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Toxoplasmosis: Advances and Vaccine Perspectives

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1. Introduction

Toxoplasma gondii was first identified more than 100 years ago in the tissues of birds and mammals. In 1908 Nicolle and Manceoux described it for the first time in the gundi (*Ctenodactylus gundi*), a North African rodent, in tachyzoite forms. At the same time, Splendore in Brazil, identified the parasite in rabbit tissues. Due to its bow-like shape (Greek: *Toxo* = Arc) the genus was named *Toxoplasma*. However, only in the 1970's was the complete life cycle known and the parasite recognized as a coccidian parasite (member of the phylum Apicomplexa). It is ubiquitous throughout the world and estimated to infect approximately half of the world's population. It is characterized by a polarized cell structure and two unique apical secretory organelles called micronemes and rhoptries.

Toxoplasma has a complex life cycle consisting of a sexual cycle in its feline definitive hosts and an asexual cycle in its intermediate hosts. The latter, including humans, can be infected by ingestion of oocysts shed in cat feces. Unlike most other Apicomplexan parasites, *Toxoplasma* can be transmitted between intermediate hosts by either vertical (via placenta) or horizontal (carnivorism) transmission.

Toxoplasma parasite is found in intermediate hosts in two interconvertable stages: bradyzoites and tachyzoites. Bradyzoites, a dormant form, are slow-growing, transmissible and encysted. Infections with bradyzoite-containing cysts occur upon ingestion of undercooked meat. The wall of these cysts is digested inside the host stomach and the released bradyzoites, which are resistant to gastric peptidases, subsequently invade the small intestine. There, they convert into tachyzoites, the rapidly growing, disease-causing form that can infect most nucleated cells, replicate inside a parasitophorous vacuole, egress, and then infect neighboring cells. These tachyzoites activate a potent host immune response that eliminates most of the parasites. Some tachyzoites, however, escape destruction and convert back into bradyzoites. In the absence of an adequate immune response, tachyzoites

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will grow unabated and cause tissue destruction, which can be severe and even fatal. However, the inflammatory immune response induced by tachyzoites can cause immune-mediated tissue destruction. Therefore, a subtle balance between inducing and evading the immune response is crucial for *Toxoplasma* to establish a chronic infection.

The success of *Toxoplasma* as a widespread pathogen is due to the ease in which it can be transmitted between intermediate hosts. Humans do not play a major role in transmission; consequently, pathogenesis in humans is the indirect result of adaptations to infection in other hosts and treatment of human infections is unlikely to lead to the spread of drug resistance.

Once inside a host, the parasite develops powerful tools to modulate its host cell and develop into a chronic infection that can evade the host's immune system as well as all known anti-toxoplasmatic drugs. The ability of the parasite to replicate within a host cell, evade immune responses and undergo bradyzoite development requires the parasite to effectively modulate its host.

Toxoplasmosis remains a major health concern in pregnancy, where it causes severe birth defects or miscarriage, and in immunocompromised hosts. Thus, new toxoplasmosis control strategies are needed. The development of effective human and veterinary vaccines against toxoplasmosis is a relevant goal for Public Health (Gazzinelli *et al*. 1996; Pifer and Yarovinsky 2011). Even if new therapeutic drugs, with less hypersensitivity and toxicity-related events, are developed, not only for acute *T. gondii* infection but also for the currently untreatable latent bradyzoite form of the parasite, a prophylactic vaccine against the disease would still be the best option from the financial, epidemiological, and social points of view. A vaccine would decrease the enormous costs of diagnosis/treatment, the premature loss of lives, the extensive rates of dissemination as well as the social impact of the disease. One major fact that suggests the possibility of vaccination against toxoplasmosis is that primary infection with the *T. gondii* parasite elicits protective immunity against re-infection in most individuals.

2. Mechanisms of protective immunity against toxoplasmosis

Immune responses during the early stages of *T. gondii* infection are characterized by activation of innate mechanisms mediated by macrophages and dendritic cells (DC) (Gazzinelli *et al*. 1996; Pifer and Yarovinsky 2011). These cells are activated in mice (not yet known how in humans) after parasite internalization, by engagement of endosomal toll-like receptor 11 (and probably others) with tachyzoite products, which drives subsequent production of interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α). In turn, IL-12 activates natural killer (NK) cells (Denkers *et al*. 1993) to secrete gamma interferon (IFN-γ) (Gazzinelli *et al*. 1994), which then acts as stimulus for T-cell activation and, in synergy with TNF-α, mediates killing of tachyzoites by macrophages through enhanced production of free oxygen radicals and nitric oxide (NO).

Acquired immunity against *T. gondii* develops afterward, and is characterized by strong $CD4^+$ and $CD8^+$ T cell activity (Gazzinelli *et al*. 1992). The cytokine IFN-γ continues to be central in resistance to the parasite during the successive acute and chronic stages of infection, driving the differentiation of $CD4^+$ T lymphocytes specific for parasite antigens to a helper T cell type (Th1) cytokine profile. More important, the newly generated $CD8^+$ T

cells become crucial to control parasite replication, not only by serving as additional sources of IFN-γ but also by developing cytotoxic activity against infected cells, eliminating parasite factories and thus preventing reactivation of infection (Denkers *et al*. 1993; Denkers and Gazzinelli 1998; Bhopale 2003). Whether B cells also play a role in protection against this parasite is not clear, but studies have generated indirect evidences that IgG antibodies may be important for protection (Kang *et al*. 2000). B cell-deficient mice have shown increased susceptibility to brain inflammatory pathology in chronic infections with the parasite, despite presenting similar levels of serum and tissue pro-inflammatory cytokines, such as IFN-γ. Furthermore, adoptive transfer of polyclonal anti- *T. gondii* IgG antibodies to these mice prevented both pathology and mortality.

3. Major toxoplasma vaccines and candidates studied to date

To reproduce what the immune system does naturally to protect hosts against *T. gondii* infection (and re-infection), researchers have attempted several strategies for vaccination. These include the use of whole parasites (attenuated in different ways), soluble parasite antigens, recombinant purified proteins (subunit vaccines) or recombinant live vectors that express heterologous antigen(s) within host organisms (figure 1). Currently, some of these tools are also being used in combination, as part of prime-boost immunization protocols. Below is a review of current's state of the art of most of these technologies.

3.1 Whole-parasite attenuated vaccines

Sporulated oocysts (sporozoite-containing cysts) from the environment or tissue cysts (bradyzoite-containing cysts) from infected animals are the two major sources of infection with *T. gondii* (figure 2). However, vaccine candidates that include sporozoites or sporozoite antigens have traditionally been less studied because of the ease of access to bradyzoites and tachyzoites, e.g. using animal brain cysts or acutely infected animal peritoneal lavage/cell cultures, respectively. As a result, the first *T. gondii* whole-parasite experimental vaccines were mainly based on attenuated tachyzoites/bradyzoites, in particular those generated by inactivation or irradiation. Inactive parasites were used for immunization of experimental animals from 1956 (Cutchins and Warren 1956) to 1972 (Krahenbuhl *et al*. 1972) with not much success. In contrast, gamma-irradiated *T. gondii* tachyzoites were successfully tested as experimental vaccines in 1975 (Seah and Hucal 1975), in part after taking the idea from the pioneering irradiated-sporozoite malaria vaccines, which were initially tested in the 1960s and 70s (Nussenzweig *et al*. 1967; Gwadz *et al*. 1979). In the 1975 report, all animals inoculated with highly irradiated *T. gondii* parasites survived, were free of tissue cysts and were solidly protected against a subsequent rechallenge. Later, a few reports (Dubey *et al*. 1996; Omata *et al*. 1996; Dubey *et al*. 1998) have also used irradiated sporozoites (under the form of sporulated oocysts) to vaccinate mice, cats and pigs against toxoplasmosis, but in contrast to tachyzoites, results were not very encouraging, though some protection was also observed.

Other attempts to induce protection against toxoplasmosis with whole-parasite vaccines included the use of live attenuated parasites (tachyzoites) such as the S-48, the cps1-1, the temperature-sensitive TS-4, the MIC1-3 knock-out or the non-replicative Δrps13 strains (McLeod *et al*. 1988; Hakim *et al*. 1991; Buxton 1993; Gigley *et al*. 2009; Lu *et al*. 2009; Hutson

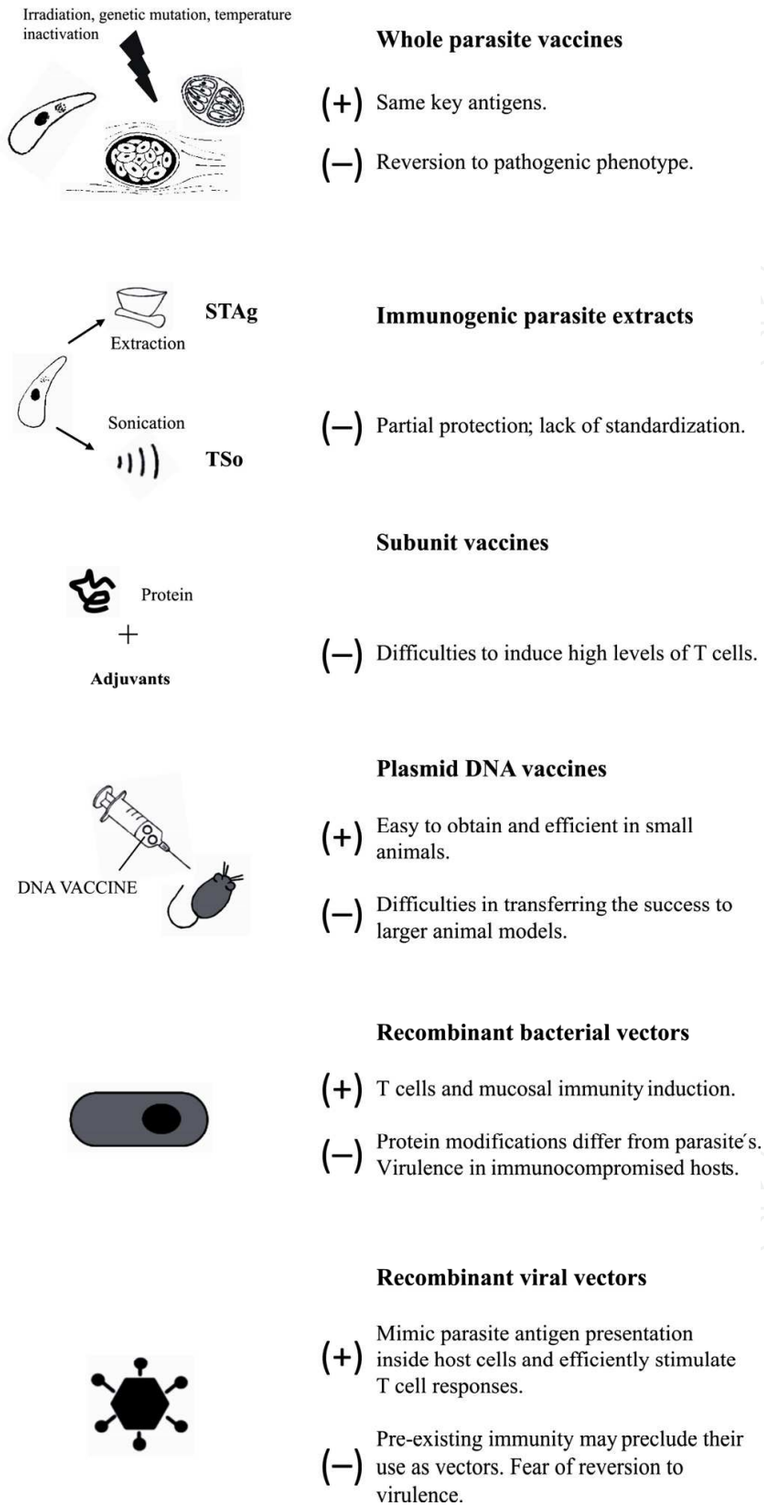

Fig. 1. Potential advantages (+) and concerns (-) of the major vaccination strategies used to immunize hosts against *T. gondii* infection. Abbreviations: STAg, Soluble Tachyzoite Antigen; TSo, Tachyzoites Sonicate.

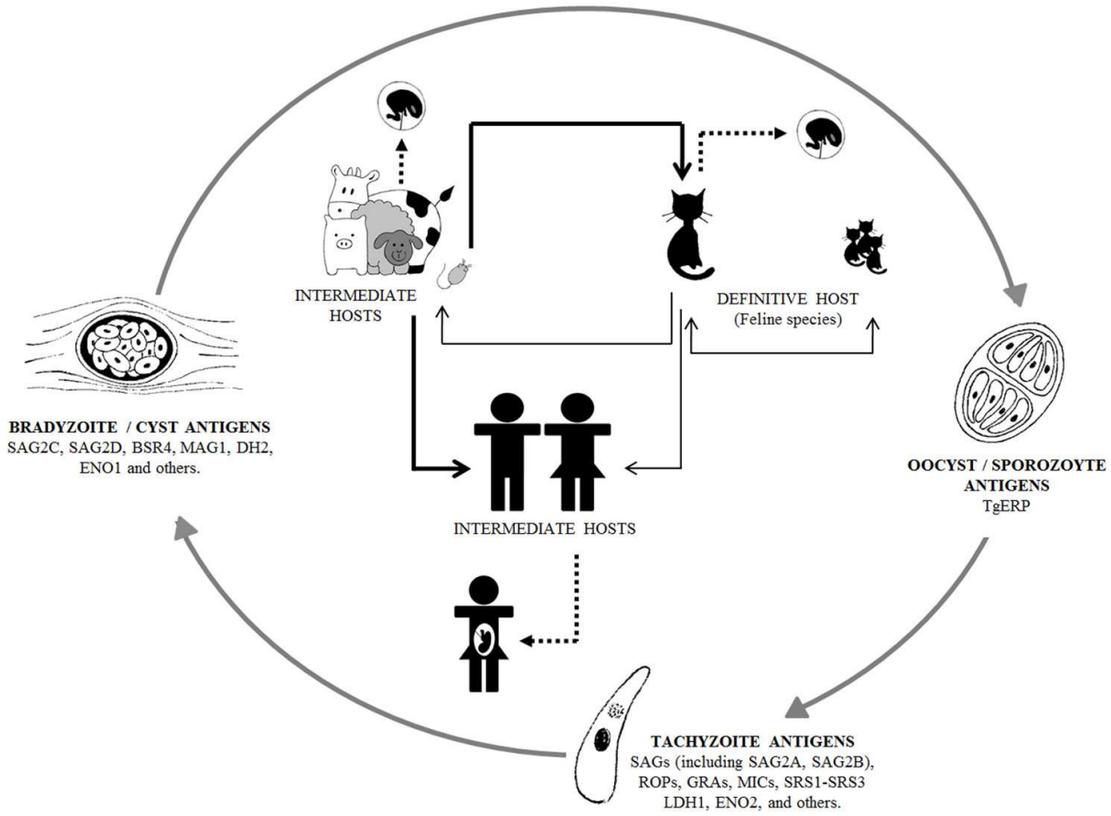

Fig. 2. Major *T. gondii* antigens identified to date in the different stages of the parasite´s life cycle and major routes of parasite transmission. Thin black arrow = horizontal transmission via oocysts; thick black arrow = horizontal transmission via tissue cysts; dotted arrows = vertical transmission via tachyzoites. Abbreviations: SAG, surface antigen; ROP, rhoptry protein; GRA, dense granules; MIC, microneme protein; SRS, SAG-related sequences; BSR, bradyzoite-specific recombinant; MAG, matrix antigen; LDH, lactate dehydrogenase; ENO, enolase; TgERP0, *T. gondii* embryogenesis-related protein.

et al. 2010; Mevelec *et al*. 2010). The only vaccine commercialized for veterinary purposes, Ovilis®Toxovax (Intervet/Schering-Plough Animal Health, UK), based on the incomplete parasite S-48 strain (not able to generate either tissue cysts or oocysts), began to be marketed in New Zealand and the United Kingdom in 1988 to control miscarriages provoked by *T. gondii* in sheep. Reduction in fetal loss and in formation of cysts in the meat used for consumption has been reported. This vaccine seems to reduce infection in sheep, which in free-range grazing are constantly exposed to oocyst contamination.

Interestingly, up to date, the most recent and technologically advanced recombinant vaccine formulations have reached, at best, the same levels of protective immunity induced by whole-parasite vaccines. Three main reasons may be responsible for that difference: (i) true protective antigens (or more plausibly antigen combinations) of the parasite have not yet been identified, (ii) while the whole organism and the recombinant vaccines contain the same antigenic sequences, the process by which the recombinant products are generated result in the loss of crucial features that are key for protein's immunogenicity (Crampton and Vanniasinkam 2007) or, finally, (iii) gamma-irradiated or otherwise attenuated parasites

maintain metabolic functions, retain the capacity to invade mammalian cells, present antigens to the host's immune system and elicit cellular immunity and cytokine responses in a highly similar way to natural infection (Hiramoto *et al*. 2002), and exogenous recombinant antigens do not.

However, even though protection has been repeatedly demonstrated after immunization with whole-parasite vaccines, real concerns also exist regarding the use of this type of vaccines, in particular for uses other than veterinary immunization. The major fear is that attenuated parasites could revert to the pathogenic phenotype. For this reason, studies towards developing a human vaccine have focused on parasite extracts or recombinant technologies that use defined immunodominant antigens and delivery strategies.

3.2 Immunogenic parasite extracts

Identification and functional characterization of proteins of the tachyzoite stage of *T. gondii* has been the focus of extensive research, because antigens within this stage are presented to the immune system effectively during natural infection, forcing the parasite to enter (in less than two weeks) into the latent bradyzoite stage seeking for protection. This strategy results in physical parasite shielding by encystation and different, and much lower, antigen availability for the immune system.

The soluble tachyzoite antigen extract (STAg) was the first protein blend identified as source of protective products, before wide-scale proteomic analyses were available (Denkers *et al*. 1993; Yap *et al*. 1998). Protection with STAg is only partial, even when the very efficient CpG oligodeoxynucleotides are used as adjuvants (Yin *et al*. 2007). Similar partial protection was also induced by the *T. gondii* sonicate of tachyzoites (TSo), even when mixed with cholera toxin (CT, a mucosal adjuvant) for oral administration (Bourguin *et al*. 1991; Bourguin *et al*. 1993).

One of the reasons why immunogenic parasite extracts render non-protective immunity may be the diversification of immune responses amongst all the different antigens (immunodominant or not) present in those extracts. Additionally, the extraction process (in the case of STAg) may have eliminated some of the innate immunity activators, namely TLR agonists, present in the whole parasite. Current proteomic analyses (high-throughput 2-dimensional electrophoresis combined with mass spectrometry) have identified nine novel vaccine candidates within STAg (Ma *et al*. 2009) and we should see some of these promising antigens being tested *in vivo* as recombinant subunit or vectorised vaccines in the near future.

3.3 Individual antigens identified and used as subunit vaccines

Three major particularities characterize the difficulties found in the development of recombinant vaccines against toxoplasmosis; these are: (i) *T. gondii* is an unicellular protozoan parasite formed by thousands of different proteins, glycoproteins, lipoproteins, and other types of molecules that can become feasible candidate antigens for a vaccine, (ii) the parasite evolves into several different stages during its life cycle, with some of those stages (cysts) particularly protected against the action of the immune system and, in addition, the antigens of one stage may not be effective vaccines for subsequent stages, and (iii) numerous strains of the parasite coexist in nature, most of them with significant differences in antigenic sequences, pathogenicity and physiological behavior within the host. Thus, protection induced against one strain may not be either cross-reactive or sufficient to prevent infection by other strain(s).

Apprehension in using attenuated whole parasites for human (and in many countries also animal) vaccination has lead research´s efforts to the development of safer vaccines by means of the identification and subsequent experimental administration of individual antigens. In principle, for an efficient immunization against *T. gondii*, the best antigens to use in vaccines should be those that are excreted/secreted (ESA) from the parasite stages that invade the host, since those have been reported as the most relevant targets of immune responses during natural infection, which, in contrast to what is seen in other diseases, controls the parasite´s ability to spread and multiply.

The surface of the tachyzoite stage was the initial source of antigens tested as vaccines because of its accessibility. The abundant surface antigen 1 (SAG-1) was the initial and most widely studied tachyzoite antigen. Multiple other antigens (see figure 2) of the tachyzoite and bradyzoite stages of the parasite subsequently entered the vaccine development pipeline, and even sporozoite-specific antigens have recently begun to be identified in mice and humans (Hill *et al*. 2011). The use of all these antigens has been carefully reviewed by Jongert *et al*. (Jongert *et al*. 2009).

In brief, up-to-date a few bradyzoite antigens, such as the abundant BAG-1, BRAD-1, CST-1, SAG4A, SRS-9, BSR-4, or the bradyzoite/tachyzoite expressed protein MAG-1 and, innumerable tachyzoite antigens, including surface antigens (SAGs), dense granule (GRAs), rhoptries (ROPs) and microneme (MICs) antigens have been identified and used as vaccine candidates with relative success and mostly in small experimental animal models.

In humans, major T-cell antigens have yet to be identified (Boothroyd 2009) although recent tests using predicted CD8 epitopes derived from the most relevant mouse antigens could identify several reactive peptides presented by HLA-A02, A03 and B07 human major histocompatibility complex (MHC) molecules (Tan *et al*. 2010; Cong *et al*. 2011). Candidate proteins include 2 surface antigens (SAG1, and SUSA1, a surface marker specific to the slow-growing, bradyzoite form of *T. gondii*) and 7 secreted proteins (GRA2, GRA3, GRA6, GRA7, ROP2, ROP16, ROP18).

Recombinant proteins were the first and most obvious tools to induce immunity against *T. gondii* and antigens produced in bacteria or yeast cells were the first finely characterized molecules inoculated into experimental animals (Jongert *et al*. 2009). However, immunization with pure proteins did not show much success in terms of induction of protective immunity. Synthetic peptides encompassing antigenic epitopes were also tested as vaccine candidates (Duquesne *et al*. 1991), but with the same unfortunate fate. New combinations of antigens/adjuvants (specially the new generation of innate-stimulating adjuvants) aiming at inducing more adequate and stronger Th1/CTL responses are the paths to follow in order to improve the results obtained to date with these types of experimental vaccines.

3.4 Genetic vaccines: DNA molecules and live vectors

Proteins are excellent inducers of antibodies, but have some difficulties to induce high levels of T lymphocytes. Genetic vaccines, on the contrary, are highly efficient to induce antigen-specific T lymphocytes. This group of vaccines represents a number of novel technologies that involve direct delivery of genes encoding antigens of interest to host cells, which then serve as antigen factories and immune-related processing plants for the resulting products. The intracellular presence of these products facilitates further induction of antigen-specific cellular immune responses by means of the easier antigen presentation in association with MHC class I molecules, which efficiently primes $CD8^+$ T lymphocyte responses. These novel

vaccine technologies have therefore being used in clinical trials against a variety of pathogens for which this cellular immune profile renders protection (Barouch 2006).

Immunization with DNA vaccines (almost exclusively bacterial plasmids because of the easy construction and multiplication, see Table 1) has traditionally been the first choice for experimental genetic vaccination against toxoplasmosis (Jongert *et al*. 2009). However, despite their successful application in many preclinical disease models, one of the most significant hurdles of DNA vaccine development has been transferring the success of inducing protective immunity in small animal models to larger animal models. The low potency of DNA vaccines in primates has so far precluded the development of most human health programs beyond Phase I clinical trials (Ulmer *et al*. 2006; Abdulhaqq and Weiner 2008). The reasons for the failure of DNA vaccines to induce potent immune responses in humans have not yet been elucidated. However, it is reasonable to assume that the low levels of overall antigen production, the inefficient cellular delivery of DNA plasmids and the insufficient stimulation of the innate immune system may be the major causes responsible for the lack of efficiency (Ulmer *et al*. 2006).

Vaccine candidate	Mouse strain	*T. gondii* chalange strain	Outcome of vaccination	References
pGRA4 + liposome, im	C57BL/6	ME49 tissue cysts, oral	Partial protection	Chen *et al*., 2009
	BALB/c	RH tachyzoites, ip	No protection	Chen *et al*., 2009
pSAG1 + pIL-18, im	BALB/c	RH tachyzoites, ip	Protection	Liu *et al*., 2010
pSAG1, im	BALB/c	RH tachyzoites, ip	Partial protection	Liu *et al*., 2010
pSAG1, im	BALB/c	RH tachyzoites, ip	No protection	Hoseinian Khosroshahi *et al*., 2011
pROP2, im	BALB/c	RH tachyzoites, ip	No protection	Hoseinian Khosroshahi *et al*., 2011
pSAG1 + pROP2, im	BALB/c	RH tachyzoites, ip	Partial protection	Hoseinian Khosroshahi *et al*., 2011
pMIC3, im	BALB/c	RH tachyzoites, ip	Partial protection	Fang *et al*. 2009
pSCA/MIC3, im	BALB/c	RH tachyzoites, ip	Partial protection	Fang *et al*. 2009
pMIC3 + pGM-CSF, im	CBA/J	76K tissue cysts, oral	Protection	Ismael *et al*. 2003; Ismael *et al*., 2009
pMIC3, sc	Kunming	RH tachyzoites, ip	Protection	Xiang *et al*. 2009
pT-ME (multi-epitope DNA), im	BALB/c	RH tachyzoites, ip	Protection	Liu *et al*., 2009
	C57BL/6	RH tachyzoites, ip	Protection	Liu *et al*., 2009
pEC2 (multi-epitope DNA), im	BALB/c	Prugniaud tissue cysts, oral	Partial protection	Rosenberg *et al*., 2009
	BALB/c	Trousseau tissue cysts, oral	No protection	Rosenberg *et al*., 2009
pEC3 (multi-epitope DNA), im	BALB/c	Prugniaud tissue cysts, oral	Partial protection	Rosenberg *et al*., 2009
	BALB/c	Trousseau tissue cysts, oral	No protection	Rosenberg *et al*., 2009
pSAG1-MIC4, in	BALB/c	RH tachyzoites, ip	Partial protection	Wang *et al*., 2009
pSAG1-MIC4 + pCTA2/B, in	BALB/c	RH tachyzoites, ip	Protection	Wang *et al*., 2009

Abbreviations: p, plasmid; im, intramuscular; ip, intraperitoneal; sc, subcutaneous; in, intranasal; pSCA, suicidal vector based on the SFV replicon; T-ME, $SAG1_{238-256}$, $SAG1_{281-320}$, $GRA1_{170-193}$, $GRA4_{331-345}$, $GRA4_{229-245}$, $GRA2_{171-185}$; EC2, MIC2-MIC3-SAG1; EC3, GRA3-GRA7-M2AP; CTA2/B, Cholera toxin A2 and B subunits.

Table 1. Major DNA vaccine candidates recently developed and tested against *T. gondii* infection

In order to increase the immunogenicity of DNA vaccines in large animal models, various methods have been tested including: (i) improvements in the design of the plasmid, e.g. by adding antigen-ubiquitination signals for improved immunoproteasome degradation and antigen presentation (Ishii *et al*. 2006), (ii) delivering multiple antigens at the same time (Beghetto *et al*. 2005; Mevelec *et al*. 2005; Jongert *et al*. 2007; Xue *et al*. 2008; Qu *et al*. 2009; Wang *et al*. 2009; Hoseinian Khosroshahi *et al*. 2011), (iii) using chemical adjuvants or immunomodulatory molecules formulated into microparticles or liposomes (van Drunen Littel-van den Hurk *et al*. 2004; Greenland and Letvin 2007), and (iv) using plasmid molecules as part of prime-boost immunization regimes (Doria-Rose and Haigwood 2003; Dunachie and Hill 2003; Dondji *et al*. 2005; Shang *et al*. 2009). However, at present, the low immunogenicity of DNA vaccines has forced researchers to find alternative immunization vectors, and recombinant bacterial or viral vectors, which carry and express DNA sequences into the host organisms more efficiently, have gradually substituted bacterial plasmids for experimental vaccination studies.

The use of bacteria as vehicles for genetic vaccination is an attractive and simple idea that derives from a number of intrinsic properties of the system. Live bacteria that contain recombinant plasmids encoding heterologous antigens of other pathogens have the potential of being oral delivery vectors for DNA vaccines in animal industry (Grillot-Courvalin *et al*. 1999; Grillot-Courvalin *et al*. 2002). In Table 2. we show two studies conducted by Qu *et al*. in which ICR mice were orally immunized with DNA vaccines encoding SAG1 and/or MIC3 antigens delivered by an attenuated *S. typhimurium* strain (Dam^- and $PhoP^-$) at different doses, and challenged with 500 tachyzoites of *T. gondii* RH strain. Those studies show that oral administration of the attenuated bacteria could induce humoral and cellular immune responses, although they just elicited partial protection of animals (a maximum of 20% improvement in survival rate). Thus, new vectors and constructs have to be tested to consider this methodology as an applicable option.

Vaccine candidate	Mouse strain	*T. gondii* chalange strain	Outcome of vaccination	References
rPRV/SAG1, im	BALB/c	RH tachyzoites, ip	Partial protection	Liu *et al*., 2008
pSAG1 (prime) + rPRV/SAG1 (boost), im	BALB/c	RH tachyzoites, ip	Protection	Shang *et al*., 2009
BV-G/SAG1, im	BALB/c	RH tachyzoites, ip	Protection	Fang *et al*., 2010
rFLU/SAG2 (prime), in + rAd/SAG2 (boost), sc	BALB/c	P-Br tissue cysts, oral	Protection	Machado *et al*., 2010
S. typhimurium/pSAG1, oral	ICR	RH tachyzoites, ip	Partial protection	Qu *et al*., 2008
S. typhimurium/pSAG1-MIC3, oral	ICR	RH tachyzoites, ip	Partial protection	Qu *et al*., 2009

Abbreviations: p, plasmid; im, intramuscular; ip, intraperitoneal; sc, subcutaneous; in, intranasal; rPRV: recombinant pseudorabies virus; BV-G: recombinant baculovirus; rFLU: recombinant influenza A; rAd: recombinant adenovirus.

Table 2. Selected examples of live attenuated vectors expressing *Toxoplasma gondii* antigens currently in tests.

Viral vectors exhibit many advantages for the development of a vaccine against toxoplasmosis. In summary, viral vectors express foreign antigens directly inside host cells very efficiently; as a result they present antigen fragments in association with MHC molecules more proficiently and, subsequently, they better stimulate the required anti-toxoplasma T cell responses (Th1 and CTL) because they act as natural adjuvants and stimulate intracellular innate immunity receptors. In addition, they can be administrated through the natural route of infection, such as via nasal mucosa, and they are able to induce effective and long lasting immune responses.

Our group has tested adenoviruses and influenza viruses as feasible vaccine vectors against toxoplasmosis (Caetano *et al*. 2006; Machado *et al*. 2010; Mendes *et al*. 2011) and they have shown significant improvement in comparison with naked plasmid vaccines. For those studies we have focused on possible formulations and immunization protocols using *T. gondii* surface antigens (SAGs). These molecules are involved in host cell attachment and invasion, and their sequences are conserved among different strains of *T. gondii*, sharing a high degree of homology even between type I (pathogenic and lethal in mice) and type II/III strains (cystogenic). However, these favorable traits do not ensure that these antigens will end up displaying sufficient protective capacities, but the proofs-of-principle obtained with their use will surely be maintained for any other antigens that display better protective properties.

In Caetano *et al*. (Caetano *et al*. 2006), we generated three recombinant adenoviruses encoding genetically modified SAG1, SAG2 and SAG3, without the 3'-end GPI anchoring motifs to ensure secretion and subsequent induction of combined Th/CTL immune responses. BALB/c mice received rAd/SAG1, rAd/SAG2, rAd/SAG3, or a combination of the three viruses (rAdMIX) and were challenged with 100 live tachyzoites of the *T. gondii* RH strain or with 20 cysts of the P-Br strain. Adenovirus immunization elicited potent antibody responses against each protein and displayed a significant bias toward a Th1 profile. When comparing the three recombinant viruses, rAd/SAG2 was the most efficient in eliciting antigen-specific antibodies. A significant reduction in cysts loads in the brain was observed in animals challenged with the P-Br strain. Vaccination with a mixture of all viruses promoted the highest level of inhibition of cyst formation, about 80%. However, no protection was observed against tachyzoites of the highly virulent RH strain (Caetano *et al*. 2006).

In the study by Machado *et al*. (2010), we generated a recombinant Influenza A vector encoding SAG2 of *T. gondii* and explored an original heterologous prime-boost immunization protocol using influenza virus (rFLU/SAG2) and a recombinant adenovirus (rAd/SAG2). Influenza A viruses are promising but currently under-explored vectors, which display some advantageous features to be used as live recombinant vaccines, such as the ability to infect and activate antigen presenting cells as well as to present high immunogenicity at mucosal and systemic levels (Rocha *et al*. 2004; Machado *et al*. 2010). BALB/c mice primed with an intranasal rFLU/SAG2 dose and boosted with a subcutaneous rAd/SAG2 dose elicited both humoral and cellular immune responses specific for SAG2. Moreover, when immunized animals were challenged with the cystogenic P-Br strain of *T. gondii*, they displayed up to 85% of reduction in parasite burden. These results demonstrate the potential use of recombinant influenza and adenoviruses in vaccination protocols to protect against oral challenge with *T. gondii* (Machado *et al*. 2010), although there is room for improvement.

Literature shows that, for other diseases, there is a good reproducibility of results when transferring experimental results obtained by immunization with some viral vectors (in particular adenoviruses) from small experimental animals to larger animal models or

humans. We expect this to be also true for the experimental vaccines generated against toxoplasmosis, so that we can see some encouraging results in the near future.

4. Conclusion

Recombinant subunit vaccines (proteins in adjuvants, DNA vaccines and recombinant live vectors) are the present trends for the development of a vaccine against Toxoplasmosis. A myriad of parasite antigens have been described and researchers are testing them in many animal models of the disease. It is our belief that, more than the description of new parasite antigens that could be used in a final vaccine formulation, one of the major issues for the next future is to develop and test highly antigenic formulations using currently known antigens. Developing this type of formulations requires a deep knowledge of the immune system's antigen processing and presentation pathways, proficiency in the use of molecular biology techniques to adapt the parasite antigen sequences to enter those pathways, and using the new generation of adjuvants and delivery vectors in a manner that can best stimulate the pretended anti-parasite Th1 cellular (and probably humoral) immune responses. The options and combinations are so broad, and yet untested, that several years of research will be needed before we can decide which combination will be more adequate [antigen(s) + adjuvant(s) + vector(s)] or what will be the most efficient immunization protocol (single dose, multiple dose, homologous or heterologous prime-boost, etc.).

Finally, we would like to call attention to the fact that a possible result of the immunization/protection tests may actually be the achievement of a cost-effective vaccine that may be suitable for large-scale production and use. Then, one key question will arise for the future, regarding the correct use of that vaccine. In principle, the vaccine should be applied to animals, because preventing oocyst shedding by cats and tissue cyst formation in meat-producing animals should have great impact on both environmental contamination and public health. But this intervention could pose a risk because of the loss of herd immunity against the parasite and the resurgence of a different profile of *Toxoplasma*-related pathologies because of the primo-infection of non-vaccinated adults traveling to other countries or regions instead of kids or young adults being infected at its home places. To solve this, a possibility would be the universal vaccination of all children against toxoplasmosis, although this might end up being not feasible in practice or even might not be considered as a priority. Researchers should include these topics amongst those to be discussed in the forthcoming years within the field of vaccine development against toxoplasmosis.

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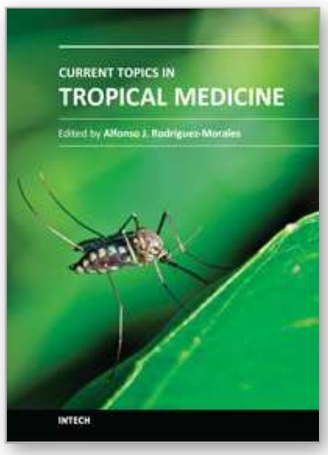

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