A Novel Polyclonal Antiserum against *Toxoplasma gondii* Sodium Hydrogen Exchanger 1

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Abstract: The sodium hydrogen exchanger 1 (NHE1), which functions in maintaining the ratio of Na\(^+\) and H\(^+\) ions, is widely distributed in cell plasma membranes. It plays a prominent role in pH balancing, cell proliferation, differentiation, adhesion, and migration. However, its exact subcellular location and biological functions in *Toxoplasma gondii* are largely unclear. In this study, we cloned the C-terminal sequence of *T. gondii* NHE1 (TgNHE1) incorporating the C-terminal peptide of NHE1 (C-NHE1) into the pGEX4T-1 expression plasmid. The peptide sequence was predicted to have good antigenicity based on the information obtained from an immune epitope database. After induction of heterologous gene expression with isopropyl-b-D-thiogalactoside, the recombinant C-NHE1 protein successfully expressed in a soluble form was purified by glutathione sepharose beads as an immunogen for production of a rabbit polyclonal antiserum. The specificity of this antiserum was confirmed by western blotting and immunofluorescence. The antiserum could reduce *T. gondii* invasion into host cells, indicated by the decreased TgNHE1 expression in *T. gondii* parasites that were pre-incubated with antiserum in the process of cell entry. Furthermore, the antiserum reduced the virulence of *T. gondii* parasites to host cells in vitro, possibly by blocking the release of Ca\(^{2+}\). In this regard, this antiserum has potential to be a valuable tool for further studies of TgNHE1.

Key words: *Toxoplasma gondii*, soluble expression, TgNHE1, polyclonal antiserum, Ca\(^{2+}\) release

**INTRODUCTION**

*Toxoplasma gondii*, a highly pathogenic intracellular parasite, causes a zoonotic disease referred to as toxoplasmosis, and infects a wide range of animals, including humans. About one-third of the global population carries dominant or recessive symptoms of toxoplasmosis, and pregnant women are highly susceptible to it [1]. Currently, the leading therapy for this disease is the combination of pyrimethamine and sulfonamide drugs [2]. However, the need for long-term treatment and untoward side effects are disadvantages of such treatment.

Membrane proteins are the first frontier of the interaction between parasites and hosts. *T. gondii* contains 4 sodium hydrogen exchangers (NHEs): TgNHE1, TgNHE2, TgNHE3, and TgNHE4. Recent studies indicate that TgNHE1 and TgNHE2 are localized in the plasma membrane and rhoptry organelle, respectively [3,4]. TgNHE3 co-localizes with the PLV/VAC TgVP1 marker [5], while the location of TgNHE4 in the parasite is still unclear. TgNHE1 functions mainly in Ca\(^{2+}\) release from intracellular pools [3]. As is known, Ca\(^{2+}\) signaling plays a pivotal role in host cell invasion by parasites. Ca\(^{2+}\)-dependent secretion from apical micronemes mediates pHi homeostasis, leading to suppression of potassium ions and promoting parasite motility [6]. Ca\(^{2+}\) ionophores ionomycin and A23187, which are 2 Ca\(^{2+}\)-elevating regents, stimulate microneme discharge of the transmembrane adhesin, MIC2 [7,8]. Apart from autogenous regulation of intracellular Ca\(^{2+}\), *T. gondii* invasion also induces significance alternations to the Ca\(^{2+}\) concentration in host cells [9,10].

To the best of our knowledge, only a few studies have focused on TgNHE1, and the detailed mechanisms it takes part in remain largely unknown. In this study, we successfully designed and expressed a C-terminal peptide of TgNHE1 (C-TgNHE1) in a soluble form using a prokaryotic expression system. A total of 2 mg of purified protein was used for generating a polyclonal antiserum against TgNHE1 by immunizing New Zealand rabbits. The specificity of the polyclonal antiserum was confirmed by western blotting and immunofluorescence as-
says. This antiserum reduced T. gondii invasion and virulence markedly, as shown by the TgNHE1 expression in intracellular T. gondii, as well as measuring the number and area of cell plaques by T. gondii parasite infection, thus indicating that TgNHE1 could be a promising therapeutic target.

**MATERIALS AND METHODS**

### Animals and reagents

Male Kunming mice weighing 25-30 g were purchased from the Laboratory Animal Center of Southern Medical University (Guangzhou, China). The animal experiments were approved by the local Animal Ethics Committee of the Southern Medical University, Guangzhou, People’s Republic of China, following the rules relating to the ethics on experimental animals. SuperScript® II reverse transcriptase was purchased from Invitrogen (Grand Island, New York, USA). PrimeSTAR® HS, restriction endonuclease, and a DNA Ligation Kit were purchased from Takara (Dalian, China). The pGEX4T-1 vector, TOP10, and BL21 (DE3) competent cells were from TIANGEN Biotech (Beijing, China). PageRuler Prest Protein Ladder was from Fermentas (Ontario, Canada). Trizol and isopropyl-β-d-thiogalactoside (IPTG) were from Sigma-Aldrich (St. Louis, Missouri, USA). Glutathione sepharose high-performance (GSH) beads were from BEAVER Nano (Suzhou, China). Goat anti-rabbit IgG-HRP antibody was from Santa Cruz Biotechnology (Dallas, Texas, USA). Alexa fluor 594 goat anti-rabbit IgG (H+L) secondary antibody conjugate was from Life Technologies (Grand Island). Bicinchoninic acid assay (BCA) protein assay kit was from Thermo Scientific (Waltham, Massachusetts, USA). Centrifugal filter units (30 kDa) were from Merck Millipore (Bedford, Massachusetts, USA).

### Parasite culture

*T. gondii*, cryopreserved in liquid nitrogen in our laboratory, was recovered at a constant 37˚C. Parasites (1 × 10^5) suspended in 200 μl PBS were intraperitoneally injected into Kunming mice. When exhibiting obvious signs of infection, the mice were humanely killed. The parasites were collected from their peritoneal exudates and added to PBS. *T. gondii* tachyzoites were purified using a method based on 3-μm filter purification, as described elsewhere [11].

### Primer design and plasmid construction

The 2 primers used for amplifying TgNHE1 cDNA were as follows. The forward primer sequence was 5’ ATGGGATCCAGGGGCAATGTCCTCGCGT 3’ (restriction sites in bold); the reverse primer sequence was 5’ AATCTCGAGAAGCTGATTCTGAAAGCTCGC 3’ (restriction sites in bold).

Total RNA was extracted from 1 × 10^7 purified *T. gondii* tachyzoites. The RNA-cDNA reaction was carried out by SuperScript® II reverse transcriptase following the manufacturer’s instructions. PCR amplification conditions were as follows: 34 cycles at 98˚C for 10 sec, 55˚C for 15 sec, 72˚C for 10 sec, and a final extension step at 72˚C for 5 min. After PCR product purification, the DNA insert and pGEX4T-1 vector were digested by *Bam*H I and *Xho* I for 1 hr, respectively. For DNA ligation, the molar ratio of the DNA insert to linearized vector was 5 to 1, respectively, and the reaction proceeded at 16˚C for 30 min.

*Escherichia coli* TOP10 competent cells were transformed with recombinant pGEX4T-1. Positive clones were confirmed by double-enzyme restriction and sequencing.

### Protein expression and purification

The recombinant pGEX4T-1-C-TgNHE1 plasmid was transformed into 50 μl *E. coli* BL21 (DE3) and cultured in Luria-Bertani solid medium with 100 μg/ml ampicillin. A single clone was grown with shaking at 220 rpm at 37˚C until the OD_{600} reached 0.6. Bacteria were induced to express C-TgNHE1 by IPTG at a concentration of 24 μg/ml for 20 hr at 18˚C to obtain the target protein in a soluble form. The bacteria were collected by centrifuging at 10,000 g for 1 min followed by 3 freeze-thaw cycles and 30 min sonication on ice under 200 W for 2 sec with 8-sec intervals. After centrifuging at 14,000 g for 20 min, the supernatants containing the target protein were separated and stored at -70˚C.

For protein purification, GSH beads (BEAVER Nano) were used. First, the beads were washed in buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) 3 times. The soluble components (1 ml) supplemented with 1× protease inhibitor cocktail were mixed with the beads vertically at 4˚C for 1 hr. After 3 additional washes with buffer A, the GST-fusion proteins that had adsorbed to the GSH beads were eluted with 500 μl of buffer B (50 mM Tris-HCl, 10 mM reduced glutathione, pH 7.4) at ambient temperature for 6 min. The eluents were filtered through 0.22-μm membranes, and the protein concentration of the eluent was measured with a BCA protein assay kit (Thermo Scientific). Finally, the base solution of recombinant proteins was replaced with normal saline using 10 kDa centrifugal filter units (Merck Millipore).
Preparation of polyclonal antiserum

New Zealand white rabbits weighing about 1.5 kg were immunized subcutaneously with 500 μg of purified recombinant C-TgNHE1 emulsified in an equal volume of Freund’s complete adjuvant. To enhance the immune response, 500 μg of recombinant protein emulsified in Freund’s incomplete adjuvant was administered twice every 2 weeks. Afterwards, a blood sample was collected from the rabbit’s auricular vein to determine the titer of the antiserum by an indirect ELISA. When the titer reached a sufficiently high level, 100 μg of the immunogen was administered, and a blood sample was collected through carotid artery intubation. Serum was separated from whole blood by standing the blood sample at 4˚C overnight, and the antiserum was purified using protein A agarose (Sigma-Aldrich).

Western blotting and immunofluorescence

Denatured samples were subjected to 10% SDS-PAGE and electrotransferred to PVDF membranes (no. 162-0177, Bio-Rad, Hercules, California, USA) at 100 V for 90 min. Membranes were blocked with 5% nonfat milk at 4˚C overnight and incubated with antiserum at a dilution of 1:1,000 for 1.5 hr. After washing with Tris-buffered saline Tween-20 (TBST) 3 times, the membranes were incubated with a goat anti-rabbit IgG-HRP antibody for 1 hr and then washed with TBST again, 3 times. Finally, chemiluminescent substrates (ECL, Thermo Scientific) were used to develop the immunoblots.

For the immunofluorescence assays, T. gondii RH tachyzoites cultured in Kunming mice were isolated and purified with a 3-μm filter, then placed on a cell culture dish and immobilized with 4% paraformaldehyde for 15 min. Parasite membranes were permeabilized with 0.1% Triton X-100 for 10 min and then washed with PBS 3 times for 5 min each time. Samples were blocked with PBST (PBS containing 1% BSA and 0.1% Tween 20) for 1 hr and incubated with antiserum at a 1:100 dilution at 4˚C overnight. The parasites were then incubated with anti-rabbit IgG Alexa Fluor 594 (red) antibody for 1 hr at ambient temperature. Unbound antibodies were washed 3 times with PBS and the cell nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). The fluorescent signals were visualized using an inverted fluorescence microscope (Leica, Brunswick, Germany).

CCK-8 assays

For the CCK-8 assays, ovine fetal turbinate (OFTu) cells were cultured in 96-well plates containing Dulbecco’s modified eagle medium (DMEM) plus 10% fetal bovine serum (FBS) (2 × 10^3/well/200 μl) for 24 hr. Different concentrations of antiserum were added to the cell medium for 72 hr. After treatment, the supernatant was removed, and the cells were washed with PBS and treated with 10 μl of CCK-8 for 2 hr. Finally, the absorbance of the cells was measured at 450 nm using a microplate reader (Bio-Rad).

Plaque assays

Plaque assays were performed as described previously [12]. In detail, 1 × 10^6 OFTu cells were cultured in 24-well plates in DMEM supplemented with 10% FBS until confluent. Cells were infected with 5 × 10^5 T. gondii RH strain pre-incubated individually with various concentrations (0.5, 1, 2, 4, and 8 mg/ml) of antiserum or with normal rabbit serum for 12 hr. Cells were washed with PBS to remove any uninvaded parasites at 4 hr post infection and then cultured in DMEM supplemented with 10% FBS. Five days later, the cells were washed, fixed in 10% buffered formalin for 24 hr, and stained with 1% toluidine blue. The cells were visualized using a light microscope, and images of the microscopic fields were captured to quantify the number of plaques (no. of areas with lysed cells) and measure the plaque area (dimension of the areas with lysed cells).

RESULTS

Design of the antigenic epitope of TgNHE1

TgNHE1 (GenBank no. AAR85890.1) contains 12 possible transmembrane helices predicted by TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The TgNHE1 internal and transmembrane domains are located mainly at the N-terminal of the amino acid (aa) sequence and the C-terminal region stretches out of the cell membrane. In consideration of the difficulty anticipated in expressing the complete high molecular-weight membrane protein (about 248 kDa) in E. coli, we selected the C-terminal antigenic determinants of TgNHE1 for antibody preparation. The B-cell epitope of TgNHE1 was analyzed by Bepipred Linear Epitope Prediction from the IEDB analysis resource (www.iedb.org). The peaks above the threshold were suggestive of promising regions with good immunogenicity. In view of avoiding the cross reaction with non-specific proteins from host cells (OFTu cells) in this research, the C-terminal sequence (2127 aa-2288 aa) with good predicted immunogenicity was determined for the follow-up expression experiments.
Construction of the recombination expression plasmid

To obtain the coding sequence of the C-terminal of TgNHE1 (C-TgNHE1), total RNA was isolated from *T. gondii* RH strain, and the corresponding cDNA was synthesized. The 486-bp C-terminal-TgNHE1 coding sequence was amplified by PCR (Fig. 1A). After double enzyme digestion with *Bam*H I and *Xho* I, the purified PCR product and linearized vector pGEX4T-1 were ligated and transformed into *E. coli* TOP10 cells. The pGEX4T-1-C-TgNHE1 recombination plasmid was identified by restriction digestion (Fig. 1B) and DNA sequencing (data not shown).

Soluble expression and purification of C-TgNHE1

The recombinant plasmid derived from *E. coli* TOP10 cells was subsequently transformed into *E. coli* BL21. A single clone was selected for IPTG induction. Compared with the mock-vehicle group, a GST fusion protein of about 43 kDa (26 kDa GST-tag plus 17 kDa C-TgNHE1) was expressed (Fig. 2A). In addition, 2 more proteins of about 35 kDa and 26 kDa (possibly derived from the GST tag) were simultaneously expressed to a lesser extent. To obtain a prokaryotic protein with approximate native conformation, we employed a method favoring soluble protein expression where the bacteria were induced at 18°C for 20 hr. After sonication, the soluble and pelleted fractions were separated by centrifuging at 14,000 g for 20 min and subjected to SDS-PAGE. The results showed that C-TgNHE1 existed in both components (Fig. 2B). The soluble fraction was then purified by GSH beads. As indicated in Fig. 3B, C-TgNHE1 was purified with high specificity and affinity. Western blotting confirmed that the purified protein of the expected size was a GST-tagged fusion protein (Fig. 2C).

Preparation, identification, and use of the polyclonal antiserum

Polyclonal antibody production was induced in New Zealand white rabbits by immunization of multiple sites by subcutaneous injections. After a booster immunization, the rabbit polyclonal anti-serum was measured by indirect ELISA. The polyclonal anti-serum bound to the coating antigen with high affinity in a dose-dependent manner (anti-serum titer: 1:640,000) (data not shown). To detect the capacity of the antibody in recognizing natural TgNHE1 in *T. gondii* RH strain, purified parasites were lysed and used for western blotting. The results showed that a single band was recognized by the antibody at the right location, while the normal rabbit serum failed to do so (Fig. 3A). We also detected cross-reaction of this antiserum using an immunofluorescence assay. TgNHE1 showed a punctate pattern of distribution in the plasma membrane, while specific signals were not seen in the control group (Fig. 3B).
Above all, these results indicate that the antiserum is a potentially valuable tool for studying TgNHE1.

The polyclonal antiserum reduced T. gondii RH strain virulence in vitro

As the TgNHE1 membrane protein is involved in Ca\(^{2+}\) egress, which is closely related to T. gondii virulence, and the polyclonal antiserum binds to this protein, we speculated that this antiserum would reduce T. gondii invasion and virulence by blocking TgNHE1. To test this hypothesis, we first investigated TgNHE1 expression in intracellular T. gondii RH strain that was pre-incubated with antiserum or normal serum at different time points post infection (p.i). Compared with controls, the expression of TgNHE1 was dramatically reduced shortly after parasites entered O/Fu cells (6h) (Fig. 4). Since T. gondii started to proliferate between 12 hr and 24 hr, sustained lower TgNHE1 expression was still observed in antiserum-treated group, indicating that the antiserum prevented the cell entry of T. gondii by
blocking TgNHE1, and lower TgNHE1 expression was possibly due to the less number of _T. gondii_ that entered the host cell (Fig. 4). To test the effect of the antiserum on _T. gondii_ virulence, we first measured the toxicity of the antiserum to host cells. Treatment with the antiserum at increasing concentrations did not reduce the cell viability using Cell Counting Kit-8 (CCK-8), which was applied to detect cell proliferation and virulence (data not shown). We then employed a plaque assay to observe the number and area of plaques changed by pretreatment with the antiserum. Parasites that were pre-incubated with increasing levels of antiserum were used to infect OFTu cells. Normal rabbit serum served as a negative control. As shown in Fig. 5A, antiserum treatment reduced the number of plaques compared with the untreated cells in a dose-dependent manner. Furthermore, the plaque areas were markedly reduced following antiserum treatment (Fig. 5B). Taken together, antiserum treatment...
reduced *T. gondii* entry and virulence to host cells.

**DISCUSSION**

*T. gondii*, a parasite of living cells, infects a wide range of warm-blooded hosts, resulting in a serious zoonosis called toxoplasmosis. *T. gondii* has high resistance to antiparasitic agents, causes severe pathogenicity in pregnant women, and is found worldwide but mainly in the USA and Europe among high-risk groups [27]. The search for an effective therapeutic target is now urgent.

The homeostasis of intracellular pH is of great importance to a variety of biological processes such as cell growth, differentiation, internal enzymatic activity, and cytoskeleton assembly in apicomplexan parasites such as *T. gondii*. NHEs, which are localized mainly in the plasma membrane, play an important role in such cellular events. TgNHE molecules possess 4 homologous series proteins: TgNHE1 to TgNHE4. TgNHE1 is a ubiquitous membrane protein involved not only in Na\(^+\)/H\(^+\) exchange, but also in Ca\(^{2+}\) egress [14]. Studies showed that Ca\(^{2+}\) signaling is crucial for protein secretion, motility, cell invasion, and differentiation of apicomplexan parasites [15-18]. When TgNHE1 is knocked out, *T. gondii* is resistant to Ca\(^{2+}\) ionophore A23187-induced egress and amiloride-induced proton efflux inhibition [14]. Compared with TgNHE1, TgNHE3 also plays a role in Ca\(^{2+}\) homeostasis and parasite invasion through mediating MIC2 secretion and maintaining intracellular Ca\(^{2+}\) concentrations [5]. In the closely related apicomplexan parasite *Plasmodium falciparum* (PF), PINHE, which has been well characterized for its resistance to an anti-malarial drug, quinine, regulates H\(^+\) egress and cytosolic Ca\(^{2+}\) concentrations [19-22]. Similarly, in both *Leishmania donovani* and *Trypanosoma brucei*, acidocalcisomal NHE facilities Ca\(^{2+}\) release from the rhoptry organelle [23,24].

There is a wealth of evidence showing the importance of Ca\(^{2+}\) release in the process of *T. gondii* invasion of host cells. Calcium ionophores, such as ionomycin and A23187, or the endoplasmic reticulum Ca\(^{2+}\) pump inhibitor thapsigargin, are capable of activate conoid extrusion of *T. gondii* tachyzoites [25]. Treatment of tachyzoites with the intracellular Ca\(^{2+}\) chelator 1,2-bis (o-aminophenoxy) ethane-N,N,N,N-tetraacetic acid/tetraacetoxymethyl ester prevented ionomycin-induced conoid extrusion and inhibited parasite invasion [25]. Ionomycin-induced conoid extrusion was also prevented by cytochalasin D, a drug that impairs *T. gondii* motility and invasion capacity into host cells [26-29]. These results suggested that Ca\(^{2+}\) release from tachyzoite internal stores has been identified as key players in activating the process of *T. gondii* invasion.

In this research, we generated a polyclonal antibody that specifically recognizes TgNHE1, a plasma membrane controller of Ca\(^{2+}\) release. We expect this antibody to be a useful tool for studying TgNHE1 and also to weaken *T. gondii* invasion by blocking Ca\(^{2+}\) release from TgNHE1. As an initial step, based on TMHMM prediction, we found that the C-terminal peptide of TgNHE1 was fully exposed to the external cell surface, while the N-terminal part was not entirely exposed. Additionally, the B-cell epitopes were more concentrated in the C-terminus and had high scores. Therefore, C-TgNHE1 was selected for expression as an immunogen for antibody production.

The GST fusion system has been used extensively as a prokaryotic expression system in bacteria for its convenience at producing soluble heterologous proteins that are easy to purify [30]. Here, GST was employed as a co-expression vehicle with C-TgNHE1, with the aim to generate a soluble functional fusion protein. After preparation of the antiserum triggered by administration of purified C-TgNHE1 in a rabbit, we identified the antibody by indirect ELISA, western blotting, and immunofluorescence. TgNHE1 was found to be embedded mainly in the cell membrane with some other dotted distribution in the cytoplasm. By indirect ELISA and western blotting analysis, we confirmed that this antibody was able to recognize natural TgNHE1 in *T. gondii* with high affinity and specificity, indicating that it has potential to be a valuable tool for studying TgNHE1. We also observed that pre-incubation of C-TgNHE1 RH strain with this antiserum reduced cell entry and plaque formation. These results show that besides binding to TgNHE1, this antibody might also block ion transport in the channel, especially invasion-related ion Ca\(^{2+}\). Thus, TgNHE1 could be a promising therapeutic target against *T. gondii*.

In conclusion, we successfully expressed a soluble C-terminal peptide of TgNHE1 and have used it as an immunogen to generate a polyclonal antiserum. The affinity and specificity of this antiserum against *T. gondii* was confirmed. We also tentatively explored the therapeutic effects of the antiserum in *T. gondii* infections in vitro. In future studies, we will explore more applications of this antiserum in research on *T. gondii*.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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