Research advances in microneme protein 3 of Toxoplasma gondii

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Abstract

Toxoplasma gondii (T. gondii) is an obligate intracellular protozoan parasite. It has extensive host populations and is prevalent globally; T. gondii infection can cause a zoonotic parasitic disease. Microneme protein 3 (MIC3) is a secreted protein that is expressed in all stages of the T. gondii life cycle. It has strong immunoreactivity and plays an important role in the recognition, adhesion and invasion of host cells by T. gondii. This article reviews the molecular structure of MIC3, its role in the invasion of host cells by parasites, its relationship with parasite virulence, and its induction of immune protection to lay a solid foundation for an in-depth study of potential diagnostic agents and vaccines for preventing toxoplasmosis.

Keywords: Toxoplasmosis, Microneme protein 3, Function, Diagnostic agents and vaccines

Review

Background

Toxoplasma gondii (T. gondii) is an obligate intracellular protozoan parasite (Fig. 1) [1]. It has extensive host populations, is prevalent globally, and can cause a zoonotic parasitic disease [2, 3]. As an opportunistic infection factor, T. gondii can cause death in patients with impaired immune function or immune suppression, such as acquired immunodeficiency syndrome (AIDS) patients, organ transplantation patients, and malignant tumor patients. Toxoplasmosis is also an important biological factor influencing human prenatal and postnatal care because if a pregnant woman is infected with T. gondii, maternal-fetal vertical transmission may occur and result in miscarriage, stillbirth or congenital defects or deformities (malformation, retardation, etc.) in fetus [4]. The major hazards after T. gondii infection in animals is the treatment cost after outbreak of toxoplasmosis and the direct loss caused by animal death; another hazard is the indirect loss caused by long-term parasite hosting to become a potential infection source and cause reproductive disorders [5].

T. gondii is highly evolved to form a complex, well-coordinated system. It regulates the secretion pathway to supplement proteins for immune regulation, gliding motility, nutritional requirements in the intracellular survival and replication process after invasion into hosts, and the final release after invasion [6]. Continuous observation of the process of invasion into hosts by T. gondii by electron microscopy showed that the invasion of host cells by T. gondii is not similar to that of other intracellular pathogens that require making full use of the phagocytosis function of host cells for invasion. T. gondii utilize an active invasion process, relying on its energy and the “gliding motility” produced by its actin-myosin system (Fig. 2) [7–11]. The completion of this process depends on a series of functions by different organelles at specific times, including microneme proteins (MIC), rhoptry proteins (ROP), and dense granule antigens (GRA) [12]. Among these, MICs secreted by the microneme play a basic role in the recognition, adhesion, and invasion of parasites into host cells during the invasion process. At the early stage of contact between parasites and host cells, MICs are first secreted from the apex of tachyzoites and facilitate adhesion through the recognition of receptors on the cell membrane of hosts; thus, they play an important role in the early stage of the invasion of host cells by parasites [13]. Currently, there are at least 19 types of known MICs (Table 1) [14–34], including MIC1-MIC12,
AMA1, M2AP, SUB1, ROM1, SPATR, PLP1, and TLN4, of which 10 types (TgMIC1-4, TgMIC6-9, TgMIC12, and SPATR) contain different adhesion domains similar to the adhesion molecules in eukaryotic cells (integrin-like domain, thrombospondin type-1 repeat (TSR), epidermal-growth factor (EGF)-like domain, chitin binding-like (CBL) domain, and the Apple domain). These proteins play a role in the process of recognition and adhesion of host cells by parasites [6]. MIC3 can bind to the receptors of a variety of host cells through its ligand structural domain; thus, it is closely associated with the invasion of host cells by parasites and the virulence of the infection. In addition, MIC3 is expressed in the tachyzoite, bradyzoite, and sporozoite stages and has excellent immune effects. Therefore, MIC3 has received extensive attention. This article reviews the structure and function of MIC3, its function in the invasion of host cells by parasites, its association with the virulence of parasites, and its induced immune protection.

**Molecular characteristics of MIC3**

The length of the mic3 gene sequence is 1,080 bp; the sequence encodes 359 amino acids and a protein with a molecular weight of 38 kD. The mic3 gene is a single copy gene; it does not have any introns, and there is not a eukaryote-specific TATA or CCAAT promoter sequence at the 5’-end non-coding region. The complete open reading frame defined by the first ATG codon encodes 359 amino acids, which contain 34 cysteine residues. There is a short hydrophobic signal peptide sequence at the N terminus, and there is no transmembrane protein domain. There is 1 potential glycosylation site and 12 phosphorylation sites. Amino acid sequence identities of MIC3 among different Toxoplasma strains are very high, more than 98.3 % (Fig. 3). In contrast, large sequence differences exist among different apicomplexan parasites (Fig. 3). MIC3 structure contains 5 partially overlapped EGF-like domains, of which 3 domains form a tandem repeat and the other 2 domains are overlapped with...
the former 3 domains, as well as a CBL domain (Fig. 4). The CBL domain may be involved in protein-protein and protein-carbohydrate interactions [18]. Cérède et al. reported that the MIC3 of T. gondii tachyzoites is a 38-kDa (monomer) end product from the proteolytic digestion of a 40-kDa precursor, which is then folded into a 90-kDa dimer [35]. These specific structures are closely associated with the function of MIC3. Amino acid mutagenesis experiments confirmed that the formation of MIC3 dimers did not rely on disulfide bonds between peptides and may be instead associated with the interaction between proteins. The dimer is necessary for the adhesion function. EGF-like domains are present in many proteins; they are distributed in the extracellular region of membrane-binding proteins or secreted proteins through a tandem repeat form. Their function is still not completely clear.

Fig. 2 Host cell invasion by T. gondii. Reprinted from [11], Copyright © 2004, with permission from Elsevier. a Schematic representation of T. gondii tachyzoite and the subcellular structures involved in gliding motility and host cell invasion. b Cycle of host cell invasion and egress by T. gondii. This multi-step process includes the attachment to host cells, the discharge by the micronemes (red), the discharge by the rhoptries (yellow), the formation and sealing of the parasitophorous vacuole, intracellular parasite replication, lysis of the PVM and parasite egress [11].
To investigate whether MIC3 interacted with the EGF-like receptors of host cells through the EGF-like domain, adhesion experiments were conducted between MIC3 and cells. The experimental results showed that regardless of whether cells had EGF-like receptors, there were no differences among the adhesion results. This finding indicates that the EGF-like domain was either not involved in adhesion between MIC3 and cells or that the EGF-like domain did not interact with the EGF-like receptors in host cells. Through the expression of different MIC3 fragments on the surface of host cells, it was elucidated that the CBL domain was necessary for the adhesion characteristics of MIC3. In contrast, the EGF-like domain could not promote adhesion of parasite MIC3 to host cells but may enable the accurate presentation of the adhesion structure from the structure formation aspect.

The MIC3 complex of *T. gondii*

During the process of secretion, transport, and release, MICs interact with other MICs to function in a complex form. Currently, the known MIC complexes include the MIC1/4/6 complex, MIC3/8 complex, MIC2/M2AP complex and AMA1/RON2/RON4/RON5/RON8. At least one protein in the complex contains a transmembrane domain and a cytoplasmic tail. The cytoplasmic tail contains a sorting signal. The sorting signal is necessary for transporting the complex from the endoplasmic reticulum (ER) to the relevant locations in the micronemes; thus, this protein is defined as an “escorter” [13, 20] and is called a transmembrane protein. MIC8 is the escorter of the MIC3/8 complex (Fig. 5) [13]. MIC3 reaches micronemes under the escort of its escorter, MIC8. It is discharged outside parasites during the process of invasion of the host cell by parasites and is then re-localized on the surface of the host cells (Fig. 6) [18]. Proteins that interact with escorters are soluble proteins. The normal execution of transmembrane protein functions also depends on its interacting soluble proteins. Only following an interaction between soluble proteins and transmembrane proteins can they be correctly folded to form a complex with biological functions; otherwise, the function of the complex will be damaged. For example, following disruption of the mic1 gene of *T. gondii*, its partner proteins, MIC6 and MIC4, remained in the Golgi apparatus [20]; when the m2ap gene of *T. gondii* was knocked out, MIC2 was retained and accumulated in the Golgi apparatus, thus causing a significant decrease in MIC expression levels [27]. Although the interactions between proteins are very important, there are some exceptions. For example, after the mic3 gene of *T. gondii* was knocked out, the transport of

| MICs   | MW(kDa)* | Domains/homologies                          | Major putative functions                                      | References |
|--------|----------|---------------------------------------------|--------------------------------------------------------------|------------|
| MIC1   | 49       | 2 TSRs                                      | Transport/folding of MIC4 and MIC6 [14, 15]                  |            |
|        |          |                                             | Binding to host cells                                        |            |
| MIC2   | 83       | 1 Integrin, 5 TSRs, and 1 TM                | Transport M2AP and adhesion [16, 17]                        |            |
| MIC3   | 38(90 dimer) | 1 CBL and 5 EGF                            | Adhesion [14, 18]                                           |            |
| MIC4   | 63       | 6 Apples                                    | Adhesion [19, 20]                                           |            |
| MIC5   | 20       | Parvulin-like PPIase motif                  | Supressing TgSUB1 activity [21]                             |            |
| MIC6   | 37       | 3 EGF-like                                  | Escorter [20]                                               |            |
| MIC7   | 36       | 5 EGF-like and 1 TM                         | unknown [13]                                                |            |
| MIC8   | 75       | 1 CBL, 10 EGF-like, and 1 TM               | Escorter [13]                                               |            |
| MIC9   | 32       | 3 EGF and 1 TM                              | unknown [13]                                                |            |
| MIC10  | 23       | unknown                                     |                                                               |            |
| MIC11  | 22       | unknown                                     |                                                               |            |
| MIC12  | 234      | 31 EGF, 4 TSRs, and 1 TM                   | unknown [24]                                                |            |
| AMA1   | 60       | Cysteine rich                               | Inhibiting secretion of the rhoptries [25]                   |            |
| M2AP   | 35       | Beta and coil                               | Transport/folding of MIC2 [26, 27]                           |            |
| SUB1   | 85       | Subtilase and GPI                           | Proteolysis [28]                                            |            |
| ROM1   | 28       | Rhomboid                                    | Proteolysis [29, 30]                                         |            |
| SPATR  | 47.5b    | 1 thrombomodulin EGF, 2 TSRs                | Trafficking of the protein [31]                              |            |
| PLP1   | 124      |                                            | Cytolysis and parasite egress [32, 33]                       |            |
| TNL4   | 256      |                                            | unknown                                                     | [34]       |

*aBased on the complete open reading frame including signal sequence or GPI anchor signal; *bPredicted molecular weight (MW) after removal of the signal peptide.

Abbreviation: TM transmembrane

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Table 1 Properties of *Toxoplasma* microneme proteins
MIC8 to the micronemes and secretion of MIC8 in the micronemes were not affected [14]. These stable complexes better explain how soluble proteins are sorted after regulation and secretion and how soluble adhesins assist the invasion process [36, 37]. However, whether the functions of these complexes in the motility and cell invasion processes of *T. gondii* are repeated or redundant are still unknown [14, 25].

**Regulatory mechanisms of MIC secretion**

Micronemes are secretion organs that are located at the apical ends of parasites. MICs are synthesized at the rough ER, are transported to micronemes through the Golgi apparatus and are secreted outside the body. It is generally accepted that the secretion of *T. gondii* MICs is only regulated by the intracellular calcium ion levels in parasites [38]. Under normal conditions, *T. gondii* tachyzoites secret MICs at a very low level; when the intracellular calcium ion level increases, the secretion of MICs can increase 10-100-fold [39]. The imbalance of calcium ions will induce the deregulation of MIC secretion.

Some reagents that can neutralize intracellular calcium ions can strongly inhibit MIC secretion by micronemes. The regulation of MIC secretion by calcium ions in parasites does not require the participation of exogenous calcium ions [40, 41]. Additionally, through the research of microneme secretion based on levels of MIC5, Carey *et al.* extrapolated that there could be two types of micronemes: one that discharges rapidly in response to elevated intracellular calcium, and one that discharges constitutively [42]. Further larger trials are needed to determine whether two types of micronemes exist.

**MIC3 protein folding and hydrolysis**

Newly synthesized proteins require structural modification at the ER. Misfolded or incompletely folded oligomeric complexes will be retained at the ER and will finally be degraded. There are some molecular chaperones and folding enzymes at the ER; they can assist the correct folding and assembly of proteins and can provide anchor points for retained immature proteins [43]. The retention functions of ER rely on different folding sensors; the N-glycans on the surface of misfolded proteins can be recognized by calreticulin and calnexin. The exposed cysteine residue plays a role in thiol-mediated...
protein retention; under this condition, thiol oxidoreductase on the ER membrane is associated with the retention of unassembled proteins. MIC3, MIC4, and MIC6 are rich in cysteine residues [35]. When the early peptides are transported to the ER, cysteine residues form disulfide conjugates to perform correct folding. Incorrectly folded proteins may form aggregates and cannot be correctly transported.

The hydrolysis processing of MICs is very common and is necessary for the smooth completion of the invasion process, which mainly occurs in 2 stages. The first stage occurs at the secretory pathway, that is, transportation to the micronemes through the Golgi apparatus after synthesis by the rough ER. The hydrolysis process at this stage is associated with the formation of specific complexes, correct targeting of complexes to organelles, and masking of enzyme active sites. MIC3, MIC5, MIC6, and M2AP are actively involved in this stage [20, 44]. The second stage occurs after extracellular secretion and discharge during the process of the invasion of host cells by T. gondii. The processing at the second stage is helpful for the completion of complex separation, host cell adhesion, and invasion of parasites. For example, the protein processing of MIC2, MIC4, MIC6, and MIC8 induce the rapid release of MICs on the cell surface, which is a key step for completion of the cell invasion. If processing is lacking, secreted MICs can establish persistent, stable bridges on the surface of host cells and parasites, thus interfering with the newly formed correct closure of parasitic vesicles and inhibiting the proliferation of parasites in vacuoles. Studies showed that unprocessed MICs increased adhesion to host cells; however, the invaded parasites decreased. Many MICs are produced as a pre-protein form and go through proteolytic processing during the secretion pathway and transport process. When crossing the Golgi apparatus, the first EGF-like domain of MIC6 is removed [13]; the function of this process is unknown, but this step does not affect the microneme targeting ability of the interaction between MIC6 and MIC1 [20]. MIC3 is also initially synthesized as a 40-kDa dimeric precursor, which then becomes a 38-kDa product through proteolysis before reaching the micronemes [45]. The removal of the leader peptides of MICs is necessary for interaction with the host cells [46]. The
enzymes that hydrolyze *T. gondii* MICs have not been completely clarified.

**The function of MIC3 in the process of invasion of the host cells by parasites**

*T. gondii* can invade almost all nucleated cells, indicating that there are multiple "receptor-ligand" interactions between parasites and host cells. Although the mechanisms underlying the invasion of host cells by *T. gondii* have not been completely clarified, current studies indicate that *T. gondii* actively invades cells, unlike the endocytosis processes of infection used by bacteria and viruses. When *T. gondii* contacts host cells, parasites first push the conoid outside the body, and then, micronemes discharge a large amount of MICs through the conoid. MICs interact with corresponding receptors on the host cell membrane and are distributed on the surface of parasites. The front end of parasites, which interacts with cells, forms a moving junction [47]; next, the actin-myosin system exerts its function. At the moving junction of parasites, MICs move from the front end of parasites to the rear end of parasites through the force produced by actin proteins and are finally hydrolyzed; parasites invade cells through the moving junctions [8, 11, 35, 47, 48]. Therefore, MICs, including MIC3, play a bridging role between parasites and host cells.

It has been shown that mic1 knockout alone affected the invasion of HFF cells by *T. gondii* and that mic3 knockout alone did not affect the invasion; the results of a mic3/mic1 double knockout did not show significant differences from the mic1 knockout. These results indicate that these 2 proteins did not have synergistic effects on *T. gondii* invasion [49]. These results contradict those showing that MIC3 was secreted outside cells during the invasion process of *T. gondii* and adhered to a variety of cells, including fibroblasts, macrophages, and epithelial cells. Cérède *et al.* [14] proposed that MICs may contain a large number of components and that different components could recognize the same receptor. For example, during the infection of HFF cells after mic3 knockout, other MICs would interact with the cell surface receptors; however, MIC3 may be indispensable for the infection of other cell types. In addition, during the invasion process, parasites may have alternative invasion routes. Furthermore, *in vitro* culture and natural infection methods may display differences; in *in vitro* culture, the disappearance of one gene function may be compensated for by other MICs or may up-regulate the secretion of other MICs to allow for normal invasion; however, this gene may be very important in natural infection conditions.

**The association between MIC3 and the virulence of parasites**

MICs contain a series of conserved adhesion domains, and some domains have been confirmed to be associated with parasite invasion [14]. Following infection of HFF cells after the *T. gondii* mic3 gene was knocked out, the adhesion characteristics were not affected, but the parasite virulence significantly reduced. *T. gondii* with gene disruption of mic1 or mic3 were inoculated into mice, and all of the inoculated mice died after 9–22 days of inoculation; mice in the control group without gene knockout all died within 7–10 days. These results indicated that after *T. gondii* mic1 and mic3 were separately knocked out, the virulence of *T. gondii* only decreased slightly. *T. gondii* with both the mic1 and mic3 genes knocked out were inoculated into mice, and the results showed that only 1 mouse among the 10 mice died and that the death time of mouse was delayed by 10 days compared to that of the control group. In addition, the dose was adjusted and inoculated into mice to cause the death of all mice on day 9; comparison of lethal doses with the control group showed that the inoculation amount of the
double gene knockout T. gondii was $2 \times 10^3$ parasite/mouse and that the dose in the control group was lower than 20 parasite/mouse. These results indicate that knockout of the mic1 and mic3 genes together significantly reduced the parasite virulence and confirmed that MIC1 and MIC3 had synergistic effects on the parasite virulence [14].

MIC3 has a very strong affinity for host cells, which is closely associated with its CBL domain [35]. The common sequences of CBL domain contain 8 disulfide bond-linked cysteine residues and several highly conserved aromatic residues. BHK-21 cells were transfected with the recombinant MIC3 plasmid, and the location of the expression product was detected. The results showed that the expression product of the recombinant MIC3 with CBL domain knockout was in the supernatant, whereas the expression product of the recombinant MIC3 without CBL domain knockout was on the cell surface. These results indicate that the CBL domain of T. gondii MIC3 is very important for cell adhesion.

In CBL domain, Cysteines determine the protein conformation, and aromatic residues react with N-acetylglucosamine. Transfection of BHK-21 cells with the recombinant MIC3 plasmid in which all the cysteine residues in CBL domain were replaced by glycine residues resulted in insufficient secretion and accumulation of the expression product, indicating that cysteine residues determine the protein conformation. In the aromatic residue replacement experiment, the F121A and Y141A mutants influenced the protein expression in BHK-21 cells; however, the adhesion characteristics of these two residues could not be confirmed. The transfection experiments of the other recombinants (Y96A, F97A, P103A, W126A, F128A, and Y135A) proved that only the expression products of the W126A and F128A mutants were in the supernatant and did not bind to the surface of transfected BHK-21 cells, indicating that the replacement of W126 or F128 alone resulted in the complete loss of adhesion function and that they were closely associated with the interaction with cell surface receptors and adhesion. The replacement of tryptophan at position 126 with glycine (W126A) and the replacement of phenylalanine at position 128 with glycine (F128A) in the CBL domain of MIC3 caused a significant reduction of virulence of the mutant strains. These studies indicated that the MIC3 CBL domain that is associated with adhesion is very important for the virulence of parasites [14].

**Induction of immune protection by MIC3**

MIC3 plays important roles in the invasion process of T. gondii; it has very strong immunogenicity and can stimulate the production of corresponding humoral immunity and cellular immunity in the body. Therefore, in studies of vaccines against T. gondii infection, MICs have received much attention. Vaccines based on MIC3 include live attenuated vaccines, subunit vaccines, and DNA vaccines.

**Gene deletion attenuated vaccine based on MIC3 of T. gondii**

Some scholars have performed studies on live attenuated vaccines by knocking out double virulence genes of parasites. A mutant strain of T. gondii (RH strain) lacking the mic1 and mic3 genes (Mic1-3KO) tachyzoites to immunize mice and that showed mice could produce a strong protective function [14, 49]; not only could the mother defend against T. gondii infection but also vertical transmission could be effectively prevented [49]. Of note, the MIC1-3KO attenuated strain induced good immune effects in murine models and also had ideal immune effects in large animal models. Subcutaneously immunized ewes with MIC1-3KO tachyzoites showed that IgG antibodies occurred in serum samples. Ewes at mid-gestation were challenged with the PRU strain of T. gondii, and the results showed that pregnant ewes in the vaccinated group only had a slight febrile response, whereas unvaccinated ewes produced a more severe, characteristic febrile response of a longer duration; all unvaccinated ewes aborted. The survival rates of newborn lambs in the vaccinated group were 62 %, 91 %, and 64 %, and there were no clinical signs of infection. A dose of $10^5$ MIC1-3KO tachyzoites was sufficient to induce protective immune responses and had very good preventive function against toxoplasmosis-induced abortion in ewes [50]. At present the strain MIC1-3KO is the main product of a French innovative company (VitamFero SA) and it is called "Toxo KO" vaccine. "Toxo KO" vaccine represents a new generation of live and attenuated vaccines obtained through the deletion of 2 genes of virulence, and the risk of reversion to virulence is totally eliminated (http://www.vitamfero.com/en/technology.html). "Toxo KO" vaccine will play an important role in preventing ovine and feline toxoplasmosis.

**Subunit vaccines based on MIC3 of T. gondii**

Subunit vaccines are vaccines developed using DNA recombinant technology to introduce antigenic genes of pathogens into prokaryotic or eukaryotic expression systems to express the corresponding antigenic proteins; these vaccines have the advantages of easy preparation of antigenic proteins and high yields. Nie et al. [51] vaccinated BALB/c mice with recombinant pseudorabies virus (PRV) expressing MIC3 (rPRV-MIC3), and the results showed that rPRV-MIC3 could induce significant humoral and Th1 cellular immune responses. After being challenged by the T. gondii RH strain at a lethal dose (100 tachyzoites), the survival rate of immunized mice was 50 %, and no protection was found in control groups. These results indicated that rMIC3 had better immunogenicity and could produce a certain protective immunity, which provided reliable bases for the development of MIC3 subunit vaccines for T. gondii. However,
current subunit vaccines still have issues such as insufficient immunogenicity and protective function and too short of a persistent time of the immune responses.

Nucleic acid vaccines based on MIC3 of T. gondii

T. gondii belongs to strictly intracellular parasitic protozoa. In induced immune responses in the body, cellular immunity is more important than humoral immunity; especially in the acute infection phase, cellular immunity usually plays a decisive role. DNA vaccines express corresponding antigenic proteins in host cells and comprehensively induce specific immune responses in the body. Compared to other vaccines, DNA vaccines have the potential advantages of safety, stability, convenience, high efficiency, and persistent immunity. Ismael et al. used the pMIC3i plasmid encoding the immature MIC3 protein to intramuscularly inoculate CBA/J mice. Immunized mice all produced high titers of IgG2a type anti-MIC3 immunoglobulin as well as IFN-γ and IL-2. Inoculation of mice with the pGM-CSF plasmid encoding the granulocyte-macrophage colony-stimulating factor gene and the pMIC3i plasmid together showed that immunized mice produced stronger humoral immune responses. Oral administration of cysts of the 76 K strain of T. gondii into immunized mice showed that the number of cysts detected in the brain was significantly lower than that in control mice [52]. Fang et al. [53] used the “suicidal” pSCA1 vector to construct a T. gondii suicidal DNA vaccine pSCA/MIC3. As conventional DNA vaccine pcDNA/MIC3, suicidal DNA vaccine pSCA/MIC3 also provides favourable efficacy. Meantime, based on the alphavirus replicon, suicidal DNA vaccines leads eventually to apoptosis of the transfected cells, which is particularly important in alleviating the concerns of potential integration and cell transformation generated by the use of conventional DNA vaccines, so it could improve safety of the conventional DNA vector. In this case, the design of vaccines against T. gondii based on suicidal DNA approach is feasible and effective. CD4 and CD8 T cells are the major effectors of the MIC3 DNA vaccine-induced protection [54, 55], both Lectin-like and EGF-like domains of MIC3 conferred protection [54]. Of note, the MIC3 DNA vaccine induced good immune effects in large animal models (sheep) [56].

Although monovalent DNA vaccines can induce a very strong immune reaction in the body against T. gondii infection, the preventive function is not comprehensive, and the immune effect is not ideal [57]. Hence, in the course of researching T. gondii vaccines, a consensus to develop multivalent vaccines that contain a combination of several antigens and target different developmental stages has been reached with the aim of overcoming the drawback of the monovalent candidate vaccines [58]. Qu et al. [59] immunized mice with the constructed composite DNA vaccine ZJ111/PSAG1-MIC3, and the results showed that mice could reduce stronger humoral and Th1-type cellular immunity than those in the control group (pSAG1, pMIC3, and pcDNA3.1). After being challenged by the T. gondii RH strain at a lethal dose for 12 days, the survival rate of mice was still above 20 %, whereas mice in the control group all died within 5–6 days. Therefore, compared to monovalent DNA vaccines, multivalent composite DNA vaccines can produce better protective immunity. Some other studies also support the view that the introduction of multiantigenic DNA vaccine is more powerful and efficient than single-gene vaccine, and deserves further evaluation and development [60, 61]. The multiantigenic DNA immunization might be an important approach to achieve an effective vaccine against T. gondii.

Cytokines are excellent adjuvants and can significantly enhance the immune effects of gene vaccines. IFN-γ is an immune upregulation factor that plays a leading role. During the process of resisting T. gondii infection by immune cell, IFN-γ plays an important immune regulation role [62]. The pcMIC3 eukaryotic expression plasmid encoding the T. gondii MIC3 and the pcIFN-γ eukaryotic expression plasmid encoding IFN-γ were mixed together at 100 μg each and were then intramuscularly injected into BALB/c mice 3 times at intervals of 2 weeks. The results showed that mice could effectively defend the challenge of a small dose of a strong virulent strain of T. gondii, indicating that the recombinant plasmids encoding the T. gondii MIC3 and IFN-γ had excellent immunogenicity to induce strong humoral immunity and cellular immunity [63]. Collectively, these results demonstrated that MIC3 is considered a very promising candidate molecule for the development of vaccines against toxoplasmosis.

Taken together, studies have indicated that immune protection of MIC3 is influenced by multiple factors. Adjuvants play an important role in the efficacy of immunizations. It is the general consensus of opinion that a type-1 response, particularly associated with CD8+ T cells producing IFN-γ, is the major mediator of immunity against T. gondii infection. Other important factors in immunization experiments are the selection of animal model (species/strain) and the selection of T. gondii strain. In general, as mice are normally susceptible to T. gondii infection, parasite strains with low virulence or low doses of virulent strains may be preferentially selected by some researchers [64]. In some studies of immunization, oral challenges were made with cystogenic strains [65]. It is important to point out that unnatural routes of infection may be key determinants of mouse survival.

Studies on diagnostic reagents based on MIC3 of T. gondii

MIC3 is expressed at all stages of the life cycle of T. gondii. Therefore, MIC3 is not only an important candidate molecule for vaccines but also a very promising candidate
molecule for the diagnosis of toxoplasmosis. Immuno-
logical diagnostic methods based on MIC3 of T. gondii in-
clude the indirect fluorescent antibody test (IFAT), latex
agglutination test (LAT), enzyme-linked immunosorbent
assay (ELISA), polymerase chain reaction (PCR), and so
on. Jiang et al. [66] used recombinant T. gondii MIC3
(rMIC3) as an antigen to establish a LAT; there was no
cross-reactivity with the standard positive sera of other
pathogens, such as classical swine fever virus and foot and
mouth disease virus, but there was a strong agglutination
reaction with T. gondii antibody-positive serum samples
from pigs. In addition, compared to the ELISA method
using rSAG1 as the antigen, the coincidence rate of sera
that tested positive using these two methods was 92.8 %,
and this LAT could detect specific T. gondii antibodies in
all experimental piglets infected with T. gondii tachyzoites
at 8 to 42 days after infection. Beghetto et al. [67] used
rMIC3 as an antigen to establish an immunoglobulin G
avidity assay, and this assay could detect low-avidity IgG
antibodies exclusively in sera collected within 2 months
after primary infection. The presence of low-avidity IgG
antibodies against rMIC3 antigen can be used to deter-
mine the point of infection with T. gondii within a 2-
month time frame after infection. Furthermore, another
research group also demonstrated that particular peptides
from MIC3 (MIC3–282:GVEVTLEKCEKEFGI; MIC3–
191:SKRGNAKCGPNGTClV) were recognized with a
significantly higher intensity by sera from acutely infected
patients than by sera from latently infected patients, and
that these peptides may be candidates for a promising
peptide panel for the diagnosis of acute toxoplasmosis in
humans [68]. These results indicated that MIC3 is a very
good marker for diagnosing recently acquired infections
and has potential clinical usefulness for diagnosing the
acute phase of T. gondii infection during pregnancy. Fur-
thermore, the mic3 gene is also a target for molecular
diagnosis of toxoplasmosis. Du et al. [69, 70] used the
T. gondii mic3 gene as a target gene to design specific
primers to establish a loop-mediated isothermal amplifica-
tion (LAMP) assay to detect T. gondii in soil. This method
had high sensitivity and could detect 5 T. gondii oocysts
in 0.5 g of soil, providing a rapid, sensitive, and specific
molecular detection method for diagnosis of toxoplasmosis.

Ethical approval
Ethical approval was obtained from the Ethical Committee
of the Lanzhou Veterinary Research Institute, Chinese
Academy of Agricultural Sciences.

Conclusions
MIC3 is a very important protein adhesion factor se-
creted by T. gondii that is expressed at the tachyzoite,
bradyzoite, and sporozoite stages. It is closely associ-
ated with the interaction with host cells and parasites,
plays important roles in the early stage of host cell
invasion by parasites and is a highly promising candi-
date factor for vaccine development and diagnosis of
toxoplasmosis. Currently, studies on MICs have made
great progress, and their structures and functions are
becoming clear. The EGF-like domain of MIC3 may
have protein adhesion and signal recognition functions
during the process of invasion of host cells by T. gondii.
The correct folding, proteolysis, and expression time of
these domains also strictly affect the function of MIC3 in
parasites. Therefore, the use of methods such as bio-
informatics analysis, fluorescence labeling, establishment of
overexpression parasites, gene knockout, gene chips, and
high-throughput genome sequencing to study the functions
of MIC3 may yield further breakthroughs in parasite-host
interaction, signal recognition, and new functions of MIC3.
Toxoplasma gondii contains EGF-like domains and function as escorters. J Cell Sci. 2002;115:563–74.

14. Céréde O, Dubremetz JF, Soëtè M, Deslée D, Vali H, Bout D, et al. Synergistic role of membrane proteins in Toxoplasma gondii virulence. J Exp Med. 2005;201:453–63.

15. Sauroos S, Edwards-Jones B, Reis M, Sawmynden K, Kota E, Simpson P, et al. A novel galec tin-like domain from Toxoplasma gondii micronemal protein 1 assists the folding, assembly and transport of a cell-adhesion complex. J Biol Chem. 2005;280:3883–91.

16. Wan KL, Carruthers VB, Sibley LD, Ajoka JW. Molecular characterisation of an expressed sequence tag locus of Toxoplasma gondii encoding the micronemal protein MIC2. Mol Biochem Parasitol. 1997;84:203–14.

17. Jewett TJ, Sibley LD. The Toxoplasma proteins MIC2 and M2AP form a hexameric complex necessary for intracellular survival. J Biol Chem. 2004;279:9362–9.

18. Garcia-Regu et N, Lebrun M, Fourmaux MN, Mercereau-Pujolat O, Mann T, Beckers CJ, et al. The microneme protein MIC3 of Toxoplasma gondii is a secretory adhesion that binds to both the surface of the host cells and the surface of the parasite. Cell Microbio. 2002;2:353–64.

19. Brecht S, Carruthers VB, Ferguson DJ, Giddings DK, Wang G, Jakle U, et al. The Toxoplasma micronemal protein MIC4 is an adhesin composed of six conserved apple domains. J Biol Chem. 2001;276:4119–27.

20. Reiss M, Viebig N, Brecht S, Carruthers MN, Soëtè M, Di Cristina M, et al. Identification and characterization of an escorter for two secretory adhesions in Toxoplasma gondii. J Cell Biol. 2001;152:563–78.

21. Noydes SD, Sherman GD, Nockemann S, Loyens A, Dubremetz JF, et al. Molecular characterisation of TgMICs, a proteolytically processed antigen secreted from the micronemes of Toxoplasma gondii. Mol Biochem Parasitol. 2000;111:51–66.

22. Hoff EF, Cook SH, Sherman GD, Harper JM, Ferguson DJ, Dubremetz JF, et al. Toxoplasma gondii: molecular cloning and characterization of a novel 18 kDa secretory antigen, TgMIC10. Exp Parasitol. 2001;97:77–88.

23. Harper JM, Zhou XM, Pozzani V, Kafasck BF, Carruthers VB. The novel coccidian micronemal protein MIC11 undergoes proteolytic maturation by sequential cleavage to remove an internal propeptide. Int J Parasitol. 2004;34:1047–58.

24. Opitz C, Di Cristina M, Reiss M, Ruppert T, Crisanti A, Soldati D, et al. The micronemal protein MIC2-M2AP adhesive protein complex. EMBO J. 2003;22:2082–92.

25. Mital J, Meissner M, Soldati D, Ward GE. Conditional expression of Toxoplasma gondii apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. Mol Bio Cell. 2005;16:4341–9.

26. Rabenau KE, Sohrabi A, Tripathy A, Reitter C, Ajioka JW, Tomley FM, et al. TgAMA1 in Toxoplasma gondii: molecular cloning and characterization of a novel 18 kDa secretory antigen, TgAMA10. Exp Parasitol. 2001;97:77–88.

27. Harper JM, Zhou XM, Pozzani V, Kafasck BF, Carruthers VB. The novel coccidian micronemal protein MIC11 undergoes proteolytic maturation by sequential cleavage to remove an internal propeptide. Int J Parasitol. 2004;34:1047–58.

28. Optiz C, Di Cristina M, Reiss M, Ruppert T, Crisanti A, Soldati D, et al. Intramembrane cleavage of microneme proteins at the surface of the apicomplexan parasite Toxoplasma gondii. Mol Bio Cell. 2002;13:21077–85.

29. Mital J, Meissner M, Soldati D, Ward GE. Conditional expression of Toxoplasma gondii apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. Mol Bio Cell. 2005;16:4341–9.

30. Rabenau KE, Sohrabi A, Tripathy A, Reitter C, Ajioka JW, Tomley FM, et al. TgAMA1 in Toxoplasma gondii: molecular cloning and characterization of a novel 18 kDa secretory antigen, TgAMA10. Exp Parasitol. 2001;97:77–88.

31. Harper JM, Zhou XM, Pozzani V, Kafasck BF, Carruthers VB. The novel coccidian micronemal protein MIC11 undergoes proteolytic maturation by sequential cleavage to remove an internal propeptide. Int J Parasitol. 2004;34:1047–58.

32. Opitz C, Di Cristina M, Reiss M, Ruppert T, Crisanti A, Soldati D, et al. The micronemal protein MIC2-M2AP adhesive protein complex. EMBO J. 2003;22:2082–92.

33. Miller SA, Binder EM, Blackman MJ. Immunogenicity and protective efficacy of two recombinant proteins of Toxoplasma gondii in mice. Vet Parasitol. 2009;166:1–9.

34. Wang and Yin Parasites & Vectors (2015) 8:384
with liposome-entrapped DNA complexes encoding *Toxoplasma gondii* MIC3 gene. Pol J Vet Sci. 2012;15:3–9.

57. Ivory C, Chadee K. DNA vaccines: designing strategies against parasitic infections. Genet Vaccines Ther. 2004;2:17–25.

58. Wang YH, Yin H. Research progress on surface antigen 1 (SAG1) of *Toxoplasma gondii*. Parasites Vectors. 2014;7:180.

59. Qu D, Yu H, Wang S, Cai W, Du A. Induction of protective immunity by multiantigenic DNA vaccine delivered in attenuated *Salmonella typhimurium* against *Toxoplasma gondii* infection in mice. Vet Parasitol. 2009;166:220–7.

60. Fang R, Feng H, Hu M, Khan MK, Wang L, Zhou Y, et al. Evaluation of immune responses induced by SAG1 and MIC3 vaccine cocktails against *Toxoplasma gondii*. Vet Parasitol. 2012;187:140–6.

61. Xu D, Han J, Du A. Evaluation of protective effect of multiantigenic DNA vaccine encoding MIC3 and ROP18 antigen segments of *Toxoplasma gondii* in mice. Parasitol Res. 2013;112:2593–9.

62. Wang YH, Wang M, Wang GX, Pang AN, Fu BQ, Yin H, et al. Increased survival time in mice vaccinated with a branched lysine multiple antigenic peptide containing B- and T-cell epitopes from *T. gondii* antigens. Vaccine. 2011;29:8619–23.

63. Jiang T, Zhou YQ, Nie H, Chen SG, Fang R, Yao BA, et al. Boost effect of interferon-gamma on immunoprotection of *Toxoplasma gondii* mic3 dna vaccine in mice. J Huazhong Agr Univ. 2007;26:519–23.

64. Daryani A, Hosseine AZ, Dalimi A. Immune response against excreted/secreted antigens of *Toxoplasma gondii* tachyzoites in the murine model. Vet Parasitol. 2003;113:123–34.

65. Hiramoto RM, Galisteo AJ, do Nascimento N, de Andrade HF. 200 Gy sterilized *Toxoplasma gondii* tachyzoites maintain metabolic functions and mammalian cell invasion, eliciting cellular immunity and cytokine response similar to natural infection in mice. Vaccine. 2002;20:2072–81.

66. Jiang T, Gong D, Ma LA, Nie H, Zhou Y, Yao B, et al. Evaluation of a recombinant MIC3 based latex agglutination test for the rapid serodiagnosis of *Toxoplasma gondii* infection in swines. Vet Parasitol. 2008;158:51–6.

67. Beghetto E, Buffolano W, Spadoni A, Del Pezzo M, Di Cristina M, Minenkova O, et al. Use of an immunoglobulin G avidity assay based on recombinant antigens for diagnosis of primary *Toxoplasma gondii* infection during pregnancy. J Clin Microbiol. 2003;41:4514–8.

68. Maksimov P, Zerweck J, Maksimov A, Hotop A, Goss U, Pleyer U, et al. Peptide microarray analysis of in silico-predicted epitopes for serological diagnosis of *Toxoplasma gondii* infection in humans. Clin Vaccine Immunol. 2012;19:865–74.

69. Du F, Feng HL, Nie H, Tu P, Zhang QL, Hu M, et al. Survey on the contamination of *Toxoplasma gondii* oocysts in the soil of public parks of Wuhan, China. Vet Parasitol. 2012;184:141–6.

70. Du F, Zhang Q, Yu Q, Hu M, Zhou Y, Zhao J. Soil contamination of *Toxoplasma gondii* oocysts in pig farms in central China. Vet Parasitol. 2012;187:33–6.

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