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MIC3, a novel cross-protective antigen expressed in *Toxoplasma gondii* and *Neospora caninum*

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Abstract Microneme protein 3 (MIC3) is an important adhesion molecule expressed by *Toxoplasma gondii* and *Neospora caninum* that plays a crucial role in invasion. In our study, we found that recombinant TgMIC3 (rTgMIC3) was recognized by both *T. gondii*-reactive sera and hyper-immune serum against *N. caninum*. Polyclonal antibodies against TgMIC3 reacted with *N. caninum* by western blot and immunofluorescence assay (IFA). These results indicate that MIC3 is a novel cross-reactive antigen expressed in *N. caninum* and *T. gondii*. To evaluate the immune-protective effect of TgMIC3, we created the eukaryotic expression vector pcDNA3.1-TgMIC3, transfected this vector into HEK293T cells by lipofection, and evaluated TgMIC3 expression in HEK293T cells using western blot and IFA. Then, groups of BALB/c mice were immunized with recombinant TgMIC3 protein, pcDNA3.1-TgMIC3, or two-vaccine immunization. The mice were challenged with *T. gondii* RH or *N. caninum* Nc-1 tachyzoites 14 days after the final immunization. The survival time of *T. gondii*-infected mice was recorded, and the parasite burden in the brain of *N. caninum*-infected mice 30 days post-infection was measured using real-time PCR. The results demonstrated that mice immunized with TgMIC3-based vaccines elicited high antibody titers. After parasitic challenge, mice immunized with pcDNA-TgMIC3 exhibited prolonged survival when infected with *T. gondii* tachyzoites and a lower parasitic burden in the brains of mice challenged with *N. caninum* tachyzoites. These results demonstrate that TgMIC3 is a cross-protective antigen expressed in *T. gondii* and *N. caninum* and could elicit some protection against toxoplasmosis and neosporosis.

Keywords MIC3 · *Toxoplasma gondii* · *Neospora caninum* · Cross-protective antigen · Immune protection

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Introduction

Toxoplasma gondii and *Neospora caninum* are known to possess highly similar biological and morphological characteristics, and toxoplasmosis and neosporosis have been both regarded as economically important diseases that cause considerable economic losses to the livestock industry (Zhang et al. 2007). Although no human *N. caninum* infections have been reported, antibodies against *N. caninum* have been detected, and whether *N. caninum* can infect humans requires further study (Zhang et al. 2010). Several cross-reactive antigens between these two parasitic species have been reported, such as apical membrane antigen 1 (AMA1), protein disulfide isomerase (PDI), ribosomal phosphoprotein (P0), ribosomal protein 1 (RP1), nucleoside triphosphate hydrolase (NTPase), heat shock protein 70 (HSP70), rhoptry protein 6 (ROP6), and BAG5 (Ahn et al. 2001; Howe and Sibley 1999; Liao et al. 2005a; McAllister et al. 1996; Zhang et al. 2011). To date, there is limited research concerning the cross-protective function of these cross-reactive antigens. Furthermore, these cross-reactive antigens could be used as candidates to develop effective vaccines or drugs to control diseases caused by *T. gondii* and *N. caninum*.

First identified in 1991, TgMIC3 has adhesive properties and is involved in invasion by acting as a bridge between the

parasite and the host cell (Achbarou et al. 1991). TgMIC3 is a 90-kDa dimeric soluble protein containing a chitin binding-like domain (CBL), two less-conserved EGF domains that overlap with the others (EGF1 and EGF5), and three tandemly repeated epidermal growth factor-like domains (EGF2, EGF3, and EGF4), which are important for host cell binding and virulence (El Hajj et al. 2008). After its discovery, TgMIC3 has been regarded as a *T. gondii* detection antigen and vaccine candidate to control toxoplasmosis. A latex agglutination test for TgMIC3 showed high accuracy and was suitable for the detection of *T. gondii* antibodies in the early stages of infection (Jiang et al. 2008).

In our study, we found that TgMIC3 could react with polyclonal antibodies against *N. caninum* by western blot and confirmed that TgMIC3 is a novel cross-reactive antigen by immunofluorescence assay (IFA) and western blot. TgMIC3 immunization has been reported to elicit specific humoral and cellular immune responses and to produce effective protection against *T. gondii* infection (Ismael et al. 2003). *T. gondii* and *N. caninum* share many hosts, such as cats, dogs, cows, pigs, and sheep, as well as others, and the clinical symptoms caused by the two parasites are very similar. The identification of a cross-reactive candidate antigen that elicits protection would be very helpful in developing effective vaccines against both toxoplasmosis and neosporosis.

Materials and methods

Parasites and mice

T. gondii tachyzoites (RH strain) and *N. caninum* tachyzoites (Nc-1strain) were maintained and passaged in African green monkey kidney (Vero) cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine, 8 % heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a 5 % CO_2 environment. Parasites were harvested and purified as described previously (Zhang et al. 2007). Tachyzoites were isolated by washing in cold phosphate-buffered saline (PBS), centrifugation, resuspension in cold PBS, and syringing with a 27-gauge needle. The parasites were then filtered through a 5.0-μm pore filter (Millipore, USA), washed twice with PBS, and pelleted at 2,000 rpm for 10 min.

Female specific-pathogen-free (SPF) BALB/c mice aged 6 to 8 weeks were purchased from the Academy of Military Medical Sciences Laboratory Animal Center (Beijing, China). All of the mice were maintained under standard conditions and provided with rodent feed and water ad libitum. Prior to experimentation, mice were acclimatized for 1 week. Animal experiments were conducted according to the institutional guidelines for animal ethics.

Expression of recombinant TgMIC3 and NcMIC3

To produce the TgMIC3 protein and anti-rTgMIC3 sera, an 882-bp fragment of the TgMIC3 open-reading frame (199–1, 080 bp) was amplified by PCR using primers that introduced *Sac*I and *Hind*III sites (underlined): 5′-CTTgagctcTCCCCCAGCAAGCAGGAGAC-3′ (TgMIC3-1) and 5′-CCCaagctt-TTACTGCTTAATTTTCTCACACGTCACG-3′ (TgMIC3-2). The product was cloned into the pET-28a expression vector (Novagen, Germany) and transformed into *Escherichia coli* for protein expression. Recombinant TgMIC3 (rTgMIC3) fused to a His₆-tag was expressed and purified using His-Trap FF purification columns (Novagen, Germany) following the manufacturer's protocol.

Additionally, to obtain the NcMIC3 protein, an 843-bp fragment of the NcMIC3 open-reading frame (244–1, 086 bp) was amplified by PCR using primers that introduced *Bam*HI and *Xho*I sites (underlined): 5′ CGggatccAGTGAAGGTCAGCCGTG 3′ (NcMIC3-1) and 5′ CCctcgagTCGAGCCGTTCCGCATT 3′ (NcMIC3-2). The product was inserted into the pGEX6P-1 expression vector (Novagen, Germany) and transformed into *E. coli* for protein expression. Recombinant NcMIC3 (rNcMIC3) fused to glutathione *S*-transferase (GST-NcMIC3) was expressed and purified using sepharose 4B purification columns (Novagen, Germany) following the manufacturer's protocol.

Rabbits were immunized subcutaneously with 1 mg/kg purified rTgMIC3 in an equal volume of Freund's complete adjuvant (Sigma) for the first injection. The second and third injections were performed 2 and 4 weeks post-primary injection using 500 μg/kg of the antigen in Freund's incomplete adjuvant (Sigma, USA). The anti-rTgMIC3 sera were collected 2 weeks after the final immunization.

Identification of TgMIC3 and NcMIC3

TgMIC3 and NcMIC3 protein were boiled in loading buffer for 10 min and then separated on a 12 % (*w/v*) reducing SDS-PAGE gel. Following electrophoresis, separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). For western analysis, the PVDF membrane was blocked in 5 % (*w/v*) skim milk diluted in PBS for 1 h at room temperature before incubation with *T. gondii*-positive serum (dilution 1:500) or *N. caninum*-positive serum (dilution 1:600). Uninfected rabbit serum was used as the negative control. After washing in PBST, the membrane was incubated with diluted goat anti-rabbit IgG horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000; Sigma, USA) for 1 h. Proteins were visualized using ECL chemiluminescence reagents (CoWin Biotech Co., LTD., Beijing, China). Both *T. gondii* and *N. caninum* tachyzoites were analyzed by IFA, which was performed as previously described by Liao et al. (Dubey et al. 2008; Liao et al. 2005a). Vero cells seeded

onto coverslips were inoculated with *T. gondii* or *N. caninum* tachyzoites. After 48 h, the coverslips were washed three times in PBS, fixed with methanol for 20 min at −20 °C, and treated with 0.2 % Triton-100 in PBS for 5 min to permeabilize the plasma membrane of both the Vero cells and the parasites. Slides with tachyzoite-infected Vero cells were blocked in PBS with 1 % BSA for 30 min at room temperature. Rabbit anti-rTgMIC3 antibody or negative serum from unimmunized rabbits was used as the primary antibody (1:100, diluted in 1 % BSA in PBS), and Texas Red-conjugated anti-rabbit IgG (Proteintech, USA) was used as the secondary antibody (1:100, diluted in 1 % BSA in PBS). We visualized the stained cells using a fluorescence microscope (Leica microsystems CMS GmbH, Germany).

Construction of eukaryotic expression plasmid

The 882-bp fragment of TgMIC3 was amplified by PCR from pET-28a-TgMIC3 using the following primers: 5′-gcc<u>AAGC TT</u>*GCCACC*ATGTCCCCCAGCAAGCAGGAGAC-3′ (TgMIC3-3) and 5′-gc<u>TCTAGA</u>TTACTGCTTAATTTTCTC ACACGTCACG-3′ (TgMIC3-4). *Hind*III and *Xba*I recognition sites (underlined) were introduced, and a Kozak sequence (in italics) was added before the ATG start codon. After purification, the PCR product was digested with *Hind*III and *Xba*I (NEB), and the fragment was ligated into the pcDNA3.1 (+) vector (Invitrogen). The resulting plasmid was named pcDNA-MIC3. The plasmid concentration was determined by spectrophotometry at OD_{260} and OD_{280}.

Expression of TgMIC3 in HEK293T cells

HEK293T cells were transfected with pcDNA-MIC3 or pcDNA3.1 by lipofection. Briefly, 2×10^5 HEK293T cells per well were plated into 24-well plates the day before transfection. For each well, 0.8 μg plasmid and 2 μL LF2000TM were diluted in DMEM without serum and incubated at room temperature separately. After 5 min, the reagents were mixed and incubated for 20 min to allow DNA-LF2000 complexes formation. Then the complexes were added directly to each well and gently mixed, and an appropriate volume of DMEM without serum was added to each well. The cells were cultured at 37 °C in a 5 % CO_2 environment for 4–6 h, and then the DMEM was replaced. Forty-eight hours after transfection, the cells were fixed in cold methanol for 20 min, and MIC3 expression was detected by IFA with the anti-rTgMIC3 antibody and a Texas Red-labeled goat anti-rabbit IgG secondary antibody (Proteintech Group Inc. Chicago). After rinsing with PBS three times, coverslips were immediately observed under a fluorescence microscope (Olympus, Japan).

Expression of TgMIC3 in HEK293T cells was also detected by western analysis. The transfected cells were maintained in DMEM for 48 h before harvest and then boiled in loading buffer for 10 min. Protein from transfected cells was separated on a 12 % (w/v) SDS-PAGE gel. Following electrophoresis, separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). For western analysis, the PVDF membrane was blocked in 5 % (w/v) skim milk diluted in PBS for 1 h at room temperature before incubation with rabbit anti-rTgMIC3 antibody (dilution 1:100). After washing in PBST, the membrane was incubated with diluted goat anti-rabbit IgG horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000; Sigma, USA) for 1 h. Proteins were visualized with ECL chemiluminescence reagents (CoWin Biotech Co., LTD., Beijing, China).

The efficacy of immunization with TgMIC3 against *T. gondii* and *N. caninum* in mice

Four groups of 6 week-old BABL/c mice (12/group) were used to test the efficacy of TgMIC3 immunization. Group I and group II were used as controls. Mice were injected with PBS or empty pcDNA 3.1(+) vector alone. Group III was injected intramuscularly in thigh skeletal muscle with 100 μg of pcDNA-MIC3 plasmid DNA suspended in 100 μL sterile PBS. Group IV was immunized by two intramuscular injections with 100 μg of recombinant TgMIC3 protein with Freund's complete adjuvant in the first immunization. The second injection was 100 μg of pcDNA-MIC3 plasmid in PBS, and the final immunization was 50 μg of recombinant TgMIC3 protein with Freund's incomplete adjuvant.

All of the mice were immunized with different materials using the same protocol on weeks 0, 2, and 4. Two weeks after the final injection, the mice in all groups were challenged intraperitoneally either with 100 *T. gondii* RH tachyzoites or 2×10^6 *N. caninum* Nc-1 tachyzoites. Blood was collected from the tail vein prior to each immunization. Sera were separated and stored at −20 °C until use. The survival time of *T. gondii*-infected mice was recorded, and the parasite burden in the brain of *N. caninum*-infected mice 30 days post-infection was calculated by real-time PCR as described previously (Hao et al. 2014). PCR, to amplify the Nc5 gene and 28S rRNA, was performed as separate assays. Amplifications were performed with an ABI PRISM® 7500 Real-time PCR system, using a SYBR® Premix Ex Taq™ II Real Time kit (TaKaRa, Dalian, China) with the recommended protocol. All of the standards and samples were analyzed in duplicate. The results were automatically analyzed with the 7500 Software (v2.0.5, Applied Biosystems, Foster City, CA) and exported to Microsoft Excel to calculate the parasitic load in the brains, which is reported as the number of tachyzoites per 1 mg of host DNA.

Detection of anti-TgMIC3 antibodies in immunized mice

TgMIC3-specific antibodies were analyzed by enzyme-linked immunosorbent assay (ELISA) as previously described (Cuppari et al. 2008). Briefly, microtiter plates were coated with recombinant TgMIC3 (200 ng/well) in 50 mM carbonate buffer (pH 9.6). After incubation overnight at 4 °C, nonspecific binding sites were blocked with 4 % bovine serum albumin (BSA) in PBS for 1 h at 37 °C. Serum samples diluted 1:100 in PBS were added to the wells and incubated for 1 h at 37 °C. HRP-conjugated goat anti-mouse IgG was used as the secondary antibody (Proteintech Group Inc., Chicago). Immune complexes were detected by incubation with ortho-phenylene diamine and 0.15 % H_2O_2 incubated for 20 min in the dark, and the optical density at 450 nm was measured (A450) using an ELISA reader (Bio-TekEL 680, USA). All of the samples were run in triplicate.

Statistical analysis

All of the data were statistically analyzed using one-way analysis of variance (ANOVA). All of the statistical analyses were processed using SPSS13.0 Data Editor Software (SPSS Inc., Chicago, IL). The differences in results between groups were considered significant when P values were less than 0.05.

Ethics statement

All experiments with animals in this study were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. All experimental procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University (The certificate of Beijing Laboratory Animal employee, ID: 18049).

Results

Purification and identification of TgMIC3 and NcMIC3

Recombinant TgMIC3, which consists of amino acids 67–360 of the full-length protein, was expressed as a His_6-tagged fusion protein in *E. coli* and purified by Ni^{2+}-affinity
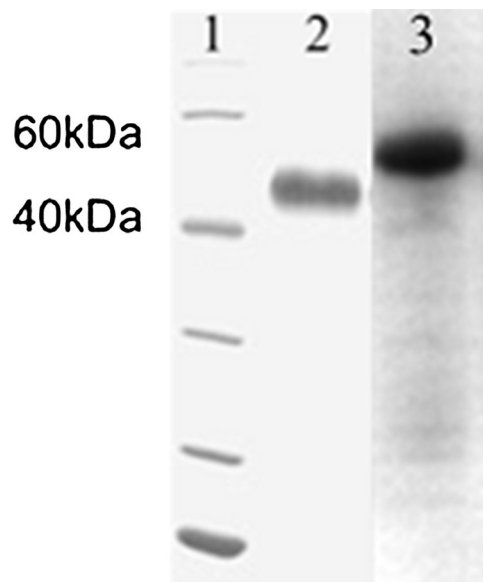
Fig. 1 Purified recombinant TgMIC3 and NcMIC3 was stained by Coomassie brilliant blue after SDS-PAGE. *Lane 1* protein marker, *lane 2* purified recombinant TgMIC3, *lane 3* purified recombinant NcMIC3
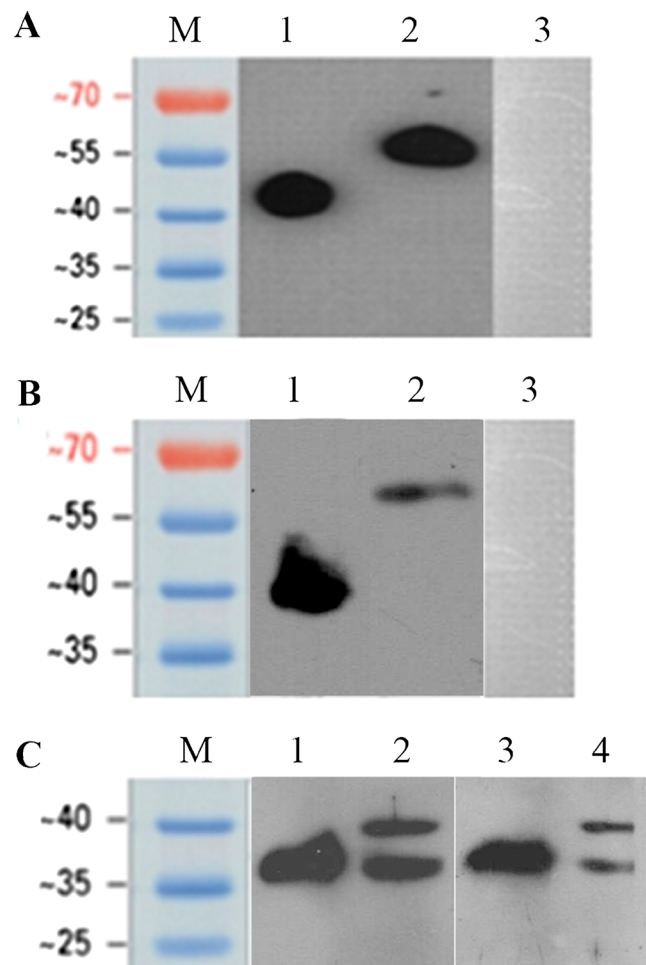
Fig. 2 MIC3 is a cross-reactive antigen expressed in *N. caninum* and *T. gondii*. **a** Both purified recombinant TgMIC3 (*lane 1*) and NcMIC3 (*lane 2*) were recognized by *N. caninum*-positive serum by western blot. Purified recombinant TgMIC3 using uninfected rabbit serum as a negative control (*lane 3*). **b** Both purified recombinant TgMIC3 (*lane 1*) and NcMIC3 (*lane 2*) were recognized by *T. gondii*-positive serum by western blot. Purified recombinant NcMIC3 using uninfected rabbit serum as a negative control (*lane 3*). **c** Endogenous MIC3 was recognized by the TgMIC3 or NcMIC3 polyclonal antibody by western blot. Total lysates of Nc-1 tachyzoite (*lane 1*) and RH tachyzoite (*lane 2*) with the TgMIC3 polyclonal antibody. Total lysates of Nc-1 tachyzoite (*lane 3*) and RH tachyzoite (*lane 4*) with the NcMIC3 polyclonal antibody

Fig. 3 The amino acid similarity between TgMIC3 and NcMIC3. An *asterisk* indicates positions which have a single, fully conserved residue. A *colon* indicates conservation between groups of strongly similar properties—scoring >0.5 in the Gonnet PAM 250 matrix. A *period* indicates conservation between groups of weakly similar properties—scoring ≤0.5 in the Gonnet PAM 250 matrix

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TgMIC3    MRGGTSALLHALTFSGAVWMCTPAEALPIQKSVQLGSFDKVVPSREVVS-ESLAPSFAVT 59
NcMIC3    MRGGASALVQALTVSGAVWMS-PAEALTVQKSSHLAQLENSQHVEGVAESLALSPSFLAS 59
          ****:***::***.******. *****.:*** :*.::::   . *.. :*:*** .:

TgMIC3    ETHSSVQS--PSKQETQLCAISSEGKPCRNRQLHTDNGYFIGASCPKSACCSKTMCGPGG 117
NcMIC3    GISSEVSYTLATDDGTLICAISSEGQPCRNRQLHTNHGYITGASCPNGLCCSKTMCGAGG 119
           *.*.    .:.: * :*******:*********::**: *****:. ********.**

TgMIC3    CGEFCSSNWIFCSSSLIYHPDKSYGGDCSCEKQGHRCDKNAECVENLDAGGGVHCKCKDG 177
NcMIC3    CGEFCSSSWVFCSTGLIYHADKSYGGDCSCEKQQHQCHKDAECVEDLNAGGSVRCRCKDG 179
          *******.*:***:.**** **.***** :*:*:*:*****:*:.***.*:*:****

TgMIC3    FVGTGLTCSEDPCSKRGNAKCGPNGTCIVVDSVSYTCTCGDGETLVNLPEGGQGCKRTGC 237
NcMIC3    FMGDGKTCKPDPCQSRGNEKCGSNGSCIVVDANRYTCACGEGEVLEPTLEGGYSCKKTGC 239
          *:* .* **. ***..*** ***.**:*****: ***:**:**.*   *** .**:***

TgMIC3    HAFRENCSPGRCIDDASHENGYTCECPTGYSREVTSKAEEESCVEGVEVTLAEKCE----K 293
NcMIC3    NAYSEYCNPGRCIDDASQPSRYTCECPQDSWRTTEGKNPMCYPTCEEMGGKDKCGPGSCR 299
          :*: * *.*********: . ****** .  * . .*  .      *: :** :

TgMIC3    EFGISASSCKCDNGYSGSASATSHHGKGESGSEGSLSEKMNIVFKCPSGYHPRYHAHTVT 353
NcMIC3    AIGKDKYACDCPAGYSRSMTSQSEE-KCVQGAEASLAER-------------------- 337
          :*  . :*.*  *** *  :: *.. *  .*:*.**:*:

TgMIC3    CEKIKHFALDGAGNHDTTTYVARRRYPASL 383
NcMIC3    CEKELGISLSASNAKCDCSKCGTAR----- 362
          ***   ::*..::. :    :   . *
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Fig. 4 The MIC3 polyclonal antibody reacted with *T. gondii* tachyzoites and *N. caninum* tachyzoites by Immunofluorescence. **a–c** Immunofluorescence microscopy of intracellular *T. gondii* tachyzoites stained with rabbit anti-TgMIC3 antibodies and Texas Red-conjugated goat anti-rabbit IgG. **d–f** Immunofluorescence microscopy of intracellular *N. caninum* tachyzoites stained with rabbit anti-TgMIC3 antibodies and Texas Red-conjugated goat anti-rabbit IgG. **g–i** Immunofluorescence microscopy of intracellular *N. caninum* tachyzoites stained with rabbit anti-NcMIC3 antibodies and Texas Red-conjugated goat anti-rabbit IgG. **j–l** Immunofluorescence microscopy of extracellular *T. gondii* tachyzoites stained with rabbit anti-NcMIC3 antibodies and FITC-conjugated goat anti-rabbit IgG. *Bar*, 5 μm
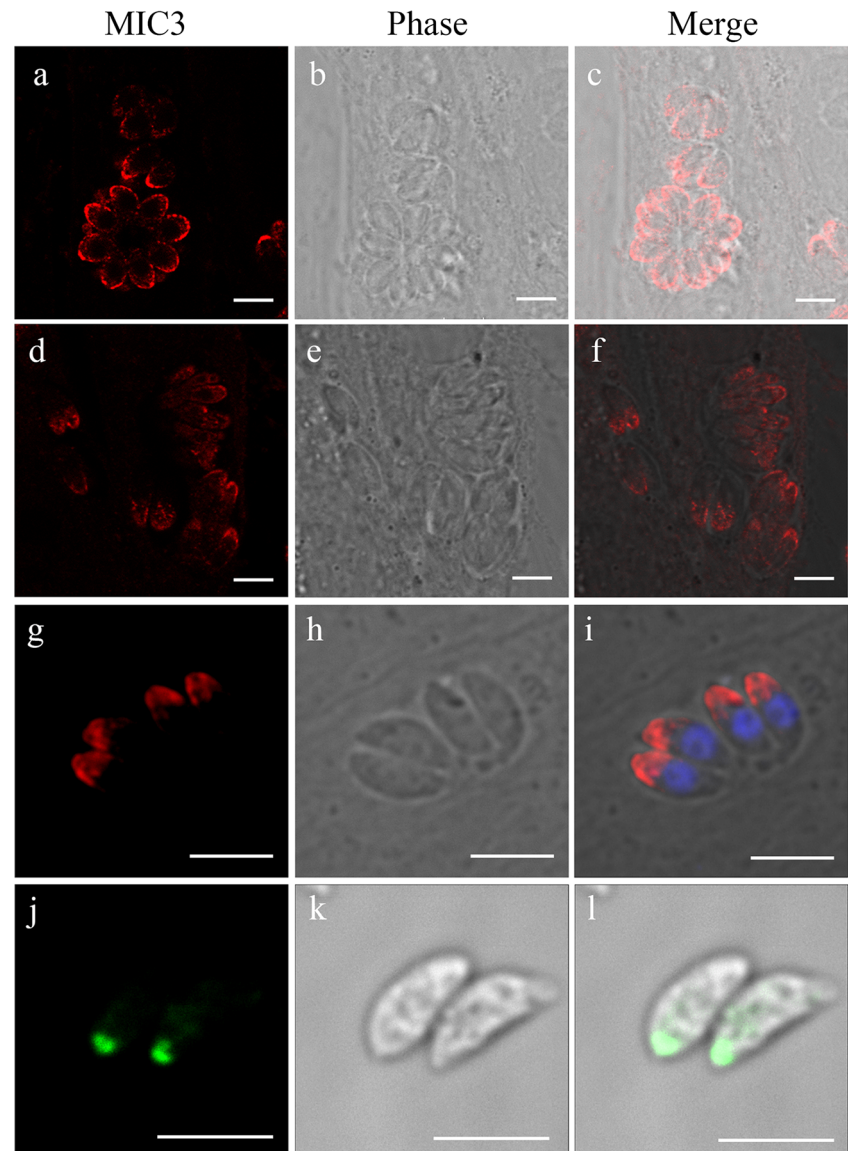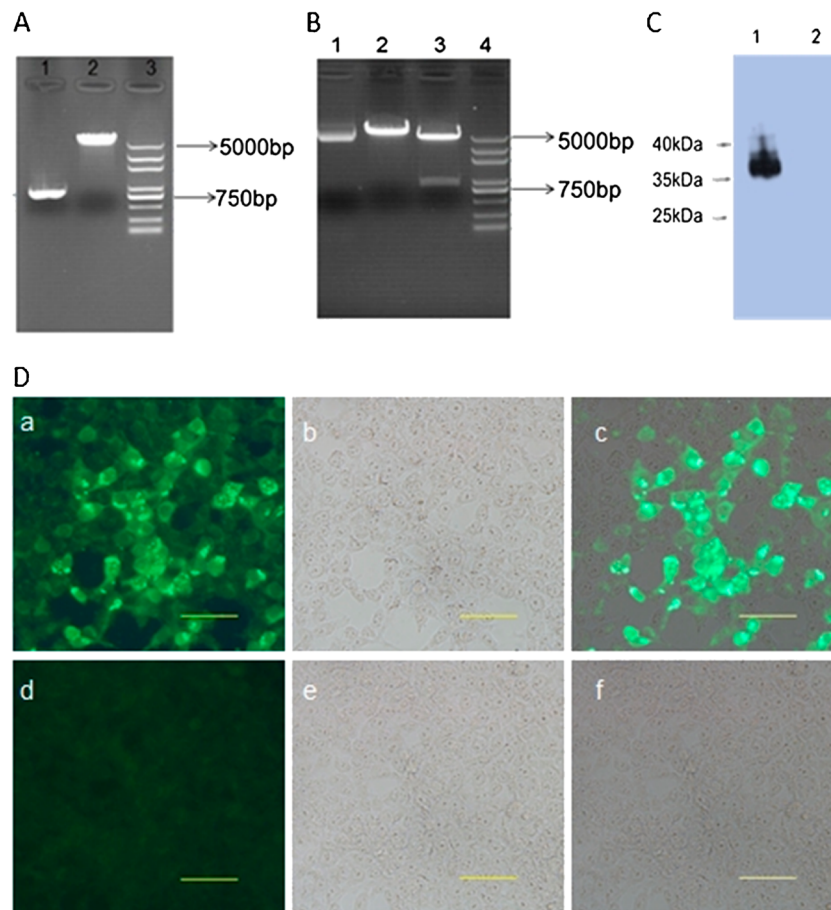
Fig. 5 Construction of the recombinant plasmid pcDNA3.1-TgMIC3 and expression in vitro. **a** Construction of pcDNA3.1-TgMIC3. *1* Recovery of the TgMIC3 gene after amplification and digestion with *Hind*III and *Xba*I, *2* recovery of pcDNA3.1 after digestion with *Hind*III and *Xba*I, *3* DNA marker. **b** Identification of the recombinant plasmid pcDNA3.1-MIC3. *1* pcDNA3.1-TgMIC3, *2* single digestion of pcDNA3.1-TgMIC3, *3* double digestion of pcDNA3.1-TgMIC3, *4* DNA marker. **c** Western blot of HEK293T cell lysates after transfection.

1 HEK293T cells transfected with pcDNA3.1-TgMIC3 react with the anti-TgMIC3 antibody, *2* HEK293T cells transfected with pcDNA3.1 do not react with the anti-TgMIC3 antibody. **d** Expression of TgMIC3 in HEK293T cell by IFA. **a–c** HEK293T cells transfected with pcDNA3.1-TgMIC3 react with the anti-TgMIC3 antibody. **d–f** HEK293T cells transfected with pcDNA3.1 do not react with the anti-TgMIC3 antibody. *Bar*, 20 μm

chromatography. One ~42 kDa protein was observed by SDS-PAGE (Fig. 1). Recombinant NcMIC3, containing amino acids 82–362 of the full-length protein, was expressed as a GST-tagged fusion protein in *E. coli* and purified by sepharose 4B chromatography (Fig. 1).

Both recombinant TgMIC3 and NcMIC3 were recognized by *N. caninum*-positive serum (Fig. 2a) and *T. gondii*-positive serum (Fig. 2b). Thus, we predicted that MIC3 is a cross-reactive antigen expressed in *T. gondii* and *N. caninum*.
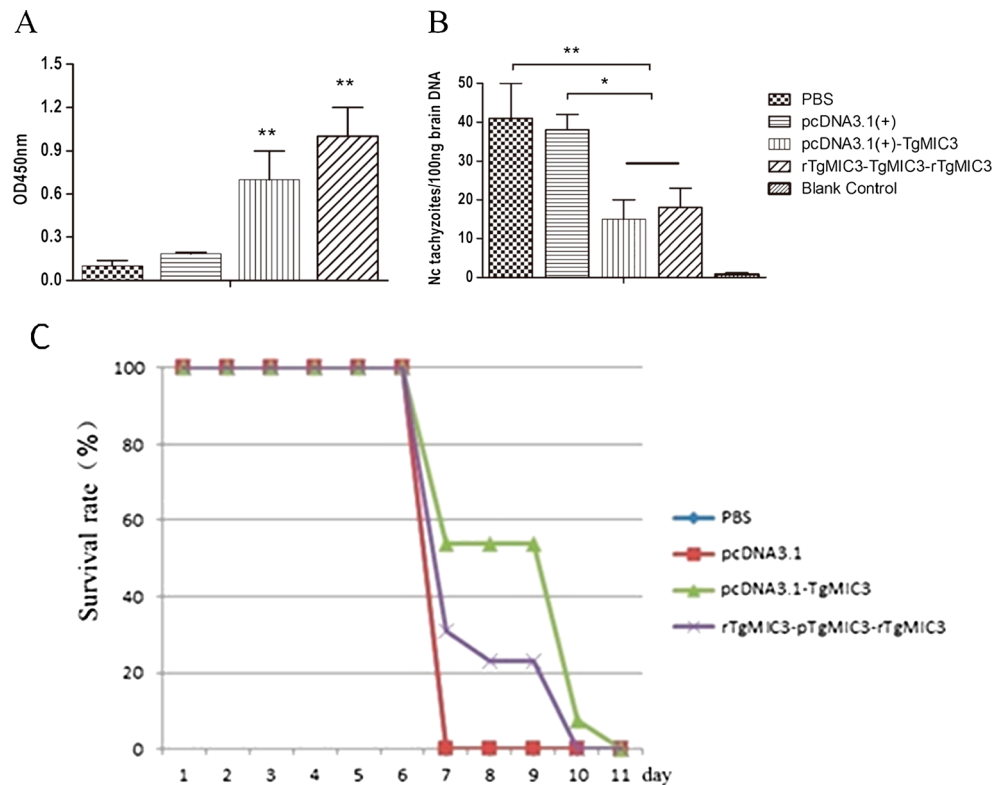
Identification of the cross-reactive antigen TgMIC3

To determine the amino acid sequence similarity of TgMIC3 and NcMIC3, the predicted amino acid sequence of TgMIC3 was aligned with that of NcMIC3 from ToxoDB. The result revealed that these two proteins have 55 % (200/363) identity, 65 % (236/363) similarity, and a 15 % (53/363) gap in their amino acid sequences (Fig. 3).

To further confirm whether TgMIC3 is a cross-reactive antigen, western and IFA analyses were performed. Western analysis indicated that the anti-TgMIC3 polyclonal antibody recognized one ~36 kDa protein when *N. caninum* tachyzoite lysates were probed. This result is consistent with the putative molecular weight of mature endogenous NcMIC3, which was approximately 36.2 kDa (Fig. 2c, lane 1). Endogenous TgMIC3 is also recognized by anti-NcMIC3 antibodies with ~40 and 38 kDa which are consistent with precursor and mature protein of TgMIC3 (Fig. 2c, lane 4).

Furthermore, IFA analysis suggested that the anti-TgMIC3 polyclonal antibody reacts with both endogenous TgMIC3 (Fig. 4a–c) and NcMIC3 (Fig. 4d–f). Red fluorescence was observed intracellularly at the apical surface in both *T. gondii* and *N. caninum* tachyzoites, whereas no fluorescence was observed in the negative control. Anti-NcMIC3 antibody could recognize endogenous NcMIC3 very well of intracellular and extracellular *N. caninum* tachyzoites (Fig. 4g–i). But

Fig. 6 The immune response and protective efficacy of TgMIC3 against *T. gondii* and *N. caninum* in mice. **a** Anti-TgMIC3 IgG levels in mice immunized with recombinant TgMIC3 protein or plasmid. *rTgMIC3* recombinant TgMIC3 protein, *pTgMIC3* recombinant TgMIC3 plasmid. **b** Parasitic burden in the brains of BALB/c mice. **c** Survival rate of BALB/c mice immunized with recombinant TgMIC3 protein or plasmid against *T. gondii* RH challenge after immunization. *rTgMIC3* recombinant TgMIC3 protein, *pTgMIC3* recombinant TgMIC3 plasmid

anti-NcMIC3 serum could only recognized endogenous TgMIC3 of extracellular *T. gondii* (Fig. 4j–l). These results confirmed that MIC3 is a cross-reactive antigen expressed in *T. gondii* and *N. caninum*.

Construction of the recombinant plasmid pcDNA3.1-TgMIC3

The 882-bp TgMIC3 ORF fragment was amplified from the pET-28a-TgMIC3 plasmid by PCR (Fig. 5a). The TgMIC3 gene was directionally inserted into pcDNA3.1 to create the pcDNA3.1-TgMIC3 recombinant plasmid. The plasmid was identified by digestion with *Hind*III and *Xba*I, and the 882 bp fragment of TgMIC3 gene was excised (Fig. 5b). The sequencing results also verified that the recombinant plasmid containing TgMIC3 ORF was constructed successfully.

Detecting pcDNA-TgMIC3 expression in vitro

The expression of pcDNA3.1-TgMIC3 in vitro was evaluated by western blot and IFA 48 h post-transfection. Western analysis showed that the anti-TgMIC3 polyclonal antibody recognizes a ~36-kDa protein in lysates from HEK293T cells transfected with pcDNA3.1-TgMIC3 but not in lysates from HEK293T cells transfected with pcDNA3.1 (Fig. 5c). Green fluorescence was observed in HEK293T cell transfected with pcDNA3.1-TgMIC3 (Fig. 5d, a–c), whereas there was no fluorescence in the pcDNA3.1-transfected cells (Fig. 5d, d–

f) using the anti-TgMIC3 polyclonal antibody. These results suggest that the TgMIC3 protein was successfully expressed in mammalian cells.

DNA vaccination induced a humoral immune response

To measure the levels of anti-TgMIC3 antibodies, all of the collected sera were evaluated by ELISA. As shown in Fig. 6a, significantly high levels of IgG antibodies were detected in the sera of mice immunized with the TgMIC3 recombinant plasmid or with a combination of TgMIC3 recombinant protein and plasmid. In contrast, mice injected with pcDNA3.1 alone or PBS did not generate specific antibodies against TgMIC3. Significant differences were observed between these groups ($P<0.05$).

Protective efficacy of DNA vaccination against *T. gondii* and *N. caninum* in mice

To test whether the DNA vaccine induced effective protection against *T. gondii* and *N. caninum* infection, the immunized mice were challenged with 100 *T. gondii* RH tachyzoites or 2×10^6 Nc-1 tachyzoites 2 weeks after the last immunization. The survival time of *T. gondii*-infected mice was recorded, and the parasitic burden in the brains of *N. caninum*-infected mice 30 days post-infection was measured using real-time PCR. Mice immunized with pcDNA3.1-TgMIC3 showed a longer survival time compared with the other groups

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(Fig. 6c), which indicated that pcDNA3.1-TgMIC3 prolonged the survival time of BALB/c mice. Mice immunized with pcDNA3.1-TgMIC3 showed a lower parasitic burden in the brains of mice challenged with Nc-1 (Fig. 6b). All of these results demonstrated that TgMIC3 is a cross-protective antigen against toxoplasmosis and neosporosis.

Discussion

In *T. gondii*, the micronemal homodimeric protein MIC3 is a potent adhesin that performs functions shared by most Apicomplexa MICs, and TgMIC3 plays important role in the invasion process and take part in forming moving junction of *T. gondii* (Cerede et al. 2002; Shen and Sibley 2012). A number of MICs act as escorters in protein complex, like MIC6 and MIC8 (Meissner et al. 2002). In *N. caninum*, NcMIC6 was verified that it interacted with NcMIC1 and NcMIC4 as an escorter and the C-terminal could bind to aldolase (Li et al. 2015). Moreover, TgMIC3 is expressed during three parasite life stages (tachyzoite, bradyzoite, and sporozoite) of *T. gondii*. Bioinformatic analysis predicted that TgMIC3 has a high antigenicity index, and thus, is a potential candidate for toxoplasmosis diagnosis and vaccine development. A previous study showed that TgMIC3 induces high levels of *T. gondii*-specific antibodies in the sera of mice and humans (Ismael et al. 2003), and TgMIC3 has not been shown to exhibit cross-reactivity with other pathogens. Thus, several kits based on TgMIC3 were developed to detect *T. gondii* infection, such as LAT, ELISA, and IFA. The coincidence rate of seropositivity between the LAT using rMIC3-sensitized latex particles and the ELISA using rSAG1 reached 92.8 %, which indicated that the rMIC3-based latex agglutination test is suitable for the detection of *T. gondii* antibodies at early stages of infection (Jiang et al. 2008). An indirect ELISA using recombinant TgMIC3 was standardized to detect antibodies against *T. gondii*, and the total coincidence rate between the ELISA and the LAT was 92.56 % (Jiang et al. 2009). Moreover, an IFA detection kit was developed based on the TgMIC3 monoclonal antibody. All of these tests suggest that TgMIC3 is a useful detection antigen.

ELISA and LAT are limited by specific antigen and complex procedure. ELISA always requires special materials and equipment and some false positive results occurred in our test, making them inconvenient for clinical or field applications. In contrast, the immunochromatographic test (ICT) is a simple, rapid method that is suitable for clinical or field applications (Liao et al. 2005b). Here, our original intent was to develop a new ICT method using rTgMIC3 to detect *T. gondii* infection. Due to the high similarity between *T. gondii* and *N. caninum*, we intended to eliminate the cross-reactivity of TgMIC3 by reacting TgMIC3 with serum against *N. caninum*. However, we did not expect the TgMIC3 antibody to react with *N. caninum*. We further confirmed that MIC3 is a novel cross-reactive antigen expressed in *T. gondii* and *N. caninum* by western blot and IFA. We selected bovine sera which had been verified natural infection of *T. gondii* to perform western blots. Those *T. gondii* positive sera could recognize rNcMIC3, but could not recognize total lysates of *N. caninum* by western blots. To date, there are ten cross antigens between *T. gondii* and *N. caninum*, but limited research on the cross protection of these cross antigens has been performed, leading us to perform this study.

In the present study, we focused on TgMIC3, a newly identified cross-reactive antigen and described the development and evaluation of a DNA vaccine based on a plasmid encoding 294 aa of the TgMIC3 protein, either used alone or combined with recombinant TgMIC3 protein. After immunization, antibodies against TgMIC3 were detected in all of the groups, and mice were subsequently challenged with *T. gondii* or *N. caninum* tachyzoites. Immunization with TgMIC3 prolonged the survival time of mice challenged with *T. gondii* and also reduced the parasitic burden in the brain of mice challenged with *N. caninum*. Taken together, these results suggest that TgMIC3 stimulates effective immune protection against toxoplasmosis and neosporosis. However, the survival time of mice challenged with *T. gondii* RH tachyzoites was increased by 4 days at most, and the parasites were still found in the brain of mice challenged by Nc-1 tachyzoites compared with the control. Generally, one cross antigen cannot generate full protection. Therefore, a new approach to develop a vaccine based on a variety of antigens against toxoplasmosis and neosporosis might be necessary.

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