Review article

CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Recent progress in microneme-based vaccines development against *Toxoplasma gondii*

Clin Exp Vaccine Res 2018;7:93-103
https://doi.org/10.7774/cevr.2018.7.2.93
pISSN 2287-3651 • eISSN 2287-366X

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Received: March 28, 2018
Revised: June 18, 2018
Accepted: July 6, 2018

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No potential conflict of interest relevant to this article was reported.

The authors would like to thank all staff of Department of Parasitology of Tarbiat Modares University, Iran.

Toxoplasmosis is a cosmopolitan zoonotic disease, which infect several warm-blooded mammals. More than one-third of the human population are seropositive worldwide. Due to the high seroprevalence of *Toxoplasma gondii* infection worldwide, the resulting clinical, mental, and economical complications, as well as incapability of current drugs in the elimination of parasites within tissue cysts, the development of a vaccine against *T. gondii* would be critical. In the past decades, valuable advances have been achieved in order to identification of vaccine candidates against *T. gondii* infection. Microneme proteins (MICs) secreted by the micronemes play a critical role in the initial stages of host cell invasion by parasites. In this review, we have summarized the recent progress for MIC-based vaccines development, such as DNA vaccines, recombinant protein vaccines, vaccines based on live-attenuated vectors, and prime-boost strategy in different mouse models. In conclusion, the use of live-attenuated vectors as vehicles to deliver and express the target gene and prime-boost regimens showed excellent outcomes in the development of vaccines against toxoplasmosis, which need more attention in the future studies.

Keywords: *Toxoplasma gondii*, Vaccines, Microneme, Immune responses, Adjuvant

Introduction

Toxoplasmosis is a cosmopolitan zoonotic disease, caused by an intracellular protozoan belonging to the Apicomplexa phylum that has a worldwide distribution [1-3]. Over one third of the human population are chronically infected worldwide [4-6]. Cats are the only definitive hosts, and several warm-blooded mammals, including humans, rodents, birds, etc. serve as intermediate hosts [3,7-9]. Additionally, *Toxoplasma gondii* was reported in snakes. Nasiri et al. (2016) [10] in Iran showed that 80.88% (55/68) of the examined snakes were positive by *GRA6* gene and sequencing revealed over 98% similarity with *T. gondii* available sequences in GenBank [10].

A wide range of risk factors are involved in the prevalence of toxoplasmosis, so that they can affect the global/regional epidemiological figure of infection including: close contact with cats or keeping them indoors as pet animals, occupation, place of residence, education level, age, eating raw meat, gender, exposure to soil, the host immune response, etc. [4,5,11-13]. In humans, toxoplasmosis is often asymptomatic in immunocompetent persons, although in immunocompromised subjects such as patients with malignancies, human immunodeficiency virus-positive individuals, and organ trans-

plant recipients may be cause severe and progressive complications with poor prognosis or even may result in death if not treated [5,13-15]. Besides, seronegative pregnant women are other risk groups for *T. gondii* infection [16,17]. Upon maternal infection, fetus is probably to be exposed with transplacental transmission. Toxoplasmosis may cause miscarriage in those pregnant mothers that acquired the infection during their pregnancy [18,19]. In general tachyzoites as an infectious form of parasite *T. gondii* are able to actively invade all nucleated cells of the intermediate host and their replication is ultimately curtailed by protective immune response [20].

The present common primary control measures for men and animals toxoplasmosis depends on chemotherapy. There are very few effective control strategies to limit infection and disease in humans and numerous warm-blooded animals throughout the globe and unfortunately the methods of therapy still could not fulfil entirely the treatment goals [21]. At the moment, the drugs for treatment of toxoplasmosis is a combination of pyrimethamine and sulfadiazine that has several side effects. Furthermore, these drugs are expensive and inadequate, which may result in toxic hypersensitivity reactions and are teratogenic on the fetus as well as they cannot eliminate bradyzoites into tissue cysts [21]. Considering the high prevalence of toxoplasmosis in the world, the resulting clinical, mental, and economical complications, as well as the current common drugs have no effect on the encysted parasites [2,21-23]; therefore, the development of a vaccine against *T. gondii* parasite can be important and necessary for preventing infection [24-28].

In recent decades, numerous advancement has been made in order to identification of vaccine candidates against both chronic and acute toxoplasmosis that could promote an effective immune response. Hence, most of the work in the development of *T. gondii* vaccines have focused on dense granule antigens (GRA), microneme antigens (MIC), rhoptry antigens, surface antigens (SAG), and some other antigens. In this regard, a wide variety of vaccines such as DNA vaccines, recombinant protein vaccines, etc. have been investigated in many countries [24-27,29-37]. Despite of the constant efforts of scientists, there is no commercial vaccine for use in human and animals.

Microneme Proteins (MICs)

During recent decades, the increasing number of articles have focused on the evaluation of the immunogenicity of several functional proteins that involve in motility, adhesion to host cells, migration, invasion, and establishment of the parasitophorous vacuole [38,39]. Among these, MICs secreted by the micronemes play a critical role in the initial stages of host cell invasion by parasites that are located at the apical end of the zoite and surrounded by a typical unit membrane [38,40]. MICs are produced at the rough endoplasmic reticulum, then are transferred to micronemes by the Golgi apparatus in order to participate in cell attachment. In the other hands, these proteins are released by micronemes after contact between parasites and host cells. Noteworthy, intracellular calcium ion levels are essential for secretion and function of *T. gondii* MICs in parasites [27,38,41].

The MICs are recognized by specific receptors on the cell membrane of hosts. Various methods (such as the proteomic and genomic approaches) have been used to detect the contents of the micronems in apicomplexan parasites. The electronic-microscope was shown micronemes are composed of electron-dense matrix due to the high protein content with secretory organelles that are important for gliding [38,42]. Overall, depending on the species of parasite and the developmental stages, the MIC family includes at least 19 types in mammals (MIC1-12, M2AP, AMA1, ROM1, PLP1, SUB1, TLN4, and SPATR), of which 10 types have been identified with adhesive motifs such as epidermal grow factor (EGF) and chitin binding-like. Noteworthy, these products are essential for adhesion to host cells by parasites [43,44]. However, MICs became famous as effective vaccine candidates against *T. gondii*, due to their basic roles in the early stage of the invasion of host cells by parasites. In this field, many kinds of MICs such as MIC2, MIC3, MIC4, MIC8, MIC11, and MIC13 have been evaluated [40,45-50].

It has been recognized that MIC11 as a soluble protein containing a α-chain and a β-chain tethered by a disulfide bond that take part the early stage of cell invasion. These data suggested that MIC11 protein is able to provoke the humoral and Th1-type immune responses, significant enhancement of interferon-γ (IFN-γ), interleukin (IL)-12, and IL-2 production along with higher survival time, compared to control groups [50], suggesting it could be a potential vaccine candidate. MIC8 is a promising vaccine candidate against acute and chronic toxoplasmosis infection that is expressed in tachyzoite stage of the life cycle and acts as an escorter for soluble adhesions to the cells. Notably, this protein plays a crucial role during the invasion of the parasite into the host cell as well as involved in the intracellular proliferation of parasite. Also, it is

 https://doi.org/10.7774/cevr.2018.7.2.93

introduced as a potent stimulator for specific immune responses [48,51]. An excellent article indicated favorable and promising results for MIC8 including increased humoral and cellular immune responses with the predominance of IgG2a over IgG1 (T helper 1 [Th1]-polarized responses), enhanced number of $CD4^+$ and $CD8^+$ T cells ($p<0.05$), high production of IFN-γ, prolonged survival time, and significant reduction in percentage of brain cyst load [51]. The specific features and main functions of some MICs have been listed in Table 1.

DNA Vaccines

During two recent decades, continuous efforts of researchers have made precious achievements in the development of DNA vaccines against acute and chronic toxoplasmosis [24,29]. DNA vaccine as a robust strategy has been developed instead of traditional approaches, because of the following reasons: long-term persistence of immunogenicity, relative stability, absence of any type of microorganism, high safety, cost benefits, ease of handling, etc. [27,72,73].

DNA vaccines in general have shown to be effective for inducing both humoral and cell mediated immune response and also stimulates dendritic cells (DCs) to be matured and makes them strong stimulators of T-cell immunity [24,27,72]. Interestingly, activation of B-cells prevent from the attachment of *T. gondii* to its host cell receptors depend on the production of specific antibodies that can eliminate the parasite with the help of macrophages (MQs) [74]. As both humoral and cellular immunity responses stimulate during toxoplasmosis infection, Th1 immune response has a critical role to limit the parasite replication and produce cytokines such as IL-2 and IL-12. Also, IL-12 is produced by innate immune cells such as DCs, MQs, neutrophils, and monocytes, which plays an important role in host resistance [24,75-77]. The nat-

Table 1. The main features and functions of some MICs

Antigen	Features or major effect on host	Reference
MIC1	MIC1 contains a tandemly duplicated domain that is distantly related to the thrombospondin 1–like domain of thrombospondin-related anonymous protein and that specificall binds lactose.	[52]
MIC2	MIC2 is essential for parasite viability. MIC2 plays roles in gliding motility of *Toxoplasma gondii*, transmigration of biological barriers, and attachment to the surface of host cell.	[53]
MIC3	MIC3 is a 90-kDa dimeric soluble protein containing a chitin binding-like domain (CBL), three tandemly repeated epidermal growth factor-like domains (EGF2, EGF3, and EGF4), and two less-conserved EGF domains that overlap with the others (EGF1 and EGF5). MIC3 is an important protein intakes during the invasion of the host cell. MIC3 is a secreted protein that is expressed in all stages of the *T. gondii* life cycle. MIC3 plays an important role in the recognition, adhesion and invasion of host cells by *T. gondii*. MIC3 plays important role in the invasion process and take part in forming moving junction of *T. gondii*.	[43,46,54-59]
MIC4	MIC4 localizes in the micronemes of all the invasive forms of *T. gondii*, tachyzoites, bradyzoites, sporozoites, and merozoites.	[47]
MIC6	The C domain of MIC6 interacts with aldolase, which binds to parasite F-actin, bridging between cell surface adhesion and the parasite actin–myosin motor.	[60]
MIC8	MIC8 is expressed in the tachyzoite stage and functions as escorters, targeting soluble adhesins to the micronemes. MIC8 is essential for the parasite to invade the host cell. When MIC8 is not present, a block in invasion is caused by the incapability of the parasite to form a moving junction with the host cell.	[48,61,62]
MIC11	MIC11 is a soluble microneme protein which is presumably considered facilitating the early stage of cell invasion. It is thought to have a role in organizing other MICs for the deployment of adhesive complexes to the apical surface to facilitate host cell invasion.	[50,63]
MIC13	MIC13 plays an important role in attachment and penetration of the host cell by *T. gondii*. MIC13 play an important role in *T. gondii* propagation, because it has three microneme adhesive repeat domains, which acts as an important determinant in host cell recognition by binding sialylated glycoconjugates on the gut epithelium.	[49,64]
AMA1	AMA1 plays an important role in attachment and invasion of host cells, and thus promoting the parasite replication. Anti-TgAMA1 antibodies have been shown to block the host cell-invasion by *T. gondii* in an *in vitro* assay of the parasite growth.	[65,66]
SPATR	It is a new member in microneme protein family, Ca^{2+}-dependently secreted during early stage of invasion and existed on the outer surface of parasites. TgSPATR is contributed to *T. gondii* invasion and virulence. Δspatr parasites were -50% reduced in invasion compared to parental strains.	[67-69]
PLP1	TgPLP1 is believed to be involved in the acute virulence of *T. gondii* in mice. TgPLP1-deficient parasites failed to exit normally after intracellular growth, resulting in entrapment within host cells.	[70,71]

MIC, microneme antigens or microneme proteins; AMA1, apical membrane antigen 1; SPATR, secreted protein with an altered thrombospondin repeat; PLP1, perforin-like protein 1.

ural killer (NK) cells and T-CD8$^+$ and T-CD4$^+$ seem to be an important source of IFN-γ in the early and chronic phases of infection. This cytokine as the adaptive cellular immunity has a key role in the controlling and restriction of the parasite as well as inhibit the reactivation of bradyzoites inside the dormant tissue cysts [76,77]. In addition, Th2 cells produce IL-4, IL-5, and IL-10, which contribute to the regulation of cell-mediated immunity response reduction [78].

With a unique design strategy of DNA vaccine, it can induce cytotoxic T lymphocytes and helper immune responses with the cooperation of major histocompatibility complex pathways. Moreover, the immunity against *T. gondii* can be stimulated, so that several antigens can be detected from different epitopes at the same time [72,73]. DNA vaccines can be injected through different routes such as, intramuscular, subcutaneous, mucosal, or transdermal [27,73]. After the injection, the naked DNA plasmid enter to the cell cytoplasm in order to express encoded proteins within the host cells. As a result, induces a strong immune response [72].

Recent studies showed that the use of genetic and non-genetic adjuvants have become popular in order to provide sufficient immunity [24,27,29,79]. It should be mentioned that adjuvants have an important role to improve the efficacy of a vaccine by enhance either the magnitude or time of DNA expression and recruiting the immune cells to the site of injection. Also, they are used in order to help the uptake of DNA into host cells as well as increase taken up by professional antigen-presenting cells [27,72]. Several publications have demonstrated that cytokines such as (IFN-γ, IL-12, IL-15, IL-21, etc.), chemokines, and costimulatory molecules (B7-1, B7-2, etc.) as adjuvant, could boost the effectiveness of DNA vaccines [24,33,46,51,71,79-81]. For instance, IL-21 synergizes with IL-15 to enhance the generation of CD8$^+$ memory T cells and NK cell activity. Thus, these cytokines can suggest as a candidate adjuvant against toxoplasmosis [51]. For this purpose, Li et al. (2014) [51] designed an investigation to evaluate the immunogenicity of pVAX-MIC8 plus pVAX/IL-21/IL-15. The findings showed co-administration of MIC8 plus mIL-15 and m-IL-21 cytokines enhanced survival time and improved protective immunity of DNA vaccine [51]. Noteworthy, as a member of germ line-encoded receptor, toll-like receptors (TLRs) have a special ability in the innate and adaptive immune responses to pathogens. Hence, they are the target of new vaccine adjuvants in order to improve the immunogenicity of DNA vaccines [82]. For instance, oligodeoxynucleotides contained CG motifs (CpG ODN) as the TLR-9 ligand and a molecular adjuvant to be effective to enhance the immunogenicity of DNA vaccines [72,83].

Recently, several papers have examined the various MICs based on DNA vaccination approach, including MIC2, MIC3, MIC4, MIC6, MIC8, MIC11, MIC13, PLP1, M2AP, AMA1, and SPATR in different mouse models [32,45-51,54,55,58,59,68,71,84-89]. Yuan et al. (2013) [49] evaluated the immunoefficacy of TgMIC13. The Kunming mice immunized with pVAX-TgMIC13 showed higher levels of IgG antibodies ($p<0.05$), T-cells proliferative response, high secretion of IFN-γ, IL-2, IL-4, and IL-10 ($p<0.05$), increased survival time ($p<0.05$), and significant reduction in the percentage of brain cysts load ($p<0.05$), compared with those mice that received phosphate-buffered saline. These data suggest that *T. gondii* MIC13 is a reasonable vaccine candidate against acute and chronic *T. gondii* infection [49]. MIC3 as a secreted protein plays an essential role in the attachment and invasion of host cells, which expressed at all stages of the life cycle of *T. gondii* (tachyzoite, bradyzoite, and sporozoite) and discharged from small secretory vesicles [84]. It has been shown that CBA/J mice vaccinated with a plasmid encoding of MIC3 (pMIC3i) produced a significant cellular immune response with the increased secretion of IFN-γ and IL-2 cytokines. The findings showed the response was increased by the pMIC3i plus the plasmid encoding granulocyte-macrophage colony-stimulating factor (pGM-CSF). Also, the immunized mice showed a dramatic reduction of brain cyst load against an oral challenge with *T. gondii* 76K cysts, compared with control mice [46]. It has been reported that CD4$^+$ and CD8$^+$ T lymphocytes have a key role in MIC3 DNA vaccine to induce protection. Furthermore, plasmids encoding the EGF-like domains and the Lectin-like domain of MIC3 are involved in the protection [90]. More examples of immunization experiments with DNA vaccines against *T. gondii* in different mouse models are listed in Supplementary Tables 1 and 2.

Recently, it has been well established that using a combination of multiple antigens to be more effective compared with single antigens, as well as improve the protective immunity against toxoplasmosis either survival duration time and/or brain cyst load [32,54,58]. For instance, Beghetto et al. (2005) [89] evaluated five distinct protein fragments MIC2, MIC4, M2AP, and AMA1 gene products that are recognized by antibodies and T cells from infected individuals. The highest protection with DNA vaccination against *T. gondii* infection was obtained by immunization of BALB/c mice with plasmid mixture and the brain cyst burden in mice vaccinated with the various proteins was significantly reduced than

 https://doi.org/10.7774/cevr.2018.7.2.93

those in control groups. The authors concluded that microneme gene fragments with the antigenic regions of cyst-specific genes could be useful in vaccination against toxoplasmosis [89]. In another study, Fang et al. [85] examined MIC3 and SAG1 proteins alone or combined together. The BALB/c mice were intraperitoneally immunized with 1×10^3 tachyzoites of RH strain. The results revealed that single-gene immunization increased humoral immune responses, increased secretion of IFN-γ, and prolonged the survival time, compared with the control groups ($p<0.05$). On the other hand, those mice that vaccinated with the multi-antigenic DNA vaccine (MIC3/SAG1), boosted the protective immunity in terms of cytokine production and survival time, compared with single gene immunized groups ($p<0.05$). These observations led to the suggestion that MIC3 with SAG1 together capable to induce long term and significant protection against toxoplasmosis, and also cocktail-vaccine immunization could be employed as an alternative way to providing effective protection against *T. gondii* infection [85].

Recombinant Protein Vaccines

Over the past decades, considerable progress has been achieved to recognize the molecular biology of the various aspects of *T. gondii* that resulted to design of different vaccine experiments against toxoplasmosis, based on the subcellular components of the parasite [27,38,43,91-93]. The family of micronemes are attractive vaccine candidates that are responsible for the host-cell invasion [43,53]. One of the alternative ways for the development of vaccine candidates against toxoplasmosis is recombinant subunit vaccines that have high potency to trigger systemic humoral and cell mediated responses as well as they are very important for large-scale production [40,94].

MICs promote Th1 response, which is critical in mediating the resistance to *T. gondii*. In order to evaluate the protective efficacy of recombinant form of MICs, Pinzan et al. (2015) [40] designed a comprehensive study on different recombinant microneme proteins (TgMIC1, TgMIC4, and TgMIC6) and combinations of these proteins (TgMIC1-4 and TgMIC1-4-6). They vaccinated the C57BL/6 (H-2b) mice subcutaneously with TgMIC1 (10 μg), TgMIC4 (10 μg), TgMIC6 (10 μg), TgMIC1-4 (5 μg of each protein), TgMIC1-4-6 (3.3 μg of each protein), or Lac+ (10 μg) emulsified in Freund's complete adjuvant. One month after the last immunization procedure, the mice were orally infected with 40 and 80 cysts of the ME49 strain for chronic and acute toxoplasmosis, respectively. The results indicated that these recombinant protein vaccines significantly enhanced IgG titers, mixed Th1/Th2 responses with the predominance of IgG2b over IgG1, high production of IFN-γ and IL-10 cytokines with strong lymphocyte proliferative responses, as well as the increased survival rate ($p<0.05$), compared with control groups. Besides, immunization with TgMIC1-4 and TgMIC1-4-6 vaccines boosted the protective efficiency, so that 70% and 80% of immunized mice survived 30-day post challenge, respectively. The brain cyst load in mice vaccinated with the different proteins was reduced than non-vaccinated groups ranging from 27.2%-67.8%. It is well known that multicomponent vaccine has better effects than single antigens. The authors declared that the use of this vaccine offers a promising strategy for conferring protection against toxoplasmosis [40]. More details can be found in Supplementary Tables 3 and 4.

Vaccines Based on Live-Attenuated Vectors

Live-attenuated vectors such as bacteria or viruses are another strategy for enhancing the antigen presentation to the immune system of the host. They can greatly mimic the intracellular niche of *T. gondii* as well as provokes a strong humoral and cell mediated immune response, due to their intrinsic adjuvant properties [85,95]. Also, these vaccines can be delivered by several routes, including intramuscular, intraoral, intranasal, subcutaneous, and intravenous in order to induce effective protection [96].

Recently, recombinant viral vectors have shown great potential and play a critical role to induce humoral and cellular immune responses. Hence, they could be a suitable vector for the development of new vaccines [85,95-99]. For instance, it is well known that pseudorabies virus (PRV) has a high capability and remarkable effectiveness to enhance vaccine potency. The study showed that a new recombinant modified PRV expressing TgSAG1 (rPRV-SAG1) and TgMIC3 (rPRV-MIC3) cocktail induced a strong IgG antibody response and significant levels of IFN-γ, IL-2, and IL-10 production as well as increased the survival rate (66.7% survival 28 days, $p<0.05$) post challenge with 100 tachyzoites of RH strain in BALB/c mice. The authors remarked that expression of protective antigens of *T. gondii* in PRV is a novel approach towards the development of a vaccine against toxoplasmosis [97].

Virus like particle (VLP) vaccines are genetically engineered complexes of multiple copies of protein antigens in a

particulate virus like structure that lacks viral genetic material and therefore cannot replicate [100]. These vaccine types have several advantages as follows [101]:

- Well-defined geometry and remarkable uniformity with repetitive and ordered surface structures
- Particulate and multivalent nature
- Preservation of native antigenic conformation
- Safety, as they are absolutely non-infectious and nonreplicating candidates
- Higher stability than soluble antigens in extreme environmental conditions
- Applicability as vectors for the presentation of foreign antigens

VLP vaccines can stimulate powerful humoral and cellular immune responses, representing one of the most appealing approaches for a vaccine platform by mimicking the main structural and functional characteristics of viruses [101,102]. Moreover, they can be produced in insect cell expression systems, where foreign antigens can be displayed [102]. It has been reported that VLPs are being useful and safe as vaccine candidates that could provide stronger and longer-lasting protection against toxoplasmosis [98]. In this case, Lee et al. (2017) [98] reported a novel recombinant VLPs carrying MIC8 and then evaluated the immune response and survival status in BALB/c mice. Interestingly, MIC8 in VLPs able to elicit significantly both humoral and cell mediated responses. After immunization, the levels of IgG antibody in sera and IgA antibody in feces elevated by MIC8 VLP vaccine, compared than controls. The enhanced survival duration (intranasal group 100% protection and intramuscular group 60% protection 16 days after challenging with 1×10^5 tachyzoites of RH strain) was observed, compared with control mice that died within 12 days. Moreover, the numbers of germinal center B cell ($B220^+$, $GL7^+$) and T cell ($CD4^+$, $CD8^+$) populations increased more obviously, in the group immunized with MIC8 VLP than in the control group. These results provide an effective approach for developing vaccines based on VLPs for protection against the highly virulent RH strain of *T. gondii* [98]. Supplementary Table 5 listed the examples of immunization with live-attenuated vectors expressing *T. gondii* antigens in mouse models.

Prime-Boost Strategies

Over the past few years, prime-boost strategy has been evaluated for vaccine development, such as DNA prime/viral vector boost, DNA prime/protein boost, and protein prime/DNA boost that are able to induce both humoral and cell-mediated immune responses against many different pathogens especially intracellular pathogens [59,72,99,103,104]. Hence, this strategy can be useful to promote the effectiveness of vaccine experiments.

Homologous prime-boost approach involves the similar formulation employed in both the prime and boost regimens, while heterologous prime-boost strategies contains different formulations used in more than one immunization [72,105]. The interval between prime and boost is very substantial for vaccine response and high efficacy. Moreover, the arrangement of vaccination schedule undoubtedly could influence the outcome of prime–boost strategies [72,106]. Noteworthy, heterologous prime-boost is more likely to be immunogenic against an antigen than the homologous prime-boost [105,107]. The advantage of heterologous prime-boost immunization is the induction of a strong cellular immune response and is associated with a higher and more specific antibody response against the vaccine target compared to homologous immunization. It has been reported that in comparison with homologous prime-boost approach with the same DNA vaccine, boosting a primary response with a heterologous vector leads to 4- to 10-fold higher T-cell responses [105]. A DNA or a viral vector, especially adenovirus for priming and a protein-based vaccine as a booster, have been used in heterologous prime-boost strategy [107]. Several different vaccine strategies have been shown to elicit different types of immune response. For example, DNA vaccines or recombinant live vector-based vaccines are able to elicit an efficient cellular immunity, but subunit vaccines commonly trigger a predominant humoral immunity [99,105]. Subunit vaccines are based on peptides, proteins or polysaccharides containing protective antigens. However, the recombinant subunit vaccines are poorly immunogenic and usually require some additional components to enhance the potency of protective immunity. Accordingly, the use of some adjuvants and also repeated boost immunizations are suggested to elevate the efficiency of subunit vaccines [105,108]. The underlying mechanisms of the effectiveness of prime-boost regimens still remain poorly understood. It is hypothesized that the lower antigen expression from DNA vaccines may preferentially prime Th cell responses with the humoral response being subsequently boosted by the protein or viral vector boost [72]. The examples of heterologous prime-boost vaccination against *T. gondii* in mouse models have been inserted in Supplementary Table 6.

 https://doi.org/10.7774/cevr.2018.7.2.93

Conclusion

During the twenty years ago, the different vaccine types with various strategies have been evaluated throughout the globe. Despite of the continuous efforts of scientists, there is no available licensed vaccine for use in human and animals. Thus, the development of an efficient vaccine urgently required to prevent and limit the infection. The use of DNA vaccines encoding MIC2, MIC3, MIC11, M2AP, and AMA1 alone or as mixture increase the survival time/rate in immunized mice (see Supplementary Tables 1 and 2). Furthermore, the use of recombinant protein vaccines showed an elevated survival rate (up to 80% protection). Nevertheless, these investigations failed to report complete protection. The use of live-attenuated vectors as vehicles to deliver and express the target gene and prime-boost regimens are excellent strategies for vaccine development, which need more attention in the future studies. Reportedly, it has been verified that the use of genetic and non-genetic adjuvants is very effective, because of their potential ability in boosting specific and long-lasting protective immunity. Collectively, the obtained findings are widely diverse, but valuable advacements have been achieved, so that they gave promising perspectives for the future investigations. However, several limitations may affect the outcome of the experimental studies such as: unsuitable immunization protocol, inadequate evaluation criterion, the strain of parasite, evaluation criterion, the vaccine construct, dosage of inoculum, the delivery route, the different mouse models, etc. The future experiments must address the all these aspects to minimize the faults. In the other hands, optimize immunization protocol and use of various types of delivery systems, traditional and/or molecular adjuvants undoubtedly would affect the outcomes.

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Supplementary Material

Supplementary materials are available at Clinical and Experimental Vaccine Research website (http://www.ecevr.org).

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http://www.ecevr.org/ https://doi.org/10.7774/cevr.2018.7.2.93

Supplementary Table 1. Baseline characteristics of included studies based on immunization experiments with *Toxoplasma gondii* DNA-encoding MICs in mouse models (single antigens)

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MIC2	Gold particles	Gene gun into abdomen	BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$)	20 Cysts of the *T. gondii* Beverley strain, orally	Induce the production of specific antibodies ↑ IFN-γ	NR	BALB/c: Increased survival rate (40%, 30-day post challenge, p=0.015) C57BL/6: Increased survival rate (37.5%, 30-day post challenge, p=0.0151)	-	[1]
MIC3	pGM-CSF	i.m	CBA/J ($H\text{-}2^k$)	70 Cysts of the 76K strain, orally	Induced a strong IgG antibody response (p<0.05) ↑ Splenocyte proliferation p<0.05) ↑ IFN-γ in mice immunized with pMIC3+pGM-CSF (111.9±11 pg/mL, p<0.05) ↑ IL-2 (210±45 pg/mL, p<0.05 and 242±57 pg/mL, p<0.05 for pMIC3 and pMIC3+pGM-CSF groups, respectively) Similar levels of IL-4 and IL-10 between all groups (p>0.05)	Reduced (p<0.05) pMIC3: 58% pMIC3+ pGM-CSF: 74%	NR	This study describes the design of a potent DNA vaccine encoding the novel *T. gondii* target antigen, MIC3 protein, that elicits a strong specific immune response as well as providing effective protection against *T. gondii* infection.	[2]
	-	Footpad	Kunming	5×10^2 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response (p<0.05) ↑ $CD4^+$ and $CD8^+$ T lymphocytes (especially $CD8^+$, p<0.05) ↓ $CD4^+/CD8^+$ ratio (p<0.05)	NR	Increased survival time (p<0.05)	A potent DNA vaccine pcDNA3-MIC3 could elicit a strong specific immune response and induce effective protection against *T. gondii* challenge in Kunming mice, suggesting that MIC3 is a potential vaccine candidate against toxoplasmosis.	[3]
	-	i.m Groups: pcDNA 3-MIC3 (the conventional DNA vaccine plasmid) pSCA1-MIC3 (the suicidal DNA vaccine plasmid) pSCA1 PBS	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response (p<0.05) No significant difference in terms of IgG responses between the groups immunized with pSCA-MIC3 and pcDNA-MIC3 (p>0.05) ↑ Splenocyte proliferation p<0.05) However, the mean lymphocyte SI of the pSCA-MIC3 vaccinated group was higher than pcDNA-MIC3 vaccinated group, but this was not statistically significant (p>0.05). ↑ IFN-γ significantly (especially in the mice immunized with pSCA-MIC3) ↓ IL-4 (p<0.05) No significant difference in terms of IFN-γ and IL-4 responses between the pSCA-MIC3 group and pcDNA-MIC3 group (p>0.05)	NR	Increased survival rate (p<0.05)	The findings demonstrated that like conventional DNA vaccine pcDNA-MIC3, suicidal DNA vaccine pSCA-MIC3 also provided favorable efficacy, but it could improve the biosafety of conventional vaccines. This result suggested that suicidal DNA vaccine pSCA-MIC3 is a potential candidate vaccine against toxoplasmosis.	[4]

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Supplementary Table 1. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
	-	i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.01$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ ($p<0.05$) Similar levels of IL-4 and IL-10 between the different immunized and unimmunized groups ($p>0.05$)	NR	Prolonged survival time ($p<0.05$)	-	[5]
	-	i.m	ICR	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response with predominance of IgG2a over IgG1 ($p<0.05$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ (346 ± 31 pg/mL, $p<0.05$) and IL-4 (51 ± 11 pg/mL, $p<0.05$)	NR	Prolonged survival time (14 days compared with 7 days in control, $p<0.05$)	Our study indicates that the introduction of multi-antigenic DNA vaccine is more powerful and efficient than single-gene vaccine.	[6]
	-	i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	↑ Levels of IgG1 and IgG2a ($p<0.05$) ↑ IFN-γ and IL-4 ($p<0.05$) ↑ Proliferation SI ($p<0.05$)	NR	Prolonged survival time (13 days compared with 6 days in control, $p<0.05$)	These results indicated DNA vaccine encoded MIC3 gene of *T. gondii* capable to induced partially protection against toxoplasmosis.	[7]
	-	i.m	BALB/c	1×10^2 Tachyzoites, RH strain, i.p	↑ Levels of IgG antibodies ($p<0.05$)	NR	Prolonged survival time (11 days compared with 7 days in control)	These results demonstrate that TgMIC3 could elicit some protection against toxoplasmosis.	[8]
	-	i.m	BALB/c	1×10^4 (high dose) and 1×10^2 (low dose) tachyzoites, RH strain, i.p	↑ Specific IgG antibody response ($p<0.05$) ↑ IFN-γ ($p<0.05$)	↓ Parasite burden in brain and liver ($p<0.01$)	High dose: increased survival time (14 days compared with 6-7 days in controls, $p<0.05$) Low dose: increased survival rate (60%, 32 days after challenge, $p<0.05$)	The present study indicates that MIC3 showed the potential as target for vaccine investigation against toxoplasmosis.	[9]
MIC4	-	i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG and IgA antibodies responses The predominance of IgG2a over IgG1 ↑ IFN-γ (632 ± 96 pg/mL, $p<0.05$) and IL-12 (415 ± 23 pg/mL, $p<0.05$)	NR	Prolonged survival time in mice (death within 11 days, $p<0.05$) All mice in the control groups died within 6-7 days.	Although this vaccine elicited humoral and cellular immune response and prolonged the life of mice which infected with the RH tachyzoites, they can't protect mice from death or unhealthy. So it is a long way for us to explore an authentic vaccine against this parasite.	[10]
	-	i.m	Kunming	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ IFN-γ (657 ± 32.74 pg/mL, $p<0.05$), IL-2 (614.33 ± 30.92 pg/mL, $p<0.05$), IL-4 (281.33 ± 14.29 pg/mL, $p<0.05$) and IL-10 (608.33 ± 17.01 pg/mL, $p<0.05$) ↑ Proliferation SI (1.61 ± 0.05, $p<0.05$)	NR	Increased survival time ($p<0.05$)	The results showed that *T. gondii* MIC4 is a potential vaccine candidate against toxoplasmosis.	[11]

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Supplementary Table 1. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MIC6	-	i.m	Kunming	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ Proliferation SI (1.50±0.07, $p<0.05$) ↑ IFN-γ (557.67±28.04 pg/mL, $p<0.05$), IL-2 (522.33±66.53 pg/mL, $p<0.05$), IL-4 (202.67±14.74 pg/mL, $p<0.05$) and IL-10 (523±37.36 pg/mL, $p<0.05$)	NR	Prolonged survival time in mice (13.3±1.2 days, $p<0.05$) Control mice were died within 5 days.	Our data demonstrate, for the first time, that MIC6 triggered a strong humoral and cellular response against *T. gondii*, and that the Ag is a potential vaccine candidate against toxoplasmosis, worth further development.	[12]
	-	i.m	Kunming	20 and 80 cysts of strain PRU, i.g	Induced a strong IgG antibody response ($p<0.05$) ↑ Proliferation SI (4.29±0.18, $p<0.05$) ↑ Proliferation SI (4.29±0.18, $p<0.05$) ↑ IFN-γ (475.8±21.2 pg/mL, $p<0.05$), IL-2 (208.0±7.2 pg/mL, $p<0.05$), IL-12 (130.5±7.51 pg/mL, $p<0.05$), IL-4 (115.5±7.9 pg/mL, $p<0.05$) and IL-10 (68.3±1.9 pg/mL, $p<0.05$)	Reduced (39.81%, $p<0.05$)	Prolonged survival time ($p<0.05$) Control mice were died within 25 days.	Immunization with the recombinant plasmid DNA encoding *T. gondii* MIC6 offers protective efficacy, and this is a promising vaccine candidate against chronic toxoplasmosis.	[13]
MIC8	-	i.m	Kunming	1×10^3 t, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ Proliferation SI (1.39±0.13, $p<0.05$) ↑ IFN-γ (484.67±25.58 pg/mL, $p<0.05$), IL-2 (359.33±61.76 pg/mL, $p<0.05$), IL-4 (189.00±18.33 pg/mL, $p<0.05$) and IL-10 (404.33±67.87 pg/mL, $p<0.05$)	NR	Increased survival time (10.3±0.9 days, $p<0.05$) Control mice were died within 5 days.	These data demonstrate that the *T. gondii* MIC8 is a potential vaccine candidate against toxoplasmosis.	[14]
	mIL-15 and mIL-21	i.m	Kunming	Acute: 1×10^3 tachyzoites, RH strain, i.p and 80 cysts PRU strain, orally Chronic: 20 cysts PRU strain, orally	Induced a strong IgG antibody response ($p<0.05$) Increased both IgG1 and IgG2a with the predominance of IgG2a over IgG1 ↑ Proliferation SI (2.37±0.14, $p<0.05$) ↑ IFN-γ (808.84±46.42 pg/mL, $p<0.05$), IL-2 (495.73±45.81 pg/mL, $p<0.05$), IL-12 p-70 (317.08±37.41 pg/mL, $p<0.05$), IL-4 (168.78±22.64 pg/mL, $p<0.05$), and IL-10 (151.75±28.28 pg/mL, $p<0.05$) ↑ Percentages of $CD4^+$ T and $CD8^+$ cells ($p<0.05$)	Reduced (63.82%, $p<0.05$)	Increased survival time 1×10^3 tachyzoites, RH strain, i.p: 16.2±1.30 days, $p<0.05$ 80 Cysts PRU strain, orally: 44.8±4.45 days, $p<0.05$	The present study demonstrates, for the first time, a synergistic effect of rIL-15 and rIL-21 genes in augmenting the efficacy of TgMIC8 DNA vaccine through induction of strong humoral and cellular immune responses which were protective against *T. gondii* challenge.	[15]
MIC11	-	i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ, IL-2, and IL-12 ($p<0.05$) Similar levels of IL-4 between mice vaccinated with pcDNA/MIC11and control groups ($p>0.05$)	NR	Increased survival rate (17%, 15-day post challenge, $p<0.05$) Control mice were died within 8-10 days.	These data suggest that *T. gondii* MIC11 is a reasonable vaccine candidate deserving further studies, and pcDNA/MIC11 is a potential strategy for the control of toxoplasmosis.	[16]
MIC13	-	i.m	Kunming	Acute: 1×10^3 tachyzoites, RH strain, i.p Chronic: 10 tissue cysts PRU strain, i.g	↑ IgG antibodies ($p<0.05$) ↑ Proliferation SI (1.74±0.05, $p<0.05$) ↑ IFN-γ (342±20.2 pg/mL, $p<0.05$), IL-2 (228.3±15 pg/mL, $p<0.05$), IL-4 (185±8.2 pg/mL, $p<0.05$) and IL-10 (358.3±33.8 pg/mL, $p<0.05$)	Reduced (57.14%, $p<0.05$)	Increased survival time (21.3±11.3 days, $p<0.05$) Control mice were died within 10 days.	*T. gondii* MIC13 is a potential vaccine candidate, worth being included in future vaccine development against acute and chronic *T. gondii* infection.	[17]

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Supplementary Table 1. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
PLP1	pIL-18	i.m	Kunming	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ Proliferation SI (4.51±0.68 and 7.95±0.87 in mice immunized with pVAX-TgPLP1 and pVAX-TgPLP1+pVAX-IL-18, respectively, $p<0.05$) ↑ IFN-γ, IL-2, IL-4, and IL-10 ($p<0.05$)	NR	Increased survival time pVAX-TgPLP1: 11.3±0.9 days, $p<0.05$ pVAX-TgPLP1+ pVAX-IL-18: 12.7±1.2 days, $p<0.05$	This study demonstrated, for the first time, that TgPLP1 induced a strong protective humoral and cellular response against *T. gondii*, indicating that it is a potential vaccine candidate against toxoplasmosis, worth further development. The murine IL-18 enhanced such immune protection. Further studies are warranted to evaluate the immune efficacy of this DNA vaccine construct in other animal host species against toxoplasmosis.	[18]
	-	i.m	Kunming	20 and 80 cysts of strain PRU, i.g	Induced a strong IgG antibody response ($p<0.05$) ↑ Proliferation SI (4.20±0.27, $p<0.05$) ↑ IFN-γ (471.5±28.9 pg/mL, $p<0.05$), IL-2 (206.3±28.2 pg/mL, $p<0.05$), IL-12 (130.3±17.7 pg/mL, $p<0.05$), IL-4 (118.5±6.4 pg/mL, $p<0.05$), and IL-10 (67.3±2.9 pg/mL, $p<0.05$)	Reduced (43.99%, $p<0.05$)	Increased survival time ($p<0.05$)	Immunization with the recombinant plasmid DNA encoding *T. gondii* TgPLP1 offers protective efficacy, and this is a promising vaccine candidate against chronic toxoplasmosis.	[13]
M2AP	Gold particles	Gene gun into abdomen	BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$)	20 Cysts of the *T. gondii* Beverley strain, orally	Induce the production of specific antibodies ↑ IFN-γ	NR	BALB/c: increased survival rate (20%, 30-day post challenge, none-significant) C57BL/6: none of the mice from pM2AP, or control groups survived the infection.	-	[1]
AMA1	Gold particles	Gene gun into abdomen	BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$)	20 Cysts of the *T. gondii* Beverley strain, orally	Induce the production of specific antibodies ↑ IFN-γ	NR	BALB/c: increased survival rate (60%, 30-day post challenge, p=0.0058) C57BL/6: increased survival rate (37.5%, 30-day post challenge, p=0.0038)	The AMA1 gene appears to generate a strong specific immune response and also provides efective protection against toxoplasmosis.	[1]
SPATR	-	i.m	BALB/c	1×10^2 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ Both IgG1 and IgG2a with the predominance of IgG2a over IgG1 ($p<0.05$) ↑ Proliferation SI (1.24±0.14, $p<0.05$) Elicited both Th1/Th2 type response ↑ IFN-γ (672.87±8.35 pg/mL, $p<0.05$), IL-2 (367.93±10.30 pg/mL, $p<0.05$), IL-4 (212±7.42 pg/mL, $p<0.05$) and IL-10 (261.8±10.03 pg/mL, $p<0.05$)	NR	Increased survival time (15.7±1.42 days, $p<0.05$) Control mice were died within 7 days.	The current study indicated that pVAX1-TgSPATR induce a *T. gondii* specific immune response and might be a promising vaccine candidate against toxoplasmosis. To the best of our knowledge, this is the first report to evaluate the immunoprotective value of TgSPATR against *T. gondii*.	[19]

MIC, microneme proteins; IFN-γ, interferon-γ; NR, not reported; pGM-CSF, plasmid encoding granulocyte-macrophage colony-stimulating factor; i.m, intramuscular; IL, interleukin; PBS, phosphate-buffered saline; i.p, intraperitoneal; i.n, intranasal; SI, stimulation index.

https://doi.org/10.7774/cevr.2018.7.2.93 http://www.ecevr.org/

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Supplementary Table 2. Baseline characteristics of included studies based on immunization experiments with *Toxoplasma gondii* DNA-encoding MICs in mouse models (mixed antigens)

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MIC2 (MIC2a and MIC2b) +MIC3+MIC4+ M2AP+AMA1	-	Plasmid, i.m	BALB/c	30 Cysts of avirulent *T. gondii* strain SSI119, orally	*T. gondii*–specific IgG against MIC2a, MIC4, M2AP, and AMA1 protein products, whereas no IgG response was detected against MIC2b and MIC3.	Reduced (84%, $p<0.0021$)	NR	The results showed that a combination of these antigenic regions should be considered in the design of potential vaccines against toxoplasmosis.	[20]
MIC3+SAG1	-	i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.01$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ ($p<0.05$) Similar levels of IL-4 and IL-10 between the different immunized and unimmunized groups ($p>0.05$)	NR	Prolonged survival time ($p<0.05$)	These results indicated that cocktail-vaccine immunization could be employed as an alternative way to develop new-generation vaccines against *T. gondii* infection.	[5]
MIC3+ROP18	-	i.m	ICR	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) Predominance of IgG2a over IgG1 (IgG2a values in the pMIC3-ROP18 immunized group were significantly higher than the single-gene immunized group, $p<0.05$) ↑ Splenocyte proliferation $p<0.05$) ↑ IFN-γ (849 ± 86 pg/mL, $p<0.05$) and IL-4 (66 ± 14 pg/mL, $p<0.05$) Higher levels of IFN-γ in mice vaccinated with pMIC3-ROP18, compared that other groups ($p<0.05$) No significant difference between multi-antigenic group and single-gene group in IL-4 production ($p>0.05$)	NR	Increased survival time (19 days compared with 7 days in control, $p<0.05$)	Our study indicates that the introduction of multi-antigenic DNA vaccine is more powerful and efficient than single-gene vaccine. These results suggested that multi-antigenic DNA immunization might be an important approach to achieve an effective vaccine against *T. gondii*.	[6]
MIC3+GRA1	-	i.m	BALB/c	1×10^4 (high dose) and 1×10^2 (low dose) tachyzoites, RH strain, i.p	↑ Specific IgG antibody response ($p<0.05$) The levels of IgG in pMIC3-GRA1 group was higher than the group of pGRA1 or pMIC3 ($p<0.05$). ↑ IFN-γ ($p<0.05$) Significantly higher levels of IFN-γ production in the mice vaccinated with pMIC3-GRA1 than pGRA1 or pMIC3 ($p<0.05$)	↓ Parasite burden in brain (57.5%, $p<0.01$) and liver (55.1%, $p<0.01$)	High dose: increased survival time (15.7 ± 1.88 days, $p<0.05$) Low dose: increased survival rate (80%, 32 days after challenge, $p<0.01$)	These observations suggest that multi-antigenic DNA vaccine is more eligible for effective anti- *T. gondii* vaccine investigation. Further studies should be considered on other potent effective antigens, suitable adjuvants and other animal models.	[9]
MIC3+GRA5	-	i.m	BALB/c	1×10^4 Tachyzoites, RH strain, i.p	↑ Levels of IgG1 and IgG2a ($p<0.05$) ↑ IFN-γ and IL-4 ($p<0.05$) ↑ Proliferation SI ($p<0.05$)	NR	Prolonged survival time (12 days compared with 6 days in control, $p<0.05$)	These results indicated DNA vaccine encoded MIC3 and GRA5 genes of *T. gondii* capable to induced partially protection against toxoplasmosis.	[7]

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Supplementary Table 2. Continued

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MIC4+SAG1	pCTA$_2$/B	i.n	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG and IgA antibodies responses ($p<0.05$) The levels of IgA and IgG antibodies were higher in the sera of mice co-immunized with pCTA$_2$/B than in the sera of mice immunized with pMIC4-SAG1 alone ($p<0.001$). The predominance of IgG2a over IgG1 IgG2a values in the pMIC4-SAG1 plus pCTA$_2$/B immunized group were significantly higher than the pMIC4-SAG1 immunized group ($p<0.01$). Similar values of IgG1 between pMIC4-SAG1 and pMIC4-SAG1 +pCTA2/B ($p>0.05$) ↑ Splenocyte proliferation ($p<0.05$) Co-administration of pCTA2/B augmented splenocyte proliferation when compared to proliferation by spleen cells from mice immunized with pMIC4-SAG1 alone ($p<0.001$) ↑ IFN-γ (1,136±152 pg/mL, $p<0.05$ and 1,874±465 pg/mL, $p<0.001$ for pMIC4-SAG1 and pMIC4-SAG1+pCTA$_2$/B groups, respectively) ↑ IL-12 (845±37 pg/mL, $p<0.05$ and 1,228±98 pg/mL, $p<0.001$ for pMIC4-SAG1 and pMIC4-SAG1+pCTA$_2$/B groups, respectively) ↓ IL-4 in mice immunized with pMIC4-SAG1+pCTA$_2$/B (23±3 pg/mL, $p<0.05$)	NR	pMIC4-SAG1: prolonged survival time (death within 14 days, $p<0.05$) pMIC4-SAG1+ pCTA2/B: increased survival rate (14%, 20-day post challenge, $p<0.05$) All mice in the control groups died within 6-7 days.	These results provided a foundation for further studies toward the use of multiantigenic DNA vaccines combined with mucosal adjuvants to develop an efficient and long-term protective immunity against *T. gondii* and other intracellular parasite infections.	[10]
MIC6+PLP1	pIL-18	i.m	Kunming	20 and 80 cysts of strain PRU, i.g	Induced a strong IgG antibody response in the sera of mice immunized with pIRESneo-MIC6-TgPLP1 or pIRESneo-MIC6-TgPLP1+pVAX-IL-18 (especially in the latter group, $p<0.01$) ↑ Proliferation SI (6.74±0.14 and 8.63±0.15 in mice immunized with pIRESneo-MIC6-TgPLP1 and pIRESneo-MIC6-TgPLP1+pVAX-IL-18, respectively, $p<0.05$) ↑ IFN-γ, IL-2, IL-12, IL-4, and IL-10 ($p<0.05$)	Reduced (61.6% and 65.43% in mice immunized with pIRESneo-MIC6-TgPLP1 and pIRESneo-MIC6-TgPLP1+ pVAX-IL-18, respectively, $p<0.05$)	Prolonged survival time pIRESneo-MIC6-TgPLP1: 42.8±2.9 days ($p<0.05$) pIRESneo-MIC6-TgPLP1+ pVAX-IL-18: 45.0±2.9 days ($p<0.05$) Control mice were died within 25 days.	Immunization with the recombinant plasmid DNA encoding *T. gondii* TgPLP1 and MIC6 offers protective efficacy, and this is a promising vaccine candidate against chronic toxoplasmosis. The application of targeted stage-specific immunization strategies and/or combination with other effective antigens should improve the protective effect of TgPLP1 or MIC6 and potentially eliminate or significantly mitigate the risks of brain cyst reactivation during chronic infection by *T. gondii*.	[13]

MIC, microneme proteins; i.m, intramuscular; IFN-γ, interferon-γ; IL, interleukin; SI, stimulation index; i.n, intranasal.

https://doi.org/10.7774/cevr.2018.7.2.93 http://www.ecevr.org/

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Supplementary Table 3. Baseline characteristics of included studies based on immunization experiments with protein vaccines against *Toxoplasma gondii* in mouse models (single antigens)

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MIC1	FCA and FIA	s.c	C57BL/6 (H-2^b)	40 and 80 cysts of the ME49 strain, orally	Induced a strong IgG antibody response ($p<0.05$) Iduced mixed Th1/Th2 immune responses with predominance of IgG2b over IgG1 ($p<0.05$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ and IL-10 ($p<0.05$)	Reduced (52%, $p<0.05$)	Increased survival rate (50%, 30-day post challenge, $p<0.05$) Control mice were died within 11 days.	The use of this vaccine offers a promising strategy for conferring protection against toxoplasmosis.	[21]
MIC4	FCA and FIA	s.c	C57BL/6 (H-2^b)	40 and 80 cysts of the ME49 strain, orally	Induced a strong IgG antibody response ($p<0.05$) Induced mixed Th1/Th2 immune responses with predominance of IgG2b over IgG1 ($p<0.05$) ↑ Ssplenocyte proliferation ($p<0.05$) ↑ IFN-γ and IL-10 ($p<0.05$)	Reduced (46.9%, $p<0.05$)	Increased survival rate (50%, 30-day post challenge, $p<0.05$) Control mice were died within 11 days.	The use of this vaccine offers a promising strategy for conferring protection against toxoplasmosis.	[21]
MIC6	FCA and FIA	s.c	C57BL/6 (H-2^b)	40 and 80 cysts of the ME49 strain, orally	Induced a strong IgG antibody response ($p<0.05$) Iduced mixed Th1/Th2 immune responses with predominance of IgG2b over IgG1 (none significant) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ and IL-10 ($p<0.05$)	Reduced (27.2%, none-significant)	Increased survival rate (40%, 30-day post challenge, $p<0.05$) Control mice were died within 11 days.	The use of this vaccine offers a promising strategy for conferring protection against toxoplasmosis.	[21]

MIC, microneme proteins; FCA, Freund's complete adjuvant; IFN-γ, interferon-γ; IL, interleukin; Th1, T helper 1.

Supplementary Table 4. Baseline characteristics of included studies based on immunization experiments with protein vaccines against *Toxoplasma gondii* in mouse models (mixed antigens)

Antigen	Adjuvant or carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MIC1+MIC4	FCA and FIA	s.c	C57BL/6 (H-2^b)	40 and 80 cysts of the ME49 strain, orally	Mixed IgG1/IgG2a response ↑ IFN-γ (9,274±2,151 pg/mL, $p<0.05$), IL-2 (50±5 pg/mL, $p<0.05$), and IL-10 (1,608±380 pg/mL, $p<0.05$)	Reduced (68%, $p<0.05$)	Increased survival rate (80%, 30-day post challenge, $p<0.05$) Control mice were died within 11 days.	The data demonstrate that MIC1 and MIC4 triggered a protective response against toxoplasmosis, and that these antigens are targets for the further development of a vaccine.	[22]
	FCA and FIA	s.c	C57BL/6 (H-2^b)	40 and 80 cysts of the ME49 strain, orally	Induced a strong IgG antibody response ($p<0.05$) Induced mixed Th1/Th2 immune responses with predominance of IgG2b over IgG1 ($p<0.05$) ↑ IFN-γ, IL-12 p-40, and IL-10 ($p<0.05$)	Reduced (59%, $p<0.05$)	Increased survival rate (70%, 30-day post challenge, $p<0.05$) Control mice were died within 11 days.	Our results demonstrate that microneme proteins are potential vaccines against *T. gondii*, since their inoculation prevents or decreases the deleterious effects of the infection.	[21]
MIC1+MIC4+MIC6	FCA and FIA	s.c	C57BL/6 (H-2^b)	40 and 80 cysts of the ME49 strain, orally	Induced a strong IgG antibody response ($p<0.05$)	Reduced (67.8%, $p<0.05$)	Increased survival rate (80%, 30-day post challenge, $p<0.05$)	Our results demonstrate that microneme proteins are potential vaccines against *T. gondii*, since their inoculation prevents or decreases the deleterious effects of the infection.	[21]

MIC, microneme proteins; s.c, subcutaneous; IFN-γ, interferon-γ; IL, interleukin; Th1, T helper 1.

Supplementary Table 5. Examples of immunization with live-attenuated vectors expressing *Toxoplasma gondii* MICs in mouse models

Antigen	Adjuvant/ Carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MIC3	-/pseudorabies virus (PRV)	i.m	BALB/c	1×10^2 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response Developed neutralizing antibodies ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ, IL-2, and IL-10 ($p<0.05$) Similar levels of IL-4 in all groups ($p>0.05$)	NR	Increased survival rate (50%, 28-day post challenge, $p<0.05$) Control mice were died within 9-10 days.	These results suggested that expression of protective Ag of *T. gondii* in PRV is a novel approach towards the development of a vaccine against txoplasmosis.	[23]
	-/baculovirus (bv)	i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.01$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ ($p<0.05$) Similar levels of IL-4 and IL-10 between the different immunized and unimmunized groups ($p>0.05$)	NR	Prolonged survival time ($p<0.05$) Control mice were died within 4 days.	These results suggest that an excellent vector-mediated vaccine strategy might be used to develop a new generation of vaccines against *T. gondii* infection.	[5]
	-/*S. typhimurium* strain SV4089 Dam– and PhoP– mutant	Orally	ICR	5×10^2 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ Splenocyte proliferation ($p<0.05$) Mixed IgG1/IgG2a response with the predominance of IgG2a over IgG1 ($p<0.05$) ↑ IFN-γ (721 ± 142 pg/mL, $p<0.01$)	NR	Prolonged survival time (11 days compared with 6 days in control, $p<0.05$)	This study preliminarily shows that attenuated *S. typhimurium* strain (Dam– and PhoP–) could be utilized as an oral delivery vector for recombinant eukaryotic expression plasmids as DNA vaccines for prevention from toxoplasmosis.	[24]
MIC8	-/VLP recombinant baculovirus (rBV) influenza matrix protein 1 (M1)	i.m, i.n	BALB/c	1×10^5 Tachyzoites, RH strain, orally	i.n mice group showed higher levels of *T. gondii*-specific IgG antibody response compared to i.m mice group ($p<0.01$). i.n mice group showed higher levels of *T. gondii*-specific IgG1 antibody response compared to IgG2a and IgG2b antibody responses, indicating that i.n administration induced Th2-dominant responses. Higher levels of *T. gondii*-specific IgG or IgA antibodies were detected in serum or feces in i.n mice groups ($p<0.01$). Significant reduction of tachyzoites recovered from abdominal cavities in i.n mice compared to i.m as neutralizing antibody response ($p<0.01$). Higher populations of CD_4^+ and CD_8^+ T cells and germinal center B cells were found in i.n and i.m mice groups compared to non-infected naïve and non-immunized control groups. Significantly higher populations of germinal center B cells (14.4%), CD_4^+ T cells (29.84%) and CD_8^+ T cells (12.55%) were observed in i.n mice group compared to that in i.m group ($p<0.05$, $p<0.01$).	NR	Increased survival rate i.n group: 100%, 16-day post challenge i.m group: 60%, 16-day post challenge Control mice were died within 12 days.	Our study shows the effective protection against *T. gondii* infection provided by VLPs containing MIC8 of *T. gondii*, thus indicating a potential *T. gondii* vaccine candidate.	[25]

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https://doi.org/10.7774/cevr.2018.7.2.93

Supplementary Table 5. Continued

Antigen	Adjuvant/ Carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
MIC3+SAG1	-/pseudorabies virus (PRV)	i.m	BALB/c	1×10^2 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response Developed neutralizing antibodies ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ, IL-2, and IL-10 ($p<0.05$) Similar levels of IL-4 in all groups ($p>0.05$)	NR	Increased survival rate (66.7%, 28-day post challenge, $p<0.05$) Control mice were died within 9-10 days.	These results suggested that expression of protective antigens of *T. gondii* in PRV is a novel approach towards the development of a vaccine against txoplasmosis.	[23]
	-/baculovirus (bv)	i.m	BALB/c	1×10^3 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.01$) ↑ Splenocyte proliferation ($p<0.05$) ↑ IFN-γ ($p<0.05$) Similar levels of IL-4 and IL-10 between the different immunized and unimmunized groups ($p>0.05$)	NR	Increased survival rate (50%, 22-day post challenge, $p<0.05$) Control mice were died within 4 days.	These results suggest that an excellent vector-mediated vaccine cocktail strategy might be used to develop a new generation of vaccines against *T. gondii* infection.	[5]
	-/*S. typhimurium* strain SV4089 Dam– and PhoP– mutant	Orally	ICR	5×10^2 Tachyzoites, RH strain, i.p	Induced a strong IgG antibody response ($p<0.05$) ↑ Splenocyte proliferation ($p<0.05$) Mixed IgG1/IgG2a response with the predominance of IgG2a over IgG1 ($p<0.05$) ↑ IFN-γ (1,089±163 pg/mL, $p<0.01$)	NR	Increased survival rate ($p<0.05$)	The current study shows that the oral multi-antigenic DNA vaccine, ZJ111/pSAG1-MIC3, produces partial protection against *T. gondii* challenge.	[24]
Encoding MAS and UMAS $ROP18_{347–396}$, $SAG3_{101–144}$, $MIC6_{288–347}$, $GRA7_{182–224}$, $MAG1_{58–125}$, $BAG1_{156–211}$, and $SPA_{142–200}$ DNA vaccine or/and Ad vaccine	Ubiquitin/Ad	DNA vaccines (p-MAS or p-UMAS plasmid, 100 μg each), i.m or recombinant Ad vaccine (Ad-UMAS virus, 3×10^8 PFU each), i.m or the combination of DNA vaccine (p-UMAS, 100 μg each) and recombinant Ad vaccine (Ad-UMAS virus, 3×10^8 PFU each).	BALB/c	Acute: 1×10^3 tachyzoites, RH strain (genotype I), i.p Chronic: 20 cysts PRU strain (genotype II), i.g via oral gavage	Induced a strong IgG antibody response in both p-MAS and p-UMAS immunized mice (especially in the p-UMAS group), compared to control groups. ↑ Splenocyte proliferation in both p-MAS and p-UMAS immunized mice (a further 30% increase in latter group) ↑ IFN-γ and IL-2 secretion in both p-MAS and p-UMAS immunized mice (especially in the p-UMAS group), compared to control groups ↑ Levels of an IgG1 and IgG2a in p-MAS and p-UMAS immunized mice (predominance of IgG2a over IgG1), compared to control groups ↑ Percentages of $CD4^+$ T and $CD8^+$ cells in p-MAS and p-UMAS groups Significantly higher levels of IFN-γ and IL-2 secretion and increased splenocyte proliferation in Ad-UMAS immunized mice compared with p-UMAS group ($p<0.05$) ↑ Percentages of $CD8^+$ T cells in immunized with Ad-UMAS compared with p-UMAS group ($p<0.05$)	Reduced ($p<0.01$). The brain cyst burden was 50% lower in p-MAS group (833±116), compared with the control groups p-UMAS (570±98) Ad-UMAS (469±103)	Increased survival rate p-MAS: 33% survival 28 days after challenge p-UMAS: 50% survival 28 days after challenge Control mice were died within 8-10 days.	Distinct humoral and cellular immunity induced by immunization with DNA vaccine and recombinant Ad vaccine encoding ubiquitin conjugated multistage Ag of *T. gondii*. The DNA vaccine had the advantage of inducing a stronger humoral response, whereas the Ad-vectored vaccine improved the cellular immune response.	[26]

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Supplementary Table 5. Continued

Antigen	Adjuvant/ Carrier	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
Encoding Ad-UMAS ROP18$_{347–396}$, SAG3$_{101–144}$, MIC6$_{288–347}$, GRA7$_{182–224}$, MAG1$_{58–125}$, BAG1$_{156–211}$, and SPA$_{142–200}$	Ubiquitin/Ad	i.m, i.n, s.c, i.o, i.v	BALB/c	Acute: 1×10^3 tachyzoites, RH strain (type I), i.p Chronic: 20 cysts PRU strain (type II), i.g via oral gavage	↑ Levels of *T. gondii*-specific IgG antibodies in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$). Highest titer of IgG antibody was observed by i.m route and followed by s.c, i.n, i.o and i.v. ↑ IgG subtypes in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$) Significantly higher values of IgG2a in i.m and s.c vaccination groups, compared with other vaccination routes Significantly higher values of IgA in i.n and i.o vaccination groups, compared with other vaccination routes ↑ Percentages of $CD4^+$ and $CD8^+$ T cells in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$). Significantly higher percentages of $CD4^+$ and $CD8^+$ T cells in i.n and i.o vaccination groups, compared with other vaccination routes ↑ IFN-γ and IL-2 in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$) Significantly higher secretion of IFN-γ and IL-2 in i.n and i.o vaccination groups, compared with other vaccination routes ↑ lymphocyte proliferation ability in the five Ad-UMAS immunization routes, compared to the controls ($p<0.05$) Significantly higher lymphocyte proliferation ability in i.n and i.o vaccination groups, compared with other vaccination routes	Reduced ($p<0.05$)	Increased survival rate i.m, i.o, and i.n vaccinated groups: 50% survival rate 28 days after challenge i.v and s.c vaccinated groups: 40% survival rate 28 days after challenge. All the control mice died within 8 days.	Ad-UMAS could be an effective and safe mucosal candidate vaccine to protect animals and humans against *T. gondii* infection.	[27]

MIC, microneme proteins; i.m, intramuscular; IFN-γ, interferon-γ; IL, interleukin; i.p, intraperitoneal; i.n, intranasal; NR, Not reported; s.c, subcutaneous; i.o, intraoral; i.v, intravenous.

https://doi.org/10.7774/cevr.2018.7.2.93 http://www.ecevr.org/

Supplementary Table 6. Examples of heterologous prime-boost immunization against *Toxoplasma gondii* in mouse models

Antigen/Adjuva nt	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
AMA1/Gold particles Prime/boost: pAMA1/Ad5AMA1 pAMA1/Ad5Null pNull/Ad5AMA1 pNull/Ad5Null	Gene gun into abdomen	C57BL/6	1×10^3 PLK-GFP of *T. gondii* tachyzoites, i.p	↑ Significantly IgG antibodies in pAMA1/Ad5AMA1, pAMA1/Ad5Null, and pNull/Ad5AMA1 groups, compared with those of control mice (immunized mice with pNull/Ad5Null) Mice that were primed with pAMA1 and boosted with Ad5AMA1 showed significant increase ($p<0.001$) in anti-TgAMA1 IgG when compared with those immunized with either pAMA1/Ad5Null or pNull/Ad5Null. The pAMA1/ Ad5AMA1-immunized mice had a robust IgG1, and IgG2c antibody response to the TgAMA1 compared with that of either pAMA1/Ad5Null or pNull/Ad5Null, and the robust responses were significantly enhanced ($p<0.001$) after the booster immunization. The levels of the TgAMA1-specific IgG2c antibody were higher in mice immunized with pNull/Ad5AMA1 than in those immunized with pAMA1/Ad5AMA1 ($p<0.05$) and pAMA1/Ad5Null ($p<0.001$). There was no significant difference in the IgG1:IgG2c ratio between pAMA1/Ad5AMA1 and pNull/Ad5AMA1 immunized groups. The pNull/Ad5AMA1 immunized mice had significantly higher levels of IFN-γ, as compared with the mice immunized with pAMA1/Ad5AMA1 ($p<0.01$) and pAMA1/Ad5Null ($p<0.001$). The pAMA1/Ad5AMA1 immunized mice had higher levels of IL-4 than those immunized with pNull/Ad5AMA1 ($p<0.05$) and pNull/Ad5Null ($p<0.01$).	The pAMA1/Ad5AMA1-immunized mice produced 23% fewer brain cysts than the pNull/Ad5AMA1-immunized mice (none-significant).	Increased survival rate pAMA1/Ad5AMA1: 50%, 30-day post challenge pNull/Ad5AMA1: 37.5%, 30-day post challenge pAMA1/Ad5Null: 12.5%, 30-day post challenge None of the mice immunized with pNull/Ad5Null survived	These results demonstrate that the heterologous DNA priming and recombinant adenovirus boost strategy may provide protective immunity against *T. gondii* infection.	[28]

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Supplementary Table 6. Continued

Antigen/Adjuva nt	Ag delivery	Mouse strain	Challenge	Immune responses	Brain cyst load	Survival	Conclusions or suggestions	Reference
Encoding MAS and UMAS ROP18$_{347–396}$, SAG3$_{101–144}$, MIC6$_{288–347}$, GRA7$_{182–224}$, MAG1$_{58–125}$, BAG1$_{156–211}$, and SPA$_{142–200}$ DNA vaccine or/and adenovirus vaccine Prime/boost: DNA/DNA (p-UMAS/p-UMAS) Ad/Ad (Ad-UMAS/Ad-UMAS) DNA/Ad (p-UMAS/Ad-UMAS) Ad/DNA (Ad-UMAS/p-UMAS)	The combination of DNA vaccine (p-UMAS, 100 μg each) and recombinant adenovirus vaccine (Ad-UMAS virus, 3×10^8 PFU each), i.m	BALB/c	Acute: 1×10^3 tachyzoites, RH strain (genotype I), i.p Chronic: 20 cysts PRU strain (genotype II), i.g via oral gavage	Highest levels of humoral antibodies and cellular immune responses were achieved in mice immunization priming with the DNA vaccine and boosting with the Ad-UMAS vaccine. Compared with p-UMAS or Ad-UMAS immunization alone, higher levels of a specific IgG (predominance of IgG2a) and higher levels of cytokines (IFN-γ and IL-2) were obtained by priming with p-UMAS and boosting with Ad-UMAS ($p<0.05$). Priming with p-UMAS and boosting with Ad-UMAS demonstrated higher proliferation activity, compared with the other immunization strategy ($p<0.05$).	Reduced ($p<0.01$) The most significant reduction of brain cyst burden was observed by the DNA prime-Ad boost approach.	Increased survival rate 67% Survival in mice vaccinated with p-UMAS prime and Ad-UMAS boost 28 days after challenge Control mice were died within 8-10 days.	Priming vaccination with DNA vaccine and boosting with the recombinant Ad vaccine encoding ubiquitin conjugated multi-stage antigens of *T. gondii* was proved to be a potential strategy against the infection of type I and type II parasite.	[26]
rTgMIC3/FCA+pcDNA-MIC3+rTgMIC3/FIA	i.m	BALB/c	1×10^2 tachyzoites, RH strain, i.p	↑ Levels of IgG antibodies ($p<0.05$)	NR	Prolonged survival time (10 days compared with 7 days in control)	These results demonstrate that TgMIC3 could elicit some protection against toxoplasmosis.	[8]

i.p, intraperitoneal; IFN-γ, interferon-γ; IL, interleukin; NR, Not reported.

https://doi.org/10.7774/cevr.2018.7.2.93 http://www.ecevr.org/

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