

RESEARCH ARTICLE

# Protective efficacy of vaccination with NcMIC3 and NcMIC8 against *Neospora caninum* infection in mice

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**Abstract** Microneme proteins (MICs) are important for Apicomplexan parasite invasion due to their adhesion to host cells. Several studies have indicated that *Neospora caninum* MIC3 and MIC8 are important adhesion factors and potential vaccine candidates against neosporosis. In this study, we evaluated the protective efficacy of recombinant proteins and DNA vaccines of NcMIC3 and NcMIC8. BALB/c mice were immunized with rNcMIC3, rNcMIC8, pcDNA3.1-NcMIC3 and pcDNA3.1-NcMIC8 respectively, and challenged with *N. caninum* tachyzoites. The immune responses were evaluated through cytokine, antibody measurements and the parasite burden in the mice brain tissues. Serological analysis showed that recombinant protein vaccines induced higher levels of immunoglobulin G (IgG) than other groups. The percentage of IgG1 and IgG2a in the recombinant protein groups was higher than the other groups, and with a predominance of IgG1 over IgG2a, suggesting that recombinant protein vaccines elicited a Th2-type immune response, while DNA vaccines mainly produce a Th1-type immune response. In addition, mice immunized with rNcMIC3 and rNcMIC8 had lower parasite burden in brain tissue compared with the other groups. These results demonstrate that rNcMIC3 and rNcMIC8 could induce humoral and Th2-type immune response, leading to a considerable level of resistance against neosporosis.

**Keywords** NcMIC3, NcMIC8, *Neospora caninum*, vaccination

## 1 Introduction

*Neospora caninum* is an obligate intracellular parasite belonging to the phylum Apicomplexa<sup>[1]</sup>. In 1988, it was originally isolated from the brain tissue of a dog with hind limb paralysis and was identified as a *Toxoplasma gondii*-

like organism<sup>[2]</sup>. It is now considered a distinct species that causes neosporosis. Neosporosis is a major cause of infectious abortion and congenital disease in cattle, persisting in cattle herds via transplacental transmission<sup>[3]</sup>. Therefore, neosporosis causes huge economic losses for the global livestock industry<sup>[4]</sup>. To date, there are no drugs licensed for the control of neosporosis in cattle<sup>[5]</sup>. Various vaccines for neosporosis have been explored, including live, killed or inactivated parasite vaccines<sup>[6,7]</sup>. However, there are safety problems and higher production costs with live vaccines. A vaccine based on whole killed tachyzoites (Bovilis-Neoguard, Intervet, Merck Animal Health, Madison, NJ, USA) was commercialized, however, it induced only partial protection against abortion and did not prevent vertical transmission<sup>[8]</sup>. Thus, the development of a next-generation vaccine is urgently required. The recent proposal of subunit vaccines and DNA vaccines has become a topic for active consideration by researchers for prevention of neosporosis.

Recent research has demonstrated that *N. caninum* microneme proteins are crucial for the initial host cell interaction and invasion process, and during the subsequent phases of infection<sup>[9,10]</sup>. Also, several studies have reported that immunization with microneme proteins, such as NcMIC1, NcMIC2, NcMIC3, NcMIC4, NcMIC6<sup>[11]</sup>, NcMIC8<sup>[12]</sup> and NcMIC10, can enhance the humoral immune responses against *N. caninum*. Lovett et al.<sup>[13]</sup> indicated NcMIC2 may be important for the attachment to and invasion of host cells by *N. caninum*. Cannas et al.<sup>[14]</sup> reported that recombinant NcMIC3 vaccine confers significant protection against experimentally-induced cerebral neosporosis in mice. In addition, Alaeddine et al.<sup>[15]</sup> immunized mice with rNcMIC1 proteins and pcDNA-MIC1 and reported that only rNcMIC1 vaccination appears to reduce cerebral infection in mice challenged with *N. caninum* tachyzoites.

Earlier studies in our laboratory have found that NcMIC3 and NcMIC8 are both important adhesion factors in parasite invasion, and NcMIC8 is present in the parasites in a precursor form and is cleaved into the mature body

Received November 26, 2018; accepted January 16, 2019

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before being transported to the microneme or on secretion. The mature body forms a complex with NcMIC3<sup>[12,16]</sup>. These adhesion factors and complexes may serve functions in the immune response and be potential vaccine candidates. Therefore, we constructed recombinant proteins and DNA vaccines encoding MIC3 and MIC8 antigen of *N. caninum* and evaluated its ability to induce an immune response and partial protective effect in BALB/c mice against neosporosis.

## 2 Materials and methods

### 2.1 Ethics statement

All animal experiments were performed in strict accordance with the recommendations of 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 (Certificate of Beijing Laboratory Animal, No: CAU20161210-2).

### 2.2 Parasite culture and preparation

The tachyzoites of *N. caninum* (Nc-1 strain) used in this study were maintained and passaged in African green monkey kidney (Vero) cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine and 8% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO<sub>2</sub> environment<sup>[16]</sup>. Parasites were harvested and purified as described previously<sup>[11]</sup>. Tachyzoites were scraped from culture flasks and released by passing the cells through a 27-gauge needle. The parasites were then filtered through a 5-µm filter (Millipore, Burlington, MA, USA), washed twice with phosphate-buffered saline (PBS) and pelleted at 2000 × *g* for 10 min.

### 2.3 Animals

Six-week-old female BALB/c mice were purchased from Peking University Health Science Center (Department of Laboratory Animal Science, Beijing, China) and maintained under specific pathogen-free standard conditions.

### 2.4 Construction of DNA vaccine plasmid

The gene sequences of NcMIC3 (NCLIV\_010600) and NcMIC8 (NCLIV\_062770) were obtained by BLAST in ToxoDB (<http://www.toxodb.org/toxo/>). Coding sequences of the NcMIC3 gene and NcMIC8 gene were amplified by PCR from *N. caninum* cDNA with synthetic primers.

NcMIC3 (672 bp): 5'-CGGGGTACCGATAAAAGC-TACGGAGGTGACT-3' (forward) and 5'-CCGGA-TATCTTATCGAGCCGTTCCGCATTGCT-3' (reverse), containing *KpnI* and *EcoRV* (NEB, Ipswich, MA, USA)

restriction enzyme sites (underlined).

NcMIC8 (453–1380 bp, which has high antigenicity and hydrophilicity, excluding the signal peptide and transmembrane region): 5'-CGGGATCCGGTGCGTATTGC-CAGTGCAACGACGG-3' (forward) and 5'-CCG-CTCGAGTTCGCAGCTTTTCACTGCTTCTGGC-3' (reverse), containing *BamHI* and *XhoI* restriction enzyme sites (underlined).

The PCR productions of MIC3 and MIC8 gene were digested with the appropriate restriction enzyme (*KpnI*/*EcoRV* and *BamHI*/*XhoI*) at 37°C overnight and purified from agarose gels. MIC3 and MIC8 gene fragments were inserted into the pcDNA3.1 vector (Novagen, Madison, WI, USA) digested with the same enzyme (*KpnI*/*EcoRV* and *BamHI*/*XhoI*). The plasmid was named pcDNA3.1-NcMIC3 and pcDNA3.1-NcMIC8. All recombinant plasmids were transformed into *Escherichia coli* strain Trans1-T1 and confirmed by restriction analysis. Endotoxin-free plasmid DNA was isolated using a plasmid purification kit (TransGen Biotech, Beijing, China). The concentrations of the purified plasmids were detected by spectrophotometry at 260 and 280 nm. For intramuscular vaccination, the plasmid was formulated in PBS (pH 7.5) at 1.0 mg·mL<sup>-1</sup>.

### 2.5 In vitro gene expression

Human embryonic kidney (HEK) 293T cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator in DMEM supplemented with 10% FBS, 100 mg·mL<sup>-1</sup> streptomycin and 100 IU·mL<sup>-1</sup> penicillin. For the transfection, HEK 293T cells were transferred into six-well plates (Costar, Corning, NY, USA). When the cells reached 80%–90% confluence they were transfected with a recombinant eukaryotic expression plasmid (pcDNA3.1-NcMIC3 or pcDNA3.1-NcMIC8) using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The empty plasmid pcDNA3.1 transfected into HEK 293T cells was used as the negative control. The transfected cells were cultured for 6 h at 37°C and 5% CO<sub>2</sub>; then the DMEM was replaced. After 48 h of transfection, the cells were fixed in cold methanol for 20 min. Plasmid expression was detected by indirect immunofluorescence assay (IFA) with rabbit anti-NcMIC3 antibody (1:50)<sup>[15]</sup> or rabbit anti-NcMIC8 antibody (1:50)<sup>[12]</sup> and FITC conjugated goat anti-rabbit immunoglobulin G (IgG) (1:50) (Proteintech, Chicago, IL, USA) as the secondary antibody. After rinsing with PBS three times, coverslips were immediately observed under a fluorescence microscope (Olympus, Tokyo, Japan).

### 2.6 Expression and purification of recombinant proteins

The recombinant plasmids pET28a-NcMIC3 and pET28a-NcMIC8 (constructed previously and preserved in our laboratory) were transformed into *E. coli* BL21 (DE3) for protein expression. rNcMIC3 and rNcMIC8 fused to a

His<sub>6</sub>-tag were expressed and purified using HisTrap FF purification columns (Novagen, Merck KGaA, Darmstadt, Germany) following the manufacturer's protocol. Expression and purification of proteins were assessed by 12% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as previously described<sup>[16]</sup>. The concentration of proteins was determined using a BCA protein assay kit (Kangwei, Beijing, China). The purified recombinant proteins were then used for immunization of mice.

## 2.7 Vaccination and challenge

Mice were randomly divided into six groups (10 mice per group). Before immunization, mice were checked serologically for the absence of anti-*N. caninum* immunoglobulins (pre-immune serum) by IFA<sup>[17]</sup>. Each group was vaccinated three times on days 0, 14 and 28 as follows. Group 1 and Group 2 were immunized by subcutaneous injections with recombinant proteins rNcMIC3 and rNcMIC8. The immunization injections were given at 100 µg per mouse for the first dose and 50 µg per mouse for the second and third doses. Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MI, USA) was added to the first injection, and Freund's incomplete adjuvant was added to the second and third immunization injections. Group 3 and Group 4 were immunized intramuscularly in their hind legs with 100 µL of PBS containing 100 µg pcDNA3.1-NcMIC3 and pcDNA3.1-NcMIC8 for the first dose and 50 µg per mouse for the second and third doses. The other two groups of mice were injected with empty vector or with PBS as a control. Blood samples were collected by retro-orbital plexus puncture on days 13, 27 and 55, and the sera were stored at -20°C for serological analysis. On day 42, all groups were challenged intraperitoneally with  $1 \times 10^6$  tachyzoites of the Nc-1 strain. All animals were sacrificed by CO<sub>2</sub> euthanasia 30 days post-infection.

## 2.8 Measurement of the levels of antibodies

To evaluate the humoral immune response, the sera of mice were collected after each immunization and after challenge to analyze for *N. caninum* specific IgG, IgG1 and IgG2a by ELISA. Briefly, the ELISA plates were coated with lysate antigen of *N. caninum* (0.5 µg per well) at 4°C overnight, washed three times with PBST (PBS plus 0.05% Tween 20, pH 7.4) and blocked with 3% BSA at 37°C for 1 h. Serum samples were diluted 1:50 in 3% BSA and incubated for 1 h at 37°C. After washing, plates were incubated with horseradish peroxidase conjugated goat anti-mouse IgG, IgG1 or IgG2a (1:8000 dilution; Proteintech) at 37°C for 1 h. Next, the diluted solution of o-phenylenediamine and H<sub>2</sub>O<sub>2</sub> were added and incubated for about 20 min in the dark. The reaction was terminated by H<sub>2</sub>SO<sub>4</sub> (2 mol·L<sup>-1</sup>, 50 µL) and the absorbance value was measured at 450 nm by a microplate reader (EL680,

BioTek, Winooski, VT, USA). All assays were performed in triplicate.

## 2.9 Assessment of serum levels of IL-4 and IFN-γ

The serum levels of IFN-gamma (IFN-γ) and interleukin-4 (IL-4) were determined using commercial mouse cytokine/chemokine kits (Dakewe Biotech, Shenzhen, China). The final immunization sera were analyzed according to the manufacturer's protocol. Cytokine concentrations were determined by referring to the standard curves constructed with known amounts of mouse recombinant IFN-γ and IL-4 (provided by the kit manufacturer). All assays were performed in triplicate.

## 2.10 Assessment of cerebral parasite burden

The parasite burden in the brain of *N. caninum*-infected mice was calculated by quantitative real-time PCR<sup>[18,19]</sup>. Mice (six mice per group) were sacrificed at the end of the experiment, and their brains were removed. DNA of brain tissues in mice was extracted using a DNA extraction kit (Aidlab Biotech, Beijing, China) according to the manufacturer's instructions. PCR, to amplify the Nc5 gene and 28S rRNA, was performed as separate assays as described previously<sup>[20]</sup>. The amplification of the Nc5 gene was used to detect the quantification of *N. caninum* DNA, and the 28S rRNA gene was chosen for the quantification of host DNA. 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 the standards and samples were analyzed in triplicate. The results were analyzed automatically by the 7500 Software (v2.0.5, Applied Biosystems, Foster City, CA, USA) and exported to Microsoft Excel (Microsoft, Albuquerque, NM, USA) to calculate the parasitic load in the brains, which was reported as the number of tachyzoites per µg of host DNA.

## 2.11 Statistical analysis

The statistical analysis was performed using the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) software package using Student's *t*-test and ANOVA. Statistical significance was reached when  $P < 0.05$ .

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# 3 Results

## 3.1 Construction of the recombinant plasmids

The NcMIC3 (Fig. 1A, lane 1) and NcMIC8 (Fig. 1B, lane 1) genes were amplified from cDNA of *N. caninum* by PCR. The NcMIC3 and NcMIC8 genes were directionally inserted into pcDNA3.1 to create the pcDNA3.1-NcMIC3 and pcDNA3.1-NcMIC8 recombinant plasmids. To ensure

the correct insert orientation, pcDNA3.1-NcMIC3 was identified by digestion with *KpnI* and *EcoRV* restriction enzymes, the 672 bp fragment of NcMIC3 gene was excised (Fig. 1A, lane 2), pcDNA3.1-NcMIC8 was identified by digestion with *BamHI* and *XhoI* restriction enzyme and the 927 bp fragment of NcMIC8 gene was excised (Fig. 1B, lane 2); the latter fragment was then characterized by restriction endonuclease digestion as well as PCR amplification. The sequencing results verified that the recombinant plasmids pcDNA3.1-NcMIC3 containing NcMIC3 ORF and pcDNA3.1-NcMIC8 containing NcMIC8 ORF were constructed successfully.

### 3.2 Expression of the DNA vaccine in HEK 293T cells

The expression of pcDNA3.1-NcMIC3 and pcDNA3.1-NcMIC8 *in vitro* was evaluated by IFA 48 h post-transfection (Fig. 1C). Specific green fluorescence was observed in the HEK 293T cells transfected with pcDNA3.1-NcMIC3 (Fig. 1C(a)) or pcDNA3.1-NcMIC8 (Fig. 1C(b)), whereas there was no fluorescence observed

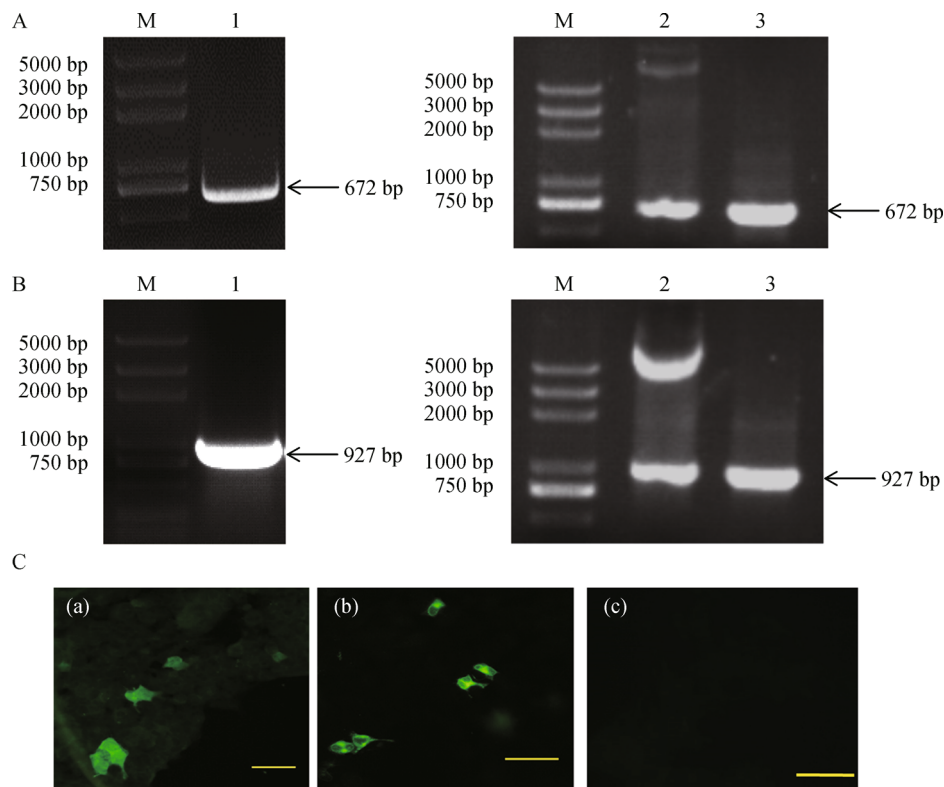
in the pcDNA3.1-transfected cells (Fig. 1C(c)). These results suggested that the NcMIC3 and NcMIC8 proteins were successfully expressed in mammalian cells.

### 3.3 Expression and purification of rNcMIC3 and rNcMIC8

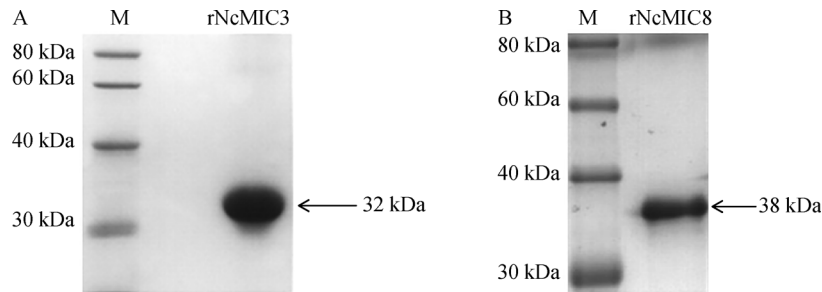
The two recombinant proteins were expressed as His<sub>6</sub>-tagged fusion proteins in *E. coli* BL21 and purified by Ni<sup>2+</sup>-affinity chromatography. Purified proteins were observed by SDS-PAGE. Purified rNcMIC3 was 32 kDa (Fig. 2A), and purified rNcMIC8 was 38 kDa (Fig. 2B). The purified proteins were then used to immunize BALB/c mice.

### 3.4 Clinical symptoms

Following vaccination, either by subcutaneous injections or intramuscular delivery, mice were inspected daily for the presence of local reactions at the inoculation sites. No such reactions were found during the experiment. This indicated that vaccination had no adverse effects and suggested that



**Fig. 1** Construction of eukaryotic expression plasmids and expression *in vitro*. A: Amplification of NcMIC3 gene (672 bp, lane 1) and identification of the recombinant plasmid pcDNA3.1-NcMIC3 by double digestion (*KpnI* and *EcoRV*, lane 2) and PCR (lane 3); B: Amplification of NcMIC8 gene (927 bp, lane 1) and identification of the recombinant plasmid pcDNA3.1-NcMIC8 by double digestion (*BamHI* and *XhoI*, lane 2) and PCR (lane 3). M: Marker. C: Expression of NcMIC3 and NcMIC8 in HEK 293T cells by IFA. (a) HEK 293T cells transfected with pNcMIC3 react with the anti-NcMIC3 antibody; (b) HEK 293T cells transfected with pNcMIC8 react with the anti-NcMIC8 antibody; (c) negative control, HEK 293T cells transfected with empty pcDNA3.1 do not react with the anti-NcMIC3 or anti-NcMIC8 antibody. Bar = 20  $\mu$ m.



**Fig. 2** The purification of NcMIC3 and NcMIC8 recombinant proteins. A: Purified rNcMIC3 (about 32 kDa) was stained by Coomassie Brilliant Blue after SDS-PAGE; B: Purified rNcMIC8 (about 38 kDa) was stained by Coomassie Brilliant Blue after SDS-PAGE. M: Protein markers.

all immunization procedures were benign and did not impose stressful conditions that would interfere with the general metabolic activity of the animals. After mice were infected with *N. caninum* tachyzoites, in the control groups we observed clinical signs of neosporosis, such as walking disorders, rounded back, apathy, and paralysis, but few mice acquired such symptoms in the recombinant protein groups and the pcDNA3.1-NcMIC3 group.

### 3.5 Humoral immune responses

Antibody-reactivity against crude *N. caninum* extract was assessed by ELISA. As shown in Fig. 3A, the levels of IgG antibodies in the experimental group gradually increased with successive immunizations. There were significant differences in the sera of mice immunized with rNcMIC3 ( $P < 0.01$ ) and rNcMIC8 ( $P < 0.05$ ) groups compared with the other groups, and high levels of IgG antibodies were detected in rNcMIC3 and rNcMIC8 groups. There was no significant difference in IgG responses between the DNA vaccine groups ( $P > 0.05$ ). These findings indicate that immunization with rNcMIC3/rNcMIC8 induced a strong IgG antibody response in mice.

The levels of IgG subclass (IgG1 and IgG2a) humoral isotype responses against *N. caninum* in immunized BALB/c mice are shown in Fig. 3B. Compared with the control groups, the IgG1 levels in the rNcMIC3 and rNcMIC8 groups increased significantly ( $P < 0.01$ ) after immunization, while there were no significant differences between the DNA vaccine and control groups. The IgG2a levels in the rNcMIC3 group were clearly higher ( $P < 0.01$ ) than the control and DNA vaccine groups, while the IgG2a levels in the rNcMIC8 group were increased ( $P < 0.05$ ) compared with the control groups (Fig. 3B, IgG2a). The levels of IgG, IgG1, and IgG2a in the rNcMIC3 group were higher than the rNcMIC8 group, although the differences were not significant. These data indicate that the protective efficacy of the rNcMIC3 was better than that of rNcMIC8. The ratio of IgG1/IgG2a indicated that IgG1 had an obvious advantage over IgG2a

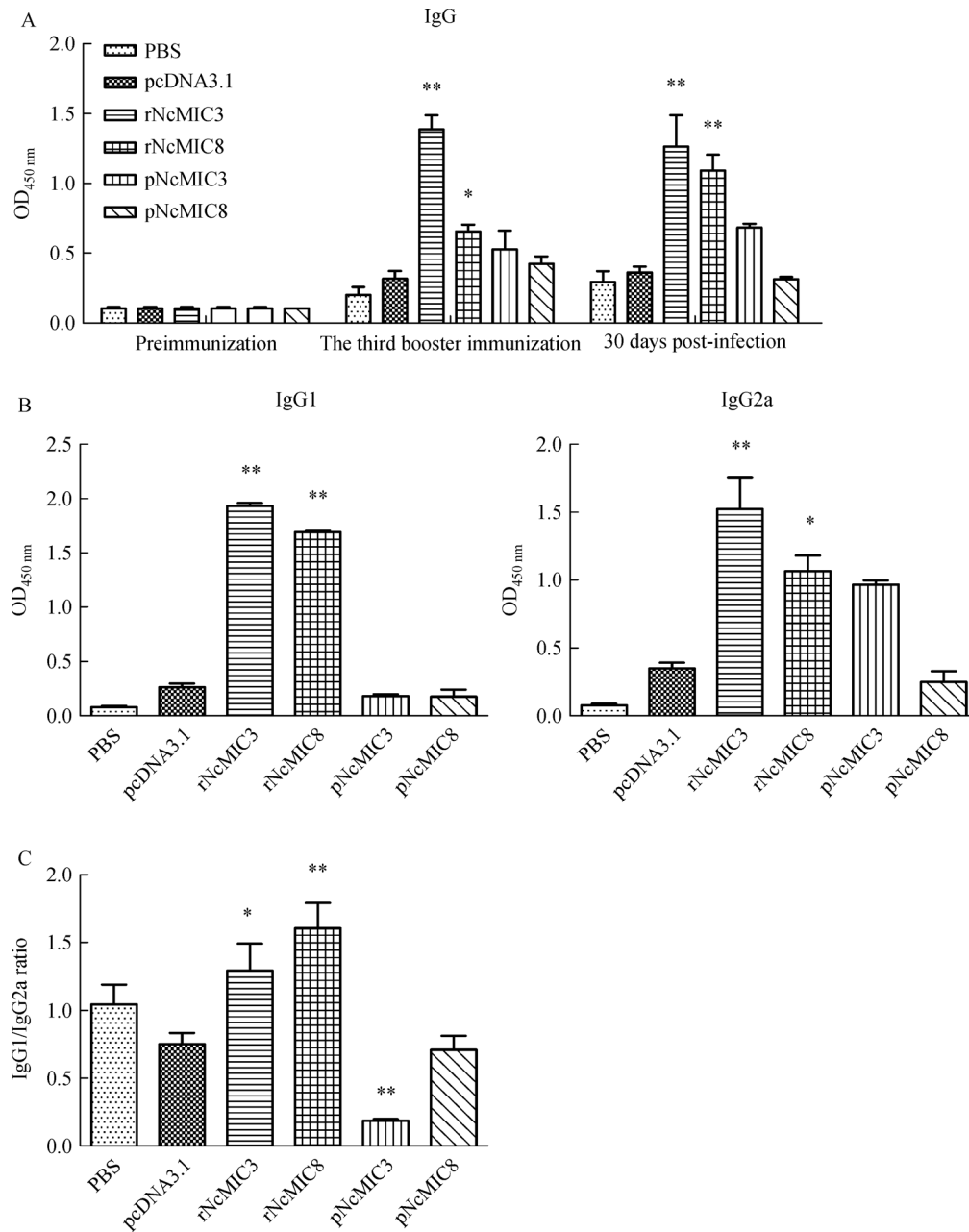
in the recombinant proteins groups (rNcMIC3: 1.073,  $P < 0.05$ ; rNcMIC8: 1.717,  $P < 0.01$ ) (Fig. 3C). Compared with the other groups, the DNA vaccine groups induced higher levels of IgG2a than IgG1, and the ratio of IgG1/IgG2a in the pcDNA3.1-NcMIC3 group ( $P < 0.01$ ) was significantly lower than in the other groups. There was no significant difference between mice immunized with PBS or the empty vector. Generally, a predominance of IgG1 over IgG2a was observed in the sera of recombinant proteins groups, which indicated that immunization with rNcMIC3/rNcMIC8 elicited a Th2 type immune response, whereas the DNA vaccine groups elicited a Th1-type immune response.

### 3.6 Cytokine expression in sera

IFN- $\gamma$  and IL-4 serum cytokine levels were determined in sera of all experimental groups. As shown in Table 1, compared with the recombinant proteins and control groups, DNA vaccines generated significantly higher levels of IFN- $\gamma$  ( $P < 0.05$ ), whereas, mice immunized with rNcMIC3 ( $829 \pm 7.3$ ,  $P < 0.01$ ) and rNcMIC8 ( $715 \pm 7.0$ ,  $P < 0.05$ ) produced large amounts of IL-4, and the production was significantly higher than in the other groups. These findings were consistent with the results obtained by the anti-*N. caninum* IgG isotype, which showed that immunization with DNA vaccines mainly produced a Th1-type immune response, while recombinant proteins elicited a Th2-type immune response.

### 3.7 Protection against *N. caninum* in mice

To test whether the vaccine induced effective protection against *N. caninum* infection, RT-PCR was used to investigate the cerebral parasite burden in vaccinated and non-vaccinated mice (Fig. 4). Quantification of the cerebral parasite burden showed a significant reduction of parasite numbers in rNcMIC3 and rNcMIC8 group, followed by the pcDNA3.1-NcMIC3 group. In contrast, the pcDNA3.1-NcMIC8 group induced a higher cerebral



**Fig. 3** *Neospora caninum*-specific antibody levels in the sera of immunized BALB/c mice. A: Levels of IgG antibodies in sera of rNcMIC3, rNcMIC8, pNcMIC3, pNcMIC8, pcDNA3.1, and PBS groups were determined using indirect ELISA. Samples were collected preimmunization, after the third booster immunization and 30 days post-infection ( $n = 10$ ); B: The levels of IgG1 and IgG2a subtypes in the sera 28 days after the last immunization were determined by ELISA; C: The IgG1/IgG2a ratio was calculated to determine whether the immunized mice induced a Th1- or a Th2-type immune responses. The results are expressed as the means $\pm$ SD from three independent experiments. The asterisk designates statistically significant differences ( $P < 0.05$ ) between groups. \*  $P < 0.05$ , compared to control groups; \*\*  $P < 0.01$ , compared to control groups.

parasite burden in mice. These data indicate that rNcMIC3 and rNcMIC8 vaccines provide partial protection over DNA vaccines by reducing the cerebral infection, and the protective efficacy of the rNcMIC3 vaccine was better than the rNcMIC8 vaccine.

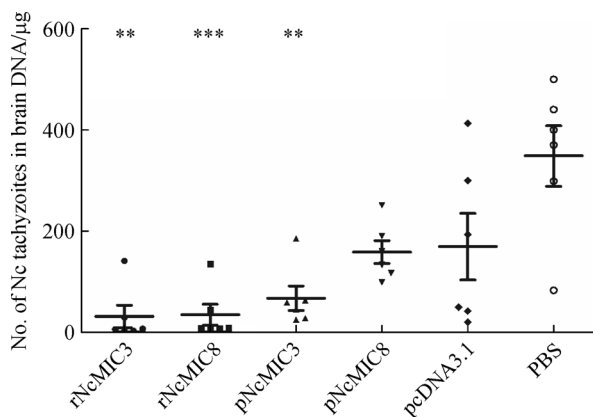
#### 4 Discussion

To date, there are no drugs available to treat neosporosis, and current control strategies rely on prevention of infection through management procedures and strict

**Table 1** Cytokines detected in sera from immunized mice

Group	IFN- $\gamma$ ±SD/(pg·mL <sup>-1</sup> )	IL-4±SD/(pg·mL <sup>-1</sup> )
rNcMIC3	126.56±2.52	828.84±7.29**
rNcMIC8	123.08±5.30	715.14±6.98*
pNcMIC3	193.16±3.73*	525.65±4.35
pNcMIC8	172.29±5.57*	696.19±3.99*
pcDNA3.1	110.16±7.21	445.12±4.05
PBS	72.38±3.91	416.70±8.64

Note: Sera from mice were obtained one week after the last immunization. Values are shown as the means±SD. \*  $P < 0.05$ , compared to control groups; \*\*  $P < 0.01$ , compared to control groups.



**Fig. 4** *Neospora caninum* burden in brain tissues of mice. The numbers on the y-axis correspond to the parasite number per  $\mu\text{g}$  of DNA, and each point represents individual animals. The results show that mice immunized with rNcMIC3 or rNcMIC8 and pcDNA3.1-NcMIC3 had reduced brain parasite loads compared to the control groups. In contrast, the pcDNA3.1-NcMIC8 group induced a higher cerebral parasite burden in mice. Values are shown as the means±SEM.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*) were statistically significant as indicated.

hygiene. Thus, a vaccine able to prevent both horizontal transmission via contaminated food or water and vertical transmission would be a cost-efficient method for the control of neosporosis<sup>[21,22]</sup>. At present, the research on recombinant subunit vaccine of *N. caninum* is mainly focused on the secreted and surface antigens. Cannas et al.<sup>[23]</sup> reported that NcSAG1 and NcSRS2 effectively prevent the invasion of *N. caninum*, and also effectively inhibit the vertical transmission of *N. caninum*. In addition, rhoptry proteins, microneme proteins and dense granule proteins all participate in the interaction between *N. caninum* and host cells and can also provide immune protection in a mouse model reducing the parasite burden in brain tissues<sup>[24]</sup>. Some *N. caninum* rhoptry and dense granule proteins have been evaluated as vaccine candidates, including NcROP2<sup>[18]</sup>, NcGRA1, NcGRA2, NcGRA6 and NcGRA7<sup>[24,25]</sup>.

Several recent studies have reported that immunization with microneme proteins such as NcMIC1, NcMIC2, NcMIC3, NcMIC4<sup>[19]</sup>, NcMIC6<sup>[11]</sup>, NcMIC8<sup>[12]</sup> and NcMIC10 can enhance humoral immune responses against *N. caninum*. Earlier studies in our laboratory found that NcMIC8 forms a complex with NcMIC3<sup>[12]</sup>. In this study, we examined the immunogenic and protective effect of the recombinant proteins and DNA vaccines.

It is informative to evaluate whether the vaccine-induced response is biased toward a Th1 or a Th2 type response. The Th1-type immune response can lead to secretion of a variety of cytokines such as interferon to promote CTL response, which is a common cellular immune response, while the Th2-type immune response, involving secretion of cytokine IL-4, which promotes antibody production, is humoral immune response. The Th1 type produces an IgG2a type antibody, and the Th2 type produces an IgG1 type antibody in mice. Therefore, we used IgG1/IgG2a to evaluate which response the vaccine-induced immune response was biased toward. By detecting the IgG antibodies and the cytokine IL-4 and IFN- $\gamma$  in the serum of mice after immunization, we found a high IgG1/IgG2a ratio in sera of recombinant proteins groups. Vaccination with recombinant proteins induced increased IgG1 levels and the mice retained the high IL-4 to IFN- $\gamma$  ratio, whereas, in mice vaccinated with DNA vaccines, IL-4 levels decreased dramatically, and in turn, IFN- $\gamma$  concentrations in the serum increased considerably, and a predominance of IgG2a over IgG1 was observed in the sera of DNA vaccine groups. Various studies have emphasized that Th1-type immune response favor the production of IgG2a, and increased IFN- $\gamma$  production. However, Th2 cytokines, such as IL-4 and IL-10, are associated with the production of IgG1, the levels of antibody subclasses reflect the *in vivo* production of cytokines and, thus, the type of immune response<sup>[26,27]</sup>. So, recombinant proteins rNcMIC3 and rNcMIC8 induced a Th2-type immune response that could reduce the brain burden in mice challenged with *N. caninum*, while DNA vaccines pcDNA3.1-NcMIC3 and pcDNA3.1-NcMIC8 elicited a Th1-type immune response.

As *N. caninum* is an intracellular parasite, not only the humoral but also the cellular immune response must be considered as an important mediator of resistance or susceptibility. Resistance to infection, either innate or following immunization, seems to depend on a Th1-type cellular immune response<sup>[23]</sup>. It is possible that a Th1 response is primarily protective against encephalitis, but it cannot effectively inhibit the vertical transmission of *N. caninum*<sup>[27]</sup>. In the late stages of *N. caninum* infection of animals, a Th2 type immune response is more important for inhibiting the proliferation and transmission of the tachyzoites<sup>[21]</sup>. During its life cycle, *N. caninum* secretes many proteins. Various studies have shown that different kinds of proteins can cause different types of immune responses following immunization in mice. For example, NcMAG1, NcBAG1 and NcSAG4 mainly cause Th1 type immune response, and rNcMIC3, NcSRS2 mainly cause Th2 type immune response<sup>[14,27,28]</sup>. However, the type of immune response is not only related to the characteristics of the protein itself, but also depends on other factors, such as the strain of mice, the state of mice at immunization, the method of vaccination and the types of adjuvants used<sup>[29,30]</sup>.

The current experimental study of candidate vaccines for *N. caninum* generally involves immunization with candidate antigens followed by challenge with *N. caninum* tachyzoites. The immunization effectiveness of the candidate vaccine is evaluated by weight change, abortion rate, survival time, survival rate and the parasite burden in the brain tissues<sup>[31]</sup>. In this study, we inoculated the tachyzoites at a lower rate of ( $1 \times 10^6$ ), consequently, the survival rate of mice was 10% and the difference in weight change was not obvious. Therefore, the parasite burden in the brain was an important factor for the evaluation of the protective effect. The mice immunized with recombinant proteins revealed a lower parasite burden compared with the DNA vaccine and control groups.

It is also important to select effective adjuvants or delivery systems, due to their significant functions in increasing the immunogenicity of antigens via induction of proper immune responses, improving the efficacy and determining the type of immune responses induced by different vaccines<sup>[32]</sup>. Several studies have reported that different combinations of antigens can improve immune protective effects. In our study, we did not evaluate combinations of protein or protein and DNA plasmids, mainly because we wanted to know the protective effect of each protein individually, and protection provided by combinations will be assessed in subsequent experiments.

In general, our study suggests that rNcMIC3 and rNcMIC8 had the ability to partly protect mice against *N. caninum* and should be considered as vaccine candidates for neosporosis. Whether these two proteins can effectively prevent the vertical transmission of *N. caninum* and whether DNA vaccine can inhibit in the

process of *N. caninum* infection are questions for further research.

**Acknowledgements** Project support was provided by the National Key Research and Development Program of China (2017YFD0501200), the National Key Basic Research Program (973 program) of China (2015CB150300), the National Natural Science Foundation of China (31772730).

**Compliance with ethics guidelines** Taotao Zhang, Xiao Zhang, Qun Liu, Jianhai Xu, and Jing Liu declare that they have no conflicts of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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