Toxoplasma gondii cathepsin proteases are undeveloped prominent vaccine antigens against toxoplasmosis

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Abstract

Background: Toxoplasma gondii, an obligate intracellular apicomplexan parasite, infects a wide range of warm-blooded animals including humans. T. gondii expresses five members of the C1 family of cysteine proteases, including cathepsin B-like (TgCPB) and cathepsin L-like (TgCPL) proteins. TgCPB is involved in ROP protein maturation and parasite invasion, whereas TgCPL contributes to proteolytic maturation of proTgM2AP and proTgMIC3. TgCPL is also associated with the residual body in the parasitophorous vacuole after cell division has occurred. Both of these proteases are potential therapeutic targets in T. gondii. The aim of this study was to investigate TgCPB and TgCPL for their potential as DNA vaccines against T. gondii.

Methods: Using bioinformatics approaches, we analyzed TgCPB and TgCPL proteins and identified several linear-B cell epitopes and potential Th-cell epitopes in them. Based on these results, we assembled two single-gene constructs (TgCPB and TgCPL) and a multi-gene construct (pTgCPB/TgCPL) with which to immunize BALB/c mice and test their effectiveness as DNA vaccines.

Results: TgCPB and TgCPL vaccines elicited strong humoral and cellular immune responses in mice, both of which were Th-1 cell mediated. In addition, all of the vaccines protected the mice against infection with virulent T. gondii RH tachyzoites, with the multi-gene vaccine (pTgCPB/TgCPL) providing the highest level of protection.

Conclusions: T. gondii CPB and CPL proteases are strong candidates for development as novel DNA vaccines.

Keywords: Toxoplasma gondii, Cathepsin proteases, Bioinformatics, Vaccine, Toxoplasmosis
immune responses, could be an option for eliminating this ubiquitous parasite has been raised [9,10].

An important design principle for DNA vaccines is the selection of parasite proteins involved in the host cell invasion process by T. gondii; therefore, many researchers are working to identify the relevant proteins that may be developed as vaccines against toxoplasmosis [11].

Cysteine proteases play many specialized roles in the body, including endocytosis-related polypeptide degradation [12], tumor invasion [13] and TNF-α-induced apoptosis [14]. More importantly, cysteine proteases are important for the growth and survival of apicomplexan parasites that infect humans. T. gondii expresses five members of the C1 family of cysteine proteases, including one cathepsin B-like (TgCPB), one cathepsin L-like (TgCPL), and three cathepsin C-like (TgCPCL1, 2 and 3) proteases [15]. Among these, TgCPB and TgCPL are mainly expressed in the vacuolar compartment, but a tiny amount of TgCPL has been identified in the late endosome [16-19]. These proteases are thought to function in protein degradation and play specialized roles in the maturation of invasion-related proteins. TgCPB is involved in ROP protein maturation and parasite invasion [16]. In contrast, TgCPL contributes to proteolytic maturation of proTgM2AP and proTgMIC3, and is also associated with the residual body in the parasitophorous vacuole after cell division [20-23].

T. gondii cathepsins are considered potential therapeutic targets based on the results of the following genetic and inhibitor studies: antisense inhibition of TgCPB expression or treatment with cathepsin inhibitors diminished parasite replication, cell invasion and infection in vivo [16,24]; genetic disruption of TgCPL diminished parasite cell invasion and growth [17]; and, the cathepsin inhibitor morpholinurea-leucyl-homophenyl-vinyl sulfone phenyl inhibited parasite invasion by blocking the release of invasion proteins from microneme secretory organelles [25,26]. To our knowledge, no studies have described the induction of protective immune responses against T. gondii CPB and CPL in the host. We propose that a DNA vaccine construct based on TgCPB and TgCPL could be a useful tool against disease caused by T. gondii.

Hence, in this study, we used bioinformatics approaches to analyze TgCPB and TgCPL, the results of which identified a large number of linear-B cell epitopes and potential Th-cell epitopes on these proteases. This suggested the possibility that TgCPB and TgCPL could be used as vaccines. Based on the results of the epitope analyses, pTgCPB and pTgCPL were constructed as single-gene vaccines and pTgCPB/pTgCPL as a multigene vaccine, and their immunogenicity, protective efficacy, and potential as vaccine candidates against T. gondii infection were examined in laboratory mice.

**Methods**

**Prediction of protein secondary structure and linear-B cell epitopes**

Epitopes are the foundation of protein antigenicity that determines antigen specificity [27,28]. There are many types of epitope prediction methods in use, including hydrophilicity, accessibility, antigenicity, flexibility, charge distribution and secondary structure [29-34]. Despite the lack of an infallible method to predict antigenic epitopes, several rules can be followed to determine which peptide fragments of a protein are likely to be antigenic. Firstly, antigenic epitopes should be located in solvent-accessible regions and contain both hydrophobic and hydrophilic residues. Secondly, peptides lying in long loops connecting secondary structure motifs should be selected preferably, while peptides located in helical regions should be avoided. Whenever possible, peptides that are in the N- and C-terminal regions of the protein should be chosen because they are usually solvent accessible and unstructured.

According to the rules outlined above, we analyzed the linear-B cell epitopes of TgCPB and TgCPL using DNAStar software and chose peptides that have good hydrophilicity, high accessibility, satisfactory flexibility and strong antigenicity. Thereafter, we used DNAMAN software to search for linear-B cell epitopes in the TgCPB and TgCPL amino acid sequences.

**Prediction of Th-cell epitopes**

T. gondii is an obligate intracellular parasite; hence, cellular immunity mediated by T cells plays an important role in T. gondii infection [35]. To develop an effective vaccine against toxoplasmosis, it is necessary to elucidate which type of Th cell-mediated immune response is necessary. Predicting Th cell epitopes is currently rather complicated and the results are ambiguous; however, there are some rules that can be used to predict Th cell epitopes [36,37]. Here, we used the Immune Epitope Database (http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html) online service to predict the half maximal inhibitory concentration (IC50) values of peptides binding to the major histocompatibility complex (MHC) class II molecules of TgCPB and TgCPL. We also used SYFPEITHI (http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm) to determine the ligation strength to a defined HLA (or H2) type for TgCPB and TgCPL. Note that such binding to MHC is necessary but not sufficient for recognition by T cells.

**Parasites and mice**

Female 6-week-old BALB/c mice were purchased from Shandong University Laboratory Animal Center. All mice were maintained under specific pathogen-free conditions. All of the animal experiments were approved by...
the Ethics Committee on Animal Experiments of the Medical School of Shandong University.

The *T. gondii* RH strain was harvested from the peritoneal fluid of the BALB/c mice 72 h after infection, and was washed by centrifugation and resuspended in sterile PBS. Half of the *T. gondii* tachyzoite suspension was used to extract total RNA and genomic DNA, while the other half was used to prepare soluble tachyzoite antigens (STAg) using an ultrasonic disintegrator. STAg preparations were aliquoted and stored at −80°C until use.

**Construction of expression plasmids**

The whole TgCPB gene was amplified from *T. gondii* total RNA by reverse transcription polymerase chain reaction (RT-PCR) using the two primer pairs shown below. TgCPB for prokaryotic expression used the following primers plasmid construction: 5′-ccGGAATTCATGGAGGGGCGAAAGTC-3′ (forward) and 5′-ccgCTCGAGCTACATTTCTCTCTCCTCTCTTGT-3′ (reverse), both of which contain EcoRI/ Xhol restriction sites (underlined). Plasmid construction for eukaryotic expression of TgCPB consisted of 5′-ataagat GCGGCCGCGATGGAGGGGCGAAAGTC-3′ (forward) and 5′-ccgCTCGAGCTACATTTCTCTCTCTCTCTTGT-3′ (reverse), both of which contain NotI/Xhol restriction sites (underlined).

The whole TgCPL gene was PCR amplified from *T. gondii* genomic DNA using the two primer pairs described below. Prokaryotic expression plasmid construction for TgCPL used the following primers: 5′-ccgGAATTCATGGAGGGGCGAAAGTC-3′ (forward) and 5′-ccgCTCGAGCTACATTTCTCTCTCTCTCTCTTGT-3′ (reverse), both of which contain NotI/Xhol restriction sites (underlined).

The PCR products for both genes were cloned into a pEASY-T1 vector (TransGen Biotech, China) to generate a recombinant cloning plasmid. After sequencing, TgCPB and TgCPL were subcloned into a eukaryotic expression plasmid pET-30a(+)(Novagen, USA) and the prokaryotic expression plasmid pET30a-TgCPB and pET30a-TgCPL constructs were used to transform *E. coli* BL21(DE3) cells, which were subsequently grown in Luria-Bertani medium with kanamycin (25 μg/mL). Synthesis of recombinant TgCPB and TgCPL proteins was induced using 1 mM isopropyl-β-D-thiogalactoside for 6 or 8 h at 25°C. The cells were then lysed and centrifuged at 4°C for 15 min at 10,000×g. Recombinant proteins were then purified via binding of their carboxy terminal histidine tags to Ni-NTA resin (Sangon Biotech, China).

Experimental mice were subcutaneously immunized with 100 μg of purified rTgCPB or rTgCPL prepared in equal volumes of Freund’s complete adjuvant for the first immunization. The second and third immunizations contained 50 μg of the purified protein in Freund’s incomplete adjuvant. Samples of antisera were collected 2 weeks after the last immunization.

**Examination of antibody specificity by western blotting**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were used to investigate antibody specificity, as described previously [38]. STAg preparations were removed from the ultra-low temperature freezer and 500 ng of each preparation was used for SDS-PAGE. The separated protein bands were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA), each of which was blocked with 5% w/v skimmed milk powder diluted in PBS for 2 h at room temperature before separate incubation with mouse anti-TgCPB or anti-TgCPL antibodies, or pre-immune mouse sera (dilution 1:600). After a wash in PBS-Tween 20, each of the membranes was incubated with diluted goat anti-mouse IgG horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000; Sigma, USA), each of which was blocked with 5% w/v skimmed milk powder diluted in PBS for 2 h at room temperature before separate incubation with mouse anti-TgCPB or anti-TgCPL antibodies, or pre-immune mouse sera (dilution 1:600). After a wash in PBS-Tween 20, each of the membranes was incubated with diluted goat anti-mouse IgG horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000; Sigma, USA) for 1 h. Parasite proteins were visualized using electrochemiluminescence reagents (Cowin Biotech, China).

**TgCPB and TgCPL expression in mammalian cells**

When the cell density reached 80–90%, HEK293 cells were transfected with pBudCE4.1-TgCPB or pBudCE4.1-TgCPL using LipoFectamine™ 2000 reagent (Invitrogen, USA). After 24-h incubation, the cells were fixed with cold methanol for 20 min and protein expression was evaluated using an indirect fluorescence antibody test as previously described [39]. Briefly, anti-TgCPB or anti-TgCPL antibodies were used as primary antibodies and a FITC-labeled goat anti-mouse IgG antibody (ZSGB-Bio, China) was used as the secondary antibody. After rinsing three times with PBS, the coverslips were immediately observed under a fluorescence microscope (Carl Zeiss, Germany). The cells were then lysed with RIPA Lysis Buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS) containing 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride, after which they were centrifuged at 12,000×g for 10 min, at either 24 h or 48 h post-transfection.
SDS-PAGE and western blot analysis
Protein production from HEK293 cells was monitored by SDS-PAGE and western blotting. About 500 ng of the purified rTgCPB or rTgCPL proteins were separated using SDS-PAGE. The separated protein bands were transferred onto PVDF membranes. The detailed procedures are the same as in the above section “Identification of the antibody specificity by western blotting”.

Animal experiments
Five groups of BALB/c mice (n = 13 each) were individually injected 4 times at two-weekly intervals with one of the following: PBS, pBudCE4.1, pTgCPB, pBudCE4.1-TgCPL or pBudCE4.1-TgCPB-TgCPL. Two weeks after the final immunization, the mice were challenged by intraperitoneal (i.p.) injection of 100 μL of PBS containing 1×10^4 T. gondii tachyzoites and the survival time of each mouse was recorded.

Antibody assays
The levels of IgG antibodies against T. gondii were analyzed using an enzyme-linked immunosorbent assay (ELISA) [40]. The microtiter plates (Costar, USA) were coated with STAg (10 pmol/well) and incubated at 4°C overnight. After washing three times with PBS-T, the plates were blocked with 1% bovine serum albumin for 1 h at 37°C. The plates were washed a further three times and incubated with the mouse sera diluted in PBS for 1 h at 37°C. After washing three times, secondary goat anti-mouse IgG, IgG1 or IgG2a conjugated with HRP (Sigma) was added and incubated at 37°C for 1 h. Immune complexes were revealed by incubating with orthophenylenediamine (Sigma) and 0.15% H2O2 for 30 min. Reactions were stopped by the addition of 2 M H2SO4 and read at 490 nm with an ELISA plate reader (EL800; Bio-Tek, USA). All samples were run in triplicate.

Cytokine assays
Cytokine levels were detected according to the previously described method [39]. Briefly, three mice per group on week 4 after the final immunization were euthanized and their spleens removed under sterile conditions. Viable splenocytes were dispensed into 96-well plates at 37°C in 5% CO2 and the cell-free supernatants were harvested and assayed for IL-4 levels at 24 h, or at 72h for IL-10, or at 96 h for IFN-γ using an ELISA kit (R&D Systems, USA).

Statistical analyses
Statistical analyses were performed using SPSS software. Antibody production and cytokine levels among the different groups were determined using a one-way analysis of variance. Survival times in the mice were compared using the Kaplan-Meier method. Values of P < 0.05 were considered statistically significant.

| Table 1 Linear-B cell antigenic epitope prediction for TgCPB |
|-------------------|-----------------|-----------------|
| Order | Amino acid position | Potential antigenic sequences | Score |
| 1 | 325–346 | LMPLSAQHTTSCNAHACASFG | 1.190 |
| 2 | 6–30 | SFRVLGLTPLFAALAILLCLGCMTY | 1.183 |
| 3 | 57–86 | AEDVLYASFVESVLDSDVXWATX | 1.176 |
| 4 | 379–407 | CWPIYEPFCAHHAKAPPPDCDATLVRK | 1.175 |
| 5 | 118–127 | GELLRLILLAD | 1.170 |
| 6 | 147–160 | RHIVRDSVLYSEA | 1.164 |
| 7 | 283–294 | AFDACKDVWG | 1.157 |
| 8 | 453–489 | GPVGAFMVYDFlSYKVSGLPVGYHAK | 1.153 |
| 9 | 196–204 | SNAVALIK | 1.130 |
| 10 | 300–306 | CGSCWAF | 1.108 |
| 11 | 243–250 | GTFLVNTK | 1.103 |
| 12 | 225–226 | EYRLVFYSLK | 1.092 |
| 13 | 272–278 | EPNAPAHF | 1.091 |
| 14 | 314–320 | DRLCIRS | 1.084 |
| 15 | 38–44 | DSLFPLS | 1.078 |
| 16 | 434–440 | TASLYR | 1.067 |
| 17 | 139–145 | FRHLTHS | 1.061 |
| 18 | 420–426 | ADWHPF | 1.059 |
| 19 | 362–367 | KGVTG | 1.055 |
| 20 | 260–265 | MPLPAK | 1.045 |
| 21 | 520–525 | MGQCGI | 1.039 |

| Table 2 Linear-B cell antigenic epitope prediction for TgCPL |
|-------------------|-----------------|-----------------|
| Order | Amino acid position | Potential antigenic sequences | Score |
| 1 | 60–82 | RAWIALVAAVSLLVSFLSFLQW | 1.205 |
| 2 | 364–372 | DHGVLLVGY | 1.168 |
| 3 | 142–151 | KNNLYYFIHTH | 1.152 |
| 4 | 303–320 | RAQSCKRVKILFGKDP | 1.152 |
| 5 | 89–100 | AVFPPSIVEDHQ | 1.152 |
| 6 | 35–44 | PFPFWVTITR | 1.152 |
| 7 | 187–209 | KSHHLGWATELLNMLPSLPAGV | 1.141 |
| 8 | 410–419 | OCGGLLDASF | 1.138 |
| 9 | 214–221 | RCIVTPW | 1.136 |
| 10 | 6–12 | THYSFL | 1.130 |
| 11 | 328–341 | KAALSPVYSAIE | 1.120 |
| 12 | 278–297 | FOYVLDSSGICSEADYPYLA | 1.119 |
| 13 | 239–255 | EGIAHKICTGKVSFLSEQ | 1.110 |
| 14 | 226–232 | CGSCWAF | 1.108 |
| 15 | 347–362 | FQHYHEGFDASCCTGD | 1.100 |
| 16 | 113–127 | FOQFAPSSQAMYAKS | 1.050 |
| 17 | 175–180 | RKYLF | 1.035 |
We chose 15 amino acids for analysis each time.

H2-Ad, H2-Ak, H2-Ed and H2-Ek alleles are mouse MHC class II molecules; the HLA-DRB1*01:01 allele is a human MHC class II molecule.

Table 3 IC50 values for TgCPB and TgCPL peptide binding to MHC class II molecules obtained using the immune epitope database

| MHC II Allele | Start-Stop | Sequence | Percentile |
|---------------|------------|----------|------------|
| H2-Ad         | 55–69      | FSAEDVLNAFVSPE | 31 |
| H2-Ad         | 76–90      | SIVAEQWATSGNL | 27 |
| H2-Ad         | 412–426    | KDCCEEQYADNHPF | 27 |
| H2-Ad         | 117–131    | AGELELILLADSEM | 26 |
| H2-Ak         | 43–57      | LSEDTSVDPRESFA | 24 |
| H2-Ak         | 72–86      | SLFSDISEAQVATS | 24 |
| H2-Ak         | 149–163    | IMRDSLVEYSEAPFS | 24 |
| H2-Ak         | 37–51      | DDSLFLPSEDTSVDP | 22 |
| H2-Ed         | 313–327    | NDLRGLSRQGKRLMP | 28 |
| H2-Ed         | 436–450    | AYSRLSRDDWKRDM | 28 |
| H2-Ed         | 229–243    | RFRYLSDLKAKLMG | 26 |
| H2-Ed         | 309–313    | TEAFNORLRCSQGK | 24 |
| H2-Ed         | 86–100     | SGNLTEASPRDRDSSA | 24 |
| H2-Ed         | 313–327    | NDLRGLSRQGKRLMP | 22 |
| H2-Ed         | 463–477    | EDFLSYSKSGVYWH | 22 |
| H2-Ed         | 37–51      | DDSLFLPSEDTSVDP | 20 |
| HLA-DRB1*0101 | 4–18      | RKSFRVLTLPFAA | 35 |
| HLA-DRB1*0101 | 13–27     | PLPFAALAILLG | 34 |
| HLA-DRB1*0101 | 470–484    | SGVYSKVHSGLPG | 33 |
| HLA-DRB1*0101 | 71–85     | ESLFSDIAEQVAT | 32 |

Table 4 Ligation strength analysis of TgCPB and TgCPL for MHC class II molecules using SYFPEITHI

| MHC II Allele | Start-Stop | Sequence | Score |
|---------------|------------|----------|-------|
| H2-Ad         | 60–74      | RAWIALVAAASLLV | 31 |
| H2-Ad         | 68–82      | AAVSLVFAFSLIQW | 30 |
| H2-Ad         | 192–206    | GVATELLNVPSLEP | 30 |
| H2-Ad         | 58–72      | TARAWIALVAAASL | 27 |
| H2-Ak         | 18–32      | GLENGEHQORRVRGA | 26 |
| H2-Ak         | 146–160    | VVTHINQQGYSYL | 22 |
| H2-Ak         | 196–210    | ELLNVPSLEPGVD | 22 |
| H2-Ak         | 256–270    | ELMDCRASCBEQCS | 20 |
| H2-Ed         | 311–325    | WKLGFKQWRPEA | 30 |
| H2-Ed         | 44–58      | RTYFVKKFLQRONF | 26 |
| H2-Ed         | 170–184    | RDEFRKKYGLPKSR | 26 |
| H2-Ed         | 369–383    | LVGYTGKESKDFW | 26 |
| H2-Ek         | 310–324    | WKLGFKQWRPEA | 24 |
| H2-Ek         | 77–91      | SFLQWQGEDDRAVF | 22 |
| H2-Ek         | 365–379    | HGHLVYGYTDKESC | 22 |
| H2-Ek         | 6–20       | THYSFGLNDEGOLE | 20 |
| HLA-DRB1*0101 | 59–73     | ARAWIALVAAASLL | 34 |
| HLA-DRB1*0101 | 60–74     | RAWIALVAAASLLV | 31 |
| HLA-DRB1*0101 | 195–209    | TELLNVPSLEPG | 31 |
| HLA-DRB1*0101 | 308–322    | EKVWKLGFQWRPEA | 30 |

1 H2-Ad, H2-Ak, H2-Ed and H2-Ek alleles are mouse MHC class II molecules; the HLA-DRB1*01:01 allele is a human MHC class II molecule.

2 We chose 15 amino acids for analysis each time.

3 TgCPB or TgCPL sequences.

4 Low percentile = high binding.
Ethics statement

All experimental procedures using animals in the present study had received prior approval by the Institutional Animal Care and Use Committee of Shandong University under Contract 2011–0015. Humane endpoints to reduce pain or distress in the experimental animals were employed via euthanasia. Mice were monitored daily over 8 weeks for signs of toxoplasmosis, which included difficulties with their food and water intake, lethargy, or severe ascites. Mice that showed signs of illness were sacrificed immediately using CO2 gas; this involved placing the mice in a chamber and administering CO2 at a concentration of 60% to 70% over a 5-minute exposure time, after which the cervical dislocation method was sometimes used to ensure that effective euthanasia had occurred.

Results

Prediction of linear-B cell epitopes and Th-cell epitopes

The results of the prediction analyses indicated the presence of 21 potential epitopes on TgCPB and 17 on TgCPL, as shown in Tables 1 and 2. The Th-cell epitopes on TgCPB and TgCPL that were identified by bioinformatic analyses are predicted to have the ability to bind strongly to MHC class II molecules (Tables 3 and 4). The binding strength of the interaction is known to influence the direction of Th cell differentiation, where, as the binding force increases, more cells tend to differentiate into Th-1 cells [41,42]. As such, we speculate that TgCPB and TgCPL are likely to induce Th-1 cell-mediated immune responses.

Prokaryotic and eukaryotic expression vectors for TgCPB and TgCPL

Recombinant plasmids pET30a-TgCPB and pET30a-TgCPL were digested with EcoRI and XhoI resulting in the correct sized fragments and indicating successful construction of the prokaryotic expression plasmids (Figure 1A). Similarly, restriction digests of pBudCE4.1-TgCPB (NotI and XhoI), pBudCE4.1-TgCPL (HindIII and XbaI), and the dual construct pBudCE4.1-TgCPB-TgCPL (NotI, XhoI, HindIII and XbaI) confirmed that the recombinant eukaryotic expression plasmids had been correctly made (Figure 1B).

Antibody specificity against rTgCPB and rTgCPL proteases

PVDF membranes from western blots of STAg preparations were incubated separately with mouse anti-TgCPB or anti-TgCPL antibodies or pre-immune mouse sera. The results showed that the mouse anti-TgCPB and anti-TgCPL antibodies recognized proteins bands of about 62 kDa and 47 kDa, which is consistent with the expected sizes of the TgCPB and TgCPL proteins, respectively (Figure 2).
Figure 3 Indirect fluorescent antibody detection of recombinant TgCPB and TgCPL proteases on the surface of HEK293 cells. (A1) pTgCPB-transfected HEK293 cells; (A2) pBudCE4.1-transfected HEK293 cells. (B1) pTgCPL-transfected HEK293 cells; (B2) pBudCE4.1-transfected HEK293 cells. (C1) pTgCPB/TgCPL-transfected HEK293 cells where pTgCPB/TgCPL expression was detected using the anti-TgCPB antibody as the primary antibody; (C2) pBudCE4.1-transfected HEK293 cells where the anti-TgCPB antibody was used as the primary antibody. (D1) pTgCPB/TgCPL-transfected HEK293 cells where the anti-TgCPL antibody was used as the primary antibody; (D2) pBudCE4.1-transfected HEK293 cells where the anti-TgCPL antibody was used as the primary antibody. High level of laser intensity was used for A1, A2, B1 and B2, lower level of laser intensity for C1, C2, D1 and D2.
Identification of protein expression in HEK293 cells using immunofluorescence assay (IFA) and western blotting

In vitro expression of pTgCPB, pTgCPL and pTgCPB/TgCPL were evaluated by IFAs at 48h post-transfection. As shown in Figure 3, green fluorescence was observed in HEK293 cells, whereas no fluorescence was observed in the pBudCE4.1 vector transfected cells. Western blotting analysis confirmed expression of rTgCPB (~62kDa) and rTgCPL (~47kDa) in HEK293 cells transfected with pTgCPB or pTgCPL. Both proteins were detected in cells transfected with the dual expression vector, pTgCPB/TgCPL (Figure 4).

Antibody responses in immunized mice

High levels of immunoglobulin G (IgG) antibodies were observed in the experimental mice immunized with pTgCPB, pTgCPL, or pTgCPB/TgCPL. The antibody levels gradually increased with successive immunizations and were higher than those of the control groups, which were immunized with phosphate-buffered saline (PBS) or pBudCE4.1 (Figure 5). A significant difference was detected between the experimental groups and the control groups (P < 0.05). IgG antibody levels in the pTgCPB/TgCPL group were higher than those of the

Table 5 Cytokine production by splenocytea cultures from immunized BALB/c mice

| Group            | IFN-γ (pg/mL) | IL-4 (pg/mL) | IL-10 (pg/mL) |
|------------------|--------------|--------------|---------------|
| PBS              | 47.59 ± 4.63 | 37.26 ± 2.84 | 44.34 ± 2.77  |
| pBudCE4.1        | 48.35 ± 1.86 | 33.70 ± 3.29 | 38.70 ± 2.70  |
| pTgCPB           | 674.93 ± 83.36 | 32.58 ± 3.72 | 34.60 ± 1.92  |
| pTgCPL           | 585.14 ± 112.03 | 32.34 ± 3.87 | 35.47 ± 1.94  |
| pTgCPBP/TgCPL    | 1182.23 ± 94.28 | 36.11 ± 3.51 | 34.57 ± 2.14  |

IFN, interferon; IL, interleukin; PBS, phosphate-buffered saline.

* Splenocytes were obtained from 3 mice per group and were collected at week 4 after the final immunization.

b Values for IFN-γ at 96 h. Values for IL-10 at 72 h. Values for IL-4 at 24 h.

* Compared with the PBS-adjuvant or pBudCE4.1 controls (P < 0.05); # compared with pTgCPB or pTgCPL (P < 0.05).
pTgCPB and pTgCPL groups \((P < 0.05)\). However, no statistical difference was detected between the pTgCPB and pTgCPL groups \((P > 0.05)\). These results indicate that the recombinant plasmids encoding TgCPB and TgCPL induced strong IgG antibody responses in the mice. Furthermore, the OD value for this group reached a high level two weeks after the third immunization.

IgG subclass (IgG1 and IgG2a) levels in all of the groups during the second week after the final immunizations were investigated to determine whether a Th1- or Th2-type response was elicited (Figure 6). An apparent predominance of IgG2a over IgG1 was observed in both the single-gene or multi-gene vaccine immunized mice, indicating a shift toward a Th1-type response. Furthermore, mice immunized with pTgCPB/TgCPL generated higher IgG2a levels than those immunized with pTgCPB or pTgCPL alone \((P < 0.05)\). However, there was no significant difference in the IgG2a levels between the pTgCPB and pTgCPL groups \((P > 0.05)\).

Cytokine production

The splenocyte supernatant was harvested at different times and used to measure cytokine production (interferon-\(\gamma\) [IFN-\(\gamma\)], interleukin-4 [IL-4] and IL-10) in the different groups. As shown in Table 5, mice vaccinated with pTgCPB/TgCPL generated significantly higher levels of IFN-\(\gamma\) than mice vaccinated with pTgCPB, pTgCPL, PBS, or an empty vector \((P < 0.05)\). The level of IFN-\(\gamma\) in the pTgCPB-immunized mice was higher than that of the pTgCPL-immunized mice, but the difference was not statistically significant \((P > 0.05)\). In addition, the low levels of IL-4 and IL-10 seen in the experimental and control groups suggested no statistically significant differences among the groups \((P > 0.05)\). IFN-\(\gamma\) and IL-2 favor Th1-type immune responses, whereas IL-4 and IL-10 favor Th2-type responses. These results show that the cellular immune response induced by the single- or multi-gene vaccines tended to be a Th1-type response in the mice.

Protective efficacy of DNA vaccination against \(T. gondii\) in mice

To evaluate the level of immunoprotection induced by the DNA vaccines, all of the mice were challenged intraperitoneally with the \(T. gondii\) RH strain and mortality was monitored daily until all of the mice showed signs of illness and were killed (Figure 7). Mice immunized with the DNA vaccines had dramatically higher survival times than did the control groups vaccinated with PBS or pBudCE4.1 \((P < 0.05)\). Mice vaccinated with pTgCPB/TgCPL showed a greater survival time than those vaccinated with pTgCPB or pTgCPL \((P < 0.05)\). However, no significant difference was observed between mice immunized with pTgCPB and those immunized with pTgCPL \((P > 0.05)\).

Discussion

Bioinformatics is an established interdisciplinary science related to mathematics, statistics, computer science, physics, biology and medicine [43]. Because of its effectiveness and low cost, it has been widely used to predict the structure, function and antigenic epitopes of proteins by estimation of the similarity of the protein of interest to a sequence of known structure or function [44,45]. As previously described, we initially used DNAStar software to analyze the secondary structures of TgCPB and TgCPL, followed by DNAMAN software to analyze their sequences from which we identified many good liner-B cell epitopes. Secondly, we used two online services (IEDB and SYFPEITHI) for analyzing Th-cell epitopes and found several potential Th-cell epitopes on TgCPB and TgCPL.

**Figure 7** Survival curves of the vaccinated BALB/c following Toxoplasma gondii challenge infections. 10 mice per group were challenged with \(1 \times 10^7\) tachyzoites of the virulent \(T. gondii\) RH strain on the 4th week after the final immunization. Survival was monitored daily for 18 days post-challenge with the parasites.
Through the animal experiments, we confirmed that both TgCPB and TgCPL can induce strong humoral and cellular immune responses and noted a significantly higher level of total IgG antibodies, IgG2a, and IFN-γ than that observed for the controls. These results show that TgCPB and TgCPL make good vaccine antigens, thus highlighting the reliability of the bioinformatics approaches that were used herein.

In the present study, we successfully constructed pTgCPB and pTgCPL single-gene vaccines and a pTgCPB/TgCPL multi-gene vaccine. Both single- and multi-gene vaccines produced humoral and cellular immune responses in the murine host. The multi-gene vaccine was superior to the single-gene vaccine; it elicited stronger immune responses and more effective protection against *T. gondii* infection. Importantly, all of the mice in the experimental groups immunized with pTgCPB, pTgCPL, or pTgCPB/TgCPL displayed strong humoral immune responses as shown by their high IgG levels. The high levels of IgG2a and IFN-γ and low levels of IL-4 and IL-10 suggest that the cellular immune responses were mediated by Th-1 cells [46,47]. These experiments were conducted in BALB/c mice; however, it would be interesting to test other strains of mice with different MCH backgrounds to investigate the range of immune responses to the vaccines.

The survival times of all of the mice in the five groups after intraperitoneal challenge with 1 × 10⁴ tachyzoites of the virulent RH strain of *T. gondii* were recorded. Compared to the mice in the control groups, the immunized mice showed protection against *T. gondii* infection: all mice in the control groups showed signs of illness and were killed within 8 days post-challenge, whereas mice immunized with pTgCPB, pTgCPL, or pTgCPB/TgCPL had significantly increased survival rates. Mice immunized with pTgCPB/TgCPL showed stronger humoral and cellular immune responses and significantly prolonged survival times than mice in the pTgCPB and pTgCPL groups. All of the mice showed signs of illness and were killed by day 16 post-challenge. The results indicate, therefore, that the DNA vaccines did not provide complete protection against *T. gondii* RH tachyzoite infection.

Finally, the pBudCE4.1 vector should be mentioned. This vector was chosen to for expression of the multi-gene vaccine because it has two promoters (CMV and EF-1a), thus ensuring that TgCPB and TgCPL can both be expressed whilst avoiding mutual interference.

Conclusions

In this study, we used bioinformatics approaches to identify antigenic epitopes on TgCPB and TgCPL proteases. Thereafter, we made single-gene (pTgCPB and pTgCPL) and multi-gene (pTgCPB/TgCPL) DNA vaccines to evaluate the level of immunoprotection induced in mice immunized with such vaccines. The experimental results are consistent with the bioinformatics predictions that the antigenic epitopes on these proteins should induce appropriate immune responses. Hence, these results show that bioinformatics analyses to predict antigenic epitopes on proteins can be a useful tool for vaccine research. When the vaccinated mice were given a challenge infection with *T. gondii* RH tachyzoites, we found that the DNA vaccine constructs did not provide complete protection against infection, however. Nevertheless, DNA vaccines merit further investigated as a strategy for controlling *T. gondii* infection.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

GZ carried out the experiments and drafted the manuscript. GL and HM revised the manuscript. YB, MS, YH, LW, OZ, HZ, HK, and XQZ conducted the experiments and revised the manuscript. AZ and SH conceived and designed the study and revised the manuscript. All of the authors have read and approved the final manuscript.

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