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ORIGINAL PAPER

E a a e ec e e ec DNA acc e e d MIC3 a d ROP18 a e e e *Toxoplasma gondii* ce

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Ab ac The high incidence and severe damage caused by *Toxoplasma gondii* infection clearly indicates the need for the development of a vaccine. In this study, we evaluated the immune responses and protection against toxoplasmosis by immunizing ICR mice with a multiantigenic DNA vaccine. To develop the multiantigenic vaccine, two *T. gondii* antigens, MIC3 and ROP18, selected on the basis of previous studies were chosen. ICR mice were immunized subcutaneously with PBS, empty pcDNA3.1 vector, pMIC3, pROP18, and pROP18–MIC3, respectively. The results of lymphocyte proliferation assay, cytokine, and antibody determinations showed that mice immunized with pROP18–MIC3 elicited stronger humoral and Th1-type cellular immune responses than those immunized with single-gene plasmids, empty plasmid, or phosphate-buffered saline. After a lethal challenge with the highly virulent *T. gondii* RH strain, a prolonged survival time in pROP18–MIC3-immunized mice was observed in comparison to control groups. Our study indicates that the introduction of multiantigenic DNA vaccine is more powerful and efficient than single-gene vaccine, and deserves further evaluation and development.

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Toxoplasma gondii is an obligate intracellular parasite with a broad host range that includes all warm-blooded animals. The parasite is an opportunistic pathogen that causes chronic infections with severe symptoms, including retinochoroiditis, encephalitis, myocarditis, and hepatitis in immunocompromised individuals, and neurological and ocular complications in congenitally infected children (Rosenberg et al. 2009; Dubey 2010). It is a significant hazard to the fetuses of mothers who acquire the infection during pregnancy, and it has been established as a cause of life-threatening disease in immunocompromised individuals (Tan et al. 2011; Ramos et al. 2011). In addition, *T. gondii* infection has economic importance due to abortion and neonatal loss in livestock, and it is a source of transmission to humans (Quan et al. 2012). Thus, a vaccine against *T. gondii* would be valuable for preventing both fetal infection and reactivation in immunocompromised individuals. The development of a vaccine might also reduce economic losses in the livestock industry.

DNA vaccines have become a major focus because they promote the specific expression of an encoded vaccine antigen by host cells and have the ability to deliver multivalent vaccines to a host in a single dose. Additionally, DNA vaccines can also elicit potent, long-lasting humoral and cell-mediated immunity (Qu et al. 2008; Belakova et al. 2007). In recent years, significant advances have been made in identifying possible new vaccines that can induce a protective immune response against *T. gondii*. For example, exocytosis secretory antigens of *T. gondii* have been shown to play an important role in stimulating an immune response to *T. gondii*. Microneme proteins, used for host cell recognition, binding, and motility, are apparently the major proteins of the

exocytosis secretory antigens (Wang et al. 2009; Liu et al. 2010). MIC3 was a component of the micronemes in *T. gondii*, and previous studies have shown that MIC3 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 (Fang et al. 2009; Ismael et al. 2003). The *T. gondii* ROP18 protein is a highly polymorphic serine-threonine kinase that is secreted into the host cell during parasite invasion of the host cell, controlling the intracellular proliferation of *T. gondii*, and ROP18 is considered one of the key virulence factors in the pathogenesis of *T. gondii* infection (Taylor et al. 2006; Saeij et al. 2006; El Hajj et al. 2007). As we all know, *T. gondii* possesses a complex life cycle with diversity of form and presents a plurality of antigenic epitopes, which varies widely among different individuals. Accumulating evidence also indicates that vaccination with stage-specific antigens only leads to partial protection (Qu et al. 2009; Zhou et al. 2007). 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 more potential protection against *T. gondii* may be achieved.

Multiantigenic DNA vaccinations have been reported to enhance protection against toxoplasmosis in a mouse model compared to single-gene vaccines (Zhang et al. 2007). In this study, we selected *T. gondii* MIC3 and ROP18 as targets for DNA antigenicity generation because they are known to be important during invasion into host cells and infection. In addition, they are potent stimulators of humoral and cellular immune responses. However, no evaluation of the protective efficacy of multiantigenic DNA vaccine expressing MIC3 and ROP18 of *T. gondii* has been reported. The objective of the present study was to evaluate the protective effect of DNA vaccine with genes encoding antigens MIC3 and ROP18 against *T. gondii* in mice.

Ma e a a d e ●d

Animals

Female ICR mice (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±10%, and a 12:12 h light/dark cycle. All procedures were in strict accordance with the PR China legislation on use.

Parasites and preparation of STAg

The tachyzoites of *T. gondii* RH strain used as challenge for immunized mice and preparations of *T. gondii* antigens were kindly provided by Prof. Zhu Xingquan. The parasites were maintained and collected from the peritoneal cavity of infected ICR mice.

Purified tachyzoites were centrifuged at 5,000×*g* for 3 min and disrupted by three cycles of freezing at −20 °C and thawing at 4 °C. Finally, the lysate was sonicated on ice at 60 W/s and centrifuged for 30 min at 3,000×*g*. The supernatants were pooled and sterile filtered, and the protein concentration was determined via the Bradford method using bovine serum albumin (BSA) as the standard. STAg was stored in aliquots at −70 °C until use.

Plasmid construction, purification, and expression

To construct the ROP18–MIC3 fusion expression plasmid, the encoding sequence of the ROP18 gene (1,026 bp, without the stop codon, encoding amino acid residues 215–556) and MIC3 gene (882 bp, encoding amino acid residues 67–360) were amplified by polymerase chain reaction (PCR) from genomic DNA of *T. gondii* (RH strain). The two kinds of PCR products were respectively digested with the above corresponding restriction enzymes and purified from agarose gel. Two independent gene fragments were ligated into the eukaryotic expression plasmid pcDNA3.1 vector, generating the pcDNA3.1–ROP18–MIC3 plasmid (pROP18–MIC3). Endotoxin-free plasmid DNA was isolated using plasmid purification kit (Qiagen). After purification, the recombinant eukaryotic expression plasmid pROP18–MIC3 was transiently transfected into Vero cells to test its expression. After 48 h of incubation, cells were lysed and the proteins were collected. Synthesis of ROP18–MIC3 protein in a eukaryotic system was tested by Western blot.

DNA immunization and challenge

Five groups of mice (20 per group) were injected intramuscularly with 100 μg of plasmid DNA suspended in 100 μl sterile PBS, whereas control mice received PBS alone. Group I was injected with PBS as control, group II with empty pcDNA3.1 vector also as control, group III with pMIC3, group IV with pROP18, and group V with pROP18–MIC3. Mice were immunized using the same protocol on days 0, 14, and 28. Two weeks after the final inoculation, immunized mice per group were intraperitoneally challenged with 1×10^3 tachyzoites of virulent RH *T. gondii*. The survival time (days) was recorded and compared in the experimental and control groups.

Evaluation of immune responses

Levels of antigen-specific IgG, IgG1, and IgG2a antibodies in serum samples were determined by standard procedures. Briefly, the 96 flat-bottom wells of microtiter plates (Costar)

were coated overnight at 4 °C with STAg at 10 mg/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 1 % BSA for 2 h at 37 °C. Individual sera were diluted in PBS–0.1 % BSA and incubated for 2 h. 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–1 % BSA, and IgG1, IgG2a (Serotec) 1:2,000. After the plates were washed in PBS–T20, 200 μl of substrate solution (10 mg of *O*-phenylenediamine and 10 μl of 30 % H_2O_2 in 25 ml of 0.1 M citrate-phosphate buffer, pH 5.0) was added. Plates then were incubated in the dark for 30 min, and the reaction was stopped by the addition of 3 N HCl (50 μl). The OD then was measured by an ELISA reader at 492 nm.

Splenocyte proliferation assay in vitro

Two weeks after the administration of the last antigens dose, the spleens were isolated from the immunized mice. A single-cell suspension of splenocytes was prepared by spleen homogenization as it was described in our previous studies (Qu et al. 2011). The number of spleen cells of each experimental and control suspension was counted and the viability was determined by the trypan blue exclusion method. The cells were seeded in triplicate at 5×10^5 cells per well in 96-well tissue culture plates (Costar) in 100 μl of culture medium, and an equal volume of the medium with STAg was added with the final concentration of 15 μg/ml of each antigen. Splenocytes cultured in medium alone or stimulated with concanavalin A (ConA) (Sigma) at a final concentration of 5 μg/ml served as negative and positive controls of lymphoproliferation, respectively. After 68 h of incubation at 37 °C in a humidified atmosphere containing 10 % CO_2, 50 μl of methylthiazolyl tetrazolium (MTT) solution (2 mg/ml) was added to each well and incubated for 4 h. The plates were centrifuged (1,400×*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 1 N HCl) was added, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min.

Cytokine production

Spleen cell proliferation was assayed as described above. Cell-free supernatants were harvested and the concentration of IL-4 or IFN-γ released by antigen-stimulated splenocytes was estimated in culture supernatants collected after 424 h or 72 h of incubation, respectively.

The IL-4, IFN-γ concentrations were evaluated using a commercial ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The sensitivity for the assays was less than 2 pg/ml for IL-4 and IFN-γ.

Statistical analysis

Results are expressed as the means ± SD for each group. The statistical evaluation of the differences including antibody responses, lymphoproliferation assays, and cytokine production were compared between the different groups by one-way ANOVA. All data were processed and analyzed by SPSS13.0 Data Editor (SPSS Inc., Chicago, IL, USA). The results in comparisons between groups were considered different if $P<0.05$.

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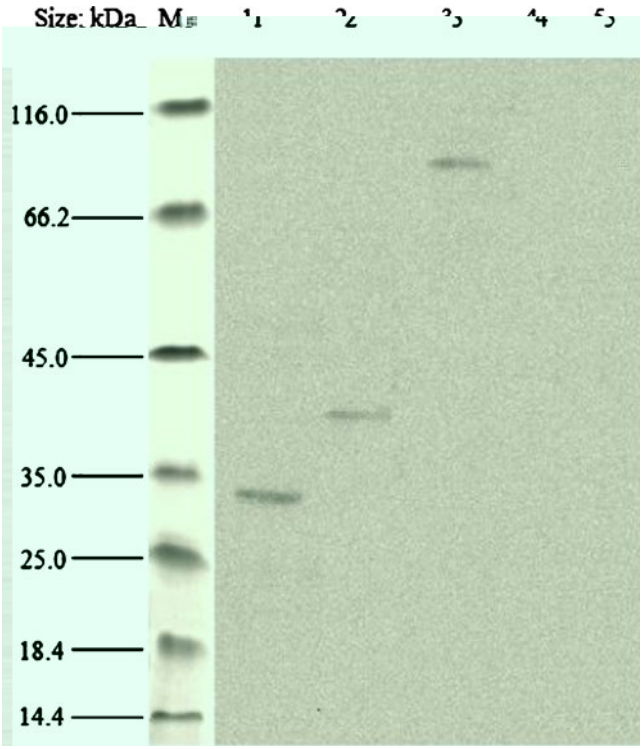
Western blot analysis of proteins synthesized in vitro

Vero cells were transfected with pMIC3, pROP18, or pROP18–MIC3. The lysates of tranfected cells were



F . 1 Western blot of *Toxoplasma gondii* SAG1gene expressed in Vero cells. *Lane 1* lysate of cells with plasmid pMIC3, *2* lysate of cells with plasmid pROP18, *3* lysate of cells with plasmid pROP18–MIC3, *4* lysate of cells with empty plasmid, *5* lysate of cells with medium. Serum obtained from STAg-immunized mice was used as antibody to probe the membrane

analyzed on immunoblots (Fig. 1). The lysate of Vero cells transfected with pMIC3, pROP18, or pROP18–MIC3 were specifically recognized by serum obtained from a *T. gondii*-infected mice. In contrast, cells transfected with pcDNA3.1 or medium not recognized.

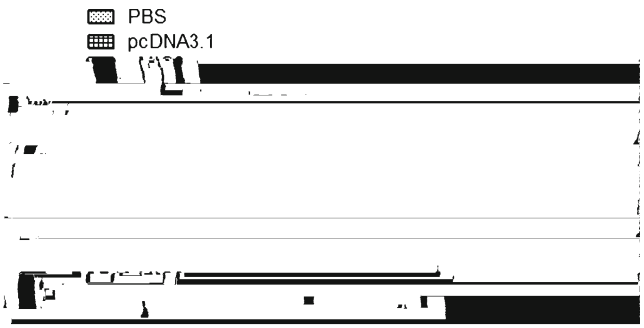
Humoral immune responses

All sera were tested by ELISA to detect the significant *T. gondii*-specific IgG production in vaccinated mice (Fig. 2). A strong antibody response was found in mice immunized with pMIC3, pROP18, and pROP18–MIC3 groups, which were significantly higher than those of the negative controls ($P<0.05$). Although anti-*T. gondii* total IgG values increased in the sera of mice in single-gene immunized groups ($P<0.05$), there were no statistically significant differences between the two groups ($P>0.05$). No antibodies augmentation was detected in the control mice and mice immunized with pcDNA3.1.

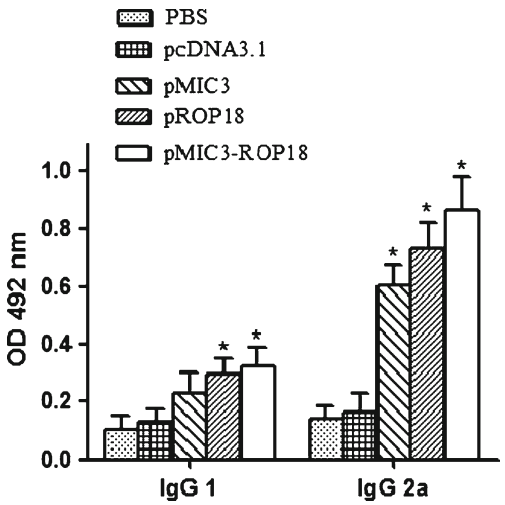
The levels of anti-*T. gondii* IgG subclasses are shown in Fig. 3. Predominant levels of IgG2a over IgG1 were observed in sera of the single- and multiple-gene immunized mice. Furthermore, IgG2a values in the pROP18–MIC3 immunized group were significantly higher than the single-gene immunized group ($P<0.05$).

In vitro splenocyte proliferation

The splenocytes from mice immunized were prepared to assess the proliferative immune responses. As shown in Fig. 4, in vitro splenocyte proliferation was significantly higher in mice immunized with pMIC3, pROP18, and pROP18–MIC3 from 2 weeks after immunization compared to mice immunized with empty vector or PBS ($P<0.05$). In addition, the levels of splenocyte proliferation were similar among all our experimental and control groups in response to ConA.



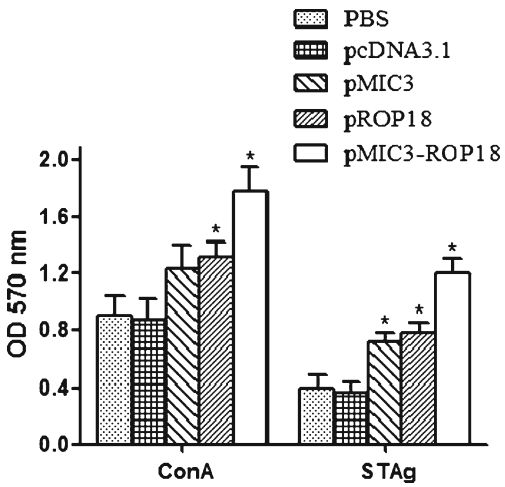
F . 2 Measurement of specific IgG in immunized mice. Serum samples were collected prior to immunization and subsequently at intervals of 2 weeks, and detected by ELISA. Results are shown as mean ± SD of three independent experiments. Significant differences with control group were designated as *$P<0.05$



F . 3 Determination of the specific anti-*T. gondii* IgG1 and IgG2a subclass antibodies in the sera of mice. Sera were collected 2 weeks after the last immunization. Results are expressed as means of the OD492±SD and representative of three experiments. *$P<0.05$

Production of cytokine by spleen cells

To investigate the possibility of cellular immune response in the immunized mice, the level of lymphocyte proliferation and cytokines in the spleen cell suspensions were evaluated using ELISA 2 weeks after the final immunization. Table 1 shows that splenocytes from mice immunized with pMIC3, pROP18, and pROP18–MIC3 secreted large amounts of IFN-γ and IL-4. Significantly higher levels of IFN-γ were observed in spleen cell cultures from mice immunized with pROP18–MIC3 compared with other groups. On the other



F . 4 Splenocyte proliferation responses of immunized mice. Mice were immunized twice at a 2-week interval with PBS, pcDNA3.1, pMIC3, pROP18, and pROP18–MIC3. Splenocytes were prepared 2 weeks after the last immunization and cultured with ConA or STAg. Splenocyte proliferation was measured by the MTT method as described in the text. Significant differences with control group were designated as *$P<0.05$

Tab e 1 Cytokine production of splenocytes from immunized mice

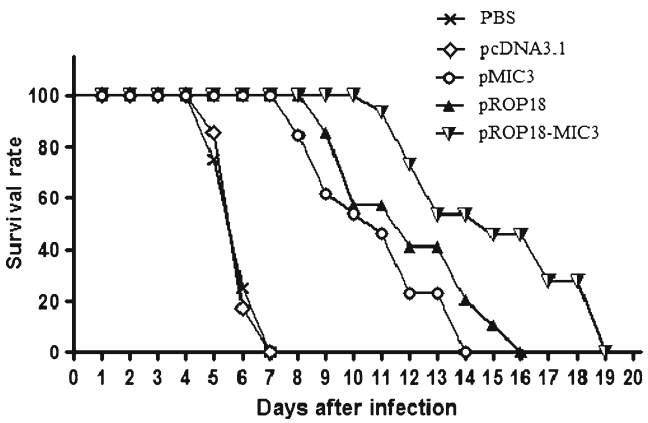
Group	Cytokine production (mean ± SD)	
	IL-4 (pg/ml)	IFN-γ (pg/ml)
PBS	29±7a	41±8a
pcDNA3.1	33±8a	38±7a
pMIC3	51±11ab	346±31b
pROP18	56±9ab	427±40b
pROP18–MIC3	66±14b	849±86c

Splenocytes from mice were harvested 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)

hand, low levels of IL-4 showed a slight but significantly proliferative response from the splenocytes from mice immunized with pROP18–MIC3 compared to those from mice immunized with empty vector and PBS (P<0.05). There was no significant difference between multiantigenic group and single-gene group in IL-4 production.

Protection against *T. gondii* in mice

Survival curves of the five groups are shown in Fig. 5. Two weeks after the last immunization, the mice were intraperitoneally infected with 10^3 tachyzoites of the highly virulent *T. gondii* RH strain (lethal challenge), and their survival was monitored. The mice immunized with pMIC3, pROP18, and pROP18–MIC3 had significantly prolonged survival (P<0.05) compared to the control mice that received either pcDNA3.1 or PBS. Among the different immunized groups, mice vaccinated with pROP18–MIC3 had significantly prolonged survival (P<0.05). In contrast, all of the mice in control groups died within 7 days of infection, while all mice in the other immunized groups died within 14–19 days of infection.



F . 5 Survival time of the immunized mice after *T. gondii* challenge. Each group of mice (n=12) was immunized (i.m.) at 2-week interval. At 2 weeks after the last immunization, mice were challenged with 10^3 tachyzoites of RH strain. Mortality was monitored daily after challenge. Mice immunized with PBS died within 7 days

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DNA vaccination can protect animals and human beings against pathogenic microorganisms, particularly intracellular parasites (Fang et al. 2012; Belakova et al. 2007). In the present study, ICR mice immunized intramuscularly with pMIC3, pROP18, or pROP18–MIC3 produced specific antibodies against *T. gondii*, and protective immunity was induced. Moreover, immunization with the multiantigenic gene plasmid pROP18–MIC3 induced a stronger humoral and cellular immune response as well as increased survival time of ICR mice challenged with RH strain tachyzoites compared with other groups.

T. gondii possesses a complex life cycle with diverse life stages and extensive invading style. Evidences indicated that vaccination with stage-specific antigens leads to stage-limited protection (Mevelec et al. 2005; Liu et al. 2009). Developing multiantigenic vaccine in connection with growth stage in different life cycle and infection process may conquer the deficiency of using single antigen as candidate vaccine. Thus, we immunized ICR mice with DNA vaccine encoding two antigens MIC3 and ROP18, MIC3 being expressed in all three infectious stages and ROP18 being considered one of the key virulence factors in the pathogenesis of *T. gondii* infection, and tested whether it could be a potent vaccine against the challenge with highly virulent *T. gondii* RH strain.

Humoral immunity seems to be important in controlling *T. gondii* invasion resulting in the production of antigen-specific IgG antibodies (Sayles et al. 2000). It has been reported that *T. gondii* infection could lead to B-cell responses resulting in production of antibodies, which limit parasite spread by inhibiting attachment of tachyzoites to host cell receptors and thus promoting intracellular killing of antibody-coated parasites by macrophages (Yu et al. 2012). Our study showed that the relative level of anti-*T. gondii* IgG antibody in mice immunized with pMIC3or pROP18 was elevated, comparing to controls, and it was further elevated by immunization with pMIC3–ROP18, suggesting that the DNA multiantigenic could produce stronger humoral immunity.

Cytokines play an important role in the activities of Th cells. It is well known that IFN-γ is the central cytokine that is responsible for resistance against *T. gondii* during both the early and late stages of infection (Hiszczynska-Sawicka et al. 2010; Wu et al. 2012). On the other hand, high levels of IFN-γ production have a deleterious effect on hosts. The presence of IL-4 in early infection inhibits protective Th1

cell differentiation likely by either direct or indirect inhibition of IFN-γ production, but later inhibits *T. gondii* replication within the central nervous system (Wurtz et al. 2004; Roberts et al. 1996). In the present study, we examined the cellular immune responses by detecting cytokines in supernatants of cultured splenocytes. Immunization with pMIC3–ROP18 efficiently increased the levels of both IFN-γ and IL-4 as compared with the control group. This result is consistent with some previous studies.

We evaluated the protection of immunization by intraperitoneally infecting the mice with 10^3 tachyzoites of *T. gondii* RH strain. An effective and highly significant degree of protection was obtained in mice immunized with DNA vaccine compared to the control group. Mice immunized with single gene died within 16 days after challenge. However, pMIC3–ROP18 vaccination resulted in longer survival of mice after a lethal challenge which suggests that multiple gene plasmids are more effective against *T. gondii* challenge than single-gene plasmid.

In this study, immunization of ICR mice with a multiantigenic DNA vaccine encoding MIC3 and ROP18 resulted in an improvement of the protective immunity against the acute phase of a RH strain infection as measured by the survival rate, in comparison with mice immunized with plasmid expressing single gene alone. These results suggested that multiantigenic DNA immunization might be an important approach to achieve an effective vaccine against *T. gondii*. Finally, it should be considered that combination with other effective antigens that generate immunity by different strategies and possible adjuvant should be also taken into account in the future.

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Re e e ce

Belakova J, Horynova M, Krupka M, Weigl E, Raska M (2007) DNA vaccines: are they still just a powerful tool for the future? Arch Immunol Ther Exp (Warsz) 55(6):387–398. doi:10.1007/s00005-007-0044-4

Dubey JP (2010) *Toxoplasma gondii* infections in chickens (*Gallus domesticus*

Sayles PC, Gibson GW, Johnson LL (2000) B cells are essential for vaccination-induced resistance to virulent *Toxoplasma gondii*. Infect Immun 68(3):1026–1033

Tan F, Hu X, Luo FJ, Pan CW, Chen XG (2011) Induction of protective Th1 immune responses in mice by vaccination with recombinant *Toxoplasma gondii* nucleoside triphosphate hydrolase-II. Vaccine 29(15):2742–2748. doi:10.1016/j.vaccine.2011.01.089

Taylor S, Barragan A, Su C, Fux B, Fentress SJ, Tang K, Beatty WL, Hajj HE, Jerome M, Behnke MS, White M, Wootton JC, Sibley LD (2006) A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. Science 314(5806):1776–1780. doi:10.1126/science.1133643

Wang H, He S, Yao Y, Cong H, Zhao H, Li T, Zhu XQ (2009) *Toxoplasma gondii*: protective effect of an intranasal SAG1 and MIC4 DNA vaccine in mice. Exp Parasitol 122(3):226–232. doi:10.1016/j.exppara.2009.04.002

Wu XN, Lin J, Lin X, Chen J, Chen ZL, Lin JY (2012) Multicomponent DNA vaccine-encoding *Toxoplasma gondii* GRA1 and SAG1 primes: anti-*Toxoplasma* immune response in mice. Parasitol Res 111(5):2001–2009. doi:10.1007/s00436-012-3047-y

Wurtz O, Bajenoff M, Guerder S (2004) IL-4-mediated inhibition of IFN-gamma production by CD4+ T cells proceeds by several developmentally regulated mechanisms. Int Immunol 16(3):501–508

Yu L, Yamagishi J, Zhang S, Jin C, Aboge GO, Zhang H, Zhang G, Tanaka T, Fujisaki K, Nishikawa Y, Xuan X (2012) Protective effect of a prime-boost strategy with plasmid DNA followed by recombinant adenovirus expressing TgAMA1 as vaccines against *Toxoplasma gondii* infection in mice. Parasitol Int 61(3):481–486. doi:10.1016/j.parint.2012.04.001

Zhang J, He S, Jiang H, Yang T, Cong H, Zhou H, Gu Q, Li Y, Zhao Q (2007) Evaluation of the immune response induced by multiantigenic DNA vaccine encoding SAG1 and ROP2 of *Toxoplasma gondii* and the adjuvant properties of murine interleukin-12 plasmid in BALB/c mice. Parasitol Res 101(2):331–338. doi:10.1007/s00436-007-0465-3

Zhou H, Gu Q, Zhao Q, Zhang J, Cong H, Li Y, He S (2007) *Toxoplasma gondii*: expression and characterization of a recombinant protein containing SAG1 and GRA2 in *Pichia pastoris*. Parasitol Res 100(4):829–835. doi:10.1007/s00436-006-0341-6