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ORIGINAL PAPER

Protective immunity induced by a DNA vaccine-encoding *Toxoplasma gondii* microneme protein 11 against acute toxoplasmosis in BALB/c mice

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Abstract *Toxoplasma gondii* is one of the most prevalent intracellular parasites and is threatening the health of both humans and animals, therefore causing incalculable economic losses worldwide. Vaccination is thought to be an efficient way of controlling toxoplasmosis. *T. gondii* microneme protein 11 (MIC11) is a soluble microneme protein which is presumably considered facilitating the early stage of cell invasion. To evaluate the protective efficacy of *T. gondii* MIC11, in the present study, a new DNA vaccine-encoding the α-chain of *T. gondii* MIC11 was constructed using the pcDNA3.1 vector. Expression of MIC11 from this vector was confirmed by indirect immunofluorescence assay following transfection into baby hamster kidney (BHK) cells. Intramuscular immunization of BALB/c mice with pcDNA/MIC11 was carried out to evaluate the immune responses by serum antibodies titers, lymphoproliferation assay, and cytokines assay. The protective efficacy was evaluated by survival rate in mice after challenging with highly virulent strain of *T. gondii*. The results demonstrated that this vaccination elicited significant humoral responses and *T. gondii* lysate antigen (TLA)-stimulated lymphoproliferation ($p<0.05$). Compared to controls, the pcDNA/MIC11 immunized mice had high production of IFN-γ, IL-12, and IL-2 ($p<0.05$), but not IL-4 ($p>0.05$), indicating that a predominant Th1 type response was developed. The vaccination also increased the survival rate of immunized mice when they were challenged with a lethal dose of tachyzoites of *T. gondii* RH strain. These data suggest that *T. gondii* MIC11 is a reasonable vaccine candidate deserving further studies, and pcDNA/MIC11 is a potential strategy for the control of toxoplasmosis.

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Introduction

Toxoplasma gondii is an obligate intracellular protozoan infecting a variety of warm-blooded animals and humans worldwide (Dubey. 2008). Although a remarkably successful pathogen, *Toxoplasma* infection is normally asymptomatic in immuno-competent individuals. However, it can cause severe complications in immune-compromised patients such as those undergoing organ transplantation or HIV infection. *T. gondii* is also able to cross the placenta following maternal infection during pregnancy, causing abortion and fetal abnormalities or fatal encephalitis (Tenter et al. 2000; Miller et al. 2009). In addition to the risk to humans' health, *T. gondii* infection of agriculturally important animals such as sheep, goats, and pigs also causes significant economic losses due to animal abortions and neonatal losses (Dubey et al. 2005). As a food-borne parasite, *T. gondii* is transmitted from animals to humans through consumption of raw or undercooked meat and other edible parts of animals (Tenter et al. 2000). Chemical treatments for acute toxoplasmosis are currently available, but they are not well acceptable due to the problems such as emergence of drug-resistant parasites and chemical residues

in food (Vercruysse et al. 2004; Kur et al. 2009). Because of these reasons, to develop an efficient vaccine against toxoplasmosis becomes very important.

So far, there is no commercially licensed *T. gondii* vaccine to be used in human. Only one commercial vaccine to limit the abortion in sheep was developed for veterinary, although the molecular mechanism for the vaccination strain is still not well understood (Buxton et al. 1993; Buxton and Innes 1995). Given the potential problems of regaining virulence or provoking iatrogenic infection of the current live vaccine, it is necessary to look for other vaccination strategies (Meng et al. 2012). For this sake, DNA vaccine is a good option because of the independence of whole-organism preparation. In addition, DNA vaccine is a promising tool for producing both specific humoral and cellular immune responses and long-lasting immunity (Kur et al. 2009).

In the last few years, many efforts and considerable advances were made in the identification of appropriate *T. gondii* antigens as vaccine candidates. *T. gondii* molecules belonging to several major protein families were thought to be ideal candidates including surface antigens (SAGs), rhoptry antigens (ROPs), and dense granule antigens (GRAs) (Sun et al. 2011; Yuan et al. 2011; Min et al. 2012; Wu et al. 2012; Pathasarathy et al. 2013). As the key roles playing during host adhesion and invasion, microneme proteins (MICs) were increasingly identified as promising vaccine targets (Soldati et al. 2001; Cérède et al. 2005; Kur et al. 2009; Wang et al. 2009; Liu et al. 2010; Yan et al. 2012). In the present study, we focused on the soluble microneme protein MIC11, which is structurally similar to *T. gondii* ROP1 and is thought to have a role in organizing other MICs for the deployment of adhesive complexes to the apical surface to facilitate host cell invasion (Harper et al. 2004). Regardless of the possible roles of *T. gondii* MIC11, no previous studies have evaluated the vaccine potential of MIC11 against *T. gondii* infection. We designed a DNA vaccine (pcDNA3.1/MIC11) encoding the α-chain of *T. gondii* MIC11 using pcDNA3.1 as a vector, and the immune responses and protective efficacy were assessed in BALB/c mice.

Materials and methods

Mice, parasites, and cells

Sixty female BALB/c mice aged 5–6 weeks were purchased from the Wuhan University Laboratory Animal Center in China. All the mice were allowed access to feeding and water ad libitum and were humanely sacrificed as required for ameliorate suffering. All the mice studies were carried out in compliance with the regulations (No. 5 proclaim of the Standing Committee of Hubei People's Congress) approved by the Standing Committee of Hubei People's Congress, P. R. China. The animal protocols were approved by Laboratory Animals Research Centre of Hubei province and the ethics committee of Huazhong Agricultural University (Permit number: 4200695757).

T. gondii RH strain tachyzoites (National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China) were inoculated peritoneally in BALB/c mice and were harvested from the peritoneal fluid 72 h after infection. The collected tachyzoites were then purified by filtration through CF-11 cellulose (Whatman Inc., USA) (Bulow and Boothroyd 1991), washed in 0.1 M phosphate buffered saline (PBS, pH 7.4), and enriched by centrifugation at 600×*g* for 10 min. The purified tachyzoites were used for the preparation of *T. gondii* lysate antigen (TLA), RNA extraction, and mice challenging.

The baby hamster kidney (BHK) cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Beijing, China) supplemented with L-glutamine, 10 % dialyzed fetal bovine serum (FBS) (Gibco, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified chamber containing 5 % CO_2 at 37 °C.

Plasmid construction

To construct the plasmid expressing recombinant MIC11, the coding sequence of the α-chain of MIC11 gene (GenBank accession no. AF539702.1) was PCR amplified from *T. gondii* tachyzoites cDNA. A pair of primers (forward primer: 5′-GAATTCATGGCCGAAGATGACAAAAGCGCC-3′; reverse primer: 5′-CTCGAGTTATCTCTCTTGAAGAAAACC-3′) was used according to the previous report by Harper et al. (2004) with the introduction of *Eco*RI and *Xho*I restriction sites (underlined). The targeted fragment of MIC11 gene was ligated into pcDNA3.1 vector (National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China), resulting in the recombinant plasmid pcDNA/MIC11. The resultant plasmid was cloned in *Escherichia coli* (DH5α strain) and was confirmed by restriction digestion and sequencing analysis. The large scale plasmid extraction was performed using Endo-Free Plasmid Maxi Kit (OMEGA bio-tek, USA) by following the manufacturer's instructions. The concentration of the extracted plasmid was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA).

Expression of MIC11 in vitro

To test the expression of recombinant plasmid in vitro, pcDNA/MIC11 was transfected into 70 % confluent BHK cell monolayer using Lipofectamine™ 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. The empty pcDNA3.1 vector was used as a negative control. Forty-eight

hours after transfection, the monolayer was fixed with chilled 3 % (*w*/*v*) paraformaldehyde (PFA) for 20 min at room temperature and then processed for indirect immunofluorescence assay (IFA) for the detection of MIC11 protein. Briefly, the fixed monolayer was permeabilized with 0.2 % TritonX-100 and incubated with porcine anti-*T. gondii* sera (National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China) at 37 °C for 60 min. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-porcine IgG (SouthernBiotech, USA) at 37 °C for 60 min and examined under a Nikon fluorescence microscope.

DNA immunization and parasite challenging

Sixty BALB/c mice were randomly divided into three groups (20 in each group). For immunization, 100 μg (for each injection) of pcDNA/MIC11 plasmid or the pcDNA3.1 empty vector were suspended in 100 μl sterile PBS and intramuscularly injected into different groups of mice, respectively. The mice injected with 100 μl sterile PBS only were served as the blank control group. All groups of mice were immunized three times at a 2-week interval using same dosages. Blood samples of mice in each group were collected from the tail vein on days 0, 14, 28, and 42 post-immunization. Sera were stored at −20 °C until used for serological tests.

Ten days after the last immunization, twelve mice from each group were randomly selected and injected intraperitoneally with 1×10^3 tachyzoites of *T. gondii* RH strain. Tachyzoites were counted with a hemocytometer and adjusted to 5×10^3 parasites/ml with 0.1 M PBS (pH 7.4). The parasite solution (200 μl) was injected into each mouse, and mice survival was subsequently monitored daily for 15 days.

Detection of humoral response

For the detection of humoral response, the IgG titer against TLA was measured using ELISA (Fachado et al. 2003). Briefly, TLA was prepared as previously described (Vercammen et al. 2000) and was used to coat ELISA plate overnight at 4 °C at the concentration of 10 μg/ml in carbonate buffer (pH 9.6). The plates were blocked with 3 % bovine serum albumin (BSA) (Equitech-Bio Inc., USA), followed by incubation with mice sera at the dilution of 1:100 and then peroxidase-conjugated goat anti-mouse IgG (EarthOx, San Francisco, USA) at the dilution of 1:6,000. The peroxidase activity was detected by the chromogenic substrate tetramethylbenzidine and was stopped by 0.25 % hydrofluoric acid (HF). The optical density was determined at 630 nm (OD_{630}) using an automated ELISA reader (Bio-Tek, ELx800, Winooski, Vermont). All sera samples were examined in triplicates.

Lymphocyte proliferation assay

The lymphocyte proliferation assay was performed according to the method described previously (Fang et al. 2009; Yan et al. 2012). Splenocyte suspensions were collected from four immunized mice of each group on day 14 after the final immunization. Collected cells were then resuspended in RPMI-1640 (Gibco) supplemented with 10 % FBS and were cultured in 96-well plates in triplicates at 5×10^5 cells/150 μl per well. Cells were stimulated with either 10 μg/ml TLA, 5 μg/ml concanavalin A (ConA) (as a positive control, Sigma), or medium alone (as a negative control) for 72 h at 37 °C in a 5 % CO2 incubator. Subsequently, 10 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (5 mg/ml, Sigma) was added to each well. After incubation for 4 h, the supernatant from each well was discarded, and cells were resuspended in 100 μl dimethysulfoxide (DMSO, Sigma). The optical density was measured at 570 nm (OD_{570}) by an automated ELISA reader. The stimulation index (SI) was calculated in order to quantitate lymphocyte proliferation response, as the ratio of the mean value of OD_{570} of antigen-stimulated cells to the mean value of OD_{570} of cells with medium only (negative control).

Cytokine assay

Splenocytes were collected from four immunized mice of each group and stimulated with TLA, ConA, or the medium alone as described in the lymphocyte proliferation assay. At different stimulation time points, supernatants of cultures were separated from the cells through centrifugation, and cytokine levels in the supernatants were measured using commercial ELISA Kits according to the manufacturer's instructions (Hermes Criterion Biotechnolog, Canada). Interleukin-2 (IL-2) and IL-4 levels were measured at 24 h stimulation, whereas IL-12 and interferon-gamma (IFN-γ) were measured at 72 and 96 h stimulation, respectively. The standard curves constructed with known amounts of mouse recombinant IL-2, IL-4, IL-12, or IFN-γ were used as a reference to determine the cytokine concentrations produced by splenocytes. All assays were carried out in triplicates. The sensitivity limitation of the assays for each cytokine was 1.0 pg/ml.

Statistical analysis

The averages were calculated in all assays, and the values have been given as means±SD. The data obtained among the different groups in the assays including humoral responses, lymphocyte proliferation assay, cytokine production, and protective immune responses were compared by analysis of variance (ANOVA). All the analyses were carried out using SPSS 11.5 software at 95 % confidence level. P values <0.05 were considered to be statistically significant.

Results

Expression of recombinant pcDNA/MIC11 plasmid in vitro

To check the expression of MIC11 from the constructed plasmid, pcDNA/MIC11 was transfected into BHK cells, and MIC11 expression was examined 48 h post-transfection using IFA analysis. As shown in Fig. 1, MIC11 expression was apparent in BHK cells transfected with pcDNA/MIC11 (Fig. 1a), but not in those control cells which were transfected with the same amount of empty vectors (Fig. 1b). These results demonstrated that the α-chain of *T. gondii* MIC11 protein was successfully expressed by pcDNA/MIC11 in BHK cells and was recognized by anti-*T. gondii* sera.

Humoral immune responses

In order to evaluate the level of humoral immune responses developed by vaccinated mice, anti-TLA IgG levels were detected in the sera collected on days 0, 14, 28, and 42 post the first immunization. As shown in Fig. 2, antibodies against TLA were first detected in the mice vaccinated with pcDNA/MIC11 2 weeks after the first immunization. More anti-TLA IgG was accumulated, and the antibody levels increased dramatically in mice group vaccinated with pcDNA/MIC11 after the second and third immunization ($p<0.05$). The mean antibody levels against TLA in the sera of mice group immunized with pcDNA/MIC11 were significantly higher than those of the pcDNA3.1 or PBS group ($p<0.05$). The sera of both two negative control groups were not detected antibodies against *T. gondii*.

Lymphocyte proliferation assay

Splenocytes were collected from mice of each group on day 14 after the last immunization to evaluate the proliferative response of lymphocytes following TLA stimulation. As expected, splenocytes from mice immunized with pcDNA/MIC11 had significant higher proliferation activity than those from the control mice immunized with PBS or pcDNA3.1 empty vector

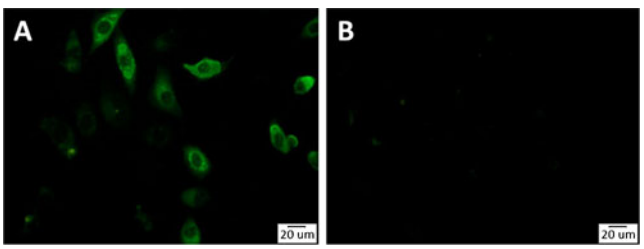

Fig. 1 Detection of the in vitro expression of the recombinant plasmid pcDNA/MIC11 in transfected BHK cells by IFA with the anti-*T. gondii* sera at 48 h post-transfection. **a** Cells transfected with pcDNA/MIC11; **b** cells transfected with the empty vector pcDNA3.1

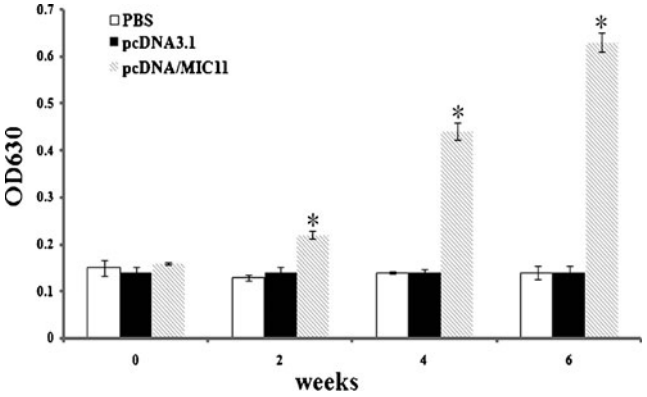

Fig. 2 Detection of specific humoral response in mice vaccinated with pcDNA/MIC11 by ELISA using TLA. Sera were collected at day 0, 14, 28, and 42 after immunization from all groups. The results were shown as means±SD of three independent experiments. Statistically significant differences (*$p<0.05$) from the control were indicated

($p<0.05$, Fig. 3). As a positive control, splenocytes from the experimental group and controls proliferated to comparable levels in response to ConA (data not shown).

Cytokine production

The cell-mediated immune responses produced by immunized mice were assessed by determining the levels of cytokines released in the supernatant by TLA-stimulated splenocyte cultures. As shown in Fig. 4, splenocyte cultures from mice immunized with pcDNA/MIC11 produced highly significant levels of IFN-γ, IL-12, and IL-2 compared with those immunized with PBS or pcDNA3.1 ($p<0.05$). In contrast, no significant difference in the levels of IL-4 was observed between different groups ($p>0.05$). The cytokine profile generated by MIC11 indicated that the cell-mediated immunity was Th1 type response.

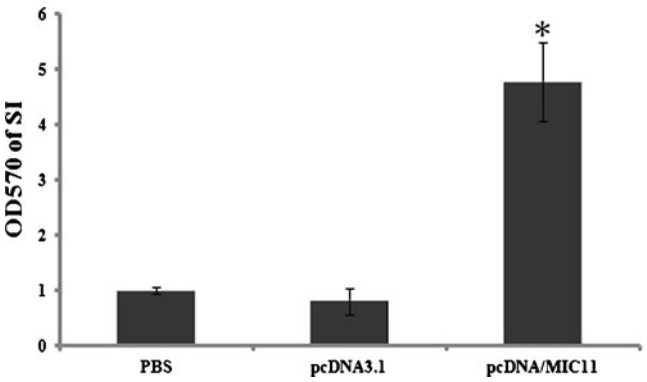

Fig. 3 Proliferation of splenocytes from each group of vaccinated mice. Splenocytes were harvested at 2 weeks after the last immunization from mice and stimulated with TLA. Results were shown as means of the SI±SD of three experiments. Statistically significant differences (*$p<0.05$) from the control were indicated

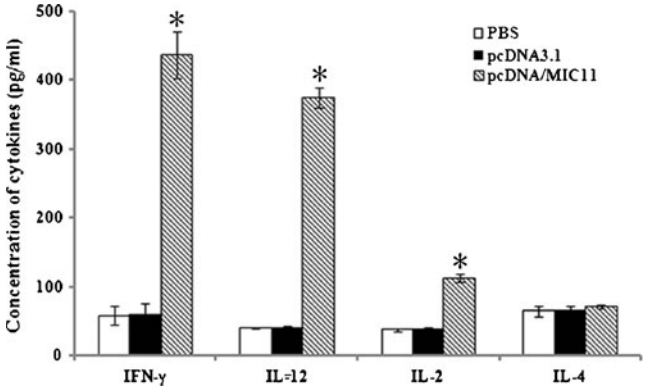

Fig. 4 Cytokine production of spleen cells from each group of immunized mice. The culture supernatants were examined for cytokine production by ELISA following the stimulation with TLA. Values for IFN-γ were taken from 96-h culture, values for IL-12 from 72 h, and values for IL-2 and IL-4 from 24 h. Statistically significant differences (*$p<0.05$) from the control were indicated

Protective effect against lethal challenge of *T. gondii* in BLAB/c mice

The protective immunity produced by pcDNA/MIC11 in mice was evaluated by giving lethal challenge of *T. gondii* tachyzoites (RH strain) to immunized mice. Mice were challenged with 10^3 tachyzoites at day 14 after the final immunization, and their survival was monitored. The mice group immunized with pcDNA/MIC11 showed significantly prolonged survival ($p<0.05$, Fig. 5). All mice in the control groups immunized with PBS or pcDNA3.1 died within 8 or 10 days post-infection, respectively. However, 50 % of the mice immunized with pcDNA/MIC11 were alive at day 10, and 17 % of them survived to day 15 after the parasite challenging.

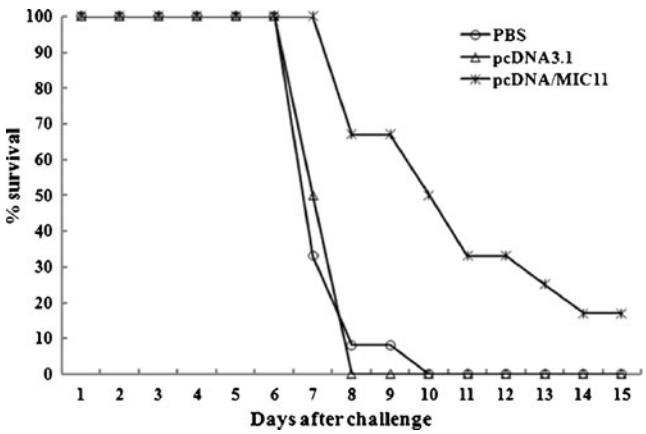

Fig. 5 Protection efficacy of the pcDNA/MIC11 was determined in BALB/c mice. The immunized mice were challenged with 1×10^3 tachyzoites of high virulent *T. gondii* RH strain at 2 weeks after the last vaccination. Survival time of challenged mice was recorded within 15 days. Each group composed of 12 mice

Discussion

DNA-based vaccination has become a novel approach for vaccine development following the initial demonstrations that plasmid DNA can generate protective immune responses in a variety of animal models (Montgomery et al. 1997; van Drunen Littel-van den Hurk et al. 2001; Donnelly et al. 2005). In the recent years, DNA vaccines have been considered a promising approach to protect animals and humans from a variety of pathogenic agents, particularly intracellular parasites (Bunnell and Morgan 1998). Various studies demonstrated that DNA vaccines induced long-lasting antibody responses and cell-mediated immunity (Gurunathan et al. 2000a, 2000b). In the present study, a DNA vaccine encoding the α-chain of *T. gondii* MIC11 was constructed and was shown the capacity to induce highly significant immune responses and protection in BALB/c mice against a lethal dose challenge of the highly virulent *T. gondii* RH strain.

Many members of micronemal family proteins are critical for host cell attachment and early invasion of *T. gondii* (Keeley and Soldati 2004; Huynh et al. 2006). Previously, several DNA vaccines encoding MICs were designed and were shown to successfully induce protective immune responses against *T. gondii*. Those include MIC2, MIC3, MIC4, MIC6, AMA1, and M2AP (Ismael et al. 2003; Dautu et al. 2007; Fang et al. 2009; Wang et al. 2009; Yan et al. 2012; Yu et al. 2012). MIC11 was recently identified as a soluble microneme protein undergoing a proteolytic maturation which is important for host cell invasion by *T. gondii* (Dowse and Soldati 2004; Harper et al. 2004). Preliminary studies indicated that MIC11 is conserved among several coccidian parasites and is secreted in a calcium-dependent manner (Harper et al. 2004). A two-step proteolytic modification removes an internal propeptide, resulting in the mature protein consisting of a α-chain and a β-chain, whereas only α-chain can be expressed stably in *E. coli*. The antibodies raised against recombinant MIC11 α-chain detected the specific band in *Toxoplasma* lysate (Harper et al. 2004). Since the detailed biological function of *T. gondii* MIC11 remains unclear, the aim of this study is to explore the immunological characteristics of MIC11 by designing a DNA vaccine expressing the α-chain of *T. gondii* MIC11 and assessing its immunogenic properties in BLAB/c mice.

The α-chain of *T. gondii* MIC11 was cloned into pcDNA3.1 so that its expression is driven by the CMV promoter, which is very active in many mammalian cells. Expression of the α-chain of *T. gondii* MIC11 from this vector was confirmed in BHK cells by using IFA analysis. As expected, the cells that contained pcDNA/MIC11 exhibited the observable bright fluorescence, which demonstrated that the antibodies against *T. gondii* can react with

MIC11 α-chain expressed in eukaryotic system. Moreover, mice immunized with pcDNA/MIC11 developed high titers of anti-TLA antibodies especially after last immunization. These results indicated that the recombinant MIC11 showed good immunogenicity and possessed the potential to induce strong humoral immune responses.

DNA-based immunization has been elucidated to elicit cellular responses and to mediate cytotoxic T lymphocytes production depending on IFN-γ and IL-12 (Gurunathan et al. 2000a, 2000b). Studies on natural infection of *T. gondii* demonstrated that a Th1 type immune response is predominant (Denkers and Gazzinelli 1998; Yuan et al. 2011). In this study, mice vaccinated with the recombinant plasmid pcDNA/MIC11 developed a significant splenocytes proliferation in response to TLA stimulation. In addition, pcDNA/MIC11 induced significantly high levels of IFN-γ, IL-12, and IL-2 production upon TLA stimulation. In contrast, MIC11 failed to stimulate the production of IL-4 ($p > 0.05$). The cytokine profile generated by pcDNA/MIC11 in the present study indicated that Th1 type response is predominant in acquiring immunity to MIC11 in BALB/c mice. Similarly, several authors revealed the occurrence of distinct pattern of Th1 response to DNA-based vaccines against *T. gondii* (Lourenco et al. 2006; Fang et al. 2009; Khosroshahi et al. 2012; Cong et al. 2013).

To evaluate the protection efficacy of the DNA vaccine, immunized BALB/c mice were intraperitoneally challenged with 1×10^3 tachyzoites of the highly virulent *T. gondii* RH strain. The survival assay demonstrated that the immunization with pcDNA/MIC11 significantly prolonged the survival time of challenged mice in comparison with those of control groups, which indicated that the immunization with pcDNA/MIC11 was able to produce certain level of protective immunity against acute *T. gondii* infection in BALB/c mice model. However, the immunization with pcDNA/MIC11 did not protect the challenged mice from obvious symptoms of toxoplasmosis at later time points and subsequent death. The current results indicated that the pcDNA/MIC11 can only induce partial protection against infection with high virulent *T. gondii* strain but not complete.

In conclusion, the present study describes the first use of *T. gondii* MIC11 as a DNA vaccine candidate against toxoplasmosis. The significant humoral and cellular immune responses in terms of high antibody titer, lymphoproliferation, and cytokines levels were elicited, respectively. Cell-mediated immune responses revealed that the DNA vaccine pcDNA/MIC11 derived an enhanced Th1 type response. The BALB/c mice immunized with pcDNA/MIC11 had prolonged survival after a lethal challenge of *T. gondii*. All these findings suggested that *T. gondii* MIC11 might be a good vaccine candidate deserving further optimization, and the DNA vaccine pcDNA/MIC11 is a potential way to battle *T. gondii* infection.

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