Evaluation of Immune Responses in Mice after DNA Immunization with Putative Toxoplasma gondii Calcium-Dependent Protein Kinase 5

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Toxoplasma gondii can cause serious public health problems and economic losses worldwide. Calcium-dependent protein kinases (CDPKs) are key mediators of T. gondii signaling pathways and are implicated as important virulence factors. In the present study, we cloned a novel T. gondii CDPK gene, named TgCDPK5, and constructed the eukaryotic expression vector pVAX-CDPK5. Then, we evaluated the immune protection induced by pVAX-CDPK5 in Kunming mice. After injection of pVAX-CDPK5 intramuscularly, immune responses, determined with lymphoproliferative assays and cytokine and antibody measurements, were monitored, and mouse survival times and brain cyst formation were evaluated following challenges with the T. gondii RH strain (genotype I) and the PRU strain (genotype II). pVAX-CDPK5 effectively induced immune responses with increased specific antibodies, a predominance of IgG2a production, and a strong lymphocyte proliferative response. The levels of gamma interferon (IFN-γ), interleukin 2 (IL-2), and IL-12(p70) and the percentages of CD3+ CD4+ and CD3+ CD8+ cells in mice vaccinated with pVAX-CDPK5 were significantly increased. However, IL-4 and IL-10 were not produced in the vaccinated mice. These results demonstrate that pVAX-CDPK5 can elicit strong humoral and cellular Th1 immune responses. The survival time of immunized mice challenged with the T. gondii RH strain (8.67 ± 4.34 days) was slightly, but not significantly, longer than that in the control groups within 7 days (P > 0.05). The numbers of brain cysts in the mice in the pVAX-CDPK5 group were reduced by ~40% compared with those in the control groups (P < 0.05), which provides a foundation for the further development of effective subunit vaccines against T. gondii.
infection with the virulent RH strain of *T. gondii* in Kunming mice.

**MATERIALS AND METHODS**

**Mice and parasites.** Six- to 8-week-old specific-pathogen-free (SPF) female Kunming mice were purchased from the Center of Experimental Animals, Lanzhou Institute of Biological Products, Lanzhou, China. All mice were handled in strict accordance with the good animal practice requirements of the Animal Ethics Procedures and Guidelines of the People’s Republic of China.

The virulent *T. gondii* RH strain and the brain cyst-forming PRU strain were used in this study. Tachyzoites of the *T. gondii* RH strain (type I) were propagated by serial intraperitoneal passage in Kunming mice. If needed, the peritoneal fluid of mice was centrifuged for 10 min at 1,000 × g at 4°C and then resuspended in sterile phosphate-buffered saline (PBS). The obtained tachyzoites were used for total RNA extraction following the instructions in the RNAprep pure tissue kit (Tiangen, China) manual and also to prepare *Toxoplasma* lysate antigen (TLA) as described in our previous study (24). Cysts of the PRU strain (genotype II) were maintained in Kunming mice. If necessary, cysts were propagated by serial intraperitoneal passage in Kunming mice. If needed, the peritoneal fluid of mice was centrifuged for 10 min at 1,000 × g at 4°C and then resuspended in sterile phosphate-buffered saline (PBS).

**Expression and purification of TgCDPK5 protein in *Escherichia coli***. Based on the corresponding sequence of the CDPK5 gene downloaded from GenBank (accession number XM_002366128), a pair of primers, pKSF (5′-GGGAGAATTCATGCCAGTCGCTGCAACAA-3′) and pKnR (5′-GGCCGGCGGCTCAGCTAATGTGGGACTCAG-3′), were designed, into which the EcoRI and NotI restriction sites (underlined) were introduced. The complete open reading frame (ORF) was amplified by reverse transcription (RT)-PCR, and the obtained products were inserted into the prokaryotic expression vector pGEX-4T-1 via the two restriction sites, to form pGEX-CDPK5. The recombinant plasmids were transformed into the *E. coli* BL21(DE3) strain and grown in Luria-Bertani (LB) agar containing kanamycin (25 μg/ml). The recombinant TgCDPK5 (rTgCDPK5) protein was expressed under the optimal conditions of 1 mmol/liter isopropyl-β-D-thiogalactopyranoside (IPTG) (Sangon, China), shaking for 6 h at 37°C. The resulting bacterial pellet containing the expressed protein was disintegrated by sonication and then centrifuged. The obtained inclusion bodies were solubilized in 8 mmol/liter urea and were visualized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Construction of the eukaryotic expression plasmid.** To obtain the eukaryotic expression plasmid, the TgCDPK5 gene was amplified using primers eKSF (5′-GGCCGGCCATGCGCAGTCGCTGCAACAA-3′) and eKnR (5′-GGCCGGCGGCTCAGCTAATGTGGGACTCAG-3′), into which the KpnI and EcoRI restriction sites (underlined) were introduced, and was inserted into pVAX I (Invitrogen). The recombinant plasmid was named pVAX-CDPK5. The recombinant TgCDPK5 (rTgCDPK5) protein was expressed under the optimal conditions of 1 mmol/liter isopropyl-β-D-thiogalactopyranoside (IPTG) (Sangon, China), shaking for 6 h at 37°C. The resulting bacterial pellet containing the expressed protein was disintegrated by sonication and then centrifuged. The obtained inclusion bodies were solubilized in 8 mmol/liter urea and were visualized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Expression of pVAX-CDPK5 in vitro.** HEK 293 cells, grown in 6-well plates, were transfected with pVAX-CDPK5 using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. Forty-eight hours after transfection, the cells were fixed with 100% acetone and then incubated with goat anti- *T. gondii* tachyzoite polyclonal antibody (1:50 dilution in PBS containing 0.05% Tween 20 [PBST]). Then, fluorosecin isothiocyanate (FITC)-labeled rabbit anti-mouse IgG antibody (1:2,000; Sigma) was added into each well. The indirect immunofluorescence assay (IFA) results were obtained using a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Germany). As a negative control, the HEK 293 cells were transfected with pVAX I.

**Immunization and challenge.** A total of 100 mice were randomly divided into four groups (25 per group). The mice in groups I, II, and III received empty pVAX I vector, PBS, and a blank control, respectively; the mice in groups IV were immunized with 100 μl pVAX-CDPK5 by intramuscular injection and were given booster immunizations 2 and 4 weeks later. Blood samples were collected from the tail vein from 3 mice in each group at weeks 0, 2, 4, and 6, and were centrifuged at 3,000 × g for 10 min. The sera were stored at −20°C until enzyme-linked immunosorbent assays (ELISA).

Two weeks after the third immunization, 10 mice in each group were challenged intraperitoneally with 1 × 10^7 tachyzoites of the virulent *T. gondii* RH strain, and the survival periods were recorded daily until all mice died. Three mice of each group were instead inoculated orally with 10 PRU strain tissue cysts, and the brain cysts were counted at 4 weeks after the challenge.

**Immuno blotting analysis of rTgCDPK5 protein.** The immunoreactivity of the rTgCDPK5 protein was detected by Western blotting using sera from immunized mice at week 6 of the immunization protocol (diluted 1:1,000). Bound antibodies were probed with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; Sigma, USA), and proteins were visualized with 4-chloro-1-naphthol (4-CN) (Sangon, China). As a negative control, sera from nonimmunized mice were used.

**Antibody assays.** The specific humoral immune response against TgCDPK5 was evaluated by an ELISA using 96-well microtiter plates. In each well, 100 μl rTgCDPK5 (10 μg/ml) was coated at 4°C overnight. After blocking of the nonspecific binding sites using 5% bovine serum albumin (BSA) for 1 h at 37°C, mouse sera were added to each well and incubated at 37°C for 1 h. Then, each well was incubated with 100 μl of horseradish peroxidase (HRP)-conjugated anti-mouse IgG diluted at 1:250 or anti-mouse IgG1 or IgG2a (1:500) for 1 h. After addition of 200 μl of substrate solution (0.1 g 3,3′,5,5′-tetramethylbenzidine, 0.03% H₂O₂) for 30 min, the reaction was stopped with 2 M H₂SO₄. All measurements were performed in triplicate. The results were read at 450 nm.

**Lymphocyte proliferation assay by MTS.** Two weeks after the final immunization, three mice per group were euthanized to harvest their spleens. The splenocytes were aseptically collected through a wire mesh, and then red blood cells (RBC) were removed using RBC lysis solution (Sigma, USA). The purified lymphocytes from each group were then cultured in triplicate at a density of 2 × 10^5 cells/well in complete medium (Dulbecco’s modified Eagle’s medium [DMEM] + 10% fetal calf serum [FCS] + 100 U/ml penicillin-streptomycin). For positive controls, the cells were stimulated with TLA (10 μg/ml) or concanavalin A (ConA) (5 μg/ml; Sigma), and cells to which medium alone (M) was added served as negative controls. The proliferative activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) method (Promega, USA) after 4 days. The proliferation index (SI) was calculated by using the
The expression of recombinant plasmid pVAX-CDPK5 in vitro was detected by IFA, and the result showed specific green fluorescence of transfected HEK 293 cells. No green fluorescence was revealed in the cells transfected with the same quantity of empty pVAX I. These results revealed that the TgCDPK5 protein has been successfully expressed in HEK 293 cells.

E. coli BL21(DE3) lysates from cells transfected with pGEX-CDPK5 were separated by SDS-PAGE. After staining with Coomassie brilliant blue, the rTgCDPK5 protein was observed at approximately 100 kDa, which coincided with the theoretical value.

**Western blotting and antibody responses.** Analysis of the immunogenicity of rTgCDPK5 protein by Western blotting indicated that the anti-CDPK5 antibody recognized the recombinant protein at ~100 kDa, whereas the protein did not react with the negative sera.

Serum samples from 3 randomly chosen mice in each group were collected to detect specific anti-CDPK5 antibodies by ELISA. Two weeks after the third immunization, the specific IgG antibodies reached their highest levels in mice immunized with pVAX-CDPK5 (0.31 ± 0.05); these levels were significantly higher than those in the pVAX I (0.15 ± 0.08), PBS (0.14 ± 0.09), and blank control (0.15 ± 0.08) groups (P < 0.05) (Fig. 1A). In order to characterize the role of humoral immunity induced by pVAX-CDPK5, the subclasses of IgG (IgG1 and IgG2a) were evaluated individually in sera of vaccinated and control mice at 2 weeks after the last immunization. As shown in Fig. 1B, the vaccine induced significant IgG1 and IgG2a antibody responses (P < 0.05) with a higher level of the antigen-specific IgG2a antibody isotype, as determined by the antibody levels. These results indicate that pVAX-CDPK5 elicited both a specific humoral response and the Th1-type immune response.

**Evaluation of splenocyte proliferation.** After 96 h of splenocyte/mitogen coculture, the proliferative response was identified by MTS. As shown in Fig. 2, the proliferation SI from the pVAX-CDPK5 vaccinated group (1.92 ± 0.21) was significantly increased compared with values from the three control groups, while the differences in splenocyte proliferation in the mice immunized with PBS (0.91 ± 0.01), pVAX I (0.89 ± 0.07), or the blank control (0.97 ± 0.01) were not statistically significant.

**Percentages of CD4⁺ and CD8⁺ T lymphocytes.** The levels of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells in each group are shown in Fig. 3. In the mice immunized with pVAX-CDPK5, the percentages of CD3⁺ CD4⁺ (17.83% ± 3.48%) (P > 0.05) and CD3⁺ CD8⁺ (8.43% ± 1.46%) (P < 0.05) T lymphocytes were higher than those in control mice. There was no difference in terms of the percentages of the two T-cell subtypes among the three control groups (P > 0.05).
Cytokine production by spleen cells. Spleen cell suspensions from individual mice at 2 weeks after the third vaccination were stimulated in vitro and were tested by ELISAs. The IFN-γ, IL-12(p70), and IL-2 levels in spleen cell cultures from mice in the pVAX-CDPK5 group were significantly increased compared with those in the pVAX I, PBS, and blank control groups (P < 0.05) (Table 1). In contrast, the levels of IL-4 produced by pVAX-CDPK5-immunized mice were similar to those in mice from the three control groups (P > 0.05), and the production of IL-10 in the immunized group was significantly lower than that of the control groups (P < 0.05).

Protection of vaccinated mice. To assess the protective immunity of pVAX-CDPK5, mice were challenged with 10³ tachyzoites of the RH strain and 10 cysts of the PRU strain at 2 weeks after the final immunization. The average survival time of mice immunized with the RH strain and 10 cysts of the PRU strain at 2 weeks after the final immunization. The average survival time of mice immunized with pVAX-CDPK5, pVAX I, PBS, and blank control groups (P < 0.05) (Table 1). In contrast, the levels of IL-4 produced by pVAX-CDPK5-immunized mice were similar to those in mice from the three control groups (P > 0.05), and the production of IL-10 in the immunized group was significantly lower than that of the control groups (P < 0.05).

DISCUSSION

The CDPK proteins were identified as key components of the signaling pathways that can regulate many crucial events in apicomplexan parasites. Previous studies showed that TgCDPK1 is involved in regulating micromere secretion, parasite motility, and host cell invasion and egress (26), TgCDPK3 is important in the calcium-dependent egress of parasites from host cells but not in motility or host cell egress (27, 28), and TgCDPK7 was crucial for parasite division, growth, and proper maintenance of centrosome integrity (29). The comparative genomic and phylogenetic analyses revealed that T. gondii harbors 11 CDPK-like genes (30), but, for the most part, their immunogenicity has not been studied. In the present study, we cloned TgCDPK5 and then evaluated the gene as a potential DNA vaccine candidate against T. gondii infection for the first time. Although this DNA vaccine did not effectively protect mice from a lethal challenge, it did prolong the survival time (8.67 ± 4.34 days) and reduced the numbers of brain cysts in immunized mice (39.13%) in contrast with values for the control mice (P < 0.05).

Specific IgG antibodies against T. gondii can inhibit the parasite, facilitating its attachment to host cell receptors and promoting macrophages to kill intracellular parasites, which may be important in controlling T. gondii infection and preventing reactivation (31). Our results showed that the level of anti-T. gondii IgG antibodies in mice immunized with pVAX-CDPK5 was statistically significantly elevated compared with levels in the three control groups. However, antibody titers in sera from the immunized mice, following three immunizations, were only twice as high as those in control mice, an antibody level which is much lower than those elicited by eukaryotic initiation factor 4A (eIF4A), rhoptry protein 18 (ROP18), microneme protein 6 (MIC6), and other T. gondii vaccine candidates (18, 22, 24, 25, 32, 33). Because IgG-dependent phagocytosis, cytotoxicity, or complement-mediated lysis effects are crucial mechanisms for resistance to tachyzoites (31), the relatively low antibody levels resulting from DNA immunization with pVAX-CDPK5 failed to prevent acute infection.

T-cell-mediated adaptive immune responses are well known to determine the course of T. gondii infection (34, 35, 36). In the present study, a significant proliferative response of splenocytes was induced following DNA immunization with pVAX-CDPK5, indicating an activated cellular immune response. Furthermore, similar to the results from DNA vaccination with eIF4A (24) and

![FIG 4](http://cvi.asm.org/Downloaded from)

![FIG 5](http://cvi.asm.org/Downloaded from)

| Mouse group       | IFN-γ (pg/ml) | IL-2 (pg/ml) | IL-4 (pg/ml) | IL-10 (pg/ml) | IL-12(p70) (pg/ml) |
|-------------------|--------------|--------------|--------------|---------------|-------------------|
| pVAX-CDPK5        | 982.68 ± 378.22 | 266.04 ± 9.13 | <15          | 136.53 ± 71.63 | 96.84 ± 4.06     |
| pVAX I            | 34.91 ± 11.23  | <15          | <15          | 322.50 ± 179.17 | 15.14 ± 7.63    |
| PBS               | 36.34 ± 4.83   | <15          | <15          | 356.13 ± 175.27 | 22.31 ± 4.40    |
| Blank control     | 47.28 ± 16.60  | <15          | <15          | 477.25 ± 87.53  | 25.29 ± 17.35   |

* Splenocytes from mice were harvested 2 weeks after the final immunization.

b Statistically significant difference (P < 0.05).
ROP18 (37), the percentages of both CD4+ T and CD8+ T cells were also increased in pVAX-CDPK5 immunized mice, which may have contributed to a T. gondii-specific CTL response.

It has been established that the Th1-type immune response plays a critical role in protective immunity against T. gondii (38). In this study, we have further evaluated the Th1 and/or Th2 immune responses in mice immunized with pVAX-CDPK5. Thus, the high ratio of IgG2a to IgG1 antibody titers and Th1-dominated immune responses. Furthermore, IL-4 and IL-10 cytokines, associated with Th2 responses, have been shown to play important roles during the early phase of acute T. gondii infection (39). However, the low levels of IL-4 and IL-10 in the immunized mice compared with those in the controls may not promote sufficient mast cell responses and B-cell proliferation, consistent with the relatively low level of specific IgG antibodies. These results may further explain the inability of pVAX-CDPK5 to protect against acute T. gondii infection.

Investigation of the expression profiles of CDPKs in three archetypal T. gondii lines by microarray analysis showed higher TgCDPK5 gene expression in the PRU strain than in the RH strain (http://toxodb.org). Further analysis of cell cycle expression profiles based on the ME49 strain revealed that the expression of TgCDPK5 fluctuated in cells, being higher during the S phase and through cytokinetic periods (S/M phases) and then declining in the G2 period (40). The expression level of the gene increased 2.7-fold in intracellular bradyzoites (12 h) compared with that in cells at 0 h (40). These expression profiles further explained the reduced occurrence of brain cysts in the vaccinated group.

Taken together, our results have demonstrated, for the first time, that immunization with pVAX-CDPK5 can generate humoral and cellular Th1 immune responses, reduce brain cyst burden significantly, and also prolong the survival time (albeit in a limited fashion). These effects suggest that this gene is a potential vaccine candidate for inclusion in a multicomponent T. gondii vaccine against toxoplasmosis.

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