Vaccines against *Toxoplasma gondii*: challenges and opportunities

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Development of vaccines against Toxoplasma gondii *infection in humans is of high priority, given the high burden of disease in some areas of the world like South America, and the lack of effective drugs with few adverse effects. Rodent models have been used in research on vaccines against* T. gondii *over the past decades. However, regardless of the vaccine construct, the vaccines have not been able to induce protective immunity when the organism is challenged with* T. gondii*, either directly or via a vector. Only a few live, attenuated* T. gondii *strains used for immunization have been able to confer protective immunity, which is measured by a lack of tissue cysts after challenge. Furthermore, challenge with low virulence strains, especially strains with genotype II, will probably be insufficient to provide protection against the more virulent* T. gondii *strains, such as those with genotypes I or II, or those genotypes from South America not belonging to genotype I, II or III. Future studies should use animal models besides rodents, and challenges should be performed with at least one genotype II* T. gondii *and one of the more virulent genotypes. Endpoints like maternal-foetal transmission and prevention of eye disease are important in addition to the traditional endpoint of survival or reduction in numbers of brain cysts after challenge.*

Key words: *Toxoplasma gondii* - vaccine models - vaccines - virulence - genotype

Toxoplasma gondii is a protozoan apicomplexan parasite with a worldwide distribution. If contracted during pregnancy, *T. gondii* infection can induce abortion or considerable morbidity of foetuses, I the form of mental and physical disabilities, and retinal inflammation. The disease is normally self-limiting in immunocompetent individuals, but inflammation of the retinae can occur. Disseminated disease, which is generally fatal if not treated, is observed in immunocompromised patients. *T. gondii* is capable of infecting most mammals and birds. Transmission to humans is either through consumption of food contaminated with tissue cysts and meat products from infected animals or by ingestion of oocysts released in the faeces of infected cats (Kijlstra & Jongert 2008). The benefits of prophylactic immunizations could therefore be threefold: (i) prevention of infection or at least of clinical disease in humans; (ii) prevention of infection in animals raised for human consumption, thereby preventing transmission; (iii) immunization of domestic cats to disrupt the zoonotic cycle and prevent contamination of the environment by oocysts. In principle, an effective recombinant vaccine against both sexual and asexual stages of the parasite should be able to address all three targets, but this is hampered by stage specific expression of *T. gondii* proteins.

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Requirements for human and veterinary vaccines are different. For instance, the use of live, attenuated vaccines in primates and humans is difficult to envisage due to side effects and risks for breakthrough infection (Escadillo & Frenkel 1991). Vaccination strategies for veterinary vaccines against *T. gondii* have recently been reviewed (Innes & Vermeulen 2006).

Adult acquired infection in immunocompetent humans probably results in lifelong infection and lifelong protection. However, congenital infections tend to relapse and can result in progressive damage, most commonly to the retina. Congenital infection is believed to only occur in immunocompetent mothers, if infected for the first time during pregnancy. However, there is now accumulating evidence that a small number of women transmit the parasite to their foetus when chronically infected (Awan 1978, Vogel et al. 1996, Silveira et al. 2003, Kodjikian et al. 2004).

In mice, chronic infection with the cyst-forming Beverley strain provided clinical immunity to superinfection with the lethal *T. gondii* strain RH. Fully virulent RH parasites could be recovered from mouse brain more than one year after challenge (Reikvam & Lorentzen-Styr 1976). We lack the tools to determine whether superinfection in humans may override the immunity induced by primary infection. It cannot be excluded that better tools may show that clinical immunity after the primary infection is not complete and that a subsequent challenge with a sufficiently high dose or different genotype may induce superinfection. Hypothetically, a mother may be susceptible to superinfection, while providing sterile immunity to her unborn child. It therefore seems that natural infection with *T. gondii* results in life long protection against clinical disease from new infection and strong

protection against congenital infection. However, natural infection might not result in sterile immunity of the healthy individual when recurrent eye disease is a problem. Therefore, despite clinical immunity against new infection, the individual would not be protected from "flare ups" due to tissue cysts, which can remain for decades after the primary infection. The apparent strong clinical immunity after a single infection suggests that a vaccine should be able to confer protection against clinical symptoms and maternal-foetal transmission.

There is no standard protocol for the evaluation of vaccines against *T. gondii* and in animal models the *T. gondii* isolate and dose used for challenge varies between studies, making comparison difficult. This review looks at studies in animal models with survival and cyst number as primary endpoints, and discusses the use of animal models with the aim of developing vaccines for humans.

Inactivated parasites, crude or purified antigens and recombinant antigens

An early study of guinea pigs immunized with inactivated whole *T. gondii* tachyzoites found some protection against challenge; animals infected with live *T. gondii* were protected against subsequent challenge (Cutchims & Warren 1956). A study using formalin fixed whole *T. gondii* tachyzoites found a significantly increased survival of vaccinated mice after challenge (Krahenbuhl et al. 1972). Later studies showed that immunization with live, non-attenuated *T. gondii* resulted in complete protection against heterologous infection (Pettersen 1988). Ten trials in animal models using inactivated *T. gondii*, crude or purified antigen extracts performed between 1972-2005 have recently been reviewed (Schaap et al. 2007).

The immunodominant stage-specific surface antigen 1 (SAG1) expressed on the surface of tachyzoites has been extensively investigated. SAG1 affinity purified from the *T. gondii* RH strain, combined with adjuvants, provided high survival rates and significant reductions in brain cyst loads in mice (Bülow & Boothroyd 1991, Khan et al. 1991, Debard et al. 1996, Velge-Roussel & Boothroyd 1997, 2000). It is worth noting that the highest protection achieved with this native antigen was obtained with intranasally delivered SAG1; 90% survival and no brain cysts in survivors when administered with the adjuvant QuilA (Khan et al. 1991) and 85% reduction in brain cyst load when adjuvanted with Cholera Toxin (Debard et al. 1996). Protection efficacy was due to the CD8+ T cell fraction (Khan et al. 1991).

Recently, MIC1 and MIC4 proteins purified from parasite extracts were shown to reduce the number of brain cysts and to produce an 80% survival rate and a 68% reduction in brain cyst load in C57BL/6 mice, after challenge with the *T. gondii* ME49 isolate (Lourenço et al. 2006). Interestingly, the most promising protection with purified parasite components was not obtained in mice but in cats, where an intranasally administered rhoptry extract, formulated in QuilA, could provide sterile immunity to oocyst shedding (Garcia et al. 2005).

With the advent of recombinant protein technology, *T. gondii* antigens produced by bacteria were evaluated in vaccines. Vaccines based on recombinant SAG1 could protect against acute toxoplasmosis (Petersen et al. 1998, Yang et al. 2004, Siachoque et al. 2006) and brain cyst formation (Letscher-Bru et al. 1998, Bonenfant et al. 2001). A study addressing protection against congenital toxoplasmosis found that recombinant SAG1 protein protected BALB/c mice against maternal-foetal transmission of *T. gondii,* but increased the number of infected foetuses by 50% in CBA/J mice, underlining the importance of different outcomes in different inbred mice models (Letscher-Bru et al. 2003). Recombinant GRA4 and ROP2 adjuvanted with Alum provided protection against brain cysts in C57BL/6 mice, while only the latter antigen provided protection in C3H mice (Martin et al. 2004). Differences in outcome depending on genetic background were also reported for a ROP2-HSP83 (*Leishmania infantum*) fusion protein where more than 80% reduction in brain cysts was observed in C3H/HeN mice, but not in C57BL/6 mice (Echeverria et al. 2006). On the other hand, a study using recombinant GRA2 and GRA6, adjuvanted with monophosphoryl A (MPL) found a significant reduction in the number of brain cysts in GRA2, but not GRA6, immunized CBA/J mice challenged with the *T. gondii* Prugniaud isolate. Combination of both antigens did not lead to enhanced protection (Golkar et al. 2007).

Recently, two studies using adjuvanted mixtures of recombinant proteins achieved a high level of protection against chronic toxoplasmosis. A mixture of SAG1, GRA1 and MAG1, adjuvanted with Freund's Complete Adjuvant, reduced brain cyst burden by 89% (Gatkowska et al. 2008) in BALB/c mice. Additionally, a mixture of GERBU adjuvanted GRA7 and a MIC2-MIC3-SAG1 chimeric protein provided a 79% reduction in brain cysts in outbred SWISS mice following challenge with *T. gondii* 76K (Jongert et al. 2008).

These studies primarily used survival and protection against brain cyst formation as the endpoint and found a significant degree of protection. Complete protection against acute disease and/or chronic toxoplasmosis in mice has only been obtained using strong adjuvants such as Freund's Complete Adjuvant and QuilA (Khan et al. 1991, Brinkmann et al. 1993, Mishima et al. 2001a). Table I shows studies using inactivated parasites or purified and recombinant antigens for antigen delivery.

Live, attenuated *T. gondii* vaccines

Immunization with live, attenuated *T. gondii* parasites conferred better protection compared to inactivated tachyzoites independent of the adjuvant used, with the exception of challenge with one mutant strain TS-1 (Waldeland & Frenkel 1983).

In 1941, a *Toxoplasma* strain was isolated from the brain of a patient with a fatal case of congenital toxoplasmosis (Sabin 1941). This strain, named RH, is defective in tissue cyst formation and was found to be extremely virulent in mice and other laboratory animals. The RH strain is the archetype of all *Toxoplasma* strains and has a Type I genome. Almost 30 years later, chemical mutagenesis of the RH strain resulted in the temperature sen-

TABLE I

Immunization with protein vaccines against *Toxoplasma gondii*

Antigen	Antigen delivery	Protection endpoint		Mouse strain	*T. gondii* challenge strain	Study
		Survival/control (%)	Cyst reduction (%)			
SAG1, ap	Protein + CFA, ip/sc	10-21/55-42	NA	BALB/c and CD1	CS, ip	Kasper et al. 1985
SAG1, ap	Protein + liposomes	93/27	NA	Swiss-webster	C, ip	Bülow et al. 1991
SAG1, ap	Protein + QuilA, intranasal	90/0	100 in CD1 survivors	CD1	ME-49 (II)	Khan et al. 1991
		33/0		C57BL/6	tissue cysts, oral	Khan et al. 1991
		67/0		A/J	tissue cysts, oral	Khan et al. 1991
$SAG1_{48-67}$	Peptide + IFA	0/0	NA	Swiss OF1	76K (II) tissue cysts, oral	Darcy et al. 1992
F3G3, ap	Protein + IFA, ip	100/0	NA	Swiss-Webster	C56 (III) tachyzoites, ip	Brinkmann et al. 1993
SAG1, ap	Protein + CT, intranasal	NA	80-85	CBA/J	76K (II) tissue cysts, oral	Debard et al. 1996
$SAG1_{125-165}$	Peptide + FCA, sc	NA	35	CBA/J	76K (II) tissue cysts, oral	Velge-Roussel et al. 1997
SAG1, rec	Protein + IL-12, sc	NA	40	CBA/J	PRU (II) tissue cysts, oral	Letscher-Bru et al. 1998
GRA4, rec	Protein + CT, oral	0-17/0	66	C57BL/6	76K (II) tissue cysts, oral	Mévélec et al. 1998
SAG1, rec	Protein + Alum	44/20	NA	NMRI	RH (I) tachyzoites, ip	Petersen et al. 1998
SAG1, ap	Protein + CT, intranasal	NA	50-60	CBA/J	76K (II) tissue cysts, oral	Velge-Roussel et al. 2000
SAG1, rec	Protein + LT, intranasal	NA	75-78	CBA/J	76K (II) tissue cysts, oral	Bonenfant et al. 2001
SAG1	Protein + FCA/IFA, ip	0/0	NA	BALB/c	Beverley (II), ip	Mishima et al. 2001a
SAG2		17/0	100	BALB/c	Beverley (II), ip	Mishima et al. 2001a
SAG3		0/0	NA	BALB/c	Beverley (II), ip	Mishima et al. 2001a
SRS1		25/0	100	BALB/c	Beverley (II), ip	Mishima et al. 2001a
P54		8/0	100	BALB/c	Beverley (II), ip	Mishima et al. 2001a
SAG1 + SAG2 + SAG3 + SRS1 + P54		18/0	100	BALB/c	Beverley (II), ip	Mishima et al. 2001a
SAG1, rec	Protein, sc	NA	Congential 33/72	BALB/c	ME49 (II) tissue cysts, oral	Letscher-Bru et al. 2003
			Congenital 53/32	CBA/J	ME49 (II) tissue cysts, oral	Letscher-Bru et al. 2003
GRA4 + ROP2, rec	Protein + Alum	NA	± 55	C57BL/6	ME49 (II) tissue cysts, oral	Martin et al. 2004
		NA	± 37	C3H/HeN	ME49 (II) tissue cysts, oral	Martin et al. 2004
SAG1-SAG2, rec	Protein, ip	73/0	NA	BALB/c	RH (I), tachyzoites, ip	Yang et al. 2004
ROP2-HSP83 (*Leishmania infantum*)	Protein, fp	0/0	NA ± 33	BALB/c	ME49 (II) tissue cysts, oral	Echeverria et al. 2006
		0/0	± 85	C57BL/6	ME49 (II) tissue cysts, oral	Echeverria et al. 2006
		NA		C3H/HeN	ME49 (II) tissue cysts, oral	Echeverria et al. 2006
SAG1	Peptides +	Yes	NA	C3H/HeN	RH, ip	Siachoque et al. 2006
MIC1, MIC4, ap	Protei, sc	80/0	68%	C57BL/6	ME49 (II) tissue cysts, oral	Lourenço et al. 2006

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Antigen	Antigen delivery	Protection endpoint		Mouse strain	*T. gondii* challenge strain	Study
		Survival/control (%)	Cyst reduction (%)			
GRA2, rec	Protein, MPL sc	NA	69.8%	CBA/J	PRU (II) tissue cysts, ip	Golkar et al. 2007
GRA6, rec		NA	± 25%	CBA/J	PRU (II) tissue cysts, ip	Golkar et al. 2007
GRA2 + GRA6, rec		NA	48.2	CBA/J	PRU (II) tissue cysts, ip	Golkar et al. 2007
SAG1 + GRA1 + MAG1, rec	Protein, FCA	NA	89%	BALB/c	DX (II) tissue	Gatkowska et al. 2008
GRA5 + GRA7 + ROP2, rec	Protein, CT intranasal	NA	0%	BALB/c	VEG (III) tissue cysts, oral	Igarashi et al. 2008
TgPI-1, rec	Protein	90/50	58%	C3H/HeN	ME-49 (II) tissue cysts, oral	Cuppari et al. 2008
EC2(MIC2-MIC3-SAG1), rec + GRA7, rec	Protein + GERBU, im	NA	60-79%	Swiss OF1	76K (II) tissue cysts, oral	Jongert et al. 2008b

ap: affinity purified; chr: chromatography; CT: cholera toxin/toxoid; FCA: Freund's Complete Adjuvant; fp: foot pad; gg: gene gun; IFA: Freund's incomplete adjuvant; im: intramuscular; ip: intraperitoneal; iv: intravenous; NA: not addressed; rec: recombinant; sc: subcutaneous; +/++/+++ = enhanced survival.

sitive, *T. gondii* isolate TS-4 (Pfefferkorn & Pfefferkorn 1976). Vaccination with this strain provided a significant protection against tissue cyst formation and partial protection against congenital toxoplasmosis (McLeod et al. 1988), as well as increased survival during acute toxoplasmosis (Gazzinelli et al. 1991). This strain provides the basis for the immunological insight that protection against toxoplasmosis is mediated by CD4+ and CD8+ IFN-γ producing T-cells (Gazinelli et al. 1991). The RH strain was shown to be safe for use in a pig model and was undetectable in the tissues as soon as three weeks post vaccination (Lindsay et al. 1993). Although promising as a vaccine strain and shown to be safe for use in immunocompetent primates, the strain failed safety tests in pregnant *Aotus* monkeys (Escajadillo & Frenkel 1991).

In 1988 a tissue cyst defective strain, S48, was isolated from an infected and aborted ovine foetus. S48 became an incomplete, attenuated strain following extensive (x 3000) passages in the laboratory (Wilkins et al. 1988). Vaccination of sheep with S-48 afforded a 75% reduction of abortion, a reduced neonatal mortality and higher birth weight (Buxton et al. 1991). The S-48 strain has since been developed into a commercial vaccine used in sheep to prevent abortions. Whether vaccination with S-48 also prevents tissue cyst formation upon challenge with cyst forming strains remains undetermined.

In recent work, intramuscular vaccination with CpG-adjuvanted RH tachyzoites could protect 52% of challenged pigs and provide sterile immunity against tissue cyst formation (Kringel et al. 2004). In 2002, a *T. gondii* uracil auxotroph mutant was found to be completely avirulent in mice, including in IFN-gamma KO mice; this mutant strain could induce complete protection against lethal injection with *T. gondii* RH (Fox & Bzik 2002).

Recently, a *T. gondii* strain attenuated by the deletion of the *mic1* and *mic3* genes was used to immunize OF1 mice. After challenge with the 76K *T. gondii* isolate, greater than 96% reduction in the number of brain cysts, as well as reduced transmission to foetuses, was observed. In spite of these promising results, no sterile immunity was obtained (Ismael et al. 2006). A review of *T. gondii* knock out strains used for immunization shows that although most offer protection against lethal challenge and can reduce cysts burden, some result in increased mortality (Schaap et al. 2007).

Sterile immunity to tissue cysts induced by live, defective *T. gondii* strains has not been obtained in mice (Schaap et al. 2007), but has been achieved in pigs (Kringel et al. 2004). In addition, sterile immunity against oocyst shedding was obtained in 84% of cats vaccinated with an oocyst deficient strain, T263. However, this vaccine has never been commercialised (Frenkel et al. 1991).

Plasmid vaccines

DNA vaccines have the particular capacity to induce $CD4^+$ T-lymphocyte and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses against the antigen of interest. As protection against *T. gondii* has been associated with a Th1 response, this vaccination strategy has received considerable attention. The choice of the vaccine vector should be further investigated, since a large number

of plasmids and live vectors have been used for antigen delivery. However, a comparative analysis between different vectors has never been performed,and thus it is very difficult to determine which is most appropriate. A discussion of the different vectors used is beyond the scope of this review.

Antigen delivery using plasmids coding for *T. gondii* SAG1 protein, or a fragment thereof, initially showed 100% protection against lethal challenge (Nielsen et al. 1999, Angus et al. 2000, Couper et al. 2003). This was correlated with a CTL response against this antigen. SAG1 has been a prime antigen because of its immunodominance. Furthermore, although plasmid vaccines based on SAG1 can provide protection against lethal infection and reduce cyst numbers, they have not been able to induce a sterile immunity against infections with cyst forming *T. gondii* isolates (Angus et al. 2000). Other proteins evaluated in DNA vaccines mostly originate from dense granules, rhoptries or micronemes. DNA vaccination with GRA1, GRA7 or ROP2 provided partial protection against acute and chronic infection with *T. gondii* 76K and IPB-G in C3H/HeN mice (Vercammen et al. 2000). GRA1 partially protected against acute toxoplasmosis by generation of a cytolytic CD8 response against this antigen (Scorza et al. 2003). DNA vaccination with GRA4 was shown to be as efficient in protection against brain cyst formation as the GRA4 protein adjuvanted with Alum (Martin et al. 2004).

Of critical importance was the discovery that the combination of multiple DNA vaccines in a single formulation could significantly enhance cellular immune responses and protection, as compared to the single gene vaccines. Prolonged protection against acute toxoplasmosis upon infection with *T. gondii* RH in BALB/c mice was reported for a cocktail DNA vaccine combining SAG1 with ROP2 (Fachado et al. 2003a), but not for the single gene DNA vaccines. Similarly, enhanced protection was observed in mice after immunization with a plasmid based combination of GRA4 and SAG1. This vaccine reduced mortality and brain cyst numbers in C57BL/6 mice, but failed to induce sterile immunity or complete protection after challenge with the type II 76K *T. gondii* isolate (Mévélec et al. 2005). A study combining the two bradyzoite antigens BAG1 and MAG1 in a cocktail DNA vaccine also found a significant reduction in the number of brain cysts (62%), but no sterile immunity (Nielsen et al. 2006). A similar study, using a combination of antigens delivered as plasmids coding for regions of microneme proteins including MIC2, MIC3, MIC4, M2AP and AMA1, resulted in a significant reduction (84%) of the number of cysts, but not sterile immunity (Beghetto et al. 2005). Recently, a GRA7 DNA vaccine was found to be correlated with high-level protection against brain cyst formation and a cocktail DNA vaccine with a combination of GRA1 and GRA7 could reduce the *T. gondii* 76K brain cyst burden by 89% in C3H/HeN mice, but no sterile immunity was obtained (Jongert et al. 2007). Interestingly, the same GRA1-GRA7 cocktail DNA vaccine was able to provide sterile protection against tissue cyst formation in two of three vaccinated pigs (Jongert et al. 2008a).

Other studies have combined *T. gondii* antigens on a single DNA vaccine plasmid. Chimeric SAG1-ROP2 and multi-antigenic DNA vaccines containing SAG1, ROP2 and GRA2 showed significant protection levels against acute toxoplasmosis in BALB/c mice. Addition of IL-12 (but not cholera toxin) as a genetic adjuvant could enhance the protective Th1 response and protection (Zhang et al. 2007a, Cui et al. 2008, Xue et al. 2008a, b). Table II shows studies using plasmid vectors for antigen delivery.

Live, attenuated vectors

Since *T. gondii* is an intracellular parasite, strategies that mimic the intracellular niche of *T. gondii* have been evaluated using live or attenuated vectors. The first report on the use of a vector for the delivery of a *T. gondii* antigen used a recombinant *Mycobacterium bovis* BCG expressing GRA1. This strategy was able to elicit a GRA1-specific cellular immune response in subcutaneously primed sheep, but not in outbred mice (Supply et al. 1999). No significant protection was reported after oocyst challenge. The same strategy using ROP2 in a BCG vector was partially successful, since vaccinated animals had significantly increased survival after challenge with the *T. gondii* RH isolate, as compared with the controls (Wang et al. 2007).

Attenuated *Salmonella* strains have been evaluated for the delivery of plasmid DNA by oral immunisation. Delivery of a SAG1-SAG2 fusion protein linked to cholera toxin A_2/B via an attenuated *Salmonella typhimurium* strain afforded 40% survival in BALB/c mice after challenge with the *T. gondii* RH isolate, compared with 100% mortality in the SAG1-2 immunized group (Cong et al. 2005). Recently, 10% and 20% survival was obtained with an attenuated *S. typhimurium* strain delivering a SAG1 DNA vaccine in ICR mice, while all naïve mice succumbed to infection (Qu et al. 2008).

In addition to intracellular bacteria, recombinant viruses have also been evaluated as vectors for vaccination against toxoplasmosis. Immunisation with recombinant adenovirus expressing SAG1, SAG2 or SAG3 has been tested in BALB/c mice and could elicit the Th1 cellular immune responses against all three antigens. Vaccination of mice with a mixture of these three adenoviruses could reduce the brain cyst burden by 80% upon challenge with *T. gondii* P-Br, but could not protect against acute toxoplasmosis after challenge with *T. gondii* RH (Caetano et al. 2006). Prime-boost experiments using a GRA4 construct delivered by a recombinant plasmid and attenuated vaccinia virus construct (Zhang et al. 2007b) showed 100% survival in the heterologous prime boost group after challenge, followed by a 70% survival in the plasmid-plasmid immunized group. The heterologous prime boost group also showed the largest reduction in brain tachyzoite counts after challenge. However, the vaccine failed to induce sterile immunity against tissue cysts. A modified vaccinia ankara vector expressing ROP2 could increase the life span after lethal challenge with *T. gondii* RH (Roque-Resendiz et al. 2004). Recently, 60% survival was obtained after vaccination

with a SAG1 expressing Pseudorabies virus, in mice challenged with *T. gondii* RH (Liu et al. 2008). Table III shows studies using live, attenuated vectors.

Adjuvants

The word adjuvant is derived from the latin word adjutants, which means ancillary. Adjuvants have generally been defined as substances that enhance the immunogenicity of the antigenic vaccine components. Adjuvants have been categorised into three groups, (i) active immunostimulants, (ii) carrier proteins that provide T cell help or (iii) vehicle adjuvants such as emulsions and vesicles that serve as a matrix for antigens, as well as stimulate the immune response (Petrovsky & Aguilar 2004). However, as components of experimental vaccines have become increasingly refined, often to the point of single proteins or small peptides, a fundamental immunological role has emerged for vaccine adjuvants consistent with category (i). The identification of this role was only possible through our increased understanding of T cell activation and the development of a 3-signal model of activation (Curtsinger et al. 1999). CD4 T cells, through their T cell receptor (TCR), encounter processed antigenic peptides bound to MHC class II on the surface of antigen presenting cells, "signal 1''. For successful activation, this must be accompanied by co-stimulation through the ligation of additional molecules on the surface of the T cell and APC, "signal 2". A third signal has been proposed that determines the nature of the T cell activation, "signal 3". Thus a "signal 3", consisting of IL-12, will directs the development of Th1 cells, whereas IL-4 will direct Th2 development (Constant & Bottomly 1997). More recently, TGF-β has been recognised as a signal for Treg cell differentiation and a combination of TGF-β/IL-6 / IL-23 has been recognised for directing the differentiation of Th17 cells (Bommireddy & Doetschman 2007, Stockinger & Veldhoe 2007). During natural infections, these signals are normally elicited through components of pathogens. These components logically have been retained in attenuated vaccines and many crude vaccine formulations. The hope is that where these are absent, in defined protein or peptide vaccine formulations, they can be replaced or mimicked by an appropriate vaccine adjuvant that elicits the appropriate "signals 2 and 3". Many of the pathogen components that elicit "signals 2 and 3" are TLR ligands, while some interact through other receptors such as retinoic acid inducible gene-like receptors or NOD-like receptors (Ishii & Akira 2007).

CD8 T cells, unlike CD4 T cells, have been reported to be activated without "signal 2t", although "signal 2" lowers the threshold for activation through TCR ligation alone. However, further studies indicate that "signal 3", normally IL-12 or type-1 interferon, is necessary for the development of the CD8 T cells'ir cytolytic ability. Importantly, and potentially relevant to *T. gondii* infection, "signal 3" is not required for their CD8 T cell expansion or IFN-γ production (Curtsinger et al. 2005, Williams & Bevan 2007). In the context of initiation of protective immunity to *T. gondii,* naïve CD8 cells have been demonstrated to require IL-2 from CD4 cells for their development (Gazzinelli et al. 1991). Another issue that has been described in the literature as important for initiating CD8 T cells, is delivery of the antigen into the endogenous antigen-processing pathway, where it can become efficiently presented in the context of MHC class 1. However, the discovery of cross-presentation, where an exogenous antigen can cross from the exogenous pathway into the endogenous pathway in dendritic cells would arguably negate this previous concern (Vyas et al. 2008).

A very efficient vaccine was developed using the ubiquitin-proteasome system to immunize mice with a chimeric DNA encoding a fusion protein between murine ubiquitin and the *T gondii* antigen SAG1. This study found that the vaccine induced strong protection, as measured by survival after challenge with the RH strain (Ishii et al. 2006). The advantage of the ubiquitin system is that it acts as a chaperone, ensuring that more of the *T. gondii* antigen is degraded and presented and thus enhances antigen presentation.

As most vaccine studies were carried out before many of the above facts were known, the selection of adjuvant has not, and could never have been completely rational. Furthermore the relatively slow progresses in adjuvant research, including the only recent realisation that adjuvants play a role in initiating "signals 2" and a precluded their rational selection. For example, it has only recently been demonstrated that ALUM, the first adjuvant licensed for use in humans, stimulates the NOD-like receptor, pyrin domain containing 3 (Nlrp3)-inflammasome (De Gregorio et al. 2008). Thus most adjuvants were chosen from empirical evidence of their ability in other systems.

The efficacy of each adjuvant employed is difficult to assess due to the many other confounding factors that varied in each reported study. For the reasons discussed, the use of adjuvants has traditionally been linked to protein vaccines, be it recombinant proteins or a protein fraction purified from the parasites, rather than killed or attenuated parasites. However, adjuvants for DNA plasmid vaccines are now being studied.

A recent review categorises adjuvants into: mineral salt adjuvants (e.g., ALUM); tensoactive adjuvants (e.g., QuilA), bacteria derived adjuvants (e.g., LPS), adjuvant emulsions (e.g., FCA) liposome adjuvants (probably better referred to as vesicular adjuvants so as to include NISV); polymeric microsphere adjuvants; carbohydrate adjuvants and cytokine adjuvants (Petrovsky & Aguillar 2004). Each of these has been used in experimental vaccines for toxoplasmosis over the years with varying degrees of success (Khan et al. 1991, Debard et al. 1996, Petersen et al. 1998, Desholme et al. 2000, Martin et al. 2004, Cong et al. 2005, Garcia et al. 2005, 2007, Mévélec et al. 2005, Golkar et al. 2007, Zhang et al. 2007a, b, Cui et al. 2008, Jongert et al. 2008b, Xue et al. 2008a, b). A common theme emerges that adjuvants do increase the immune response to the antigen administered and usually, but not always, induce a degree of protection to a subsequent challenge. However, the inability of any antigen-adjuvant combination to afford complete sterile protection most likely indicates that antigen/epitope identification is insufficiently advanced.

Since many vaccine studies have explored the use of plasmid DNA for vaccination, other adjuvants systems

TABLE II

Immunization with plasmid DNA vaccines against *Toxoplasma gondii*

Antigen	Antigen delivery	Protection endpoint: Survival/control (%)	Protection endpoint: Cyst reduction (%)	Mouse strain	*T. gondii* challenge strain	Study
SAG1	Plasmid, im	100/30	NA	C3H/HeN	RH (I) tachyzoites, ip	Nielsen et al. 1999
		8/20	NA	BALB/c	RH (I) tachyzoites, ip	Nielsen et al. 1999
SAG1	Plasmid, im	90-100/0	70	C57BL/6	ME-49 (II) tissue cysts, oral	Angus et al. 2000
		0/0	NA	C57BL/6	RH (I) tachyzoites, ip	Angus et al. 2000
GRA4	Plasmid, im	62/0	NA	C57BL/6	76K (II) tissue cysts, oral	Desolme et al. 2000
GRA4 + GM-CSF	Plasmid, im	75/0	NA	C57BL/6	76K (II) tissue cysts, oral	Desolme et al. 2000
GRA4 + IL-12	Plasmid, im	25/0	NA	C57BL/6	76K (II) tissue cysts, oral	Desolme et al. 2000
GRA1	Plasmid, im	70/10	54	C3H/HeN	IPB-G (II) tissue cysts, oral	Vercammen et al. 2000
		No protection	NA	C57BL/6 and BALB/c	IPB-G (II) tissue cysts, oral	Vercammen et al. 2000
GRA7		90/10	63	C3H/HeN	IPB-G (II) tissue cysts, oral	Vercammen et al. 2000
		No protection	NA	C57BL/6 and BALB/c	IPB-G (II) tissue cysts, oral	Vercammen et al. 2000
ROP2		90/10	67	C3H/HeN	IPB-G (II) tissue cysts, oral	Vercammen et al. 2000
		No protection	NA	C57BL/6 and BALB/c	IPB-G (II) tissue cysts, oral	Vercammen et al. 2000
ROP2	Plasmid, im	0/0	NA	BALB/c	RH (I) tachyzoites, ip	Leyva et al. 2001
SAG1	Plasmid, im	67/25 Survival pups	100 infected pups:	BALB/c	Beverley (II) tissue cysts, oral	Couper et al. 2003
		77 (vacc)/71 (cntrl)	43 (vacc)/32 (cntrl)	BALB/c	Beverley (II) tissue cysts, oral	Couper et al. 2003
MIC3	Plasmid, im	NA	45-58	CBA/J	76K (II) tissue cysts, oral	Ismael et al. 2003
MIC3 + GM-CSF	Plasmids, im	NA	67-74	CBA/J	76K (II) tissue cysts, oral	Ismael et al. 2003
Genetic library	Plasmid, im	0/0	NA	BALB/c	RH (I) tachyzoites, ip	Fachado et al. 2003a
SAG1 + ROP2	Plasmids, im	0/0	NA	BALB/c	RH (I) tachyzoites, ip	Fachado et al. 2003b
GRA1	Plasmid, im	75-100/0-25	NA	C3H/HeN	IPB-G (II) tissue cysts, ip	Scorza et al. 2003
GRA4	Plasmid, im	NA	± 50	C3H/HeN	ME49 (II) tissue cysts, oral	Martin et al. 2004
		NA	± 50	C57BL/6	ME49 (II) tissue cysts, oral	Mohamed et al. 2003
HSP-70	Plasmid, gg	NA	Yes	C57BL/6	ME49 (II) tissue cysts, oral	Mohamed et al. 2003
HSP30		NA	Yes	BALB/C	ME49 (II) tissue cysts, oral	Mohamed et al. 2003
SAG1		NA	Yes	BALB/C	ME49 (II) tissue cysts, oral	Mohamed et al. 2003
MIC2 + MIC3 + MIC4 + M2AP + AMA1	Plasmid, id	NA	84	BALB/c	SSI119 tissue cysts, oral	Beghetto et al. 2005
GRA4	Plasmid, im	62/0	NA	C57BL/6	76K (II) tissue cysts, oral	Mévélec et al. 2005
SAG1		62/0	NA	Swiss OF1	76K (II) tissue cysts, oral	Mévélec et al. 2005
SAG1 + GRA4		75/0	NA	Swiss OF1	76K (II) tissue cysts, oral	Mévélec et al. 2005

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Antigen	Antigen delivery	Protection endpoint: Survival/control (%)	Protection endpoint: Cyst reduction (%)	Mouse strain	*T. gondii* challenge strain	Study
SAG1 + GRA4 + GM-CSF	Plasmids, im	87/0	NA	Swiss OF1	76K (II) tissue cysts, oral	Mévélec et al. 2005
			± 66	Swiss OF1	76K (II) tissue cysts, oral	Mévélec et al. 2005
SAG1 + GRA4 + GM-CSF		NA	Less infected/ dead pups/litter	Swiss OF1	76K (II) tissue cysts, oral	Mévélec et al. 2005
MAG1 + BAG1	Plasmids, im	NA	62	C3H/HeN	SSI119 tissue cysts, oral	Nielsen et al. 2006
MIC2	Plasmid, id	37.5/0; 40/0,	NA	C57BL/6 and BALB/c	Beverly (II) tissue cysts, oral	Dautu et al. 2007
M2AP		0/0	NA	C57BL/6 and BALB/c	Beverly (II) tissue cysts, oral	Dautu et al. 2007
AMA1		37.5/0; 60/0	NA	C57BL/6 and BALB/c	Beverly (II) tissue cysts, oral	Dautu et al. 2007
BAG1		12.5/0	NA	C57BL/6 and BALB/c	Beverly (II) tissue cysts, oral	Dautu et al. 2007
GRA1 + GRA7 + ROP2	Plasmids, im	100/44	81-84	C3H/HeN	76K (II) tissue cysts, oral	Jongert et al. 2007
GRA1 + GRA7			89	C3H/HeN	76K (II) tissue cysts, oral	Jongert et al. 2007
GRA7 + ROP2			79	C3H/HeN	76K (II) tissue cysts, oral	Jongert et al. 2007
GRA1 + ROP2			57	C3H/HeN	76K (II) tissue cysts, oral	Jongert et al. 2007
GRA1			31	C3H/HeN	76K (II) tissue cysts, oral	Jongert et al. 2007
GRA7			80	C3H/HeN	76K (II) tissue cysts, oral	Jongert et al. 2007
ROP2			43	C3H/HeN	76K (II) tissue cysts, oral	Jongert et al. 2007
SAG1-ROP2		0+/0	NA	BALB/c	RH (I) tachyzoites, ip	Zhang et al. 2007a
SAG1-ROP2 + IL-12	Plasmid, im	0++/0	NA	BALB/c	RH (I) tachyzoites, ip	Zhang et al. 2007a
GRA4	Plasmid, gg	71/28	NA	C57BL/6	PLK (II), ip	Zhang et al. 2007b
GRA4 heterologous	Plasmid, vaccinia, gg	100/28	NA	C57BL/6	PLK (II), ip	Zhang et al. 2007
SAG1-ROP2	Plasmid, im	0/0	NA	BALB/c	RH (I) tachyzoites, ip	Xue et al. 2008a
SAG1-ROP2+CT		0/0	NA	BALB/c	RH (I) tachyzoites, ip	Xue et al. 2008a
SAG1-ROP2+IL-12		0+/0	NA	BALB/c	RH (I) tachyzoites, ip	Xue et al. 2008a
SAG1-ROP2	Plasmid, im	0+/0	NA	BALB/c	RH (I) tachyzoites, ip	Xue et al. 2008b
SAG1-GRA2		0+/0	NA	BALB/c	RH (I) tachyzoites, ip	Xue et al. 2008b
SAG1-ROP2-GRA2		0++/0	NA	BALB/c	RH (I) tachyzoites, ip	Xue et al. 2008b
SAG1-ROP2-GRA2 + IL-12		0+++/0	NA	BALB/c	RH (I) tachyzoites, ip	Xue et al. 2008b
SAG1-ROP2-SAG2	Plasmid, im	0+/0	NA	BALB/c	RH (I) tachyzoites, ip	Cui et al. 2008
SAG1-ROP2-SAG2 + IL-12		0+/0	NA	BALB/c	RH (I) tachyzoites, ip	Cui et al. 2008
SAG1-GRA1-ROP2-GRA4	Plasmid, im	0+/0	NA	BALB/c	RH (I) tachyzoites, ip	Cong 2008
		20+/0	NA	BALB/c	RH (I) tachyzoites, ip	Cong 2008
SAG1-GRA1-ROP2-GRA4-CT		20+/0	NA	BALB/c	RH (I) tachyzoites, ip	Cong 2008

CT: cholera toxin; gg: gene gun; im: intramuscular; ip: intraperitoneal; NA: not addressed; +/++/+++: enhanced survival.

TABLE III
Immunization with live attenuated vectors expressing *Toxoplasma gondii* antigens

Antigen	Antigen delivery	Protection endpoint		Mouse strain	*T. gondii* challenge strain	Study
		Survival/control (%)	Cyst reduction (%)			
SAG1	RMA.S cell line	40/0	NA	C57BL/6	RH (I) tachyzoites, ip	Aosai et al. 1999
GRA1	BCG	13/0	NA	Swiss-OF1	76K (II), oral	Supply et al. 1999
ROP2	MVA	0/0, +	None	Swiss	RH (I) tachyzoites, ip Me49	Roque-Reséndiz et al. 2004
SAG1-SAG2-CT	*S. typhimurium*, oral	40/0	NA	BALB/c	RH (I) tachyzoites, ip	Cong et al. 2005
SAG1, SAG2, SAG3	Adenovirus	0/0	NA	BALB/c	RH (I) tachyzoites, ipP-Br	Caetano et al. 2006
		NA	80%	BALB/c	RH (I) tachyzoites, ipP-Br	Caetano et al. 2006
GRA4	Vaccinia, gg	14/28	NA	C57BL/6	PLK (II), ip	Zhang et al. 2007b
ROP2	BCG	0/0, +	NA	BALB/c	RH (I) tachyzoites, ip	Wang et al. 2007
SAG1	*S. typhimurium*, oral	80-90/0	NA	ICR	RH (I) tachyzoites, ip	Qu et al. 2008
SAG1	Pseudorabies virus, im	60/0	NA	BALB/c	RH (I) tachyzoites, ip	Liu et al. 2008

gg = gene gun; ip = intraperitoneal; im = intramuscular; NA = not adressed; +/++/+++ = enhanced survival.

have been considered. Again many of these rely on TLR ligands, including CpG which is a ligand of TLR9 (Zimmermann et al. 2008), or genes for cytokines (e.g., IL-12 or GM-CSF) that when expressed, function directly as the third signal, or induce the third signal.

Recently, a number of components from *T. gondii* have been found to activate TLRs. For example, *T. gondii* has a variety of GPI-anchored proteins, which in theory can interact with TLR2 and 4 (Debierre-Grockiego et al. 2007). *T. gondii* HSP70 can also interact with TLR4 to induce maturation of dendrites (Aosai et al. 2006). Profilin from *T. gondii* can ligate TLR11 and induce IL-12 production (Yarovinsky et al. 2005). TLR11 is only present in certain mammalian species such as mice, but absent from others including humans. However *T. gondii* cyclophilin 18 has been demonstrated to bind the CCR5 receptor and induce IL-12 production (Aliberti et al. 2003). These molecules could be considered as "endogenous" adjuvants and may have promise as vaccine adjuvants, especially for *T. gondii* vaccines. An early study using purified SAG1 as a vaccine in mice found that it could function in the apparent absence of adjuvant, a fact that can now be retrospectively explained by this molecule containing a GPI-anchor, making it an "endogenous" adjuvant (Khan et al. 1991).

The use of IL-12 as an adjuvant and the use of chimeric administration systems like the ubiquitin-SAG1 construct to enhance antigen processing and presentation needs further attention, combined with *T. gondii* profilin and cyclophilin 18 as TLR binding antigens.

Other than providing or eliciting the second and third signals, vaccine adjuvants that are capable of mucosal delivery may be of particular interest in *T. gondii* vaccines as infection most often occurs through this route. In this respect bilosomes, a mixture of non-ionic surfactant and bile salts in a vesicular formulation may hold promise (Conacher et al. 2001). Such systems may require additional components to generate appropriate second and third signals.

DISCUSSION

Protein vaccines are known to induce primarily B-cell responses and thus are highly efficient for competing infections that can be antagonized by neutralizing antibodies. DNA vaccines are now known to induce primarily strong cytotoxic T cell responses and have been demonstrated to have limited capacity in overall protection, especially when used in primates. Immunological restriction very likely limits the development of a vaccine, either protein or DNA, against *T. gondii* since this is a parasite with various life cycles and antigenic variation. The immunological response against *T. gondii* clearly is a strong cytotoxic T cell response (Gazzinelli et al. 1991, 1992, Hakim et al. 1991, Denkers et al. 1993). However, whether the humoral response against the parasite is of diagnostic value or may add to protection remains under debate (Sayles et al. 2000, Johnson et al. 2004).

The importance of stimulating both the humoral and the cellular immunity when applying a *T. gondii* vaccine might be best reflected by 90% survival achieved in a study where a DNA prime regimen was followed by a

TABLE IV

Immunization of other animal models against *Toxoplasma gondii* infection

Antigen	Antigen Delivery	Protection endpoint/negative control group (%)	Animal	*T. gondii* challenge strain	Study
S48, bradyzoites deficient strain, live tachyzoites	im	72-80/17 viable lambs, almost 66 of viable lambs were infected	sheep	Oocysts, orally	Buxton et al. 1991
$GRA1_{170-193}$	Peptide+FCA/IFA, iv	50/0 survival	fisher rats	RH (I) tachyzoites, ip	Duquesne et al. 1991
TS-4 tachyzoites	sc	19++/0 survival 1/25 foetuses infected with TS-4 during pregnancy	*Aotus* monkeys	T265, T163	Escajadillo & Frenkel 1991
T263, oocyst deficient strain, live bradyzoites	oral	84 of cats protected from oocyst shedding	cat	T265, oral	Frenkel et al. 1991
GRA1	BCG	No difference in febrile response	sheep	76K (II), oral	Supply et al. 1999
GRA2, GRA5, chr	Protein+IFA, sc	64-69 reduction in foetal infection	fisher rats	76K (II) tissue cysts, oral	Zenner et al. 1999
SAG1+GM-CSF	Plasmid, im	60 brain cyst reduction	sprague-dawley rats	VEG (III) oocysts, oral	Angus et al. 2000
ROP2	Feline herpesvirus, in	100/0 *Toxoplasma* free brain, oocyst shedding not affected	cat	Beverley (II) tissue cysts, oral	Mishima et al. 2002a, b
RH Tachyzoites	IFA + CpG, im	52/0 *Toxoplasma* free	pig	VEG (III) oocysts, oral	Kringel et al. 2004
RH tachyzoites	PLG microspheres, intranasal	no protection	sheep	M3 oocysts, oral	Stanley et al. 2004
LIV-5 rhoptry extract RH tachyzoites, live	ISCOM, sc im	20/0 *Toxoplasma* free 30/0 *Toxoplasma* free	pig	VEG (III) oocysts, oral	Garcia et al. 2005
SAG1	Protein	lower C56 parasite load in circulation and brain; lower 76K parasite load in lungs	guinea pig	C56 (III) tachyzoites ip, 76K (II) tissue cysts oral	Flori et al. 2006
LIV-5 tachyzoites ropthry extract	QuilA, in	67 of cats protected from oocyst shedding	cat	VEG (III) tissue cysts, oral	Garcia et al. 2007
GRA1 + GRA7 + GM-CSF	Plasmid, id	67/0 *Toxoplasma* free	pig	76K (II) tissue cysts, ip	Jongert et al. 2008b

id: intradermal; in: intranasal; im: intramuscular; iv: intravenous; sc: subcutaneous.

protein boost (Zhang et al. 2007a, b). However, sterile immunity was not achieved by this heterologous prime-boost regimen, or by any of the other up-to-date experimental settings.

The apparent complete protection against new infections in immunocompetent individuals after natural infection, and the cross protection in animals after natural infection, show that protective immunity can be developed. Furthermore, this evidence also suggests that a vaccination derived from a single clonal lineage of *T. gondii* could protect against challenge with any of the other lineages. However this is still open to debate, as we are lacking tools to evaluate the immune response and parasite dissemination after reinfection. The studies discussed here illustrate that most antigens can induce some degree of protection measured both by increased survival after lethal infection with a virulent parasite and by a reduction in the number of brain cysts after challenge with a cyst forming *T. gondii* isolate. However, meaningful comparison of the overall protective efficacy between the different studies in mice is clearly hampered by the heterogeneity of experimental protocols being used over the last years. Sterile immunity and complete protection against acute transplacental infection and protection against congenital disease has not been obtained in mice, despite intensive efforts.

It important to note that sterile immunity against tissue cyst formation in mice was obtained by Mishima et al. (2001b**)** and Couper et al. (2003), but these mice were not absolutely protected against lethal challenge or congenital infection, respectively. These data beg the question of whether the same immunological mechanism is correlated with protection against acute, chronic and congenital protection.

In sharp contrast, sterile immunity against tissue cyst formation and oocyst shedding has been obtained in pigs (Kringel et al. 2004, Garcia et al. 2005, Jongert et al. 2008a) and cats, respectively (Frenkel et al. 1991, Garcia et al. 2007). The highest protection against congenital disease, however, was obtained in a rat model (Zenner et al. 1999). However, in these models, the correlates of protection have not been elucidated. Most studies have been performed in inbred mice, simply due to the simplicity and cost of the model. However, better protection in outbred pigs, which are closer to humans, raises the question of whether the mouse model is the most appropriate for studies aimed at developing a human vaccine.

Standardisation of several experimental parameters would be helpful in order to derive conclusions from studies using varying antigens and/or varying adjuvants. Challenge protocols should use the route of administration that is the most commonly found in mammals: the oral route, either by feeding tissue cysts or oocysts. It must be considered whether the usage of brain derived tissue cysts equivalently reflects the ingestion of cysts from meat.

Moreover, a number of different *T. gondii* strains are used for challenge experiments in vivo. It is now established that *T. gondii* strains differ in their virulence in mammals (Sibley & Boothroyd 1992, Boyle et al. 2006). Hence, it is important for comparison and standardization of infection challenge protocols, and to agree on the use of a number of strains displaying different virulence and to test these strains in parallel experiments under standardized read-out conditions. In particular, now that Brazilian *Toxoplasma* strains are emerging as a distinct and more virulent type than the European type I/II/III strains, and many other *Toxoplasma* genotypes are emerging in human congenital cases and animal reservoirs worldwide, it is important to use strains originating from different parts of the world in protection assays to ensure cross-type and cross-virulence protection against toxoplasmosis.

The genetic background of experimental animals influences the outcome of vaccination protocols and challenge infections. The use of inbred mouse strains for challenge experiments does not reflect the genetic situation in animals or humans. If inbred mouse strains are used, the same vaccine should be tested in a number of possible H2 settings, for example BALB/c for H2k-d, C57Bl/6 for H2k-b and C3H for H2k-k. The use of outbred strains might better overcome immunological restrictions of the major histocompatibility complex. Moreover, when thinking of the development of a *T. gondii* vaccine for human use, genetically engineered mice expressing human rather than mouse H2 (Taneja & David 1998) might be helpful in order to draw conclusions of vaccine efficacy in humans. Despite the high number of vaccine studies in mice, only a few have addressed protection against congenital infection.

There is an urgent need to compare the efficacy of different adjuvants for use in vaccines against *T. gondii* using standardized protocols i.e., antigen formulation and animal models. The importance of this is illustrated by a study of different adjuvants used for immunization against hepatitis B using a recombinant hepatitis B SAG (Vandepapelierea et al. 2007). The authors found that CpG was inferior to MPL, suggesting that adjuvants that help signal antigen processing through TLR4 are more efficient compared to adjuvants that direct antigen processing through TLR9.

For the development of a vaccine that can prevent toxoplasmosis, there have been a number of recent technological and knowledge-based advances that may prove important. First, the completion of the *T. gondii* genome project should provide the complete "antigenome" of *T. gondii*. This will also allow comparison of the "antigenome" between major *T. gondii* lineages. Second, the mode of action for traditional adjuvants and how this relates to T cell activation will contribute to the development of new and improved adjuvants based on TLR and other (innate receptor) ligands. Third, the development of improved read outs of vaccine efficacy, such as parasite multiplication, using advanced imaging techniques (e.g., Xenogen camera and luciferase expressing parasites) will improve our ability to identify effective vaccines. Finally, the ability to analyse the immune response in relatively high through put systems such as quantitative Real Time PCR arrays and cytokine bead arrays will further guide vaccine development strategies.

Currently, studies have not been able to induce sterile immunity in mice. Varying degrees of protective immunity against lethal challenge and reduction in brain cyst loads have been achieved with most antigens.

Thus it seems that the answer may not lie in the choice of antigen and studies of different adjuvants therefore must have high priority.

Protocols of vaccine studies need be standardized in the future and we propose the following guidelines as a minimum: (i) vaccine constructs aiming at protection against cyst forming *T. gondii* in animal models should have brain cyst load as a main end point, not survival; (ii) infection with cysts simulating oral infection can be done using brain emulsion, but this needs to be administered by the oral route; (iii) challenges should be performed in parallel, using at least two *T. gondii* isolates belonging to two different lineages. Protection against the least pathogenic genotype II is not enough to demonstrate protective immunity against other genotypes; (iv) immunization should be performed in a number of inbred mouse strains, preferably BALB/c and C57BL/6 or C3H/HeN; (v) the vaccine efficacy should also be tested in outbred mice, since this more closely reflect the genetic situation of animals and humans.

In general, next generation studies should address standardizing the immunization protocol and comparing the immunogenicity of a fixed antigen delivered with different vectors and adjuvants in order to solve the complex puzzle of the *T. gondii* vaccine.

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