported detection of parasitemia and isolation of *T. gondii* from peripheral blood in 15.2% of children with congenital toxoplasmosis, which is an excessively large number considering the difficulty of detecting the parasite in blood.[12]

Additionally, in spite of highly effective antiretroviral therapy, the estimated incidence of toxoplasmic encephalitis is 15.9% in patients infected with the human immunodeficiency virus.[13] Before highly effective antiretroviral therapy, encephalitis caused by *T. gondii* was the most frequent opportunistic infection complicating acquired immunodeficiency syndrome.[14] Ocular toxoplasmosis is another concern with *T. gondii* infection, and may occur following congenital and acquired transmission;[15] the risk of ocular toxoplasmosis is highly variable depending on geographic region, ranging from 2% in Europe and North America to 18% in southern Brazil.

Moreover, there are reports that *T. gondii* may be associated with psychiatric disorders,[16] and may affect human behavior, personality, and other phenotypic traits. This parasite may change the behavioral phenotype of the host (mouse) to increase its likelihood of transmission to a new host (cat) by predation.[17]

Correspondence: João Luis Garcia
Departamento de Medicina Veterinária Preventiva, Centro de Ciências Agrárias, Universidade Estadual de Londrina, CP 6001, CEP 86051-990, Londrina-PR, Brazil
Email jlgarcia@uel.br

The main infection sources for humans are consumption of vegetables or water contaminated with sporulated *T. gondii* oocysts and undercooked meat infected with tissue cysts. The risk of becoming infected depends on culinary practices, such as regular consumption of raw or undercooked meat and living in an environment with a higher risk of oocyst contamination. Those relative risks have been shown in research isolating *T. gondii* from meat and the environment.[18–21] Hill et al[22] recently suggested that cats were an important source of *T. gondii* infection in the USA, based on finding a low frequency of *T. gondii* in meat samples.[23] In addition, Boyer et al[24] showed that environmental contamination by *T. gondii* oocysts contributes substantially to acquisition of *T. gondii* and subsequent disease in people. These authors went on to suggest systematic screening of pregnant women and developing a vaccine that would have the potential to prevent the fetal disease caused by acquisition of *T. gondii* during gestation in North America.

Another important factor in the epidemiology of toxoplasmosis is the definitive host. Cats have an important role due to their close interaction with humans, and infected cats shed millions of oocysts in their feces that contaminate the environment.[25] The risk of infection via sporulated oocysts in human populations has been well documented.[20,21] One study showed seropositivity for immunoglobulin (Ig)G and IgM antibodies to *T. gondii* in human populations living in Erechim, Rio Grande do Sul, Brazil, where there was a high risk (odds ratio >2.08) of infection in individuals who have had prior contact with soil.[21] In addition, an environmental survey suggested that 22.58% (7/31) of soil samples obtained from gardens in public schools in the state of São Paulo, Brazil, were positive for *T. gondii*, and found that oocysts are widely distributed in areas belonging to public schools located in the region.[20] An outbreak of human toxoplasmosis was described in Santa Isabel do Ivaí, Paraná, Brazil, where about 426 people were infected, and water contaminated with *T. gondii* oocysts was considered to be the primary transmission route.[26] These data demonstrate the need to control oocyst shedding by cats; however, few studies have been conducted in this regard.

Like several other parasites, *T. gondii* can be controlled by education about sanitation, management of animals, including livestock and cats, treatment, and vaccination. Treatments are available to reduce clinical signs, but there are no drugs available that kill the parasite or cure the host of infection. There is just one commercial vaccine available (Toxovax®, MSD Animal Health, Summit, NJ, USA) that is used in the UK, New Zealand, France, and Ireland. This vaccine comprises live tachyzoites of the incomplete S48 strain that has lost the ability to differentiate into tissue cysts in animals. The vaccine is licensed only for use in sheep and goats and should be administered prior to mating; however, there are some concerns about its safety because the vaccine can infect humans, and because it is a live vaccine, it has a short shelf-life.[27]

The aim of this paper is to discuss recent advances in the epidemiology, biology, parasite diversity, and host–parasite interactions of *T. gondii*, and to review current vaccination strategies further to the recent papers by Garcia et al[25] and Innes et al[6] and the prospect of future vaccines against this parasite.

Invasion of cells by *T. gondii*

Apicomplexan parasites, which include *T. gondii*, are obligate intracellular parasites.[28] Invasion of cells by *T. gondii* is a very complex event and key to the survival of the parasite inside hosts, since the organism cannot multiply outside cells.[29]

The mechanisms via which tachyzoites invade host cells are regulated by many factors, including conoid movements, motility, and secretion of organelles (micronemes, rhoptries, and dense granules), and are calcium-dependent.[30–32] This invasion is extremely rapid (15–40 seconds), depending on the sequential secretion of proteins by organelle complexes and is very important for survival of the parasite within the host cell.[33] After the tachyzoite finds the host cell, it starts to excrete proteins, first from micronemes and then from rhoptries, that enable attachment of the parasite to the cell, leading to formation of a moving junction, through which the parasite enters the cell.[34] Carruthers and Sibley[35] compared the kinetics of secretion from apical organelles, and observed that microneme proteins are initially secreted to form the host–parasite junction. Secretion from rhoptries is then involved in formation of the parasitophorous vacuole membrane, and secretion of dense granules starts only after the parasite is inside the parasitophorous vacuole. The parasitophorous vacuole membrane is considered to be a hybrid membrane, which means that secreted-excreted proteins from the parasite will be part of this membrane, and the parasitophorous vacuole does not fuse with host endosomes or lysosomes.[31]

Inside the parasitophorous vacuole, the tachyzoites multiply via a process called endodyogeny. This intracellular growth by the parasite leads to rupture of infected cells and subsequent infiltrates of neutrophils, eosinophils, and mononuclear cells at the site of infection. Progression of the infection, usually by day 6,[36] leads to development

of diffuse damage interrupted by multifocal necrosis. By about the third week after infection, tachyzoites start to disappear from visceral tissues and may localize as tissue cysts in neural and muscular tissues. Tachyzoites may persist longer in the spinal cord and brain than in visceral tissues because immunity is less effective in neural organs, and this persistence varies depending on the strain of *T. gondii* and the host species.[37] The host immune system is activated at a very early stage after infection, with involvement of natural killer cells, interferon gamma (IFN-γ), and interleukin (IL)-12 macrophages, and the innate immune system creating the right microenvironment for stimulation of the specific adaptive immune system.[38] As a consequence of the host immune response, the parasites are retained in tissue cysts and transform to bradyzoites, which multiply slowly within the cyst. In sheep and other livestock species, the tissue cysts can remain for the lifetime of the host, and this is also thought to be the case with humans.[39] *T. gondii* tissue cysts can be observed in the nervous system, eyes, organs, and muscles. When definitive hosts, ie, members of the cat family, become infected they shed unsporulated oocysts in their feces after a sexual phase in their intestinal cells. In the environment, unsporulated oocysts start sporogony and become sporulated (two sporocysts with four sporozoites each) and become infectious in 1–5 days depending on appropriate moist temperate environmental conditions.[1,5]

Host cell invasion by sporozoites differs from infection by tachyzoites and bradyzoites. Following entry of sporozoites into cells, a large parasitophorous vacuole known as PV1 is formed initially. Sporozoites are not able to replicate inside PV1, because it lacks channels allowing absorption/uptake of nutrients. Therefore, a second vacuole, PV2, is formed in the cytoplasm, the parasite transfers across, and multiplication of the parasite takes place within VP2.[40]

Potential proteins for vaccine use

About 1,360 specialized protein families have been described in *T. gondii*, which is a high number compared with related coccidian parasites.[34] Surface antigen glycoproteins (SAGs) are important for host cell attachment and host immune evasion, and *T. gondii* possesses 182 SAG-related sequences distributed across 14 chromosomes at 57 genomic loci.[41] Additionally, the most abundant SAGs in the stages of *T. gondii* (SAG1 in tachyzoites, BSRS4 in bradyzoites, and sporoSAG in sporozoites) are significantly divergent.[35,42] Although SAG1 and BSR4 have different expression patterns, they are both members of the SAG1 family. Despite abundant expression of SporoSAG in infectious sporozoites, serum from patients does not contain antibodies against SporoSAG, which is a member of the SAG2 subfamily and is significantly divergent from SAG1 and BSR4.[35,39]

The main SAGs in *T. gondii* are SAG1 and SAG2, which are the most abundant proteins in tachyzoites.[43,44] SAG1 is detected in the parasitophorous vacuole and in the intravacuolar membranous network; it is hydrophobic and has an acidic pH.[45] SAG1 has a role in penetration of the host cell by the tachyzoite during the initial stage of invasion, and along with SAG2 is involved in cell adhesion and invasion.[38,46] Other stages of the parasite, ie, sporozoites and bradyzoites, lose all their surface proteins during stage differentiation to the tachyzoite and show specific proteins that mediate invasion of host cells.[38,47] The antigenic differences in different stages of the parasite are not fully understood, but it is speculated that surface proteins from different stages interact with different tissues/cells in the host.[40] SAG1 and SAG2 have also been identified in the parasitophorous vacuole membrane and tubular vesicular network.[48] Several researchers have investigated SAG1 as a candidate vaccine.[49–57]

As a member of the Apicomplexa phylum, *T. gondii* has an apical complex with specialized organelles used for host cell invasion, such as micronemes, rhoptries, and dense granules. Micronemes include adhesion proteins and a class of four aspartyl proteases (toxomepsin). Apical membrane antigen 1 (AMA1), a protein that is produced during intracellular multiplication of tachyzoites and initially localized in the microneme,[58] and together with RON proteins forms a ring-like structure known as the moving junction, leads to internalization of the parasite into a parasitophorous vacuole.[59] AMA1 plays a central role in host cell invasion, and antibodies against this protein block invasion of the parasite.[60] The importance of this protein during the process of host cell penetration was demonstrated when a *T. gondii* AMA1 knockout strain was unable to invade host cells.[61] Because of this, AMA1 is considered to be a potential vaccine candidate.

About 20 dense granule proteins from *T. gondii* have been previously described.[62–64] These proteins are secreted from the parasite into the parasitophorous vacuole where they participate in the remodeling and maintenance of this compartment. Dense granule proteins may be found in the intravacuolar membranous network and the parasitophorous vacuole membrane,[65] and are of fundamental importance to the survival of the parasite within the cell.[42] GRA4 and GRA7 have been described also as candidate proteins for vaccine development.[62,66,67]

The amino acid sequence deduced for GRA4 (40 kDa) indicates a proline-rich product with an internal hydrophobic

region and a potential site of N-glycosylation.[68] This protein was detected in serum from infected mice, providing evidence of the antigenicity of GRA4 protein synthesized from cells transfected with the plasmid DNA construct.[69]

Alaganan et al[70] demonstrated that there is an interaction between ROP18, ROP8/2, and GRA7 leading to an increase in the virulence of *T. gondii* in mice. GRA7 has been shown in vitro to increase, by biochemical mechanisms, the turnover of immunity-related GTPases, thus potentially complementing the functions of other known virulence determinants, ROP18 and ROP5. This example reveals multiple layers of defense used by the parasite to counteract the innate immunity response in the host.[70]

There are several rhoptry proteins described to date,[71] and the most abundant is the ROP2-related family, which includes ROP2, ROP3, ROP4, ROP7, and ROP8. These share antigenic determinants, a very similar molecular weight (between 55 kDa and 60 kDa) and hydrophobic region(s).[62,72] ROP2 is thought to serve as the molecular link between host cell mitochondria and the parasitophorous vacuole membrane. A study, which used ROP2-depleted parasites, observed that ROP2 was essential for multiplication and invasion of the parasite.[73] ROP2 was recognized by a human T cell clone and isolated from an immune donor, and is specific for the parasite protein and produces high levels of IFN-γ.[74] Additionally, ROP2 has been seen in all subgroups (types I, II, and III) and stages of the parasite.[75] For these reasons, ROP2 was identified as a candidate vaccine, and plasmids encoding the ROP2 antigen have been used to vaccinate mice.[49,76]

Howe and Sibley[77] showed that 95% of *T. gondii* strains from Europe and North America (isolates from humans with and without acquired immune deficiency syndrome and from animals) belonged to three clonal lineages (genotypes I, II, and III), indicating low genetic diversity, which is explained by infrequent recombination during replication of diploid sexual stages in the definitive host. This may be applicable in Europe and North America, but in other parts of the world, such as South America, the *T. gondii* strains are highly diverse.[78] A recent study[79] examined more than 950 isolates representing worldwide diversity, and genotyped these using three independent sets of polymorphic DNA markers, sampling 30 loci distributed across all chromosomes as well as the plastid genome. The strains clustered into 138 unique genotypes, 15 haplogroups, and six major clades. This study also indicated that a small number of ancestral lineages gave rise to the existing diversity, the predominant highly clonal lineages in the northern hemisphere, and a selection of less common genotypes showing greater evidence of recombination in parts of South America.

There are marked phenotypic variations between the genotypes; for example, while the RH strain (type I) is more virulent, ME49 (type II) and VEG (type III) are less virulent in mice.[80] Analysis of complex quantitative phenotypes such as virulence has revealed that chromosome VIIa controls differences in virulence between the highly virulent type I and avirulent type III strains. Also, members of the polymorphic protein kinases, such as ROP5, ROP16, and ROP18, contribute to phenotypic differences between the genotypes.[81–83] However, the importance of these variations for development of vaccines remains unknown.

Immunity against *T. gondii*

T. gondii infection rarely produces clinical signs in the host, and severity of the disease is dependent on species, age of the host, sex hormones, pregnancy, immunological status, nutritional status of the host, strain (including differences between strains[84]), parasite stage, and concomitant infection.[85] The mechanisms involved in host protection against infection are components of the innate and adaptive immune responses.

Animals (laboratory, domestic, livestock) and humans are clearly not identical, and many aspects of their anatomy, physiology, and genetics are different, including the development and function of their immune systems. Basically, the process by which an immune response is generated against the parasite is the same in all species, and involves induction and function of the innate and adaptive immune responses.

Toll-like receptors (TLRs) are a family of germ line-encoded receptors of the innate immune system, via which the host recognizes pathogen-associated molecular patterns.[86] In vertebrates, TLRs are essential for the recognition of parasites via the innate immune response and for induction of a clonally polarized, antigen-specific response by the adaptive immune system, mediated by B and T cells. TLRs are considered to be a conserved system, and there is no obvious interspecies variation between mammals. The nucleotide homology of human, livestock (cattle, small ruminants, pigs, horses), and domestic animal (cats, dogs) TLR genes ranges from 65% to 77%.[87]

Since TLRs are associated with the adaptive immune response, study of adjuvants and specific antigens from *T. gondii* in livestock and domestic animals and their association with TLRs might help to develop vaccines for these species.[86] TLRs are essential for the development of the acquired immune response characterized by stimulation

of naïve CD4+ helper T cells toward the Th1 or Th2 phenotype. TLR-2 contributes to the resistance to high challenged doses of *T. gondii* in mice,[88] and TLR-11 and TLR-12 are very important in regulation of the immune response.[89–92] Prominent expression of TLR-4 in the first trimester of pregnancy in humans has been reported, and lipopolysaccharide expressed in the cell wall of Gram-negative bacteria is recognized as the classic TLR-4 ligand, suggesting a possible protective role for maternally derived cells expressing these receptors.[93] TLR-11 plays a dominant role in recognition of *T. gondii* in mice; however, TLR-11 is represented in humans only by a pseudogene, so the major question of how innate and adaptive immune responses occur in the absence of TLR-11 remains unanswered.[92] Despite the lack of this functional receptor in humans, studies in knockout mice (TLR-11$^{-/-}$) show that neutrophils are a crucial source for IFN-γ, which is required for protection against *T. gondii*.[94]

Monocytes, neutrophils, and dendritic cells are recruited locally during the initial phases of infection and are important for resistance to *T. gondii*. This innate immune response produces the cytokine IL-12, which stimulates natural killer cells and T cells to produce the cytokine IFN-γ.[95] *T. gondii* antigens are processed by major histocompatibility complex class II antigen-presenting cells, and their peptides are presented to CD4$^+$ cells to stimulate the adaptive immune response.

Studies using knockout mice deficient in B cells, CD4+, CD8+, and cytokines have demonstrated the importance of the adaptive immune response in resistance to toxoplasmosis.[96,97] In mice, extremely elevated levels of IFN-γ and IL-18 result in lethal toxoplasmosis, whereas moderate levels of these cytokines cause nonlethal infection. Tumor necrosis factor (TNF)-α has an important role in resistance to toxoplasmosis, but high levels of this cytokine may contribute to pathogenesis. Elevated levels of IL-18, IFN-γ, IL-12, and TNF-α increase vascular permeability, and can lead to multiple organ failure and death of the animal.[98]

CD4+ cells and CD8+ T lymphocytes are essential for the development of protective immunity and long-term survival during persistent infection, and this property is due to their ability to produce IFN-γ, a proinflammatory cytokine that is known to be a major mediator of resistance to *T. gondii*.[99] Depletion of this cytokine during the persistent phase of *T. gondii* infection in mice showed that continued production of IFN-γ is necessary for long-term survival.[100] Extracellular tachyzoites can be destroyed in the presence of specific antibodies and complement pathway.[101] Anti-*T. gondii* antibodies may also prevent entry of the parasite into cells.

There are many questions regarding induction of innate and adaptive immune responses during *T. gondii* infection. Most studies that have evaluated immunity against this parasite were done in murine models. However, one important issue is the clear difference between animal species regarding their susceptibility to infection, and it is unclear how we could extrapolate results from one species to another.[39] Susceptibility to *T. gondii* infection may depend on differences in host–parasite interactions. This has been demonstrated in the development of toxoplasmic encephalitis in humans and mice. Human toxoplasmic encephalitis is associated with a loss of T cell function, whereas in mice it is associated with a defect in the CD8$^+$ T cell response.[102] Further, information on the immune response obtained from mice should not be extrapolated to pigs because of differences in their immune response mechanisms, such as the lack of an active nitric oxide pathway in pigs.[103,104] Another important difference between animal species is found in IgM and IgG, eg, cats are not able to activate the classical complement pathway.[105] The major differences between the immune systems of animal species have been described in a recent review,[106] and included natural killer cells, distribution of Peyer's patches, immunoglobulin subclasses, and T cell receptor distribution.

Vaccines against *T. gondii*

Data obtained from Medline (PubMed, National Center for Biotechnology Information) using the key words "*Toxoplasma gondii*" and "vaccine", focusing on the years 2009, 2010, 2011, 2012, through September 2013, yielded approximately 109 papers addressing vaccines against *T. gondii*, comprising 26 in 2009, 15 in 2010, 26 in 2011, 20 in 2012, and 22 in 2013. Most of these studies used mice (78%, 85/109) as an experimental model, six (5.5%) used sheep, and only two (1.8%) were in pigs. Interestingly, only one study attempted to reduce oocyst shedding in the feces of domestic cats,[107] given that these animals are considered to be the key in the transmission cycle of *T. gondii*.[27] Most of the mouse studies (Table 1) were conducted to test DNA vaccines (37.7%), with the main vector used being pVAX1 (a eukaryotic expression vector), and the main vaccine candidates inserted were the SAG1, ROP2, GRA4, and MIC3 genes. Some live virus vaccines expressing recombinant proteins were evaluated, and these studies showed promising levels of protection since anti-viral responses are similar to the immune responses required to control this parasite.[50,108]

An important feature of *T. gondii* is that the main route of host infection is oral, with oocysts being the main form of infection in herbivores and tissue cysts being a route of

Table 1 *Toxoplasma gondii* vaccine studies in mouse models

Animal	Antigen and adjuvant	Route of immunization	Challenge	Aim	Results	Author(s)
C57BL/6, CD4 KO B cell and IL-10 KO	TS-4 (temperature-sensitive mutant) 2×10^4 TS-4 strain	Intracameral	10^2 tachyzoites of RH strain ocularly	OT	Live TS-4 vaccine can be used for the prevention of OT	Lu et al[153]
NIH	TLA and TCP + *Lactobacillus casei* as adjuvant	Intraperitoneal	Orally 20 cysts of Me49 strain	BCB	Results suggest that *T. gondii* cytoskeleton proteins with *L. casei* are a good vaccine candidate	Martınez-Gomez et al[154]
CBA/J mice (*H-2k*)	Exosomes secreted by DC pulsed with TLA	Subcutaneous	25 cysts of 76K strain orally	CT	This approach may be an effective alternative candidate vaccine against congenital toxoplasmosis	Beauvillain et al[155]
BALB/c mice	pcDNA3.1-SAG1-MIC4 plasmid	Intranasally	Intraperitoneally 10^3 tachyzoites of RH strain	MR	Increase in survival rate and 14% higher survival rate than mice immunized with single-gene vaccines	Wang et al[156]
CBA/J	*Eimeria* antigen (rEA) + TLA	Intranasally	Orally 70 cysts of the 76K strain	BCB	Weak humoral and cellular immune response after intranasal immunization, However, mice had a 50% reduction in brain cysts	Hedhli et al[52]
CBA/J	Mic1–Mic3 KO and parental WT tachyzoites	Intraperitoneally/orally	ND	MR	The KO strain derived from type I strain behaved like type II strain	Moiré et al[157]
C3H/HeJ	Recombinant proteins ROP2 and ROP4	Subcutaneously	Intraperitoneally/five cysts of DX strain	BCB	Both antigens generated a strong systemic mixed Th1/Th2 response polarized toward IgG1 antibody isotype. In the immunized group brain cyst number was reduced by ≅ 46%	Dziadek et al[158]
C57BL/6, B6.MRL	Replication-deficient parasites that express the model antigen OVA	Intraperitoneally	Intraperitoneally/ 10^3 RH strain	MR	Immunization provided resistance to rechallenge; the RH parental strain was also protective	Jordan et al[159]
ICR mice	Multiantigenic DNA vaccine by attenuated *Salmonella typhimurium* (ZJ111/pSAG1-MIC3)	Orally	Intraperitoneally/ 500 tachyzoites of RH strain	MR	The vaccine produced partial protection against *T. gondii* challenge	Qu et al[160]
C57BL/6	Attenuated type I vaccine strain	Intraperitoneally	Intraperitoneally or orally/ ten or 100 cysts of ME49 strain	BCB	Immunization elicited CD8-immune T cells able to inhibit recrudescence of brain cysts	Gigley et al[161]
BALB/c	DNA vaccine (pcDNA–SAG1) and pseudotype baculovirus expressing SAG1 (BV-G-SAG1)	Intramuscularly	Intraperitoneally/ 10^3 tachyzoites RH strain	MR	Compared with DNA vaccine (pcDNA/SAG1) BV-G-SAG1 induced higher levels of specific *T. gondii* antibodies and IFN-γ and survival rate of mice with BV-G-SAG1 was significantly improved	Fang et al[54]
Kunming	DNA vaccine (pVAX-MIC8)	Intramuscularly	Intraperitoneally/ 10^3 tachyzoites of RH strain	MR	Partial protection; MIC8 is a potential vaccine candidate against toxoplasmosis	Liu et al[53]
BALB/c Swiss-Webster	Recombinant virus + SAG2, influenza virus (FLU + SAG2) Boosted with adenovirus (Ad + SAG2)	Intranasally/subcutaneously	Orally/20 cysts of P-Br strain	BCB	Authors observed 85% of reduction in parasite burden	Machado et al[108]
C57BL/6 B6/J ($IFN^{-/-}$)	KO strain/KU80 pyrimidine starvation	Intraperitoneally	Intraperitoneally/ 200 tachyzoites of RH strain	MR	A single immunization was able to produce a protective immunity to lethally challenge infection	Fox and Bzik[162]
C3H, C57BL/6	Extract of tobacco leaves expressing SAG1	Subcutaneously/orally	Orally/20 cysts of ME49 strain	BCB	Animals immunized by the parenteral route were better protected; use of plant has a potential for protecting humans and animals against toxoplasmosis	Laguía-Becher et al[163]

C57BL/6 C3H/HeJ	Recombinant proteins ROP2, ROP4, SAG1, and GRA4	Subcutaneously	Intraperitoneally/five cysts of DX strain	BCB	A significant reduction, ranging from 59% to 71%, in the formation of brain cysts in immunized animals	Dziadek et al[164]
BALB/c	DNA vaccine, SAG1, and ROP2	Intramuscularly	Intraperitoneally/ 10^4 tachyzoites of RH strain	MR	Partial protection; vaccinated animals had longer survival time than controls	Hoseinian Khosroshahi et al[55]
Kunming	DNA vaccine, pVAXROP16	Intramuscularly	Intraperitoneally/ 10^3 tachyzoites of RH strain	MR	pVAX-ROP16 dramatically increased the survival time (21.6–9.9 days) compared with control mice, which died 7 days after challenge	Yuan et al[165]
C57BL/6 MyD88-deficient	DNA vaccine, pME18100/HSP70	Gene gun vaccination	Orally/ten cysts of Fukaya strain	BCB	All groups of deficient mice showed lower brain cysts than controls unvaccinated	Makino et al[166]
Swiss-Webster, C57BL/6 and KO mice	Adenovirus expressing SAG1 (AdSAG1), SAG2 (AdSAG2), or SAG3 (AdSAG3)	Subcutaneously	Orally/ten cysts of ME49 strain	MR	Vaccination with Adenovirus expressing SAG1 showed protective immunity in the highly susceptible C57BL/6 mice	Mendes et al[167]
C3H/HeN	Recombinant proteins, rROP2, rGRA4 plus CpG-ODN	Intramuscularly	Orally/20 cysts of ME49 strain	BCB	Immunized animals had reduced brain cysts by 62%–64%	Sánchez et al[168]
BALB/c mice	Recombinant pseudorabies virus, SAG1 or MIC3	Intramuscularly	Intraperitoneally/ 10^2 tachyzoites of RH strain	MR	A partial protection of 66.7% was obtained in the mice immunized with a cocktail of SAG1-MIC3 rPRV	Nie et al[56]
CBA/J	DNA vaccine, RON4	Intramuscularly	Orally/70 cysts of 76K strain	BCB	RON4 showed no significant reduction in brain cyst load	Rashid et al[169]
Kunming	DNA vaccine, pVAX-ROP18	Intramuscularly	Intraperitoneally/ 10^3 tachyzoites of RH strain	MR	immunization increased survival time (27.9 days) compared with control mice (7 days)	Yuan et al[170]
BALB/c	DNA vaccine, encoding GRA1 and SAG1	Intramuscularly	Intraperitoneally/ 10^5 tachyzoites of RH strain	MR	Immunized animals showed a strong humoral and cellular immune response and had enhanced protection against challenge	Wu et al [57]
Kunming	DNA vaccine, encoding ROP13/IL-18	Intramuscularly	Intraperitoneally/ 10^3 tachyzoites of RH strain	MR	ROP13 and IL-18 increased the survival time (24.9 to 32.3 days) of mice	Wang et al[171]
BALB/c	DNA vaccine, encoding Immune mapped protein 1	Intramuscularly	Intraperitoneally/ 500 tachyzoites of RH strain	MR	Immunized mice showed an increased survival time (15.8 days)	Cui et al[172]
BALB/c	DNA vaccine, encoding *Toxoplasma* rhomboid protein 1	Intramuscularly	Intraperitoneally/ 10^3 tachyzoites of RH strain	MR	Immunized mice showed an increased survival time (12.5 days)	Li et al[173]
BALB/c	Recombinant proteins (rROP2, rROP4, rGRA4, rSAG1) incomplete Freund's adjuvant	Subcutaneously	Intraperitoneally/five cysts of DX strain	BCB	The animals immunized with rROP2 + rGRA4 + rSAG1, rROP2 + rROP4 + rGRA4 and rROP2 + rROP4 + rSAG1 showed reduction in the brain cyst number, 46%, 84%, and 77% respectively	Dziadek et al[49]
BALB/c	Recombinant protein SAG1 and SAG 2 encapsulated in PLG microparticles	Intraperitoneally	Subcutaneously/ 10^4 tachyzoites of RH strain	MR	PLG-rSAG1/2-immunized animals showed a higher survival rate (83%)	Chuang et al[174]
Kunming	DNA vaccine, encoding MIC13	Intramuscularly	Intraperitoneally/10^3 tachyzoites of RH strain and orally/ten cysts of PRU strain i	MR and BCB	Immunized mice showed an increased survival time (21.3 days) and reduction in the brain cyst number (900 cysts)	Yuan et al[175]
Kunming	Ultraviolet irradiation tachyzoites plus pidotimod adjuvant	Intraperitoneally	Intraperitoneally/ 10^2 tachyzoites of RH strain	MR	Immunized mice showed an increased survival time (7 to over 30 days, in which 50% of the mice survived by day 30)	Zhao et al[176]

Abbreviations: BCB, brain cysts burden; DC, dendritic cells; OT, ocular toxoplasmosis; TLA, *Toxoplasma* lysate antigen; TCP, *Toxoplasma* cytoskeleton proteins; CT, congenital toxoplasmosis; KO, knockout; NIH, National Institutes of Health; IL, interleukin; MR, mortality rate; WT, wild-type; OVA, ovalbumin; ND, not done; IFN-γ, interferon-gamma; ICR, imprinting control region; CD, cluster of differentiation.

infection for pigs and humans, so local immunity in the gut via lymphocytes (mainly intraepithelial lymphocytes with $CD8^+$ activity) and IgA is of fundamental importance in host resistance to the parasite.[109] However, after initial infection by sporozoites, the parasite transforms into tachyzoites, the rapidly dividing form of the parasite, and the sporoSAG protein is nonimmunogenic during natural infection.[110]

Livestock

Sheep, goats, and pigs are more susceptible to disease following *T. gondii* infection than other livestock species and can harbor tissue cysts for life, whereas horses and cattle are less susceptible to infection and cysts containing bradyzoites are rarely detected in their tissues.[1,39] However, animals raised for human consumption, including sheep, goats, chicken, cattle, horses, and pigs, are intermediate hosts and may serve as sources of *T. gondii* infection if their meat contains tissue cysts and is ingested raw or undercooked. This form of infection is described as carnivorism,[85,111] and is thought to be a major transmission route for humans. Additionally, the disease burden of congenital toxoplasmosis, as represented by disability-adjusted life years, is the highest of all the food-borne pathogens.[112]

Toxovax is the only commercial vaccine available for congenital toxoplasmosis and is licensed for use only in sheep and goats. The manufacturer's recommendation is to use $\geq 10^5$ live tachyzoites of the S48 strain per animal. This dose should be given in a single injection of 2 mL via the intramuscular route at least 3 weeks prior to mating. Animals may be vaccinated from 5 months of age. The vaccine induces long-lasting immunity of at least 18 months without natural challenge. A rise in body temperature can be observed in animals following vaccination, and their meat should not be eaten for 42 days. The potential of Toxovax to prevent formation of *Toxoplasma* cysts in the ewe after a challenge is currently unknown.

Falcon and Freyre[113] studied the capacity of a relatively low virulent strain to protect against formation of tissue cysts in sheep. Lambs were dosed orally with 10^6 sporulated oocysts of the ME-49 strain, and after 45 days, were challenged orally with 4×10^6 sporulated oocysts of the M3 strain. Unvaccinated control animals were infected with 4×10^6 sporulated oocysts of the M3 strain. Using a bioassay, the authors did not observe cysts in muscle or brain tissue from lambs that were previously infected with ME49; however, unvaccinated challenged control animals showed a high burden of *T. gondii* cysts in muscle and brain tissue.

Sheep have been used as a large animal model to determine the immune response to *T. gondii* induced by a DNA vaccine.[66,114–117] The antigen genes tested were expressed in dense granules and rhoptries, ie, GRA1, GRA4, GRA6, GRA7, and ROP1. These were used as DNA vaccines in different types of adjuvant formulations, such as liposomes. Ovine CD154 was added at the time of vaccination to enhance stimulation of the immune response. CD154 is expressed as a type II integral membrane protein on the surface of activated T cells, basophils, and mast cells. The results showed that intramuscular injection of sheep with DNA liposomes formulated to contain plasmids coding for GRA proteins is an effective system that induces a significant mixed Th1/Th2 response, while intramuscular injection of ROP1 induced a Th1-specific immune response against *T. gondii.* Li et al[51] investigated the immune response in sheep after injection of plasmids encoding *T. gondii* SAG1 and ROP1. Injection of ROP1 produced a better immune response than SAG1. However, these studies did not indicate sufficient protection against *T. gondii* challenge.

Mevelec et al[118] showed the effectiveness of a mic1 and mic3 gene knockout (Mic1–3 KO) RH strain against *Toxoplasma* abortion in sheep. Sheep were inoculated subcutaneously with 10^5 Mic1–3 KO tachyzoites, and showed a mild febrile and parasite-specific IgG antibody response. Ewes were mated 2 months after vaccination and were challenged orally at mid gestation with different doses of oocysts from the PRU strain (100–400 sporulated oocysts). After challenge, the animals showed a slight febrile response, whereas unvaccinated ewes developed a more severe febrile response. All unvaccinated ewes aborted, while 62%–91% of lambs born to the vaccinated sheep were viable, and did not show clinical signs of infection. The authors tested the Toxovax (S48 strain) vaccine as well, and showed that the mutant strain was as effective as the S48 strain. The mutant strain, at a dose of 10^5 tachyzoites, induced a level of protection comparable with that of 2×10^6 S48 tachyzoites.

There are only three studies that have attempted to vaccinate goats against toxoplasmosis.[119–121] Two of these studies[119,120] used goats with related parasites (*Hammondia hammondi*) and *H. heydorni* to protect against congenital toxoplasmosis in does, and reported partial protection using *H. hammondi* but no promising effect using *H. heydorni.* The last study[121] show good efficacy of the S48 strain in protecting does against congenital infection in France, one of the countries in which Toxovax is licensed. It is still necessary to conduct more studies using this host in vaccine experiments, given that goats are one of the species most

susceptible to *T. gondii* infection.[122] Moreover, consumption of *T. gondii*-infected raw or undercooked goat meat and unpasteurized goat milk containing tachyzoites constitutes a significant infection risk in humans.[123–125]

Clinical signs of toxoplasmosis in pigs are considered rare, and abortion in sows is not common but can occur.[126,127] Tissue cysts in pork can persist for more than 2 years, and these are one of the most important sources of *T. gondii* infection in humans.[23,128] Because of the potential public health risk from undercooked infected pork, a vaccine for pigs needs to be able to reduce or prevent formation of tissue cysts.

Verhelst et al[129] showed that GRA7 and MIC3 were able to induce a good humoral immune response in pigs experimentally challenged with *T. gondii* tissue cysts. Cunha et al[130] evaluated protection against formation of tissue cysts in pigs immunized intranasally with a crude rhoptry protein preparation of *T. gondii* plus Quil-A®. The vaccinated and challenged group showed 41.6% protection against tissue cyst formation versus 6.5% protection in the adjuvant control group.

Excreted-secreted antigens (proteins discharged from organelles of the parasite during invasion of host cells) from *T. gondii* mixed with Freund's adjuvant were used as a vaccine in pigs to evaluate the humoral and cellular immune response and protection against an intraperitoneal challenge with 10^7 tachyzoites of the GJS strain.[131] The authors showed a cellular immune response associated with production of IFN-γ and IL-4, and a humoral response mainly against antigens with molecular masses of 34–116 kDa. Following challenge, the immunized pigs remained clinically normal except for a rise in temperature, while the control pigs developed higher fever and clinical signs of toxoplasmosis. A reduction in formation of tissue cysts in muscles was found in the vaccinated animals.

Cats

Felines are the only definitive hosts able to shed oocysts via their feces, and domestic cats play an important role in the transmission of *T. gondii*. Cats are widespread globally and are considered to be among the worst non-native invasive species in the world. Moreover, they live in close proximity to humans.[132] Infected cats can shed millions of oocysts in their feces,[25] that may contaminate soil, water, and food. The significance of the environmental spread of oocysts produced by wild cats is unknown.

The first studies to immunize cats to prevent oocyst shedding were reported by Frenkel et al[133] and Freyre et al.[134] These authors, along with Mateus-Pinilla et al,[135] used a T-263 strain that had been transformed in the laboratory and lost the ability to complete the sexual cycle in enteroepithelial cells in cats as a vaccine. The main concern about use of the T-263 strain as a vaccine is its short shelf-life and need for production in mice. 60Co-irradiation tachyzoites were tested in cats in order to prevent/reduce oocyst shedding; however, the authors did not observe a significant reduction in oocysts in the vaccinated cats.[136,137] Garcia et al,[25] and more recently Zulpo et al,[107] tested a vaccine based on rhoptry proteins administered by the intranasal and rectal routes in cats and found that the immunized animals eliminated 90.8% and 98% fewer oocysts than the control group, respectively.

Mice

Mice are the experimental model of choice for *Toxoplasma* vaccine studies, as shown in Table 1. They are easier to manipulate and inexpensive to maintain compared with domestic and livestock animals, and their immunology is very well characterized. Many studies have used mice to investigate vaccines (Table 1). However, comparison of their results is difficult because of the different types of immunization, challenge routes (with different forms of parasite), and mouse strains used. Several types of approach have been used, ie, attenuated live vaccines, knockout strains, DNA vaccines, attenuated recombinant viruses, and recombinant proteins, and all described partial protection, mainly against mortality due to acute infection and tissue cyst burden.

Biological models for vaccine studies

Mice and rats are the main biological models used to study toxoplasmosis in vivo. While mice are sensitive, rats, like humans, are relatively resistant to *T. gondii*. Murine susceptibility to *T. gondii* is under multigenic control, with at least one of the genes linked to the major histocompatibility complex (H2 locus).[138] In addition, reduction of cyst numbers in the brain and encephalitis is mediated by the L^d gene in the D region of the H2 complex in mice.[139]

Sequencing the mouse genome has shown that approximately 99% of mouse genes have a homolog in the human genome.[140] However, there are differences between the immune systems of mice and humans[141,142] and between mice and domestic animals.[86,106] These differences need to be considered before data obtained from mice can be extrapolated to humans or domestic animals.

Mice are useful for in vivo vaccine studies against *T. gondii*, and with the availability of knockout gene mouse

strains, they will continue to be the first choice for vaccine trials. Other animal models of human toxoplasmosis have been used, including the rabbit,[143] hamster,[144,145] sheep,[146,147] and pig.[130,148,149] However, extrapolation of the results observed in experiments with animals is a difficult task, and a good biological model for ocular, congenital, recrudescence, and acute and persistent *T. gondii* infection would be very helpful in the prevention of human toxoplasmosis.

Prospects for future vaccines

Vaccination against bacterial and viral diseases is widespread, routine, and successful, but only a few vaccines for veterinary protozoan diseases have been developed successfully, and thus far none are available for human use. Future studies aiming to develop *T. gondii* vaccines should focus on parasite antigens able to trigger a protective immune response and investigate immunization routes and vaccine delivery strategies. To be effective, a protective vaccine needs to stimulate both humoral and cellular immune responses, which is difficult to achieve with killed vaccines. Concerns regarding live vaccines are mainly to do with their safety and short shelf-life. It would also be desirable to create a vaccine that would enable differentiation between vaccinated and infected animals. Thus, the use of viruses as vectors and knockout strains could be a good strategy to develop vaccines against this parasite.

Based on disability-adjusted life-year papers, *T. gondii* is considered one of the most significant food-borne pathogens,[112,150] and there is no treatment that is able to eliminate the parasite from human tissue; this is important when considering the brain (psychiatric disorders and toxoplasmic encephalitis) and eye (ocular disease), and what enforces the development of a vaccine against this parasite.

Use of relevant animal species is important in the study of protective immune responses to *T. gondii*, because they address questions relating to specific host–parasite interactions. More studies should be done using cats in order to answer important questions, such as: Which approach should be used to achieve an intestinal immune response against *T. gondii* to diminish oocyst shedding, ie, live or killed vaccines? What is the best route of immunization, ie, systemic or oral? Which proteins should be used in recombinant vaccines? What is the potential of cats to reshed oocysts after reinfection? Do different genotypes induce/require a different immune response?

Goats are one of the most susceptible domestic animal species with respect to toxoplasmosis, and the main clinical signs in adult goats are reproductive disorders, although some animals may develop anorexia, prostration, fever, and mastitis.[151,152] Unfortunately, very few studies that have attempted to produce a vaccine against *T. gondii* for goats. Further, despite Toxovax being licensed for use in does, it is only available in a limited number of countries, so there is a need for more studies looking at developing vaccines in goats.

Information obtained so far indicates that future vaccines for *T. gondii* should be developed against several different targets, ie, to prevent oocyst shedding by cats, prevent formation of tissue cysts in animals raised for human consumption, and prevent abortion or fetal malformation in sheep, goats, and women.

A vaccine against ocular toxoplasmosis as a consequence of acquired infection in regions with a high level of ocular toxoplasmosis, such as southern Brazil, should also be addressed. Development of a type of immunomodulator to be used in patients with chronic ocular toxoplasmosis could be a good approach to avoid future reactivation.

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The authors report no conflicts of interest in this work.

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