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Immunization with *Toxoplasma gondii* aspartic protease 3 increases survival time of infected mice

Guanghui Zhao[a,1], Xiaojie Song[b,1], Xiangnan Kong[a], Ning Zhang[a], Shaoling Qu[a], Wei Zhu[a], Yanyan Yang[a], Qian Wang[a,*]

[a] Clinical Laboratory Medicine Center, Qilu Hospital of Shandong University, Jinan, Shandong Province 250012, China
[b] Department of Respiratory, Qilu Hospital of Shandong University, Qingdao, Shandong Province 255036, China

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ABSTRACT

Aspartic proteases in the *Toxoplasma gondii*, called TgASP1, 2, 3, and 5, play essential roles in the life cycle. In a previous study, we have demonstrated that TgASP1 is an antigen that prolongs survival time of infected mice. As an in-depth study, we have investigated the protective immunity of TgSAP3. A bioinformatic analysis was used to predict the linear B-cell epitopes and potential Th-cell epitopes on TgASP3, the results suggested that it has a large number of excellent epitopes. Mice were inoculated with a recombinant eukaryotic expression vector to evaluate the immune protection against an infection with the virulent RH strain of *T. gondii*. The enhanced immune response and increased survival time (up to 18 days) were observed for vaccinated mice, showing that the TgASP3 antigen can provides partial protection.

1. Introduction

Toxoplasma gondii, a coccidian apicomplexan, is an obligate intracellular parasite of humans and other warm-blooded animals (Liu et al., 2015; Foroutan-Rad et al., 2016). *T. gondii* infection normally causes mild symptoms or is asymptomatic in humans, But for the special groups that suffer from impaired immune function, including AIDS patients and patients receiving long-term chemotherapy, *T. gondii* infection is often a fatal risk (Cuervo et al., 2016; Cong et al., 2015). However, no drug treatments are available. Therefore, other options for controlling the disease are required. DNA vaccines are an option worth considering because they can induce continuous and strong protective immune responses against this ubiquitous parasite (Zhang et al., 2015; Lu et al., 2015; Lim and Othman, 2014). However, searching for potent antigens has been a difficult problem. The potential value of the protease has attracted more and more attention. The parasite encodes five cysteine proteases, including one cathepsin B-like (TgCPB), one cathepsin L-like (TgCPL), and three cathepsin C-like proteins (TgCPC1, 2 and 3). *T. gondii* also contains genes encoding four aspartic proteases, designated toxomepsin 1, 2, 3, and 5 (TgASP1, 2, 3, and 5, respectively) and two subtilases (TgSUB1 and TgSUB2) (Kim, 2004).

Proteases play several key roles in *T. gondii* infection, including in host cell invasion, nutrient acquisition, avoidance of the host's protective immune responses, escape from the parasitophorous vacuole, parasite differentiation, and the regulation of pathogenesis (Pszenny et al., 2012; Donnelly et al., 2011; Stewart and Tonkin, 2015; Dou and Carruthers, 2011). As an obligatory intracellular protozoan parasite, *T. gondii* must invade the host cell as a prerequisite to establishing and maintaining a life-long infection in its host. Proteases are also proved to be potential therapeutic targets, studies have indicated that protease inhibitors can reduce the activities of protease, significantly reduce the replication ability and cell invasion by apicomplexan parasites (Monzote et al., 2013; Teo et al., 2007).

In our previous research work, we have proved that TgCPB and TgCPL are partially protective vaccine antigens against toxoplasmosis (Zhao et al., 2013a). TgASP1 also has been identified and characterized as a novel vaccine candidate (Zhao et al., 2013b). TgASP3 is another vaccine antigen we're interested in. It is worth mentioning that application of bioinformatics significantly improved the screening efficiency of vaccine antigen. Now, we can use bioinformatics software to analyze the potential epitopes on protein surface, and to exclude those proteins that are not likely to be excellent antigens.

* Corresponding author.
E-mail addresses: zgh2009100@163.com (G. Zhao), songxiaojie53@163.com (X. Song), alicekong1988@sina.com (X. Kong), 406882081@qq.com (N. Zhang), qdsnlq@126.com (S. Qu), 1565201908@qq.com (W. Zhu), 0307-yan@163.com (Y. Yang), sd.wangqian@163.com (Q. Wang).
[1] Equal contribution.

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In this study, we constructed a TgASP3 gene vaccine to evaluate the protective immune responses induced against toxoplasmosis in laboratory mice. We show that it is a novel candidate vaccine that induces substantial humoral and cellular immune responses against *T. gondii* infections in mice.

2. Materials and methods

2.1. Bioinformatic analysis of TgASP3

TgASP3 nucleotide and amino acid sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and were analyzed using DNAMAN software and BLAST (protein-protein). Physical and chemical properties of the TgASP3 protein were analyzed by ProtParam (http://web.expasy.org/protparam/), whilst GENSCAN was used to search for the open reading frame (ORF) structure of the TgASP3 gene (http://genes.mit.edu/GENSCAN.html). TMHMM server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and SignalP server (http://www.cbs.dtu.dk/services/SignalP/) were used to predict the transmembrane structure and signal peptides respectively. B-cell epitopes and T-cell epitopes on TgASP3 were predicted using DNASTAR, Gene Runner, DNAMAN software and IEDB server (http://tools.immuneepitope.org/main/html/tcell_tools.html). Finally, the 3D model was predicted by SWISS-MODEL (http://swissmodel.expasy.org/), SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and Vector NTI software.

2.2. Parasites and mice

The *T. gondii* RH strain was maintained by passage in vitro in human malignant epithelial cells (HeLa cells), which were cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), L-glutamine(2 mM), penicillin (100U/ml) and streptomycin (100 μg/ml). Cell cultures were maintained at 37 °C with 5% CO_2 and were changed every 2–3 d. The *T. gondii* RH strain was harvested and purified as previously described (Quan et al., 2012; Yuan et al., 2011). In brief, tachyzoites were collected after washed by cold phosphate buffered saline (PBS), centrifuged, resuspended in cold PBS and syringed three times with a 27-gauge needle. The parasites were filtered through a 5.0 μm pore size filter (Millipore, USA), washed with cold PBS and pelleted at 3000 × g for 10 min. Total RNA was extracted from tachyzoites and reverse transcription into cDNA.

Six-week-old female BALB/c mice were purchased from the Shandong University Laboratory Animal Center. All the mice were maintained under specific-pathogen-free conditions when the first immunizations were performed. All the animal experiments were approved by the Animal Ethics Committee of Shandong University Qilu Hospital.

2.3. Expression plasmid construction

The TgASP3 gene (GenBank ID: AY592973) was amplified by polymerase chain reaction (PCR) using the following primer pairs. The first pair of primers was for cloning into the prokaryotic expression vector pET-30a: 5′-cggGGTACCATGTCTC-CGTCGTCGCG-3′ (forward) and 5′-ccgCTCGAGTCATCAATTCACGCATCGAC-G-3′ (reverse), which contains *Kpn*I and *Xho*I restriction sites (underlined); the second pair of primers was for cloning into the eukaryotic expression vector pEGFP-C1: 5′-ccgCTCGAGATGGAGGGCCGGACTACC-3′ (forward), 5′-cggGGTACCTCACAATTCACGCATCGACG-3′ (reverse), which contains *Xho*I and *Kpn*I restriction sites (underlined). PCR products were cloned into the pEASY-T1 simple vector (TransGen Biotech, China) to generate a recombinant cloning plasmid. After sequencing, TgASP3 was subcloned into pET-30a(+) (Novagen, USA) and pEGFP-C1 (Clontech, USA) to produce pET-30a-TgASP3 and pEGFP-TgASP3 (pTgASP3), respectively.

2.4. Preparation of anti-rTgASP3 sera

The recombinant prokaryotic expression plasmid pET-30a-TgASP3 was transformed into E.coli BL21(DE3) cells and grown in Luria Bertani medium (LB) with kanamycin (25 μg/ml). Recombinant TgASP3 (rTgASP3) protein was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 6 h at 25 °C. The cells were lysed with 50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA containing 1 mM of the protease inhibitor PMSF (phenylmethanesulfonyl fluoride) and then centrifuged at 4 °C at 10,000 × *g* for 15 min, after which the protein was purified by binding of its carboxy terminal histidine (His) tag to Ni-NTA resin (Sangon Biotech, China).

BALB/c mice were immunized subcutaneously with 100 μg of purified rTgASP3 mixed with equal volume of Freund's complete adjuvant (Sigma) for the first injection. After 3 weeks, The second injection consisted of 50 μg of purified protein in equal volume of Freund's incomplete adjuvant (Sigma), and third immunization was performed with the same dose as the second injection. Antisera were collected from the mice two weeks after their last immunizations. Immunoglobulin G (IgG) was purified from the antisera using protein A chromatography columns (GE Healthcare, USA), and the IgG containing fractions were identified by SDS-PAGE and western blotting as previously described (Peng et al., 2009). Approximately 500 ng of rTgASP3 protein was used in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the separated protein bands were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA), which were blocked with 5% (W/V) skimmed milk diluted in PBS for 2 h, and then were incubated with antisera prepared above (dilution 1:500) for 24 h at 4 °C. After washing, the membrane was incubated with diluted goat anti-mouse IgG horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000; Sigma, USA) for 1 h. Protein bands were detected with ECL chemiluminescence reagents (Cowin Biotech, China).

2.5. Immunolocalization experiments

As previously described (Cortez et al., 2008; Pastor-Fernández et al., 2016), intracellular and extracellular parasites were prepared for Indirect IFAs. Briefly, the cells and parasites were fixed on slides with 4% paraformaldehyde for 20 min, after washing, which were permeabilized with 0.2% Triton X-100 in PBS for 20 min, and then blocked in 10% FBS for 20 min. The primary antibody (anti-rTgASP3, 1:500, diluted in PBS-FBS) was added and incubated for 1 h at 37 °C, after washing, the secondary antibody (Alexa Fluor 647-Labeled Goat anti-mouse IgG, Beyotime, China) diluted in 1% PBS-FBS (dilution 1:1000) was added and incubated for 1 h. After washing, the slides were mounted in Antifade Mounting Medium (Beyotime, China) and observed under a laser scanning confocal microscope (Carl Zeiss LSM700, Germany).

2.6. TgASP3 expression in mammalian cells

The recombinant plasmid pEGFP-TgASP3 was transformed into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen, USA) as previously described (Kamyingkird et al., 2014). HEK293 cells were maintained at 37 °C in 5% CO_2 and then transferred into 6-well culture plates (Costar, USA), when the density of the cells reached approximately 80%. Liposomes (10 μl) were diluted in 240 μl of DMEM and then incubated at room temperature for 5 min, while