Peroxiredoxin 3 promotes IL-12 production from macrophages and partially protects mice against infection with *Toxoplasma gondii*

Ragab M. Fereig[a,b] and Yoshifumi Nishikawa[a,*]

[a]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan.*

[b]*Department of Animal Medicine, Faculty of Veterinary Medicine, South Valley University, Qena City, Qena 83523, Egypt.*

***Correspondence**

Yoshifumi Nishikawa, PhD

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

Tel.: +81-155-49-5886

Fax: +81-155-49-5643

E-mail: nisikawa@obihiro.ac.jp

ABSTRACT

Toxoplasmosis remains a life-threatening infection of humans and various domestic and wild animals worldwide. It is caused by the obligatory intracellular protozoan parasite *Toxoplasma gondii*. Peroxiredoxins (Prxs) are a family of antioxidant enzymes that protect cells from oxidative stress from hydroperoxides. In the recent years, several studies have reported the potential use of *T. gondii*-derived enzymes in triggering protective immunity against *T. gondii* infection. Therefore, this study was conducted to investigate the immunogenicity and protective efficacy of TgPrx3. *In vitro* stimulation of peritoneal macrophages with recombinant TgPrx3 protein fused to glutathione-S transferase (TgPrx3-GST) enhanced IL-12p40 production, indicating the immune-stimulating potentials of TgPrx3. Next, protective efficacy was investigated by subcutaneous inoculation of mice with TgPrx3-GST (25 pmol), and recombinant GST or PBS were used as the controls. Mice immunized with TgPrx3-GST exhibited a significant elevation of specific antibodies in terms of IgG1 and IgG2c isotypes. Moreover, interferon-gamma production and spleen cell proliferation dramatically increased in the TgPrx3-GST-sensitized cells from mice immunized with the same antigen. The severity of the *T. gondii* infections tended to be attenuated in the TgPrx3-GST-immunized mice, as evidenced by their higher survival rates and lower parasite burdens in the brain. Altogether, TgPrx3 immunization induced specific humoral and cellular immune responses and partially protected the mice against lethal toxoplasmosis. Our results suggest the possible use of TgPrx3 as a vaccine candidate against *T. gondii* infections.

Keywords

Toxoplasma gondii; Peroxiredoxin 3; Vaccine; Immunization

1. Introduction

Toxoplasma gondii is a very successful parasite in its ability to infect almost all mammals and induce lifelong infections in a third of the world's human population [1–3]. Contracting an infection with this parasite is of great concern for AIDS patients and pregnant women because of the high mortality and foetal losses induced by it, respectively [4]. Animals play a key role in human toxoplasmosis by acting as reservoir hosts for *T. gondii* and transmission of the infection via the oral route [5]. Moreover, animal toxoplasmosis can result in great economic losses to farmers because of the high morbidity and mortality it causes in sheep, goats and pigs [2,6].

It is difficult to control toxoplasmosis using the drugs currently available because of their toxic effects and limited efficacies [7,8]. Vaccination against toxoplasmosis will be advantageous not only in minimizing the infection rate, but also in attenuating the severity of an established infection. Despite an attenuated vaccine being successfully deployed in the veterinary field to avoid abortions in ewes [9], it cannot be used at large scale because of its inadequacy of protection in other animals and worries about reversion of parasite virulence [10]. In the last decade, numerous research trials have demonstrated that recombinant protein as a subunit vaccine can induce variable protection against *T. gondii* infection, especially in the mouse model [11]. However, more research is needed to validate the utility of these genetically modified vaccines before field application.

Peroxiredoxins are ubiquitously recognized antioxidant enzymes whose functions combat oxidative stressors and free radicals such as hydrogen peroxide and hydroxyl molecules. The catalytic mechanism of these enzymes involves a redoxactive cysteine, which is highly conserved in the vicinity of the 47th position of the amino acid sequence [12]. To date, the following three tachyzoite-stage *T. gondii* peroxiredoxins have been identified: 2-

cys cytosolic Prx1, 1-cys cytosolic Prx 2, and 2-cys mitochondrial Prx 3 [13,14]. There have been several studies published on TgPrx1 and TgPrx2, particularly structural and functional analyses [13-18]. Additionally, immunological investigation of TgPrx1 revealed its strong ability to activate macrophage function and cytokine production [19]. Although TgPrx3 may have an important role in the redox process and in protecting the parasite from cellular damage by free radicals [20,21], the immunomodulatory effect of TgPrx3 remains unknown.

Therefore, we aimed to investigate the immunological characteristics of TgPrx3 by evaluating the protective efficacy of the recombinant antigen. Here, we confirmed the specific humoral and cellular immune response triggered by immunization of mice with TgPrx3.

2. Materials and methods

2.1. Ethics statement

We strictly followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers 24-16, 24–17, 25–66, 26-68, 27-30). Surgical operations and painful procedures were applied under general anesthesia induced with isoflurane.

2.2. Animals

Seven-week-old female C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). The animals used in the study were treated under the guiding principles for the care and use of research animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

2.3. Parasites and cell cultures

In the current study, the avirulent type II *T. gondii* PLK strain was used. The parasites were maintained in Vero cells (African green monkey kidney epithelial cells) cultured in Eagle's minimum essential medium (Sigma, St Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan) and 1% streptomycin–penicillin (Sigma). For tachyzoite purification, the parasites and host cell debris were washed with sterile phosphate-buffered saline (PBS), and the infected cell monolayer was removed from the plate with a cell scraper (BD Biosciences, San Jose, CA,

USA). The final cell pellet was resuspended in Roswell park memorial institute (RPMI) 1640 medium (Sigma) and passed through a 27-gauge needle and a filter with a pore size of 5.0 μm (Millipore, Bedford, MA, USA).

2.4. Amplification and cloning of the TgPrx3 gene

To clone the TgPrx3 gene (GenBank accession number, AY251021), a pair of oligonucleotide primers containing *Bam*HI and *Xho*I restriction enzyme sites was designed and used to amplify the gene from cDNA from the *T. gondii* PLK strain. A *Bam*HI site (underlined) in the forward primer 5′ AA GGA TCC ATG GCG GCT TGC CTT CGA GCG -3′ and an *Xho*I site (underlined) in the reverse primer 5′- GC CTC GAG TTA GTT TTT CAG TTG TCC AAG -3′ were used. The PCR product was ligated to the cut *Bam*HI and *Xho*I restriction sites of the pGEX-4T-3 expression vector (Amersham Pharmacia Biotech, Madison, CA, USA). The resultant plasmid construct was checked for accurate insertion of the PCR product by sequencing using a Big Dye Terminator Cycle Sequencing Kit (AB Applied Biosystems, Carlsbad, CA, USA), and an ABI PRISM 3100 genetic analyzer (AB Applied Biosystems). Nucleotide sequence of the obtained clone and target sequence from GenBank was checked and analysed with GENETYX software (GENETYX Co., Tokyo, Japan) (Fig. 1A).

2.5. Expression and purification of TgPrx3 recombinant protein

The TgPrx3 recombinant protein was expressed as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli* BL21 (DE3) cells (New England BioLabs Inc., Ipswich, MA, USA). Protein expression was achieved at 37°C for 8 h after induction with 1 mM

isopropyl β-D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan). The resulting *E. coli* cells were harvested in TNE buffer (100 mM Tris-HCl, pH 8; 100 mM NaCl, 5 mM EDTA) by high-speed centrifugation (10,000 × *g*, 4°C, 30 min), lysed with 1% Triton in PBS and 50 mg/mL of lysozyme, sonicated on ice, and then centrifuged as in the previous step. The supernatant was purified with Glutathione Sepharose 4B beads (GE Healthcare Life Sciences Buckinghampshire, UK), according to the manufacturer's instructions. Briefly, the supernatant–beads mixture was incubated for 1 h at room temperature with rotation, and the GST-fused protein was eluted with elution buffer (100 mM Tris-HCl, pH 8; 100 mM NaCl, 5 mM EDTA, 20 mM reduced glutathione powder; Wako Inc.). The protein obtained was dialyzed in PBS and endotoxins were removed with a Detoxi-Gel Endotoxin Removing Column (Thermo Scientific, Waltham, MA, USA). Proteins for use in cell culture were filtered with a 0.45-μm low-protein binding Supor® membrane (Pall Life Sciences, Ann Arbor, MI, USA). The endotoxin levels in the TgPrx3-GST and GST preparations were estimated with Limulus Amebocyte Lysate (LAL) reagents (Seikagaku Inc., Tokyo, Japan). The purities of the TgPrx3-GST and GST proteins were assessed to be single bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue R250 (MP Biomedicals Inc., Illkirch-Graffenstaden, France) (Fig. 1). The protein concentrations were measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

2.6. Preparation and culture of murine peritoneal macrophages

Four days after intraperitoneal injection of the C57BL/6J mice with 2 mL of 4.05% BBL™ Brewer modified thioglycolate medium (Becton Dickinson, Sparks, MD, USA), the peritoneal macrophages were harvested by lavage with 5 ml of ice-cold PBS, twice. The cells

were centrifuged at 1,300 × *g* for 10 min and then suspended in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% FBS. Red blood cells were removed with lysis buffer (0.83% NH_4Cl; 0.01 M Tris-HCl, pH 7.2) and their remnants then washed away with medium. The macrophage suspension in an amount of 100 μl was added to a 96-well microplate at 3×10^5 cells/well and then incubated at 37°C in a 5% CO_2 incubator for 4 h. The wells were washed with FBS-free DMEM to remove any floating cells, and then incubated with recombinant TgPrx3, lipopolysaccharide (LPS, positive control), or medium alone for 20 h. To confirm the effects of the resident LPS, polymixin B (Sigma) was used.

2.7. Indirect enzyme-linked immunosorbent assay (ELISA) to detect TgPrx3-specific antibodies

The concentrations of recombinant TgPrx3-GST and GST proteins were adjusted to 0.1 μM each in 0.05 M carbonate buffer (pH 9.6), added to the ELISA plate wells, and the plates were incubated overnight at 4°C. The plates were washed twice with washing buffer (0.05% Tween 20 in PBS) and then blocked with PBS containing 3% skimmed milk (PBS-SM) for 1 h at 37°C. The plates were washed twice and 50 μL of the test serum, or the positive or negative control serum (diluted 1:100 with PBS-SM), was added to duplicate wells. The plates were incubated at 37°C for 1 h. After the plates were washed six times, they were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG1, or IgG2c antibodies (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:4,000 with PBS-SM, at 37°C for 1 h. The plates were washed six times, and 100 μL of substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, 0.3 mg/mL 2,2′-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]; Sigma) was added to each well. After sample incubation at room temperature in the dark for 1 h, sample absorbance at 415 nm was

determined with a plate reader (Corona Microplate Reader MTP-120; Corona, Tokyo, Japan). The ELISA results for TgPrx3 were determined by measuring the mean optical density of the GST readings subtracted from that of TgPrx3-GST coated antigen, at a wavelength of 415 nm.

2.8. Spleen cell proliferation and cytokine responses

Spleens from the immunized mice were septically dissected two weeks after the last immunization, and then treated as reported previously [22], with slight modifications. Each mouse spleen was crushed between two sterile glass slides, and the cells were suspended thoroughly in RPMI 1640 medium (Sigma) supplemented with 10 % FBS. Red blood cells were destroyed by addition of lysis buffer and the cell suspension was then washed with medium. The cells were transferred to the individual wells of a 96-well microplate at a density of 2.5 x 10^5/well in 100 µl RPMI 1640 medium. Spleen cells were stimulated with the TgPrx3-GST and GST recombinant proteins, and concanavalin A (ConA; Sigma-Aldrich, St Louis, MO, USA) acted as a positive control and stimulant-free medium as a negative control. The plates were incubated for 48 h at 37°C in 5% CO_2, and then 100 µl samples from the culture supernatants were collected and assayed for cytokines. To investigate the proliferation of the previously stimulated spleen cells, 10 µl of Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well. After 2 h incubation at 37°C in 5% CO_2, the optical densities of the cells were measured using the plate reader set at 450 nm.

2.9. Cytokine production measurements using sandwich ELISAs

The supernatants from the cultured macrophage or spleen cells were collected to measure the cytokine levels with commercial ELISAs (Pierce Biotechnology Inc., Rockford, IL, USA), according to the manufacturer's recommendations. The cytokines IL-10 and IL-12p40 from macrophages and IFN-γ and IL-4 from splenocytes were checked in the current study. The cytokine concentrations were calculated from standard cytokine curves constructed from samples run on the same plate.

2.10. Immunization regime and infection

Three groups of mice were used to evaluate the protective efficacy of TgPrx3. Mice were subcutaneously inoculated with 25 pmol of recombinant TgPrx3-GST or GST proteins, or with PBS alone (each 100 μL) three times at 2-week intervals (total number = 18 mice per group from 3 independent trials). Two weeks after the third immunization, the mice were challenged via the intraperitoneal route with 1×10^3 tachyzoites of the *T. gondii* PLK strain. The survival rates of the mice were measured for 30 days after challenge. Serum samples (20 μL) were collected from each mouse at 14, 28, and 42 days post-immunization, via the tail vein, to determine if specific antibodies had developed against TgPrx3 using indirect ELISAs. An absence of antibody responses in each unvaccinated and uninfected mouse was confirmed by checking the control sera collected from all the animals on day 2 before immunization. After 30 days post-challenge, mouse brains and serum samples were harvested from all the surviving animals.

2.11. Quantitative PCR analysis of parasite DNA

To measure the parasite numbers in the brain tissues from the experimental groups, parasite DNA from the brains was extracted, purified, and quantified as described formerly [23], with slight modifications. Brain DNA was extracted by incubation with extraction buffer (0.1 M Tris-HCl, pH 9.9; 1% SDS, 0.1 M NaCl, 1 mM EDTA, 1 mg/mL proteinase K) at 55°C. Phenol–chloroform extraction and ethanol precipitation were used to purify the DNA. The parasite DNA was amplified with primers specific for the *T. gondii* B1 gene (5′-AAC GGG CGA GTA GCA CCT GAG GAG-3′ and 5′-TGG GTC TAC GTC GAT GGC ATG ACA AC-3′), which have already been shown to detect all known parasite strains [24]. The PCR mixture (25 μl total volume) contained 1 × SYBR Green PCR buffer, 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dATP, dCTP, and dGTP), 400 μM dUTP, 0.625 U of AmpliTaq Gold DNA polymerase, 0.25 U of AmpErase Uracil-*N*-Glycosylase (AB Applied Biosystems), 0.5 μmol of each primer, and 50 ng of genomic DNA. Amplification was performed using a standard protocol recommended by the manufacturer (AB Applied Biosystems, 2 min at 50 °C, 10 min at 95° C, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min). Amplification, data acquisition, and data analysis were performed with the ABI Prism 7900HT Sequence Detection System (AB Applied Biosystems), and the calculated cycle threshold (Ct) values were exported to Microsoft Excel for analysis. A standard curve was established from *T. gondii* DNA extracted from 1×10^5 parasites using 1 μL of a serial dilution ranging from 10,000 to 0.01 parasites. The parasite numbers were calculated by interpolation on the standard curve, with the Ct values plotted against known concentrations of parasites. After amplification, the melting-curve data for the PCR products were acquired using stepwise increases in temperature from 60°C to 95°C. The data were analyzed using Dissociation Curves version 1.0 F (AB Applied Biosystems).

2.12. Statistical analyses

Data processing and analysis were performed by the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical analyses were performed with one-way or two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for group comparisons. The significance of the differences in mouse survival was analyzed with a χ^2 test. The levels of statistical significance are presented with asterisks or letters and are defined in each figure legend, together with the name of the statistical test that was used. A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. Protein expression

Recombinant TgPrx3-GST and GST proteins were expressed in highly pure forms (Fig. 1B). The recombinant TgPrx3-GST and GST proteins were obtained with apparent molecular weights of 53-kDa and 26-kDa, respectively, which is consistent with the expected molecular sizes of each protein. Measurements of the endotoxin levels in each protein preparation using LAL reagents revealed undetectable levels of both proteins.

3.2. Response of murine peritoneal macrophages against TgPrx3

The ability of TgPrx3 to stimulate the innate immune response was investigated via *in vitro* stimulation of murine peritoneal macrophages with recombinant TgPrx3-GST. Macrophage treatment with recombinant TgPrx3-GST at concentrations of 10 and 100 nM significantly triggered the production of IL-12p40 compared with the mock- or GST-treated cells (Fig. 2A). The IL-12p40 levels after stimulation with TgPrx3-GST did not change in the presence of polymixin B. Treatment of the cells with LPS as a positive control also enhanced the production of IL-12p40, but polymixin B treatment significantly reduced its production. These results indicate the potential immune-stimulating activity of TgPrx3. Moreover, the production of IL-10 was estimated from macrophages treated with TgPrx3-GST, GST, LPS or medium only. The LPS triggered the production of IL-10 from the cultured macrophages while this production was significantly reduced after addition of polymixin B (Fig. 2B). However, TgPrx3-GST did not stimulate the IL-10 production (Fig. 2B).

3.3. Humoral and cellular immune responses

Mice immunized with recombinant TgPrx3-GST produced significant levels of specific antibodies against it, including IgG1, and IgG2c antibodies (Fig. 3). The levels of

antibodies directed against recombinant TgPrx3 were significantly higher than those of the other control groups. The IgG1 response was obvious after the second immunization and persisted at a high level until challenge with *T. gondii* tachyzoites. The peak IgG2c response was noticable after the third immunization. This result indicates the high efficiency of TgPrx3 in the induction of humoral immunity in the immunized mice.

To determine the potency of recombinant TgPrx3 in inducing specific cellular immune responses, the mitogenicity of the spleen cells and IFN-γ and IL-4 cytokine secretion were measured after *in vitro* stimulation of the spleen cells collected from the mice after their third immunization (Fig. 4). Splenocyte proliferation in the TgPrx3-GST-immunized mice was very obvious following stimulation with the same antigen, but not with GST alone (Fig. 4A). IFN-γ levels were higher in the spleen cells from the mice immunized with recombinant TgPrx3-GST than those from animals immunized with PBS or GST in all the stimulant groups, except for those stimulated with 5 μg/ml of ConA (Fig. 4B). Moreover, the spleen cells isolated from the mice immunized with PBS or GST when incubated with TgPrx3-GST recombinant antigen at 10 and 50 μg/ml enhanced the production of IFN-γ compared with the nontreated cells, but this effect was not statistically significant (Fig. 4B). Regarding IL-4 production, the spleen cells from mice immunized with recombinant TgPrx3-GST showed a marked increase in IL-4 production, but only when enhanced by stimulation with the immunization antigen (50 μg/ml) (Fig. 4C). Overall, these results reveal the enhanced antigen-specific cell-mediated immune responses in mice vaccinated with recombinant TgPrx3.

3.4. Survival rates and cerebral parasite burdens

The protective potential of recombinant TgPrx3 was evaluated by measuring mouse survival rates and cerebral parasite burdens. The survival rate at 30 days post-infection (dpi)

in mice immunized with recombinant TgPrx3-GST (55.6%) was higher than that with GST (38.9%) or PBS (27.8%) (Fig. 5A). Although the survival rates were not significantly different at 30 dpi among the experimental groups, the survival rate of the TgPrx3-GST-immunized group was significantly higher than that of the PBS-immunized group at 15 dpi, the critical period for acute infection with *T. gondii* ($P<0.05$). To further confirm the protective efficacy of recombinant TgPrx3 in the immunized mice, the brains of the surviving mice in all the groups were collected at 30 dpi to quantify the parasite loads (Fig. 5B). The number of parasites in the TgPrx3-GST-immunized group was lower than that of the GST- and PBS-immunization groups, although the differences were not statistically significant.

4. Discussion

In this study, we attempted to exploit previous reports of the success of Prxs from other parasites as potent vaccine candidates in different animal models by evaluating the protective efficacy of recombinant TgPrx3 against *T. gondii* infection. Peroxiredoxins are successful at inducing protective immunity against *Leishmania major* infections in mouse and nonhuman primate models [25], and in microfilaria *Brugia malayi* infections in mice [26] as well as in infections with *Fasciola hepatica* in goats [27]. The protective mechanism relates to the induction of both Th1- and Th2-mediated immune responses. Because of the potency of Prxs as vaccine candidate in different animal models for multiple parasite species, the protective efficacy may be predominantly related to enzymatic activity of these antigens [25-27]. However, in *T. gondii*, most studies investigating the protective potential of *T. gondii* molecules have been focused on the following four major protein families: surface proteins, dense granule proteins, rhoptry proteins, and microneme proteins [28]. Numerous reports have affirmed the success of *T. gondii*-derived enzymes at conferring a remarkable degree of protective immunity, and these include protein disulfide isomerase [29], calcium dependent protein kinase 3 [30] and glutathione reductase [31]. Abundant IFN-γ production and proliferation of effector immune cells are hallmarks of the protective immune response induced by these vaccine antigens.

Prior to evaluating the prophylactic potential of recombinant TgPrx3, we investigated its effect against macrophages. The potent role of TgPrx3 in the host-parasite interaction was demonstrated by recombinant TgPrx3 triggering the release of IL-12p40 in macrophages. Macrophages are the first defense line of the innate immune response against many pathogens. IL-12, which is produced by macrophages and other immune cells in response to antigen stimulation, is critical for the endogenous secretion of IFN-γ [32]. Enhancement of

IL-12 production in macrophages may be the triggering mechanism required for TgPrx3-induced host resistance against *T. gondii* infection, particularly during the acute phase. On the other hand, the anti-inflammatory cytokine IL-10 was not produced from TgPrx3-stimulated macrophages. A previous study has revealed the effect of TgPrx1 as an immunomodulator, and showed that it mainly stimulates the alternatively activated macrophage and induces secretion of IL-10 [19]. This conflict with our study might be due to the functional differences between TgPrx1 and TgPrx3 [13,14].

The current study has verified the potential candicacy of the TgPrx3 recombinant subunit vaccine as an effective strategy for combating toxoplasmosis. Humoral immunity (Th2) has an essential role in restricting *T. gondii* invasion via development of specific IgGs against parasite molecules and compartment proteins. These antibodies effectively respond to *T. gondii* infection by restricting the parasite's spread by activating the complement system or by opsonizing the parasites for phagocytosis and killing by macrophages [33-36]. To determine whether TgPrx3 stimulates the production of specific antibodies, the sera of mice infected with PLK were tested with an indirect ELISA based on the TgPrx3 antigen and TgGRA7, an antigenic protein [37]. Although TgPrx3-specific antibodies were detected, the levels of the antibodies were significantly lower than thoes of TgGRA7-specific antibodies (Fig. S1). These results indicate that TgPrx3 is less antigenic than TgGRA7. On the contrary, immunization of mice with recombinant TgPrx3 strongly induced specific antibody production, especially the IgG1 subclass, an effect that was more pronounced after the second immunization. This result indicates that TgPrx3 is able to induce strong humoral immunity and a subsequent increment of mouse resistance against *T. gondii* infection. We observed that stimulation with recombinant TgPrx3-GST (50 μg/ml) induces IL-4 production in the spleen cells of the TgPrx3-GST-immunized mice, which suggests an induction of humoral immunity by recombinant TgPrx3.

Additionally, TgPrx3 was also able to induce cellular immunity (Th1), where it robustly enhanced the proliferation of spleen cells as well as IFN-γ production. Th1-mediated immunity is the key player for resistance against *T. gondii* infection and its role in the vaccine candidate we developed may exceed its role in humoral immunity because the control of natural *T. gondii* infection is mediated primarily by Th1 cells and cytokines [38,39]. Resistance to toxoplasmosis is largely dependent on IFN-γ released from $CD4^+$ and $CD8^+$ T lymphocytes [40-42]. IFN-γ exerts anti-*Toxoplasma* activity by generating nitric oxide (NO) by inducible NO synthase [43-45], by disrupting the parasitophorous vacuole via immunity-related GTPases and p65 guanylate-binding proteins [46], by inducing tryptophan starvation via up-regulation of indoleamine 2,3-dioxygenase [47], and by upregulating the production of oxygen radicals [48] and the activity of P2X7 receptors [49]. We have shown that recombinant TgPrx3 not only induces high IFN-γ secretion in the spleen cells of TgPrx3-immunized mice, but also induced IFN-γ production in the spleen cells collected from the control mice. Because we did not use any adjuvant (eg. Complete Freund's adjuvant, alum) for injection with TgPrx3, this effect suggests the utility of TgPrx3 for use as antigen with immune-stimulating activity.

To evaluate the level of protection conferred by recombinant TgPrx3, parasite DNA in the brain tissue of the mice challenged with a lethal dose of *T. gondii* was quantified and the survival rates of these mice were monitored. The TgPrx3-immunized mice had higher survival rates than the GST-immunized or the PBS-immunized controls. Additionally, we noted a tendency for lower parasite burdens in the brains of the TgPrx3-immunized mice in comparison with the GST-immunized and PBS-immunized groups. The induction of both humoral and cellular immunity by immunization with recombinant TgPrx3 should partially protect mice against lethal infections with *T. gondii*.

5. Conclusion

This study shows the first immunological characterization of TgPrx3. Notably, recombinant TgPrx3 possessed potent immune-stimulating activity. Mouse immunization with TgPrx3 induced partial protection against a lethal dose of *T. gondii* tachyzoites. The mutual protective immune responses from the humoral and cellular immunity induced by vaccination with recombinant TgPrx3 may indicate its candidacy as a potent vaccine against *T. gondii* infection. Subsequent studies are required to improve the protection conferred by TgPrx3, either by reformulation with an appropriate adjuvant, or by employing an antigen-delivery system.

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Conflict of interest

The authors declare that they have no financial or competing interests concerning this study.

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Figure Legends

Fig. 1. Panel (A) shows the sequence alignment between sequence of the cloned product (query sequence) and reference sequence (Accession number: AY251021). The sequence analysis showed 100% identity. The sequence of the cloned product includes recognition sites for restriction enzymes (underlined sequences); BamHI (GGATCC), and XhoI (CTCGAG) at the sites of forward and reverse primers, respectively. Panel (B) shows the recombinant TgPrx3-GST and GST proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis image of the recombinant proteins, with Coomassie Blue staining. Lanes: M, molecular mass marker; lane 1, TgPrx3–GST (53 kDa); lane 2, GST (26 kDa).

A

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B

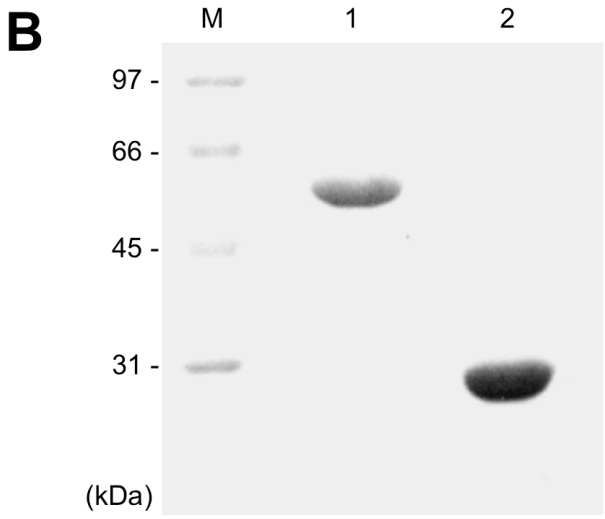

Fig. 1. Fereig et al.

Fig. 2. IL-12p40 (A) and IL-10 (B) production by mouse peritoneal macrophages. Murine peritoneal macrophages were treated with 1 ng/mL of LPS, TgPrx3-GST or GST recombinant proteins for 20 h in the presence or absence of 1 μg/mL polymixin B (PB). The IL-12p40 and IL-10 levels were measured in the culture supernatant. No treatment was used as the mock control. Each value represents the mean ± standard deviation of quadruple samples. The different letters above the bars in the graphs indicate statistically significant differences among the test groups and the mock group (one-way ANOVA plus Tukey–Kramer *post hoc* analysis, $P < 0.05$).

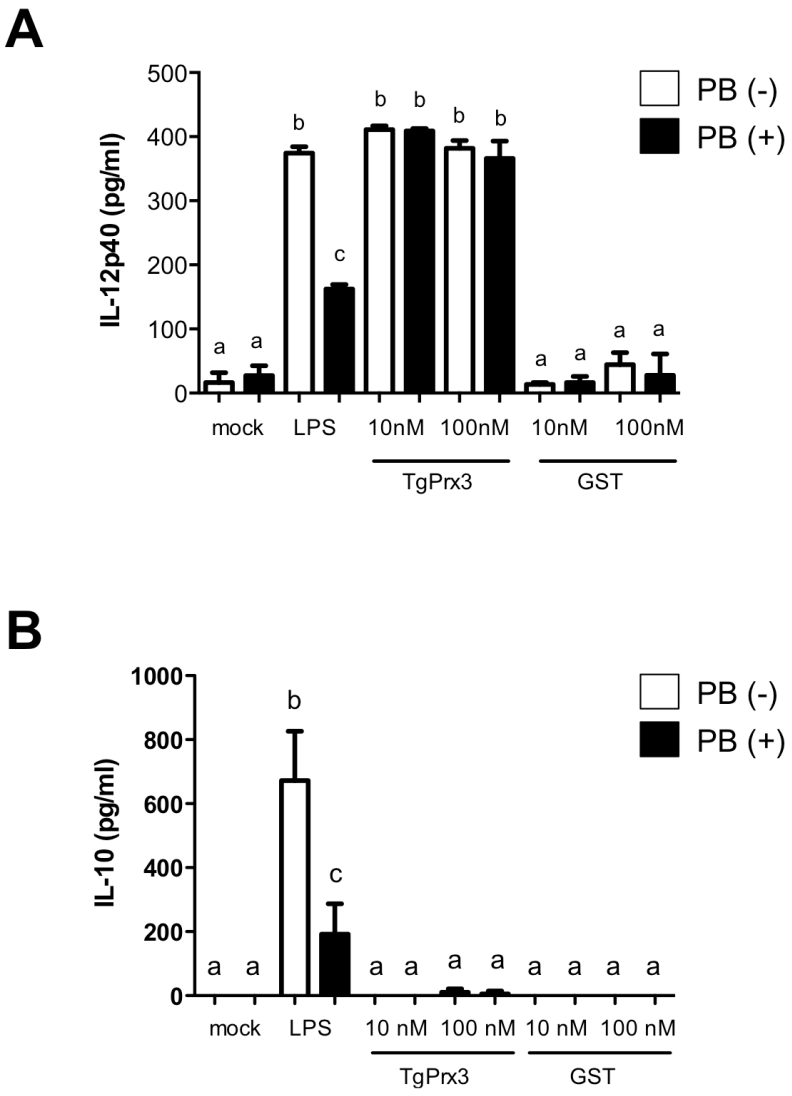

Fig. 2. Fereig et al.

Fig. 3. Antibody production against recombinant TgPrx3. A 25 pmol quantity of recombinant TgPrx3-GST protein or recombinant GST protein, or PBS alone was subcutaneously inoculated into mice. Panel (A) shows IgG1, and panel (B) shows IgG2c responses in the mice against recombinant TgPrx3, GST, and PBS prior to immunization and after the first, second and third immunizations. The specific antibody responses in each experimental group were tested against the TgPrx3 and GST recombinant proteins using serum samples collected from all the mouse groups 2 days before immunization and 12 days after each immunization, as tested by indirect ELISAs. The mean optical density (OD) was determined at a wavelength of 415 nm. The readings for the GST protein were subtracted from those of the TgPrx3 antigen. Each bar represents the mean ± standard deviation for the mice used per group (n = 6) and the results are representative of two independent experiments with similar results. The different letters above the bars in the graphs indicate statistically significant differences among the same immunization group (one-way ANOVA plus Tukey–Kramer *post hoc* analysis, $P < 0.05$).

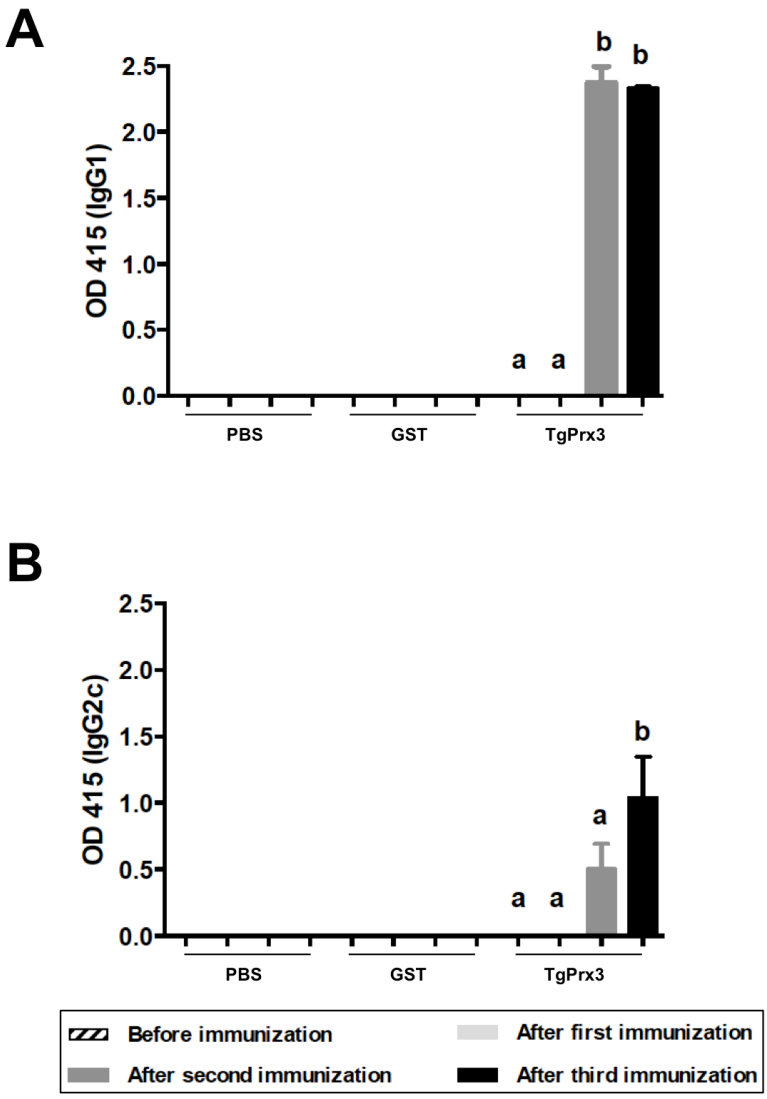

Fig. 3. Fereig et al.

Fig. 4. Proliferation and cytokine production in spleen cells. Monolayers of spleen cells from mice immunized with TgPrx3-GST, GST or PBS were cultured in the presence of TgPrx3-GST or GST recombinant proteins, Con A, or without any stimulator (medium alone). (A) Cell proliferation was measured at 48 h. The culture supernatants were assayed for production of IFN-γ (B) and IL-4 (C) using ELISAs. Each bar represents the mean ±standard deviation (PBS; n = 3, TgPrx3 and GST; n = 4). *, statistically significant differences were observed between the TgPrx3-immunized group and each other group with same stimulator using a one-way ANOVA and Tukey–Kramer *post hoc* analysis, $P < 0.05$.

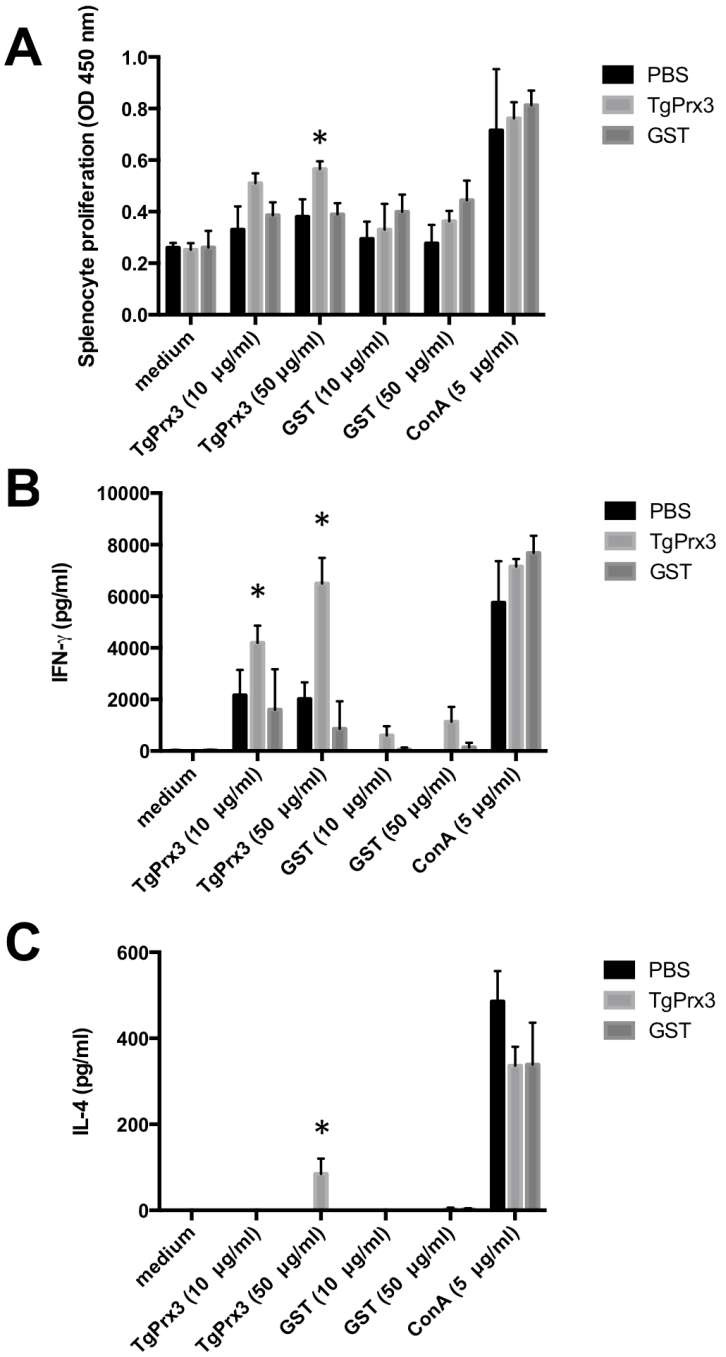

Fig. 4. Fereig et al.

Fig. 5. Mouse survival and parasite numbers in the brains of the surviving mice. (A) Mice immunized with recombinant TgPrx3, GST, or PBS (n=6 per trial) were challenged with *T. gondii* PLK-tachyzoites and then monitored daily until 30 days postinfection (dpi). The survival rates (surviving mice/total mice) were calculated from three successive independent experiments. A χ^2 test was used to estimate the differences in the survival rates at 15 dpi and 30 dpi between the experimental groups. At 15 dpi: PBS, 6/18 (33.3%); TgPrx3, 12/18 (66.7%); GST, 8/18 (44.4%). At 30 dpi: PBS, 5/18 (27.8%); TgPrx3, 10/18 (55.6%); GST, 7/18 (38.9%). Although a significant difference was noticed between recombinant TgPrx3- GST- and PBS-immunized mice at 15 dpi (P=0.046), the differences were not statistically significant at 30 dpi among the groups. (B) Quantified parasite DNA in the brains of the surviving mice at 30 dpi. ßted. Results are from three pooled independent experiments (PBS, n = 5; TgPrx3, n = 10; GST, n = 7). The results were analyzed with a one-way ANOVA plus a Tukey–Kramer *post hoc* analysis, but there were no significant differences.

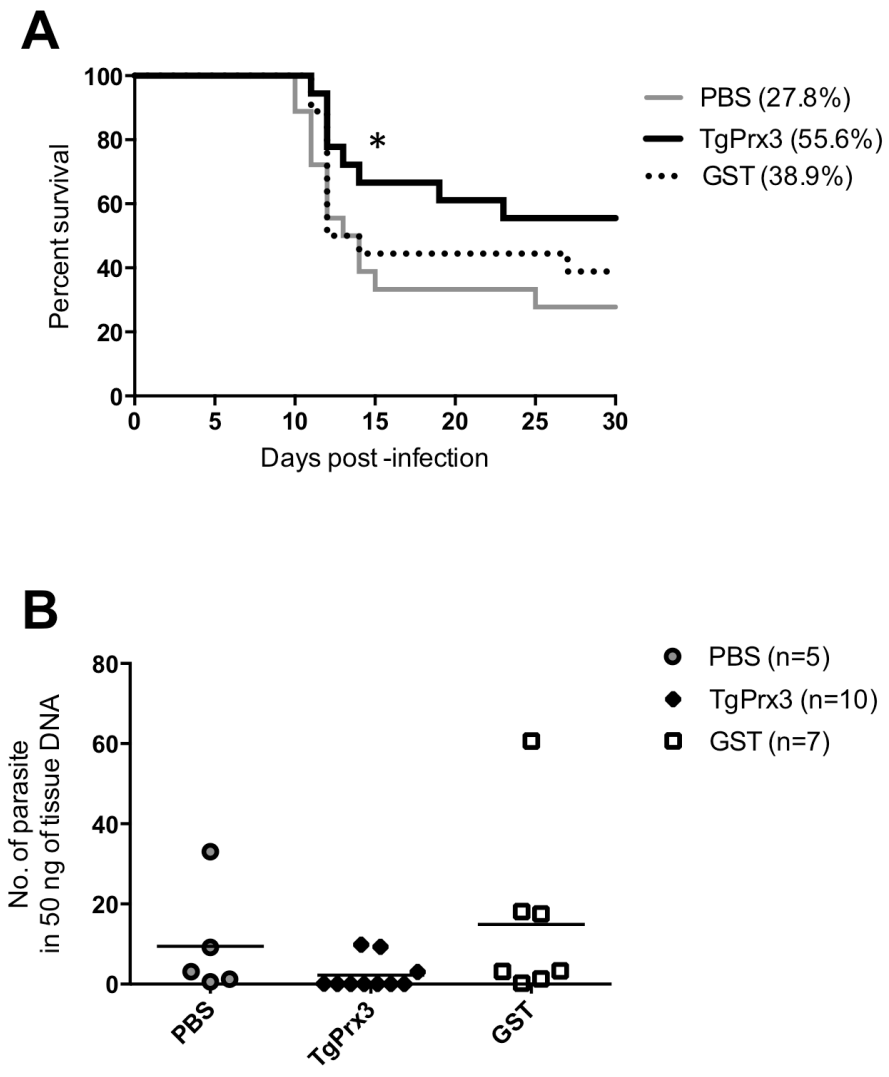

Fig. 5. Fereig et al.

Fig. S1. Production of specific IgG antibodies against TgPrx3 in chronically infected mice. C57BL/6 mice (n = 5) were intraperitoneally infected with 10^3 *T. gondii* PLK tachyzoites. Serum samples were collected from the mice 4 weeks after infection and tested with indirect ELISAs, using recombinant TgPrx3 and TgGRA7 antigens. The mean optical density (OD) was determined at a wavelength of 415 nm. Each bar represents the mean ± standard deviation. Sera of uninfected mice (n = 4) were used as the negative control. *, statistically significant differences were observed between the uninfected and infected mice with the Student's *t* test ($P < 0.05$).

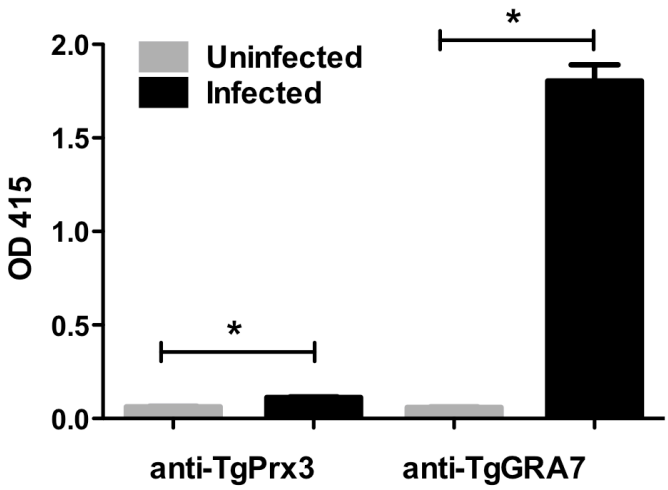

Fig. S1. Fereig et al.