Veterinary Parasitology 166 (2009) 220–227

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Induction of protective immunity by multiantigenic DNA vaccine delivered in attenuated *Salmonella typhimurium* against *Toxoplasma gondii* infection in mice

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ARTICLE INFO

Article history:
Received 11 February 2009
Received in revised form 19 July 2009
Accepted 15 August 2009

Keywords:
Toxoplasma gondii
SAG1
MIC3
DNA vaccine
Attenuated *Salmonella typhimurium*

ABSTRACT

Toxoplasma gondii, capable of infecting all warm blooded animals, is one of the most successful parasites worldwide. It was reported that the single-gene vaccine with SAG1 or MIC3 could only produce partial protection against *T. gondii* and multiantigenic vaccines were more effective than single ones. In the present study, a multiantigenic DNA vaccine delivered by attenuated *Salmonella typhimurium* (ZJ111/pSAG1-MIC3) was constructed, which expresses surface protein SAG1 and micronemal protein MIC3. The safety and stability of ZJ111/pSAG1-MIC3 were evaluated and immune response with ZJ111/pSAG1 and ZJ111/pMIC3 were compared. The results of lymphocyte proliferation assay, antibody and cytokine determination show that mice immunized with ZJ111/pSAG1-MIC3 elicited stronger humoral and Th1-type cellular immune responses than other groups. The mice immunized with ZJ111/pSAG1-MIC3 also exhibited significant higher survival time after challenged with *T. gondii* RH strain. The current study shows that the oral multiantigenic DNA vaccine, ZJ111/pSAG1-MIC3, produces partial protection against *T. gondii* challenge.

1. Introduction

Toxoplasma gondii, an obligate intracellular protozoan parasite, has the ability to infect and propagate in virtually all nucleated host cells (Joiner and Dubremetz, 1993; Saeij et al., 2006). The parasite belongs to the phylum Apicomplexa that is responsible for a wide variety of illnesses such as toxoplasmosis (*Toxoplasma*), malaria (*Plasmodium*), crytosporidiosis (*Cryptosporidium*) and many veterinary diseases (Dubey et al., 2006, 2003; Weiss and Kim, 2004). Human infection by *T. gondii* is generally asymptomatic and induces a self-limiting disease in immunocompetent individuals. In contrast, primary *T. gondii* infection acquired during gestation can be transmitted to the fetus through the placenta and may cause miscarriage, permanent neurological damage, premature birth, and visual impairment (Beghetto et al., 2006; Wallon et al., 2004). Congenital toxoplasmosis is also of considerable economic importance in the farming industry, because it is one of the principal causes of abortion, fetal death, and stillbirths in all types of livestock, particularly in pigs, goats and sheep (Ismael et al., 2006). Reports have indicated that a high proportion of meat products were contaminated with *T. gondii*, and that a major risk of infection is associated with meat from animal-friendly production systems (Aspinall et al., 2002; Jongert et al., 2007; Kijlstra et al., 2004). Consumption of raw or undercooked meat products is perceived as a major source of infection to pregnant women (Cook et al., 2000). This suggests that control of the disease by vaccination would

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doi:10.1016/j.vetpar.2009.08.016

be valuable for preventing both fetal infection and reactivation in the farming industry.

Plasmid DNA vaccine against *T. gondii* is a potent strategy for the induction of protective cellular immune responses (Jongert et al., 2007; Mevelec et al., 2005). Vaccination studies in mice have focused on the selection of protective antigens and the most promising experimental vaccines now combine proteins from membrane associated surface antigen, excreted–secreted dense granule proteins, rhoptry proteins and micronemal proteins (Jongert et al., 2008). Among these vaccine candidates, the main surface antigen 1 (SAG1), highly conserved in *T. gondii* strains, is best characterized and was shown to induce both humoral and cellular immune responses in mice (Cong et al., 2008; Qu et al., 2008; Zhang et al., 2007). The micronemal protein MIC3 looks particularly promising because it is a potent adhesin of *T. gondii*. It is expressed in all three infectious stages of *T. gondii* (tachyzoites, bradyzoites, and sporozoites) and elicits early and powerful immune responses in mice and humans (Garcia-Reguet et al., 2000; Ismael et al., 2006, 2003). *T. gondii* possesses a complex life cycle with diverse life stages and extensive invading style. Accumulating evidence also indicates that vaccination with stage-specific antigens only leads to partial protection (Alexander et al., 1996; Zhou et al., 2007). So, if the epitopes of the two molecules are simultaneously presented to the immune system, the immunopotentiating properties of the two antigens can be utilized, and effective protection against *T. gondii* may be achieved.

However, DNA vaccination by direct intramuscular injection is time-consuming, laborious and stressful to animals, and thus not suitable to livestock industry where mass vaccination is often practiced (Li et al., 2006). Recent studies have shown that attenuated *Salmonella typhimurium* is a promising carrier to deliver plasmid DNA vaccines encoding immunogens of pathogenic microorganisms or tumors (Avogadri et al., 2005; Fensterle et al., 2008; Xu et al., 2007). This strategy allows administration of DNA vaccines via mucosal surfaces as well as delivery of the plasmid DNA directly to antigen presenting cells (APC), which could elicit strong humoral and cellular responses against the pathogens, and this system has been proven successful in human immunodeficiency virus, hepatitis B virus and coccidiosis in chickens (Du and Wang, 2005; Tsunetsugu-Yokota et al., 2007; Woo et al., 2001). However, a multiantigenic DNA vaccine in *T. gondii* delivered by attenuated *S. typhimurium* has not been reported much.

In this study, we constructed a multiantigenic DNA vaccine expressing SAG1 and MIC3 antigens carried by attenuated *S. typhimurium*, and compared the immunogenicity and protective efficacy of the DNA vaccine with the ones expressing single gene in ICR mice.

2. Materials and methods

2.1. Experimental animals

Female ICR mice (Grade II, 6 weeks old) weighing 18–22 g were purchased from Zhejiang Experimental Animal Center (Certificate No. 22-2001001, Hangzhou, China). Rodent laboratory chow and tap water were provided ad libitum and maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of $50 \pm 10\%$, and a 12/12 h light/dark cycle. All procedures were in strict accordance with the PR China legislation on use and care of laboratory animals and the guidelines established by Institute for Experimental Animals of Zhejiang University and were approved by the university committee for animal experiments.

2.2. Bacterial strains and parasites

Attenuated *S. typhimurium* strain SV4089 (kindly provided by Dr J. Casadesus, Department of Molecular Genetics, University of Sevilla, Spain) is a double mutant (Dam^- and $PhoP^-$) derived from wild-type *S. typhimurium* strain SL1344. The mutant is not pathogenic to mice via oral delivery at dose level of as high as 1010 colony forming units (CFU).

Tachyzoites of *T. gondii* RH strain were kindly provided by Prof. Zhu Xingquan (College of Veterinary Medicine, South China Agricultural University) and were harvested from the peritoneal fluid of ICR mice that had been intraperitoneally infected 3–4 days earlier.

2.3. Preparation of STAg

Soluble tachyzoite antigen (STAg) was prepared from RH strain tachyzoites as previously described. Briefly, the obtained tachyzoites were washed with 0.01 mol/l phosphate-buffered saline (PBS, pH 7.4) and sonicated for three 10-min periods at 60 W/s. The toxoplasma sonicate was centrifuged at $2000 \times g$ for 30 min. The protein concentration was determined in the supernatant, which was later used as the source of antigen, according to Bradford method. The STAg was stored at −70 °C before use.

2.4. Plasmid construction and transformation

To construct the multiantigenic SAG1-MIC3 fusion eukaryotic expression plasmid, the coding sequences of the SAG1 and MIC3 genes were amplified by polymerase chain reaction (PCR) from genomic DNA of *T. gondii* (RH strain). We designed two pairs of oligonucleotide primers: SAG1, forward primer: 5′-CGGGGTACCATGTCGGTTTCGCTGCACCAC-3′ and reverse primer: 5′-CCGGATATCTCCCGCGACACAAGCTGCG-3′, introduced KpnI and EcoRV recognition sites (underlined) respectively; MIC3, forward primer: 5′-TCTGATATCTCCCCCAGCAAGCAGGA-3′ and reverse primer: 5′-GTAATCTAGAGACTCCAGCTCACTGCTTAAT-3′, introduced EcoRV and XbaI recognition sites (underlined) respectively. The two kinds of PCR products were respectively digested with the above corresponding restriction enzymes and purified from agarose gel, and then introduced into the eukaryotic expression plasmid pcDNA3.1 vector (Invitrogen). Attenuated *S. typhimurium* was grown at 37 °C in LB broth overnight to an OD 600 of 0.6–0.8 and made competent by resuspending the bacteria in ice-cold ultrapure H_2O (Sangon Biological Technology and Service Co., Ltd., Shanghai, China), and then on ice for

20 min. The purified plasmid pSAG1, pMIC3, pSAG1-MIC3 or control vector pcDNA3.1 was transformed into *S. typhimurium* competent cells by electroporation at 2.5 kV, 25 μF and 200–400 Ω. The positive transformants were selected on LB agar containing 50 μg/ml ampicillin and verified by PCR amplification of the target gene SAG1, MIC3 and digestion with restriction enzymes. The *S. typhimurium* strains containing plasmid pSAG1, pMIC3, pSAG1-MIC3 or pcDNA3.1 were named ZJ111/pSAG1, ZJ111/pMIC3, ZJ111/pSAG1-MIC3 or ZJ111/pcDNA3.1, respectively.

2.5. Immunization

Female ICR mice were divided into five groups, each consisting of thirty mice. Thirty minutes prior to oral inoculation, mice were administered with 100 μl 10% sodium bicarbonate to neutralize stomach acidity. Group I, saline control. Mice in groups II–V were orally immunized with *S. typhimurium* ZJ111/pSAG1, ZJ111/pMIC3, ZJ111/pSAG1-MIC3 and ZJ111/pcDNA3.1 in 108 CFU dosages per mice, respectively. Mice in vaccinated groups were boosted with the same dose 2 weeks later.

2.6. Bacterial colonization and plasmid stability in organs

The immunized mice were monitored daily for clinical changes. The body weights of all groups were recorded pre-immunization and 6 weeks post-immunization. Two mice in each group were euthanized every week post-immunization, and fecal samples, spleens and livers were collected and homogenized in PBS, respectively. The bacterial counts were determined by plating serial dilutions of the homogenized samples on LB agar plates containing 50 μg/ml ampicillin. Ten colonies of bacteria were picked randomly at each time point for PCR identification of the target gene and digestion by restriction enzymes.

2.7. Splenocyte proliferation assay

The spleens were aseptically removed from mice (three per group) 2 weeks after the last immunization and pressed through stainless steel mesh to obtain homogeneous cell suspension, and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (1500 × *g* at 4 °C for 10 min), the pelleted cells were washed three times in PBS and resuspended in complete medium [RPMI 1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS]. Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as previously described (Sun and Pan, 2006). Briefly, splenocytes were seeded into 4–5 wells of a 96-well flat-bottom microtiter plate (Costar) at 5 × 106 cells/ml in 100 μl complete medium, thereafter Con A (final concentration 5 μg/ml), LPS (final concentration 10 μg/ml), STAg (final concentration 15 μg/ml), and medium were added to give a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2. After 68 h, 50 μl of methyl thiazolyl tetrazolium (MTT) solution (2 mg/ml) was added to each well and incubated for 4 h. The plates were centrifuged (1400 × *g*, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well 200 μl of a DMSO working solution (192 μl DMSO with 8 μl 1N HCl) was added, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min.

2.8. Measurement of humoral antibody responses

Levels of antigen-specific IgG, IgG1 and IgG2a antibodies in serum samples were determined by standard procedures (Chiani et al., 2009). Briefly, the 96 flat-bottom wells of microtiter plates (Costar) were coated overnight at 4 °C with STAg at 10 μg/ml in 50 mM sodium carbonate buffer (pH 9.6). The plates were washed with PBS containing 0.05% Tween 20 (PBST-20), pH 7.4; nonspecific binding sites were blocked with PBS containing 5% FCS for 2 h at 37 °C. Individual sera were diluted 1:200. After the plates were washed, bound antibodies were detected by incubation for 2 h at 37 °C with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Serotec) diluted 1:10,000 in PBS-5% FCS, and IgG1, IgG2a (Serotec) 1:2000. After the plates were washed in PBS-T20, the peroxidase activity was assayed as follows: 100 μl of substrate solution (10 mg of P-phenylenediamine and 37.5 μl of 30% H_2O_2 in 25 ml of 0.1 M citrate–phosphate buffer, pH 5.0) was added to each well. The plate was incubated for 10 min at 37 °C, and enzyme reaction was terminated by adding 50 μl/well of 2N H_2SO_4. The optical density (OD) was measured in an ELISA reader at 492 nm with a 595 nm reference.

2.9. Cellular immune response induced in mice

For evaluation of cellular immunity, splenocytes from immunized mice were isolated and used to detect production of interleukin-4 (IL-4) and gamma interferon (IFN-γ) by ELISA. Briefly, spleen cell proliferation was assayed as described above. Cell-free supernatants were harvested and assayed for IL-4 activity at 24 h, and IFN-γ activity at 96 h. The concentration of IL-4 and IFN-γ were determined with ELISA kit (R&D) as specified by the manufacturer. The sensitivity for the assays was less than 2 pg/ml for IL-4 and IFN-γ.

2.10. Challenge infection

ICR mice (ten per group) were challenged intraperitoneally with 500 tachyzoite forms of *T. gondii* RH strain 2 weeks after the second immunization. The mice were observed and the time of death was recorded.

2.11. Statistical analyses

Antibody responses measured by ELISA, lymphocyte responses measured by MTT, body weight gains were expressed as the mean ± SD and evaluated by analysis of variance (ANOVA) and Duncan's multiple range tests using the SPASS 13.0 software. Significant level of the tests was set at 0.05.

Table 1
Effects of vaccine on body weight in mice.

Group	Number of mice	Pre-immunization (g)	Post-immunization (g)
Saline	10	19.26 ± 0.62 a	31.86 ± 1.63 a
ZJ111/pcDNA3.1	10	19.50 ± 0.74 a	30.10 ± 2.12 a
ZJ111/pSAG1	10	18.87 ± 0.57 a	32.13 ± 1.76 a
ZJ111/pMIC3	10	19.16 ± 0.70 a	31.25 ± 1.17 a
ZJ111/pSAG1-MIC3	10	19.22 ± 0.53 a	30.96 ± 1.57 a

Mice were immunized by the oral route on days 1 and 14 with ZJ111/pcDNA3 .1, pSAG1, pMIC3 or pSAG1-MIC3 of different dosages and were monitored daily for clinical changes. The body weight in all groups was recorded pre-immunization and 6 weeks post-immunization. Values with same letter are no significant difference between groups ($P > 0.05$). The values are presented as means ± SD ($n = 10$).

3. Results

3.1. Safety and stability of recombinant S. typhimurium in mice

No clinical aberrations were observed of the test mice receiving *S. typhimurium* ZJ111/pSAG1, ZJ111/pMIC3, ZJ111/pSAG1-MIC3, ZJ111/pcDNA3.1 during the 6-week observation period. No significant difference in body weights among the groups was detected before immunization and week 6 after the booster immunization (Table 1). The bacteria were eventually eliminated from the spleen and liver on week 6 post-immunization (PI) (Fig. 1). PCR amplification and enzyme digestion revealed constant presence of the target gene in recovered bacterial isolates, which were performed on the pooled DNA extracted from the 10 randomly selected bacteria (Figs. 2 and 3).

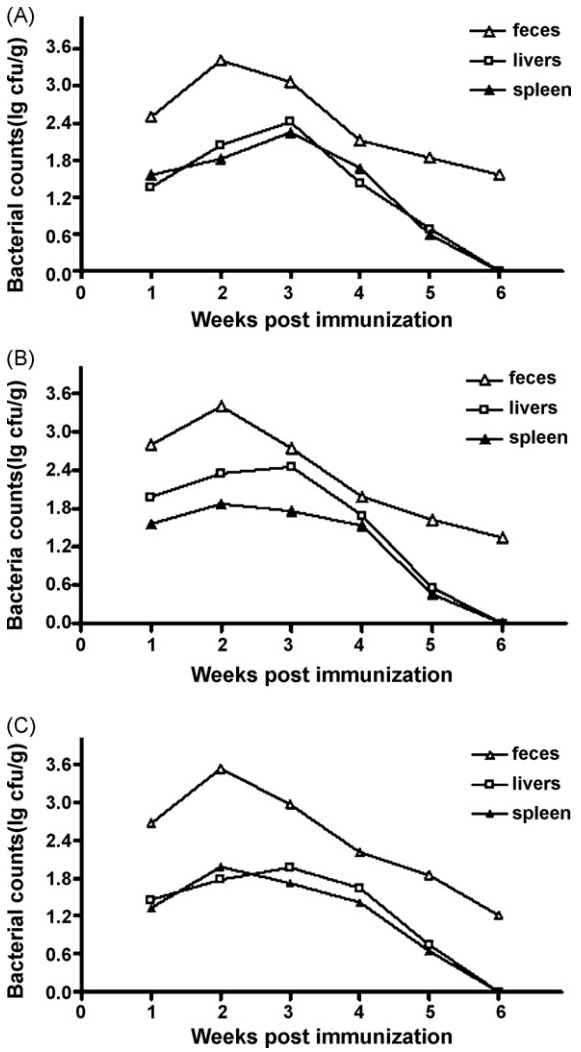

Fig. 1. Recovery of Salmonella from feces, spleens and livers of mice orally immunized with ZJ111/pSAG1 (A), ZJ111/pMIC3 (B), ZJ111/pSAG1-MIC3 (C) and boosted in the same dosage 2 weeks later. Fresh feces, spleens and livers were collected axenically and uniformed in PBS for bacteria CFU determination every week.

3.2. Proliferation of lymphocytes

The splenocytes from immunized mice were prepared 2 weeks after the last immunization to assess the proliferative immune responses. As shown in Fig. 4, the lymphocyte proliferation response was significantly higher in all vaccinated groups than the control mice on week 2 PI ($P < 0.05$). Con A-stimulated splenocyte proliferation in the mice immunized with ZJ111/pSAG1-MIC3 was significantly higher than the other vaccinated groups ($P < 0.05$). ZJ111/pSAG1 and ZJ111/pMIC3 were both significantly higher than that in ZJ111/pcDNA3.1 and control group ($P < 0.05$). Splenocytes isolated from ZJ111/pSAG1, ZJ111/pMIC3 and ZJ111/pSAG1-MIC3 stimulated

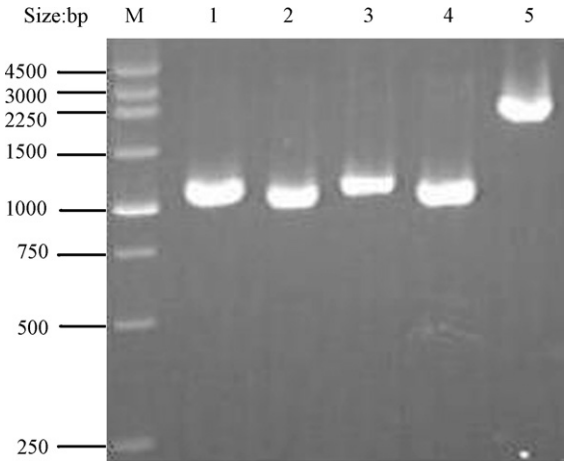

Fig. 2. PCR identification of ZJ111/pSAG1-MIC3 in organs. *S. typhimurium* carrying recombinant plasmid was isolated from liver (lanes (1) and (2)), spleen (lanes (3) and (4)) and feces (lane (5)) after immunization. The genes of PCR and digestion in lanes (1) and (3) are *mic*3, *sag*1 in lanes (2) and (4) and *sag*1-*mic*3 in lane (5) respectively.

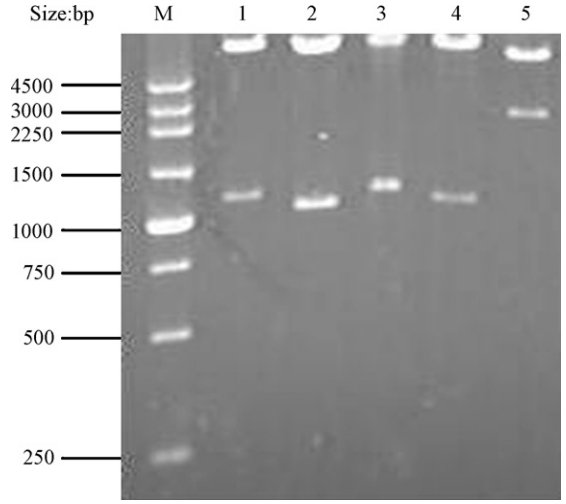

Fig. 3. Identification of *sag*1-*mic*3 gene carried by recombinant *Salmonella typhimurium* isolated from liver (lanes (1) and (2)), spleen (lanes (3) and (4)) and feces (lane (5)) after immunization. The genes of PCR and digestion in lanes (1) and (3) are *mic*3, *sag*1 in lanes (2) and (4) and *sag*1-*mic*3 in lane (5) respectively.

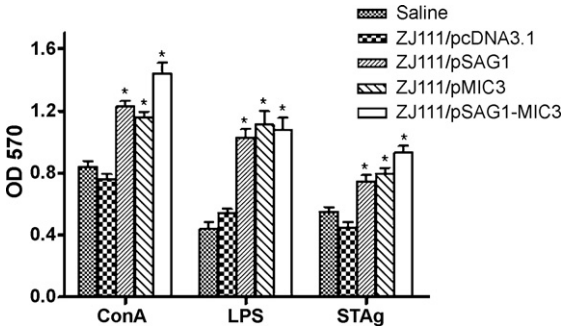

Fig. 4. The effect of DNA vaccine stimulated splenocyte proliferation in vivo. Five groups of ICR mice were orally immunized with saline, ZJ111/pcDNA3.1, ZJ111/pSAG1, ZJ111/pMIC3 or ZJ111/pSAG1-MIC3 respectively. Splenocytes were prepared 2 weeks after the last immunization, and cultured with Con A, LPS, STAg, or RPMI 1640 for 72 h. Splenocyte proliferation was measured by the MTT method as described in the text. The values are presented as means of the OD570 ± SD ($n = 3$), and significant differences with control group were designated as $^{*}P < 0.05$.

by LPS showed a greater proliferative response than that observed for the mice of control groups ($P < 0.05$). There was no statistically significant difference among the three immunized groups ($P > 0.05$). STAg-induced splenocyte proliferation of ZJ111/pSAG1-MIC3 was significantly higher than that of ZJ111/pSAG1 ($P < 0.05$), while not significantly higher than ZJ111/pMIC3 ($P > 0.05$). The three immunized groups were significantly higher than that in control groups ($P < 0.05$).

3.3. Specific IgG and IgG subclass induced by vaccination

A specific IgG antibody response was generated 2 weeks after the first immunization and the level of antibodies increased with the boost immunization (Fig. 5). Serum STAg-specific IgG levels in the immunized mice were enhanced significantly compared with those of control groups ($P < 0.05$). The mice immunized with ZJ111/pSAG1-MIC3 presented the highest antibody response and the serum levels were significantly higher than those in other groups during weeks 4–8 PI ($P < 0.05$). There were no significant differences between the groups ZJ111/pSAG1 and ZJ111/pMIC3 ($P > 0.05$), both of which were significantly higher than control groups during weeks 2–8 PI ($P < 0.05$). No antibodies were detected in the control mice (mice immunized with saline and ZJ111/pcDNA3.1).

Anti-*T. gondii* subclasses (IgG1, IgG2a) were analyzed 2 weeks after the last immunization (Fig. 6). Both IgG1 and IgG2a were found in the sera of immunized mice and there was no statistically significant difference among the three groups ($P > 0.05$), except that ZJ111/pSAG1-MIC3 induced a higher IgG2a level compared with ZJ111/pSAG1 ($P < 0.05$). The predominance of IgG2a over IgG1 suggests that a Th1 type of response was elicited.

3.4. Cytokine production

The levels of IFN-γ and IL-4 produced in splenocytes from immunized mice stimulated with STAg are shown in

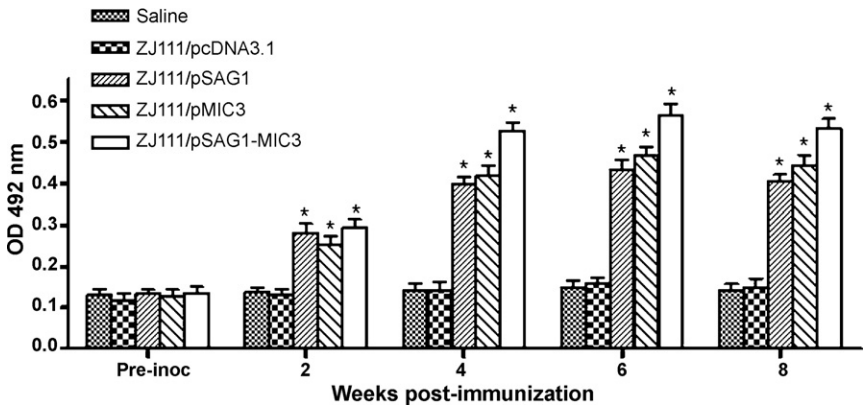

Fig. 5. The effect of DNA vaccine immunization on the antibody response. Serum responses in mice immunized with *Salmonella typhimurium* ZJ111/pcDNA3.1, ZJ111/pSAG1, ZJ111/pMIC3 or ZJ111/pSAG1-MIC3. Serum samples were prepared at weeks 0, 2, 4, 6, and 8 post-primary vaccinations. Results are expressed as means of the OD492 ± SD ($n = 3$) and significant differences with control group were designated as $^{*}P < 0.05$.

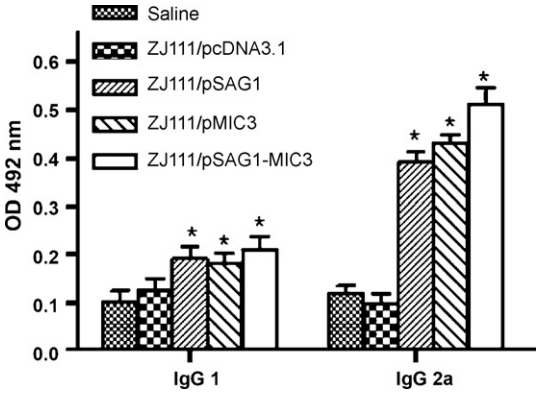

Fig. 6. Determination of the specific anti-SAG1 IgG subclass profile in the sera of immunized ICR mice. Sera were collected on day 28 after first immunization and analyzed by ELISA using STAg.

Table 2
Production of cytokines by spleen cells following in vitro antigenic stimulation.

Group	Cytokine production (mean ± SD)	
	IL-4 (pg/ml)	IFN-γ (pg/ml)
Saline	49 ± 11	78 ± 8
ZJ111/pcDNA3.1	78 ± 23	106 ± 17
ZJ111/pSAG1	71 ± 16	696 ± 126**
ZJ111/pMIC3	85 ± 21	721 ± 142**
ZJ111/pSAG1-MIC3	79 ± 28	1089 ± 163**

Mice were immunized by the oral route on days 1 and 14 and spleen cells were collected 2 weeks after the last immunization. Cell-free supernatants were harvested and assayed for IL-4 activity at 24 h, and IFN-γ activity at 96 h. The values are presented as means ± SD ($n = 3$).

** Significant differences with control group were designated as $P < 0.01$.

Table 2. Significantly higher levels of IFN-γ were observed in spleen cell cultures from immunized mice compared with those of control groups ($P < 0.05$). And the levels in ZJ111/pSAG1-MIC3 group were markedly enhanced compared with the other groups ($P < 0.05$). On the other hand, low levels of IL-4 were observed from any culture supernatants, and no statistically significant differences could be found among the five groups ($P > 0.05$).

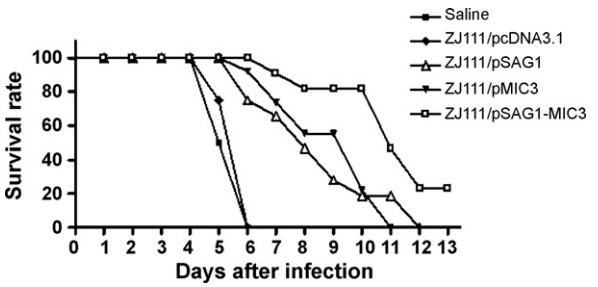

Fig. 7. Survival rate of mice immunized with oral vaccination after challenging with *T. gondii*. The mice were immunized with ZJ111/pcDNA3.1, ZJ111/pSAG1, ZJ111/pMIC3 or ZJ111/pSAG1-MIC3. Mice immunized with saline and ZJ111/pcDNA3.1 was included as negative control. The mice were intraperitoneally challenged with 500 tachyzoite of *T. gondii* 2 weeks after last immunization and observed for mortality ($n = 10$ mice per group).

3.5. Effective protection of vaccination in mice

To evaluate whether this vaccination protocol could induce protection against *T. gondii*, immunized mice were challenged with tachyzoites of the virulent RH strain. Survival rates of different groups of mice are shown in Fig. 7. From our results, the survival time of mice was significantly increased by oral immunization with ZJ111/pSAG1, ZJ111/pMIC3 and ZJ111/pSAG1-MIC3. Substantially longer survival times were found in the group vaccinated with ZJ111/pSAG1-MIC3. Mice in control groups died within 5–6 days.

4. Discussion

DNA immunization has been shown to be the most effective way of inducing specific humoral and cellular immune responses in a number of vertebrate host species. While the success of a DNA vaccine invariably depends on several parameters, such as adjuvanticity of the formulation component(s), excipients, vaccine carriers, not to mention the route of administration, in addition to the active antigen itself. Among the formulation variables, the route of plasmid of DNA delivery and vaccine carriers play an important part in achieving good responses (Innes and Vermeulen, 2006). It has been reported that live antigen delivery systems such as *Salmonella*, *Shigella* and *Listeria* have been used as oral carriers to elicit immune responses against a wide range of pathogens (Baillie et al., 2008; Kulkarni et al., 2008). And live bacterial vectors offer many potential clinical advantages. They are stable and easy and relatively cheaper to produce in large scales. They are also able to deliver large or multiple antigens and oral delivery increases the safety and ease of administration (Massis et al., 2008). In this study, we constructed three DNA vaccines encoding the target antigens of *T. gondii*, namely SAG1, MIC3 and multiantigenic SAG1-MIC3, delivered by attenuated *S. typhimurium*. And then we evaluated their stability in vitro, compared the effects in inducing protective immune responses.

The safety of a live vaccine and stability of an expression plasmid in a vaccine vector may potentially affect the efficacy of the vaccine and alter the outcome of vaccination (Coulson et al., 1994). In this experiment, no mice displayed post-immunization clinical aberrations. There was no significant difference in the body weight among the groups. The three plasmids were retained by attenuated *S. typhimurium* from mice organs (liver, spleen) or feces for 42 days and the mice eliminated the bacteria within 6 weeks after oral vaccination. The above results indicated that use of attenuated *S. typhimurium* as the oral delivery vector for DNA vaccine was safe and relatively stable.

Furthermore, we have shown that the recombinant proteins SAG1, MIC3, SAG1-MIC3 expressed in vivo by oral vaccination were immunogenic, because all the immunized mice produced specific anti-STAg IgG antibodies. A specific antibody was developed in immunized mice as determined by ELISA, especially after the last immunization. The mice immunized with ZJ111/pSAG1-MIC3 exhibited a higher antibody response in comparison with

ZJ111 expressing SAG1 or MIC3 alone. For naturally occurring *T. gondii* infection, it has been demonstrated that a Th1-based response is required for effective protection (Dautu et al., 2007). This suggests that a good vaccination protocol will direct T-helper cells toward a Th1 rather than a Th2 response. We evaluated the nature of the IgG subclass achieved during immunization to determine the T-helper response of immunized mice. Both IgG2a (Th1) and IgG1 (Th2) were detected in immunized mice, with a slight increase in the anti-STAg Abs of IgG2a than IgG1 isotype. A significant predominance of IgG2a over IgG1 antibodies was observed in the mice immunized with ZJ111/pSAG1-MIC3. This indicates that the response was oriented toward a Th1-type response, which was confirmed by analysis of the cytokine production. As a result, large amounts of IFN-γ were produced in the supernatants of restimulated splenocyte cultures from all immunized mice. The splenocytes from mice immunized with ZJ111/pSAG1-MIC3 exhibited stronger specific proliferation when stimulated with STAg, and also a higher production of IFN-γ was observed. Some studies indicate that bacteria naturally possess immunostimulatory molecules such as lipopolysaccharide (LPS) that can function as adjuvant to stimulate immune responses (Roland et al., 1999). Most importantly, live bacterial vectors are able to induce systemic immune response associated with Th1-type cytokine contribution. Thus, both the antibody response and the cytokine production pattern were indicative of a predominant Th response to the vaccine antigens.

We evaluated the protection of oral vaccination by infecting intraperitoneally the vaccinated mice with 500 tachyzoite of *T. gondii* RH strain. A partial protection was obtained in immunized mice, and all mice except ZJ111/pSAG1-MIC3 eventually succumbed. Furthermore, the ZJ111/pSAG1-MIC3 vaccinated mice had significant longer survival time. No prior vaccine has been shown to completely protect against intraperitoneally challenge with the RH strain of *T. gondii* (Cong et al., 2008), maybe it is associated with the powerful virulence of RH strain of *T. gondii*. As we all know, RH strain belongs to type I strain, which causes lethal infection in mice even at low inoculate [lethal dose (LD100) ≈ 1] (Taylor et al., 2006), so, it would have been preferable to look for protection against oral challenge with oocysts. In future experiments it would be helpful to confirm the efficacy of DNA immunization by comparing the parasite levels in different tissues in vaccinated and control groups, using a moderately virulent *T. gondii* strain.

In conclusion, this study preliminarily shows that attenuated *S. typhimurium* strain (Dam^- and $PhoP^-$) could be utilized as an oral delivery vector for recombinant eukaryotic expression plasmids as DNA vaccines for prevention from *Toxoplasmosis*. The introduction of two functional genes encoding SAG1 and MIC3 delivered by attenuated *S. typhimurium* induces more effective protection than the two single-gene vaccines. Because oral rather than intranasal immunization presents many advantages such as labor, time-saving and stress-minimizing, this vaccine candidate deserves more extensive study to assess its safety and protective efficacy in livestock.

Acknowledgements

This study was supported by the National Special Research Programs for Non-Profit Trades (Agriculture) (No. 200803017), the Science and Technology Department of Zhejiang Province (No. 2006C22043) and General Administration of Quality Supervision, Inspection and Quarantine of China (No. 2006IK016).

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