Serosurveillance and vaccine development as a strategy for control of *Toxoplasma* infection

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Ragab Makhlouf Mahmoud FEREIG

Doctoral Program in Animal and Food Hygiene

Graduate School of Animal Husbandry

Obihiro University of

Agriculture and Veterinary Medicine

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帯広畜産大学大学院畜産学研究科

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List of abbreviations and units

BCA	- bicinochoninic acid
CCK-8	- cell counting kit-8
CD	- cluster of differentiation
cDNA	- complementary deoxyribonucleic nucleic acid
CI	- confidence interval
Con A	- concanavilin A
Ct	- cycle threshold value for quantitative PCR analysis
Cys	- amino acid cysteine
DMEM	- Dulbecco's modified Eagle medium
dpi	- days post infection
ELISA	- enzyme linked immunosorbent assay
EMEM	- Eagle's minimum essential medium
FBS	- fetal bovine serum
GRAs	- proteins secreted from dense granule organelles
GST	- glutathione S- transferase
HRP	- horseraddish peroxidase
iELISA	- indirect ELISA
IFN-γ	- interferon gamma
IL	- interleukin
IgG	- subtype of immunoglobulins
kDa	- kilodalton unit
LAL	- limulus amebocyte lysate
LAT	- latex agglutination test
LPS	- lipopolysaccharides
ng	- nanogram unit
nM	- nanomolar concentration
NF-kB	- nuclear factor of kappa-light-chain-enhancer of activated B cells
NO	- nitric oxide
NPV	- negative predictive value
OD	- optical density

OR	- odd ratio
PB	- polymixin B
PBS	- phosphate-buffered saline
PBS-SM	- PBS containing 3% skimmed milk
PCR	- polymerase chain reaction
PLK	- type II low virulent strain of *T. gondii*
PPV	- positive predictive value
PV	- parasitophorus vacuoles
Prx	- peroxiredoxin
RAW 264.7	- a kind of mouse macrophage cell line
RH	- type I highly virulent strain of *T. gondii*
RONs	- proteins secreted from neck region of rhoptry organelles
ROPs	- proteins secreted from bulb region of rhoptry organelles
RPMI 1640	- Roswell park memorial institute 1640 medium
SDS-PAGE	- sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SEAP	- secreted alkaline phosphatase
STAT6	- the signal transducer and activator of transcription 6 signaling pathway
TLA	-*Toxoplasma* lysate antigen
TLR	- toll-like receptor
TNF-α	- tumor necrosis factor alpha
TgPrx	- *Toxoplasma gondii*-derived peroxiredoxin
Th1	- T helper 1 mediated (cellular) immunity
Th2	- T helper 2 mediated (humoral) immunity

General introduction

1. Ultrastructure and biology of *Toxoplasma gondii*

Toxoplasma gondii (*T. gondii*) is an obligate unicellular and intracellular apicomplexan protozoan parasite. There are four developmental stages capable of inducing infection: tachyzoite, bradyzoite, merozoite and sporozoite. Cell invasive stages (Zoites) have a crescent shape and almost similar in basic structure. Although *T. gondii* is a single celled-organism, it possesses well structured and accommodated organelles rendered it as a model for investigating host-parasite interactions and immunomodulatory responses. Apical secretory organelles such as rhoptries, micronemes and dense granules are considered of special concern in *T. gondii* because of their professional role in development, invasion and survival of the parasite inside the host cell [Blackman and Bannister, 2001].

Rhoptries are club-shaped apical organelles which occupy the apical end of zoites and secrete two categories of proteins; rhoptry proteins (ROPs) which have numerous targets in the host cell including the host cell nucleus and the parasitophorous vacuole, and another subset of rhoptry proteins are called RONs which have been demonstrated to target the moving junction, the crucial phenomenon in invasion and parasitophorous vacuole formation [Bradley and Sibley, 2007].
Micronemes are the smallest of the apical end-aggregated secretory organelles. In addition to rhoptries, secretory materials of microneme provide crucial keys and strategies for cellular processes including gliding motility, active cell invasion and migration through cells [Carruthers and Tomley, 2008].

The successful establishment of infection is relied on two peculiarities of *T. gondii*; rapid penetration of host cell membrane and residing in a parasitophorus vacuole (PV), which is a well protected zone inside the host cell. Rhoptry and microneme products are synchronizing the initiation and establishment of infection, whilst the maintaining and resistance of parasite inside the PV in the

host cell is well controlled with various proteins released from abundantly distributed organelles in the zoite cytosol called dense granules [Carruthers and Sibley, 1997].

Apicoplast is a characteristic organelle for most apicomplexan parasites including *T. gondii*. It seems to be crucial organelle for the survival of parasite because all experimental trials which have been induced its structural or functional disorders led to the parasite death [Fichera and Roos, 1997, Waller et al., 1998]. The accurate machinery behind this effect is still anonymous, although some studies revealed the involvement of apicoplast in lipid metabolism and some other aspects of vital cellular processes [He et al., 2001, Karnataki et al., 2007].

In addition to the highly specified apical secretory organelles, basic organelles such as mitochondrion, Golgi bodies, endoplasmic reticulum and others which are common with other living organisms are also well developed and efficiently exert their basic functions essential for growth, multiplication and development of *T. gondii* either *in vivo* or *in vitro* [Black and Boothroyd, 2001, Pieperhoff et al., 2013, Coffey et al., 2016, Tahara et al., 2016, van Dooren et al., 2016]. Cellular organelles of *T. gondii* have been shown in (Fig. 1).

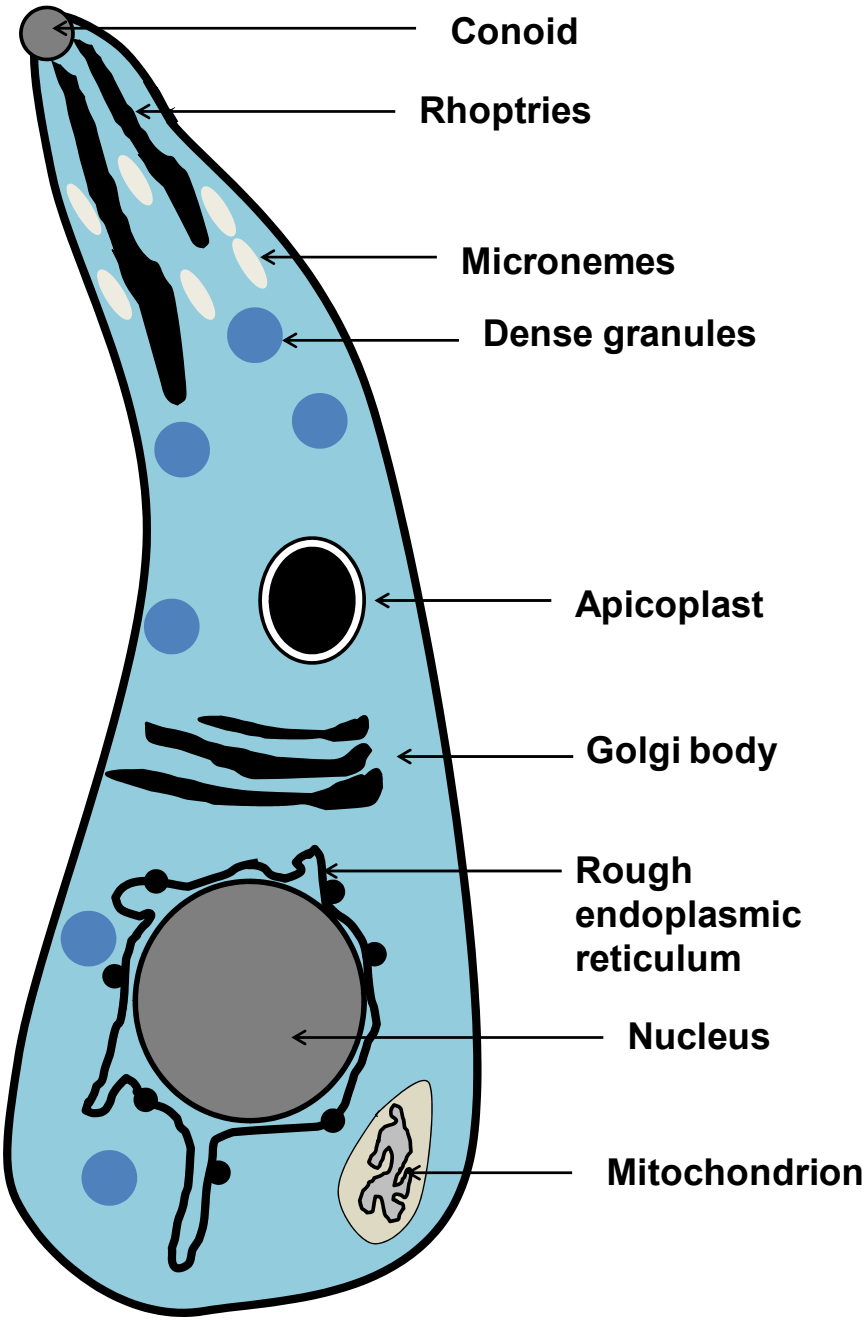

Fig. 1. An illustration showing the vital cell organelles in the tachyzoite of *T. gondii*.

2. Distribution and life cycle

Although the cosmopolitan distribution and wide range of susceptible hosts to toxoplasmosis are reported, the only known definitive hosts of *T. gondii* are feline species. *T. gondii* can infect all warm-blood vertebrates including humans and birds [Dubey, 2010]. For human population, around one-third of the world's population is estimated to have the specific antibodies against *T. gondii* [Tenter et al., 2000]. Approximately, specific anti-*T. gondii* antibodies were recorded in all countries and regions of the world and among most animal species.

The life cycle of *T. gondii* is described as a facultative heteroxenous cycle because of the possibility to develop in multiple animal hosts (Fig. 2.). It includes sexual stage which develops only in the definitive host such as the domestic and wild felids, and asexual stage that can develop in various warm-blooded animals including human and cat itself [Dubey, 2010]. In case of ingestion of cat for foods contaminated by infectious sources such as oocysts, tissue cysts or pseudocysts, the infective phase of the parasite as sporozoites, bradyzoites or tachyzoites are released. Then, the aforementioned stages invade the intestinal cells and differentiate into five morphologically distinguished asexual forms of schizonts. Within 2 days in the intestine of cats, *T. gondii* develops through all five stages of schizonts and transforms into merozoites, the first sexual stage. Merozoites proliferate for two to four doublings, and then differentiate into macrogametes and microgametes. The macro- and microgametes fuse together to produce diploid oocysts, which develop thick impermeable walls and are excreted in the feces. Cats shed between 2 and 20 million oocysts per day in their feces and shed from 3 to 10 days postinfection. [Dubey and Frenkel, 1972, Speer and Dubey, 2005]. Under favorable environmental conditions of temperature and humidity, unsporulated oocysts become sporulated and infectious [Dubey et al, 1970].

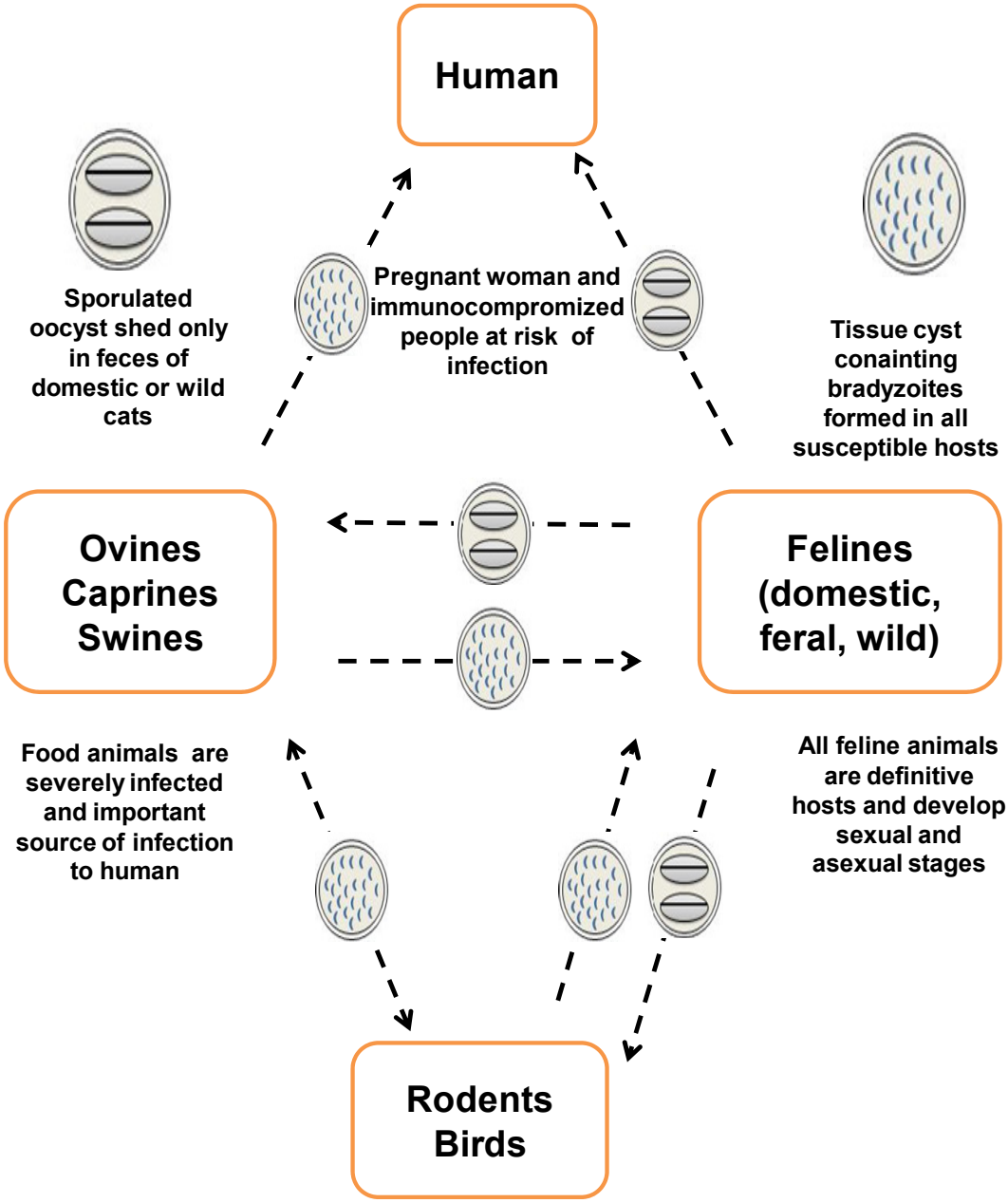

Fig. 2. A schematic illustration for life cycle and host range of *T. gondii*.

3. Clinical and immunological pathology

There are three common strains of *T. gondii* in North America and Europe. Type I strains (e.g. RH, GT-1) are the most virulent while type II strains (e.g. ME49 and its derivatives (PDS, PLK, PTg), Prugniaud) are moderately virulent and type III strains (e.g. CEP, VEG) are avirulent [Black and Boothroyd, 2000]. In the definitive or intermediate hosts, after ingestion of the *T. gondii* cysts or oocysts, the outer wall is ruptured by the acid digestion and proteolytic enzymes in the stomach and small intestine (e.g. pepsin, trypsin and erepsin), and bradyzoites and sporozoites are released into intestinal lumen. The tachyzoites are rapidly formed in the epithelium of intestinal cells where they distributed and disseminated via blood or lymphatic system [Dubey et al., 2002, Bhopale, 2003].

Toxoplasmosis is usually inapparent but the symptomatic case is usually characterized by different forms of clinical disorders. The eye and brain are preferred organs for *T. gondii* infection because of the immunosuppressive properties to avoid the extensive tissue damage [Bhopale, 2003]. Pneumonia, myocarditis, hepatitis and lymphdenitis are common sequelae of *T. gondii* infection in relevant organs. Moreover, in congenital infection, hepatic and pulmonary disorders are followed by central nervous system involvement resulting in hydrocephalus, retinochoroiditis and cerebral calcifications [Jackson and Hutchison, 1989].

Toxoplasma gondii has a unique relationship with the host immune system, where it efficiently manipulates the effector immune cells and molecules to maintain its survival and to keep the host alive. Both cellular and humoral immunity is involved in response against the parasite to control its progression and dissemination. Macrophages are crucial for resistance against *T. gondii* because of the production of interleukin 12 (IL-12). The cytokine IL-12 is essential for regulating the immune response against invading *T. gondii*. It performs this effect by promoting the function of natural killer cells and T-cells to produce interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) which act synergistically to mediate the parasite killing by macrophages [Sibley et al., 1991,

Gazinelli et al., 1993]. Furthermore, humoral immune responses play critical roles in increase of host resistance against the parasite. The production of specific antibodies IgG, IgA, IgM and IgE are generated against specific antigens of *T. gondii*. The specific antigen-antibodies reaction is adequate to kill the extracellular parasites through activation of complement system [Schreiber and Feldman, 1980].

4. Current status of vaccine development against toxoplasmosis

Toxoplasma gondii has a complex life cycle and multiple developmental forms of different infective pathways, making the development of a potent vaccine to reduce the hazards of toxoplasmosis far from straightforward [Elmore et al., 2010, Sullivan and Jeffers, 2012]. Establishment of novel control and preventive strategies for toxoplasmosis is essential for protecting public health and livestock production. Currently, only one commercial vaccine (Toxovax®, Intervet), based on live attenuated tachyzoites of the *T. gondii* S48 strain, is available for veterinary use in a limited number of countries, where it is used primarily for minimizing the incidence of abortion in sheep [Kur et al., 2009]. This vaccine has certain disadvantages and cannot be used for humans because live vaccines possess the capacity to restore parasite virulence and provoke iatrogenic infection [Innes et al., 2009b]. Moreover, most available drugs used for treatment and control of toxoplasmosis have several limitations; hence, discovery of highly effective and safe chemotherapies remains an essential goal. Whereas the most commonly used anti-*Toxoplasma* drugs such as sulfadoxine/pyremethamine or clindamycin are only partially effective in treating acute infections, their efficacy is abrogated for treatment of chronic infections, and also have high toxicity [Rodriguez and Szajnman, 2012]. Also, there are currently no effective treatments that can prevent the severe neurological, ocular, cardiac and cerebral anomalies characteristic of congenital toxoplasmosis contracted during pregnancy [Paquet and Yudin, 2013]. This situation makes the

development of an effective and safe vaccine against *T. gondii* critical for controlling this parasitic infection in humans and animals. In the last few years, the most extensive vaccine trials have been focused on the use of recombinant subunit vaccines (DNA and protein subunit vaccine). The merits of such vaccines have been investigated in terms of their potential to elicit long lasting cellular and humoral immunity, as well as their safety and overall costs. DNA and protein subunit vaccines are based on certain parasite molecules, particularly those participating in host-parasite interactions. With DNA vaccines, the target parasite gene is inserted into a eukaryotic vector that has the capacity to express the parasite antigen inside the immunized host. In contrast, recombinant protein vaccines comprise a pre-prepared parasite antigen previously expressed in a prokaryotic or eukaryotic vector in each host cell. In last decade, recombinant DNA vaccines have been achieved significant advances in triggering potent immune responses and inducing high levels of protection [Dautu et al., 2007, Döşkaya et al., 2007, Wang et al., 2012, Gong et al., 2013, Hassan et al., 2014]. Similar successes were reported for recombinant protein vaccines [Golkar et al., 2007, Huang et al., 2012, Zheng et al., 2013, Wang et al., 2013].

Additionally, a revolution is taking place in the processing and use of recombinant protein vaccines by blending them with adjuvant substances that can act as antigen agonists, thereby improving their protective efficacy. Numerous types of adjuvant have been evaluated in immunization experiments, including chemically formulated ones such as Provax, Freund, poly (Lactide-co-glycolide) microparticles and oligomannose-coated liposomes, and those of natural origin as Ginsenside Re. Most of the adjuvants used in such vaccines have succeeded in potentiating the antigen's protective efficacy and increasing the safety of the vaccine [Döşkaya et al., 2007, Flori et al., 2006, Chuang et al., 2013, Qu et al., 2013, Tanaka et al., 2014].

4. Peroxiredoxins of *T. gondii*

Toxoplasma gondii has a well developed anti-oxidant system including various enzymes such as catalase, superoxide dismutase, glutathione peroxidase and peroxiredoxins (Prxs) which work independently or synergistically to enable the parasite to survive under adverse conditions *in vivo* or *ex vivo*. The genome of *T. gondii* contains three Prx genes, which were already identified and characterized. Depending on the number of active redox cysteine residue and the expression sites, the Prxs named as 1-cys-Prx (TgPrx1) and 2-cys-Prx (TgPrx2) were identified in the cytoplasm of the parasite, while 2-cys-Prx (TgPrx3) is localized in the mitochondria; all of them were expressed in tachyzoite and bradyzoite stages [Ding et al., 2004].

Peroxiredoxin-linked detoxification of reactive oxygen species is identified as an efficient antioxidant system in *T. gondii*, protecting it against the resultant oxidative stress during its intracellular life. Using of high dosage of certain endogenous and exogenous oxidative stressors increased the susceptibility and induced a remarkable destruction to the tachyzoites of *T. gondii*. This indicates that slight changes in the redox balance of the parasites lead to deterioration of their oxidant homeostasis and parasite damage. Simultaneously, these stressors specifically affect the parasite but no obvious effects were noticed on the host cell. This interesting phenomenon renders the peroxiredoxins as suitable targets for developing therapies and vaccinations against *T. gondii* [Son et al., 2001, Akerman and Muller, 2005, Sautel et al., 2009].

A previous study investigated the immunomodulatory role of TgPrx1-derived from RH strain using an *in vitro* model of macrophage. The recombinant TgPrx1 promoted the function of alternatively activated macrophages manifested in production of IL-10 and high expression of arginase-1 which was dependent or independent on the signal transducer and activator of transcription (STAT) 6 signaling pathway. In additions, TgPrx1 induces downregulation of IL-1β production and non significant increase of IL-12p40 production. This result suggests the role of TgPrx1 in enhancement of Th2-mediated immunity during infection with *T. gondii* [Marshall et al.,

2011].

5. Aims of the present study

Toxoplasmosis is caused by zoonotic transmission and induces health problem and economic losses in infected human and animal population, respectively. Approximately a third of world population was identified to have specific antibodies against *T. gondii*. Infection in human induces abortions, congenital anomalies and general illness. Similar disorders were recognized in economically important animal species particularly sheep, goats and pigs [Dubey, 2010]. To establish an efficient strategy for control of an infection or a disease, accurate data and information about the current prevalence and distribution is required. Accordingly, in this study, we attempted to estimate the current status of *T. gondii* infection in Egypt as an indispensable step for establishing preventive and control strategies towards *T. gondii* infection. The specific antibodies to *T. gondii* among multiple animal species in various regions in Egypt were firstly screened to establish a comprehensive record for prevalence of such parasite in this country. Second, after navigating effector molecules derived from *T. gondii,* peroxiredoxins are targeted as promising candidates because they were recognized as crucial molecules for parasite survival and trigger the host immunity [Ding et al., 2004, Akerman and Muller, 2005, Marshall et al., 2011], and then their biological roles in host-parasite interactions were assessed to confirm their immunomodulatory effects. Third, evaluation of the protective efficacy of TgPrx1 and TgPrx3 was executed against challenge infection with lethal dose of *T. gondii* PLK strain in a murine model.

Chapter 1

Seroprevalence of *Toxoplasma gondii* in farm animals in Egypt

1-1. Introduction

Toxoplasma gondii is a protozoan parasite that infects virtually all warm-blooded animals, including humans, livestock, birds, and marine mammals. Sheep, goats, and cattle are intermediate hosts of *T. gondii*, and are infected by the ingestion of food or water contaminated with oocysts shed by cats. The raw or undercooked meat from these animals is potentially hazardous if ingested by humans or other animals [Dubey, 2010]. Toxoplasmosis has a severe economic impact on the sheep and goat industries because it induces abortion, still birth, and neonatal losses [Tenter et al., 2000].

Previous studies that estimated the seroprevalence of anti-*T. gondii* antibodies in Egypt focused predominantly on human surveillance. These studies have shown that 59.6% of asymptomatic blood donors [Elsheikha et al., 2009], 51.5% of pregnant women [Ibrahim et al., 2009a), 67.5% of pregnant women [El Deeb et al., 2012] and 46.1% of women suffering spontaneous abortion [Tammam et al., 2013] were seropositive, and that 45.8% and 41.4% of these pregnant and nonpregnant women, respectively, had been in contact with animals [Ghoneim et al., 2010]. These results imply the strong presence of *T. gondii* in Egypt, which may present a risk to pregnant women.

In farm animals in Egypt, anti-*T. gondii* antibodies were detected in 10.8% of the cattle sera tested by enzyme-linked immunosorbent assay (ELISA)-based on truncated surface antigen 2 (TgSAG2t) [Ibrahim et al., 2009a], and in 43.7% or 41.7% of sheep sera, when a modified

agglutination test or ELISA was used, respectively [Shaapan et al., 2008], and in 98.4% of sheep and 41.7% of goats when an ELISA was used [Ghoneim et al., 2010]. A high seroprevalence of 65.6% was recorded in donkeys [El-Ghaysh, 1998] and 48.1% in horses [Ghazy et al., 2007]. Anti-*T. gondii* antibodies were detected in 17.4% of 166 camels [Hilali et al., 1998]. When poultry were tested, 47.2% of chickens, 59.5% of turkeys, and 50% of ducks were positive for anti-*T. gondii* antibodies [El-Massry et al., 2000], and in another study, 40.4% of chickens and 15.7% of ducks [Dubey et al., 2003]. Therefore, in Egypt, the seroprevalence of *T. gondii* antibodies is high, not only in highly susceptible animals such as sheep and goats, but also among other animals, such as cattle, donkeys, horses, camels, and domestic birds. Among farm animals in Egypt, sheep and goats are considered the most highly susceptible hosts of toxoplasmosis. The high rate of transmission from dams to offspring, remarkable fetal losses and abnormalities, and the high viability of cysts in the meat of infected animals are characteristic of *T. gondii* infections in sheep and goats [Buxton, 1998, Buxton et al., 2007, Dubey, 2009, Innes et al., 2009a]. However, *T. gondii*-infected cattle and donkeys are of negligible importance because the infections are not clinically significant in these animals and they have no severe complications. However, specific anti-*T. gondii* antibodies have been detected in serum samples and parasite DNA has been detected in the meat and milk from these infected cattle and donkeys, so they play an important role in the epidemiology of the infection [Dubey, 1986, Dubey and Thulliez, 1993, Opsteegh et al., 2011, Alvarado-Esquivel et al., 2015].

The latex agglutination test (LAT) is widely used as a reference test for the seroprevalence of toxoplasmosis in different animal species [Matsuo and Husin, 1996, Shahiduzzaman et al., 2011, Kyan et al., 2012, Matsuo et al., 2014]. However, the TgGRA7-based ELISA shows higher potency, sensitivity, and specificity than other reference serodiagnostic tests, including LAT, the direct agglutination test, the modified agglutination test, and the indirect fluorescent antibody test, which

are used to detect anti-*T. gondii* antibodies in serum samples from different animals [Terkawi et al., 2013, Wang et al., 2014a, Wang et al., 2014b, Gu et al., 2015, Ichikawa-Seki et al., 2015].

The main aim of this study was to establish a comprehensive record of the seroprevalence of *T. gondii*-specific antibodies in Egypt using several animal hosts at different locations and to identify the risk factors associated with toxoplasmosis, using a cross-sectional epidemiological study. Moreover, LAT and TgGRA7-based ELISA were used for further field validation of these detection systems against various animal species in Egypt.

1-2. Materials and methods

Animals and geographic distributions

Serum samples (n = 652) were collected in the period between May 2014 and June 2015. Cattle (n = 301), sheep (n = 111), goats (n = 94), and donkeys (n = 146) from different geographic locations in Egypt were screened for anti-*T. gondii* antibodies in this study. The availability of sampling animals with the adequate relevant data and the cooperation of animal owners determined the current animal grouping and distribution in this study. The cattle were divided into four groups: group 1 - randomly sampled male and female cattle of different ages from individual owners (less than five cattle per owner) and smallholder farms (5–20 cattle per farm), in different villages in the Qena governorate; group 2 - adult cows (over 3 years of age) that were bred in an intensive farming system (> 2000 cattle) in Qena governorate; group 3 - adult bulls (over 3 years of age) that were admitted to the Qena slaughter house from individual owners and smallholder farms; and group 4 - randomly sampled cattle of different ages and genders from individual owners and smallholder farms from different villages in the Sohag governorate. Because serum samples from investigated governorates except cattle samples from Qena were collected from animals from individual owners

and smallholder farms located in a limited geographical area with similar environmental and husbandry conditions, they were categorized as one group. The data for the different ages, sexes, breeding systems, and localities of the cattle sampled were used in a risk factor analysis for *T. gondii* infection.

The governorates investigated in this study are representative of all the regions in Egypt, including diverse climatic and ecological features. Qena and Sohag are located in the southern part, characterized by hot and dry weather. Giza and Minoufiya are in the middle region, where the weather is usually humid and temperate. Kafr El Sheikh, in the far northern area, is located in a coastal region and the weather is humid, rainy, and temperate for most of the year. Although all the governorates investigated are rural areas, Matrouh is a coastal semi-desert area, with predominantly heavy rains and cold climate in the winter, but dry and temperate weather in the summer. Details of the animal species investigated, their locations, and the numbers of samples collected are shown in Table 1 and Fig. 3.

Blood sampling

Blood samples were collected from each animal in the field with venal puncture, into glass tubes without anticoagulant. These samples were kept in an icebox, then sent to the laboratories at University of South Valley for samples of Qena and Sohag, University of Cairo for samples of Giza Zoo, University of Sadat City for samples of Minoufiya and Matrouh and University of Kafr El Sheikh for samples of Kafr El Sheikh. These serum samples were centrifuged to harvest the sera and kept at − 20 °C until used.

Latex agglutination test (LAT)

The sera were tested with LAT to detect *T. gondii* infections using Toxocheck-MT (Eiken Chemical, Tokyo, Japan), according to the manufacturer's instructions. Samples were considered positive when agglutination was observed at a dilution of 1:32.

Recombinant protein expression

The recombinant TgGRA7 was expressed with previously described methods [Terkawi et al., 2013], with slight modifications. The purity and quantity of the proteins were confirmed with the detection of 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). The protein concentration was measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

Indirect ELISA (iELISA)

Purified antigen (50 µl) at a final concentration of 0.1 µM was coated onto ELISA plates (Nunc, Roskilde, Denmark) overnight at 4 °C in a carbonate–bicarbonate buffer (pH 9.6). The plates were washed once with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 3% skimmed milk (PBS-SM) for 1 h at 37 °C. The plates were washed once with PBS-T, and 50 µl serum samples, diluted 1:100 with PBS-SM, were added to the wells. The plates were incubated at 37 °C for 1 h. After the plates were washed six times with PBS-T, they were incubated with horseraddish peroxidase (HRP)-conjugated anti-IgG antibody of the corresponding species (Bethyl Laboratories, Montgomery, TX, USA), diluted 1:4,000 for the sheep, goat, and cattle sera and 1:6,000 for the donkey sera with PBS-SM and incubated 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 sulfonic acid];

Sigma-Aldrich, St Louis, MO, USA) was added to each well. After incubation for 1 h at room temperature, the absorbance at 405 nm (A450) was measured with an Infinite® F50/Robotic ELISA reader (Tecan Group Ltd., Männedorf, Switzerland). The cut-off point was determined as the mean A405 value for standard *Toxoplasma*-negative sera kept in our laboratory (cattle, $n = 10$; sheep, $n = 5$; goat, $n = 5$; donkey, $n = 10$) plus three standard deviations. The negative serum samples were confirmed with both LAT and the TgGRA7-based iELISA.

Statistical analysis

The significance of the differences in the prevalence rates of different species and risk factors was analyzed with a chi-square ($\chi 2$) test. A P value of < 0.05 was considered statistically significant. The kappa values, specificity, sensitivity, and 95% confidence intervals were calculated with www.vassarstats.net. The strength of agreement was graded with kappa values of fair (0.21–0.40), moderate (0.41–0.60), and substantial (0.61–0.80). $\chi 2$ values and odds ratios were calculated with the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

1-3. Results

In total, 652 animal sera were tested in a comprehensive survey of specific anti-*T. gondii* antibodies in different localities in Egypt among cattle, sheep, goats, and donkeys. The overall seropositive rate in all investigated animals was 26.7%. The seroprevalence among sheep was 47.8%, 51.4%, and 38.8% according to LAT, iELISA, and both tests, respectively, and the highest positive rates were in Kafr El Sheikh, at 69.6%, 69.6%, and 56.5%, respectively (Table 2). In goats, the overall seroprevalence was 35.1%, 39.4%, and 28.7% according to LAT, iELISA, and both tests, respectively, which were lower than those in sheep (Table 3). The highest seropositive rates for goats

were also in Kafr El Sheikh (66.7%, 66.7%, and 60.0% according to LAT, iELISA, and both tests, respectively). The prevalence of anti-*T. gondii* antibodies in less susceptible hosts, such as donkeys and cattle, were 22.6% and 23.6%, respectively, in the animals positive on both LAT and iELISA (Table 4 and Table 5).

To examine the predisposing factors for *T. gondii* infection, the animal species, locality, and climatic conditions were analyzed. The seroprevalence rates were significantly higher in sheep than in cattle or donkeys (Table 6). The seropositive rates in Kafr El Sheikh were higher than those in other locations (Table 6). However, no obvious difference was observed according to climate (Table 6). The influence of age (< 3 and > 3 years of age), sex (cow or bull), location (Qena or Sohag), and type of breeding (individual breeding or mass farming system) were analyzed to identify the potential risk factors for *T. gondii* infection in cattle in the southern part of Egypt (Table 7). The seroprevalence of anti-*T. gondii* antibodies did not differ significantly between animals of different ages, sexes, localities, or breeding systems (Table 7).

The efficacy of the TgGRA7-based iELISA was compared with that of LAT (reference test) to validate the iELISA (Table 8). The recorded kappa values were 0.645, 0.682, 0.649, and 0.726 for sheep, goats, cattle, and donkeys, respectively. Therefore, there was substantial agreement between the TgGRA7-based iELISA and LAT in all the animals tested. High levels of sensitivity, specificity, and concordance were also observed for all the tested animals.

1-4. Discussion

Cattle, sheep, goats, and donkeys are the main components of the agricultural sector in Egypt, and are used for different purposes. Cattle, sheep, and goats are the main sources of animal protein and play a crucial role in maintaining the food security and health status of Egypt. This study

provides the first record of the serological prevalence of *T. gondii* antibodies in Qena, Sohag, and Matrouh governorates for all the animals investigated.

A high prevalence of *T. gondii* infection was demonstrated among all investigated animals in the south, middle and the far north regions of Egypt, indicating that the distribution of toxoplasmosis is ubiquitous across the Egyptian governorates. This result might be attributed to the frequent transportation of animals between different locations and the relatively similar condition for animal husbandry and environment across the Egyptian regions. The significantly higher prevalence rates of anti-*T. gondii* antibodies in the Kafr El Sheikh governorate than in the other governorates might be attributable to its highly favorable environmental conditions for the development of oocysts, particularly its humidity and temperature [Dubey, 1998], because as a coastal agricultural region, this governorate has humid temperate weather for most of the year. Kafr El Sheikh is also famous for its large animal populations, which are bred in large groups.

Also, valuable data were presented on the sera collected from donkeys, which are slaughtered and used as food for carnivorous animals at Giza Zoo, the largest and oldest zoo in Egypt. Because at least 27.6% of the donkey sera tested were positive for *T. gondii* antibodies, the meat of the donkeys supplied to the zoo animals might be an important source of infection. The transmission of *T. gondii* infection via this route may also facilitate human infections through the handling of meats from infected animals. Stray cats and rodents exposed to the infected meats might be another source of infection.

The highest seroprevalence rates were observed in sheep and goats, rather than in cattle or donkeys, indicating that they are susceptible hosts of *T. gondii* infection. A further epidemiological investigation was conducted in cattle to assess whether age, sex, locality, or breeding system is a predisposing factor for *T. gondii* infection. Although the greatest difference was reported between the cattle sera from an individual breeding system (25.6%) and from intensive farming systems (18.8%),

it was not significant. These results are similar to those reported in previous studies [Klun et al., 2006, Qiu et al., 2012, Zhou et al., 2012], although Berger-Schoch et al. (2011) and Lopes et al. (2013) reported that the age of the animal is a risk factor for *T. gondii* infection.

The results of this study were compared with those of previous reports of the seroprevalence of *T. gondii* infection in Egypt. Ghoneim et al. (2010) demonstrated 98.4% and 41.7% seroprevalence in sheep and goats, respectively, in the Al-fayium governorate in the middle region of the country using an ELISA, which are higher than those in the current study. In Dakahlia, nearby governorate to Minoufiya, Younis et al. (2015) revealed that 41.7% and 62% for sheep, 49.4% and 50.6% for goat and 44.3% and 68.4% for donkey as seropositive samples using LAT and ELISA, respectively. Ibrahim et al. (2009a) reported 10.7% seropositive cattle in Sharkia in northern Egypt using a TgSAG2t-based ELISA, whereas this study showed 23.6% seropositive cattle in southern Egypt with both LAT and the TgGRA7-based ELISA. In the study of El-Ghaysh (1998), 65.6% of donkeys from Minoufiya were seropositive when tested with ELISA, indicating a higher infection rate than those observed in the current study (22.6%) and 45.0% positive reported by Haridy et al. (2010). These variations might be attributable to differences in the times and places of sampling, the numbers of animals tested, and/or the detection systems used.

In the same context, regional and global comparison of these results for *T. gondii* prevalence was additionally applied for more comprehensiveness of this study. Regarding the prevalence in sheep, 38.74% seropositive, highest prevalence rate among screened animals in the current study, was lower than 57.5% seropositive using LAT in Sudan [Khalil and Elrayah, 2011] and 53.3% seropositive in Brazil using modified agglutination test (MAT) [Cosendey-Kezenleite et al., 2014]. On the other hand, 23.4% seropositive using LAT in Saudia Arabia [Sanad and Al-Ghabban, 2007] and 17.8% seropositive by indirect haemagglutination test in Kuwait [Alazemi, 2014] showed a markedly lower prevalence than recorded in this study. Dumetre et al. (2006)

showed 38% seropositive using direct agglutination test in France, which is similar result to this study. For seroprevalence in goat, lower prevalence rates in China (17.6%, indirect haemagglutination test, Zou et al., 2015) in Myanmar (11.4%, LAT, Bawm et al., 2016) were reported compared with the current result (28.7% seropositive). Otherwise, Hamilton et al. (2014) recorded markedly higher seropositive rates using ELISA in Dominica and Grenade of Caribbean islands as 58% and 57% seropositive, respectively. The 23.6% seropositivity against *T. gondii* in cattle of this study was higher than the seropositivity 9.4% by LAT and 17% by ELISA in Thailand [Inpankaew et al., 2010], 13.3% by ELISA in Sudan [Elfahal et al., 2013], 7.3% by LAT in Japan [Matsuo et al., 2014] and 7.4% by TgGRA7-based ELISA in Indonesia [Ichikawa-Seki et al., 2015]. Compared with the current result, higher seropositive was observed in Sudan using LAT as 32% [Khalil and Elrayah, 2011]. Except for ruminant animals, the seropositive rate in donkeys in this study was 22.6%, which was markedly higher than that in different regions of the world such as 8% using LAT in Italy [Machacova et al., 2013], 6.4% using MAT in United States of America [Dubey et al., 2014], 10.9% using MAT in Mexico [Alvarado-Esquivel et al., 2015]. A similar result with this study was seen in northeastern China as 23.6% seropositive using MAT [Yang et al., 2013] and markedly higher seropositive was reported in Brazil as 43.3% seropositive using indirect fluorescent antibody test [de Oliveira et al., 2013]. Collectively, all these data concerning the seroprevalence of *T. gondii* in different animal species in multiple regions indicates the worldwide distribution of *T. gondii* infection and toxoplasmosis in farm animals.

Although LAT is widely used as a reference test [Matsuo et al., 2014] and the iELISA based on TgGRA7 has been validated for the serodiagnosis of toxoplasmosis in different countries throughout the world [Gu et al., 2015, Ichikawa-Seki et al., 2015, Wang et al., 2014a and Wang et al., 2014b], further field validation studies are required to confirm the success of both tests in most animal species and in different regions of the world. The current study is the first to use the

TgGRA7-based iELISA to examine the seroprevalence of *T. gondii* antibodies in Egypt. As shown in Table 8, the high performance of LAT and the TgGRA7-based iELISA was confirmed, with reliable kappa values and concordance, indicating their excellent potential for use in seroprevalence studies in the field. Given the high serological prevalence of *T. gondii* in several animals and at different localities in Egypt, specialists must establish new control and preventive policies to reduce the risk of toxoplasmosis in both the medical and veterinary contexts.

1-5. Summary

Toxoplasmosis is a cosmopolitan protozoan disease that has been recorded in a wide range of vertebrate hosts, including humans. This chapter was undertaken to comprehensively establish the seroprevalence of *T. gondii* among various farm animals in different localities of Egypt. The LAT and TgGRA7-based ELISA were used to screen the investigated animals for anti-*T. gondii* IgG antibodies. When only samples with simultaneously positive results for both the LAT and the TgGRA7-based ELISA were considered positive, 174 (26.7%) of 652 serum samples from different animals were seropositive. The prevalence of antibodies according to species was: sheep 38.7%, goats 28.7%, cattle 23.6%, and donkeys 22.6%. Thus, prevalence rate was significantly higher in sheep than in cattle or donkeys. The prevalence was also significantly higher in Kafr El Sheikh than in the other governorates investigated (Qena, Sohag, Minoufiya, and Matrouh). No significant differences were observed in age, sex, locality, or breeding system when evaluated as predisposing factors for *T. gondii* infection in cattle. In conclusion, this study demonstrates the high prevalence for *T. gondii*-specific antibodies among different animal species in southern and northern localities of Egypt, and provides valuable new data on the prevalence of *T. gondii* in donkeys, which are used as a food for carnivorous animals, particularly in the feline family, at Giza Zoo, Egypt.

Table 1: Geographic distributions and numbers of animal samples tested in this study.

Geographical regions	Sampling area	Cattle	Sheep	Goat	Donkey
Southern region	Qena	225	37	27	-
	Sohag	76	-	-	-
Middle region	Giza	-	-	-	58
	Minoufiya	-	28	37	43
Northern region	Kafr El Sheikh	-	46	30	-
North western region	Matrouh	-	-	-	45
Total number		301	111	94	146

Table 2: Seroprevalence of *T. gondii* in sheep in different governorates of Egypt.

	LAT		ELISA		LAT/ELISA positive		
Governorate	No. of tested	No. of positive (%)	No. of tested	No. of positive (%)	No. of tested	No. of positive (%)	95% CI
Qena	37	18 (48.7)	37	21 (56.8)	37	16 (43.2)	27.5 - 60.3
Kafr El Sheikh	46	32 (69.6)	46	32 (69.6)	46	26 (56.5)	42.2 - 69.7
Minoufiya	28	3 (10.7)	28	4 (14.3)	28	1 (3.6)	0.2 - 20.2
Total	111	53 (47.8)	111	57 (51.4)	111	43 (38.7)	29.7 - 48.4

LAT = Latex agglutination test, CI = Confidence interval

LAT/ELISA positive = samples that were simultaneously positive on both tests

95% CI calculated according to method described by (http://vassarstats.net/)

Table 3: Seroprevalence of *T. gondii* in goats in different governorates of Egypt.

Governorate	LAT		ELISA		LAT/ELISA positive		
	No. of tested	No. of positive (%)	No. of tested	No. of positive (%)	No. of tested	No. of positive (%)	95% CI
Qena	27	10 (37)	27	13 (48.2)	27	7 (25.9)	11.8 - 46.6
Kafr El Sheikh	30	20 (66.7)	30	20 (66.7)	30	18 (60)	40.7 - 76.7
Minoufiya	37	3 (8.1)	37	4 (10.8)	37	2 (5.4)	0.9 - 19.5
Total	94	33 (35.1)	94	37 (39.4)	94	27 (28.7)	20.1 - 39.1

LAT = Latex agglutination test, CI = Confidence interval

LAT/ELISA positive = samples that were simultaneously positive on both tests

95% CI calculated according to method described by at http://vassarstats.net/

Table 4: Seroprevalence of *T. gondii* in donkeys in different governorates of Egypt.

Governorate	LAT		ELISA		LAT/ELISA positive		95% CI
	No. of tested	No. of positive (%)	No. of tested	No. of positive (%)	No. of tested	No. of positive (%)	
Giza	58	16 (27.6)	58	22 (37.9)	58	16 (27.6)	17 - 41.1
Minoufiya	43	13 (30.2)	43	11 (25.6)	43	11 (25.6)	14 - 41.4
Matrouh	45	10 (22.2)	45	9 (20)	45	6 (13.3)	5.5 - 27.4
Total	146	39 (26.7)	146	42 (28.8)	146	33 (22.6)	16.2 - 30.4

LAT = Latex agglutination test, CI = Confidence interval

LAT/ELISA positive = samples that were simultaneously positive on both tests

95% CI calculated according to method described at http://vassarstats.net/

Table 5: Seroprevalence of *T. gondii* in cattle in different governorates of Egypt.

Cattle group	LAT		ELISA		LAT/ELISA positive		95% CI
	No. of tested	No. of positive (%)	No. of tested	No. of positive (%)	No. of tested	No. of positive (%)	
Group1 Qena	100	34 (34)	100	31 (31)	100	29 (29)	20.5 - 39
Group2 Qena	90	22 (24.4)	90	23 (25.6)	90	17 (18.9)	11.7 - 28.8
Group3 Qena	35	10 (28.6)	35	11 (31.4)	35	9 (25.7)	13.1 - 43.5
Group4 Sohag	76	22 (29)	76	20 (26.3)	76	16 (21.1)	12.8 - 32.2
Total	301	88 (29.2)	301	85 (28.2)	301	71 (23.6)	18.9 - 28.8

Group 1, 2, 3 were collected from Qena governorate but from different locations.

LAT = Latex agglutination test, CI = Confidence interval

LAT/ELISA positive = samples that were simultaneously positive on both tests

95% CI calculated according to method described by at http://vassarstats.net/

Table 6: Analysis of the influence of animal species, localities and climates on the distribution of *T. gondii* in Egypt.

Analyzed factor	No. of tested	No. of positive (%)	OR (95% CI)	*P -value*
Animal species				
Sheep	111	43 (38.7)		
Goat	94	27 (28.7)	1.56 (0.87-2.81)	0.13
Cattle	301	71 (23.6)	2.04 (1.28-3.26)	0.002
Donkey	146	33 (22.6)	2.16 (1.25-3.73)	0.004
Localities				
Qena	289	78 (27)	3.71 (2.2-6.28)	<0.0001
Sohag	76	16 (21.1)	5.15 (2.52-10.54)	<0.0001
Giza	58	16 (27.6)	3.3 (1.73-7.52)	<0.0004
Minoufiya	108	14 (13)	9.23 (4.48-19.02)	<0.0001
Kafr El Sheikh	76	44 (57.9)		
Matrouh	45	6 (13.3)	8.93 (3.37-23.64)	<0.0001
Climate				
Hot and dry	365	94 (25.8)	0.897 (0.633-1.27)	0.54
Temperate and humid	287	80 (27.9)		

OR = Odds ratio, CI = Confidence interval

χ2 test was used to detect the difference between variables

χ2 test and odds ratio were calculated by GraphPad Prism 5 software

Table 7: Univariable analysis of risk factors associated with *T. gondii* infection in cattle in Egypt.

Analyzed factor	No. of tested	No. of positive (%)	OR (95% CI)	*P -value*
Age				
< 3 years old	61	16 (26.2)	1.19 (0.62-2.27)	0.58
> 3 years old	240	55 (22.9)		
Gender				
Male	61	16 (26.2)	1.19 (0.62-2.27)	0.58
Female	240	55 (22.9)		
Location				
Qena	225	55 (24.4)		
Sohag	76	16 (21.1)	0.98 (0.52-1.84)	1
Breeding				
Individual	211	54 (25.6)		
Mass Farming	90	17 (18.8)	1.47 (0.8-2.72)	0.2

OR = Odds ratio, CI = Confidence interval

χ2 test was used to detect the difference between variables

χ2 test and odds ratio were calculated by GraphPad Prism 5 software

Table 8: Specificity and sensitivity of TgGRA7-based iELISA in detecting IgG to *T. gondii* infection in the field animal sera compared with the reference test LAT.

Animal	Kappa value	Sensitivity %	Specificity %	Concordance %	PPV %	NPV %
Sheep	0.645	83	83	83	75	88
Goat	0.682	82	88	86	75	92
Cattle	0.65	84	88	86	68	94
Donkey	0.726	85	91	90	75	95

PPV = positive predictive value

NPV = negative predictive value

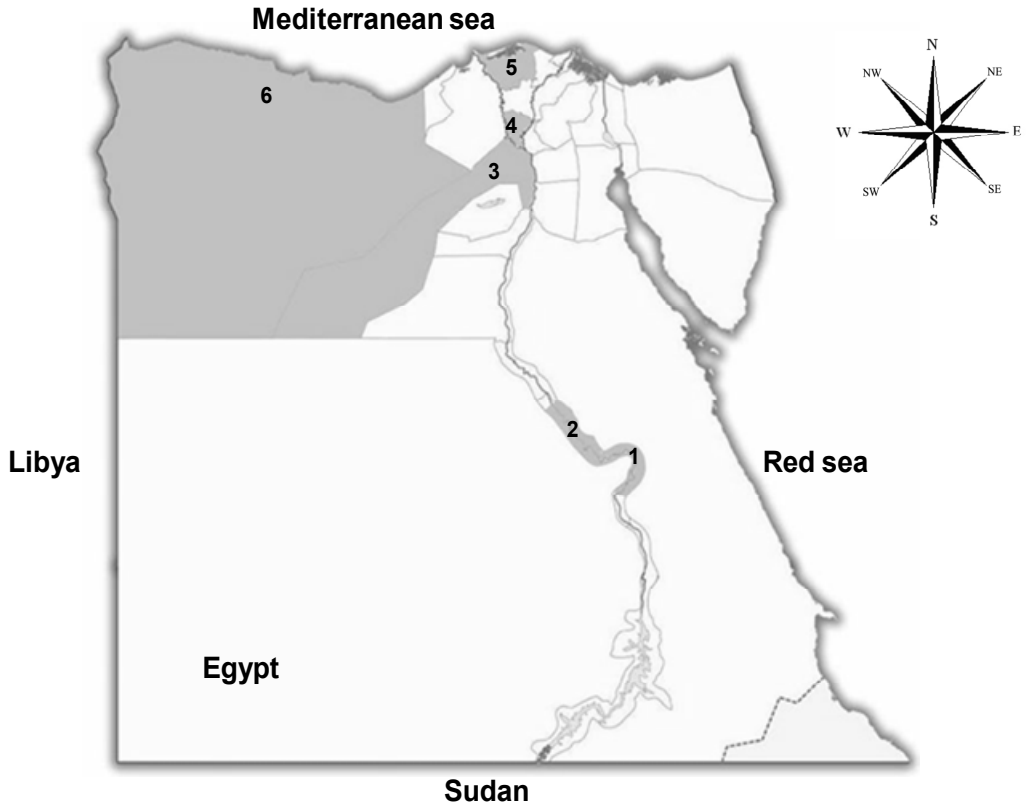

Fig. 3. Geographic distribution of the sampling sites in Egypt used in this study. Dark-colored areas with different numbers indicate the investigated governorates. 1 Qena, 2 Sohag, 3 Giza, 4 Minoufiya, 5 Kafr El Sheikh, and 6 Matrouh.

Chapter 2

Immunogenicity and protective efficacy of TgPrx1

2-1. Introduction

Toxoplasmosis is a zoonotic disease caused by the protozoan parasite *Toxoplasma gondii*, which infects approximately one third of the world population [Flegr et al., 2014]. It also invades almost all endothermic animals. Human infection by *T. gondii* is generally not apparent and induces a self-curing disease in immunocompetent individuals. However, the effects of infection are much more severe in immunocompromised patients [Dupont et al., 2012]. Toxoplasmosis in animals, mainly sheep, goats, and pigs, is of great economic importance because it causes abortion, still birth, and neonatal losses. The consumption of food contaminated with the tissue cysts of *T. gondii*, such as meat from infected livestock, is the main route of transmission of the parasite to humans [Jones and Dubey, 2012, Robertt-Gangneux and Darde, 2012].

Numerous *Toxoplasma*-derived molecules have been identified as immune-synchronizing effectors that induce dramatic and diverse immunomodulatory pathways. A rhoptry protein of *T. gondii*, ROP16, suppresses the expression of the proinflammatory cytokine IL-12 by the host macrophages [Saeij et al., 2007]. *Toxoplasma gondii* cyclophilin is reported to enhance macrophage nitric oxide production by binding to C-C chemokine receptor type 5 [Ibrahim et al., 2009b], and its profilin protein has been identified as a critical molecule that stimulates IL-12 production via a Toll-like receptor 11 (TLR11)-dependent pathway [Yarovinsky et al., 2005]. In the same context, the dense granule protein GRA15 of the *T. gondii* type II avirulent strain significantly increases the secretion of IL-12 [Rosowski et al., 2011].

The peroxiredoxins are a recently described family of antioxidants that are identified in eukaryotes and prokaryotes [McGonigle et al., 1998]. Prx acts as an antioxidant enzyme by sweeping hydrogen peroxide and hydroxyl radicals. The catalytic mechanism of the enzyme involves a redox-active cysteine (cys), which is highly conserved in the vicinity of the 47th position of its amino acid sequence [Chae et al., 1994]. Three Prxs have been identified in *T. gondii* tachyzoites, protecting them from oxidative stress: 2-cys Prx1, 1-cys Prx2, and 2-cys Prx3 [Ding et al., 2004, Akerman and Muller, 2005]. Recombinant TgPrx1 promotes an alternative activated macrophage pathway and induces IL-10 secretion via STAT6-dependent and -independent mechanisms, while reducing IL-1β production via caspase 1 [Marshall et al., 2011]. In contrast, *Plasmodium berghei* ANKA Prx strongly induces the macrophage secretion of proinflammatory cytokines TNF-α and IL-12p40 [Furuta et al., 2008]. In the same way, human Prx1 enhances the production of IL-6 and TNF-α from macrophage cells by binding to TLR4 [Riddell et al., 2010], and induces the secretion of inflammatory IL-23 by activating TLR2 and TLR4 [Shichita et al., 2012].

Macrophages constitute the first line of innate immunity, which contributes to the effective elimination of *T. gondii*. This action is mediated by IL-12, which is critical for the endogenous secretion of IFN-γ [Morgado et al., 2014]. Cellular immune response and IFN-γ production are the most successful strategy for development of potent vaccine candidates because immune protection against *T. gondii* in mice is primarily related to Th1 cell mediated immunity and IFN- γ secretion [Gazzinelli et al., 1993, Henriquez et al., 2010]. In addition, several studies unveiled a robust linkage between the canonical signaling pathway of nuclear factor-kappa B (NF-kB) and infection with *T. gondii* either by activation or inhibition [Butcher et al., 2004, Tato et al., 2003, Denkers et al., 2004, Shapira et al., 2005]. However, the mechanism of interaction between *T. gondii* and NF-kB signaling pathway is still deeply unknown. Although numerous *T. gondii* effector molecules were already

described as potent immunomodulators, some molecules interact with NF-kB transcription factors or relevant effectors [Rosowski et al., 2011, Dobbin et al., 2002, Du et al., 2014, Yang et al., 2015].

The establishment of novel control and preventive strategies for toxoplasmosis is critical in reducing the risk to public health and livestock production. Currently, the only commercial vaccine (ToxoVax®, Intervet B.V.), based on live attenuated tachyzoites of *T. gondii* strain S48, is available for veterinary use in a limited number of countries to minimize the incidence of abortion in sheep [Buxton, 1993, Kur et al., 2009]. This vaccine has certain limitations and cannot be used in humans because live vaccines can potentially recover their virulence and induce infection [Innes et al., 2009b]. Moreover, most available drugs used for the treatment and control of toxoplasmosis are only effective in acute case, whereas others, such as sulfadoxine/pyremethamine, have highly toxic effects on the treated individuals, including teratogenic effects and cutaneous lesions [Peters et al., 2007, Rodriguez and Szajnman, 2012]. Therefore, the development of an effective and safe vaccine against *T. gondii* would be extremely valuable in controlling this parasitic infection in humans and animals.

The molecular and biochemical properties of the TgPrx1 have been extensively investigated. TgPrx1 is expressed in the cytosol and protects cells against the free radicals generated as the byproducts of vital processes that occur in the cytoplasm [Akerman and Muller, 2005, Deponte and Becker, 2005]. Moreover, alignment of amino acid sequence between TgPrx1 and those of many other living creatures indicates the high specificity of TgPrx1, suggesting that TgPrx1 is a potent vaccine antigen and a candidate of drug target [Son et al., 2001]. Therefore, in the present study, the immunological and protective potentials of TgPrx1 were investigated. Only one study has discussed relevant research into TgPrx1 [Marshall et al., 2011], and no study has yet clarified the immunoprophylactic potential of this antigen.

2-2. Materials and Methods

Ethics statement

In this study, the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan were strictly followed. The protocol was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers 25–66, 26-68, 27-30, 28-49). All painful experimental treatments and surgical operations were implemented under general anesthesia induced with isoflurane.

Animals

Female C57BL/6J mice aged 6–7 weeks were purchased from Clea Japan (Tokyo, Japan) and allocated to the immunomodulatory or immunization experiments. Seven-week-old female BALB/c mice and female white Japanese rabbits were obtained from Clea Japan for the preparation of polyclonal antibodies against recombinant protein TgPrx1. All 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.

Parasites and cell cultures

Toxoplasma gondii PLK (avirulent type II strain) and RH (virulent type I strain) were used in this study. The parasites were maintained in Vero cells (African green monkey kidney epithelial cells) cultured in Eagle's minimum essential medium (EMEM; Sigma) supplemented with 8% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan) and 1% streptomycin–penicillin (Sigma). For the purification of tachyzoites, the parasites and host cell

debris were washed with sterile PBS and the infected cell monolayer was peeled from the plate with a cell scraper (BD Bioscience, San Jose, CA, USA). The final cell pellet was resuspended in Roswell park memorial institute 1640 medium (RPMI; Sigma) and passed through a 27-gauge needle and a filter with a pore size of 5.0 μm (Millipore, Bedford, MA, USA).

Expression and purification of recombinant proteins

The *TgPrx1* gene (GenBank accession number, XM_002371315.1) was amplified from cDNA of *T. gondii* PLK strain with PCR using oligonucleotide primers that included a *Bam*HI site (underlined) in the forward primer 5′-TA GGA TCC ATG CCG GCC CCG ATG GTG TCT-3′ and an *Xho*I site (underlined) in the reverse primer 5′-AG CTC GAG TTA CTT GCT TCC GAG ATA CTC-3′. The PCR products digested with *Bam*HI and *Xho*I, were inserted into the pGEX-4T3 plasmid vector treated with the same restriction enzymes (Amersham Pharmacia Biotech, Madison, CA, USA). Recombinant TgPrx1 was expressed as glutathione S-transferase (GST) fusion protein (TgPrx1-GST) in *Escherichia coli* BL21(DE3) (New England BioLabs Inc., Ipswich, MA, USA). The expression 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) with high-speed centrifugation (10,000 × *g*, 4°C, 30 min), lysed with 1% Triton in PBS and 50 mg/mL lysozyme, sonicated on ice, and then centrifuged as in the previous step. The supernatant was purified with Glutathione Sepharose 4B beads (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. In brief, the supernatant–beads mixture was incubated overnight at 4°C 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 yield of protein was dialyzed in PBS and the endotoxins were removed with a Detoxi-Gel Endotoxin Removing Column (Thermo

Scientific, Waltham, MA, USA). For use in cell culture, the proteins were filtered with a 0.45-μm low-protein binding Supor® membrane (Pall Life Sciences, Ann Arbor, MI, USA). The endotoxin levels in the TgPrx1-GST and GST preparations were estimated with Limulus Amebocyte Lysate reagents (Seikagaku Inc., Tokyo, Japan), and no endotoxin was detected in the tested protein lots. The purity and quantities of the proteins were determined as a single band on SDS-PAGE followed by staining with Coomassie Brilliant Blue R250 (MP Biomedicals Inc.). The protein concentrations were measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The recombinant TgPrx1–GST and GST proteins were isolated with apparent molecular weights of 49 kDa and 26 kDa, respectively, which were consistent with the expected molecular size of each protein (Fig. 4).

Generation of polyclonal antibodies against TgPrx1 and purification of immunoglobulin G (IgG)

Seven-week-old female BALB/c mice were intraperitoneally injected on day 0 with 100 μg of purified recombinant protein TgPrx1-GST emulsified in Freund's complete adjuvant (Sigma). The same protein in Freund's incomplete adjuvant (Sigma) was injected into the mice on days 14, 28, and 42 after the first immunization. Sera were collected from the tail veins of the mice at −2, 12, 26, and 40 days and with heart puncture after 49 days. Sera from the inoculated mice that displayed the highest sensitivity to and specificity for *T. gondii* were used in subsequent experiments. Purified recombinant protein (1 mg) was also subcutaneously injected into a female Japanese white rabbit. The presacrifice serum was collected from the ear vein. The sacrifice serum sample was collected after the antibody titers were measured. The titer of IgG was estimated with an ELISA, with the method discussed below. IgG was purified from 2 mL of the rabbit serum using protein A chromatography columns (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Aliquots of IgG were checked for protein purity and quantity with

SDS-PAGE. The protein concentration of the purified rabbit polyclonal IgG were measured with a BCA protein assay kit.

Western blot analysis

The protein lysates from purified *T. gondii* tachyzoites (15 μg/10 μl) were mixed with 10 μl of 2×SDS gel reducing loading buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 140 mM 2-mercaptoethanol, 10% (w/v) glycerol and 0.02% (w/v) bromophenol blue). Samples were heated at 95°C for 5 min and separated on a 15% polyacrylamide gel. After SDS polyacrylamide gel electrophoresis, the protein bands in the gel were transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). After washing twice with 0.05% PBS-T, the membranes were blocked with 3% PBS-SM for 12 h at 4°C. After two further washes, the membranes were incubated with anti-TgPrx1 mouse or rabbit serum (1:200) for 1 h at room temperature. After washing three times, the membranes were incubated with HRP-conjugated immunoglobulin G (1:2,000; Amersham Pharmacia Biotech, Piscataway, NJ, USA) diluted in PBS-SM, for 1 h at 37°C. After washing five times, the proteins were visualized using ECL™ western blotting detection reagents (GE Healthcare) by VersaDoc™ imaging system (Nippon Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer's recommendations. To confirm the reactivity of anti-TgPrx1 mouse or rabbit serum, western blot using *Toxoplasma* lysate antigens (TLA) was performed. As shown in Fig. 4B, protein with apparent molecular weights of 22 kDa was detected from the TLA of PLK and RH strains, which were consistent with the expected molecular size of TgPrx1 [Ding et al., 2004].

Measuring the levels of secreted antigen using double-antibody sandwich (DAS)-ELISA

Rabbit IgG directed against TgPrx1 was diluted to 10 μg/mL in 0.05 M carbonate buffer (pH 9.6). IgG (0.5 μg per well) was used as the capture antibody to coat microtiter plates (Nunc,

Inc.) at 4°C overnight. Blocking solution (200 μL; 3% PBS-SM) was added to each well and the plates were incubated at 37°C for 2 h. After the addition of the test samples in duplicate and the recombinant protein as the standard, the plates were incubated at 37°C for 30 min. The test samples were collected from the peritoneal fluid of C57BL/6J mice infected with the RH or PLK *T. gondii* strain. The plates were washed six times with washing solution (0.05% Tween 20 in PBS). Anti-TgPrx1 mouse serum, diluted at 1:3,000 in blocking solution, was added to each well and the plate was incubated at 37°C for 1 h. After six more washes, the plates were incubated with HRP-conjugated anti-mouse IgG antibody, diluted 1:5,000 in blocking solution, at 37 °C. Substrate solution (100 μL; 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 to visualize binding. The absorbance at 415 nm was measured with an ELISA reader (Corona MTP-120 microplate reader; Corona, Tokyo, Japan). The concentration of TgPrx1 antigen was calculated for each sample by standardization against the purified recombinant protein.

Indirect ELISA to detect TgPrx1- specific antibody

Recombinant TgPrx1-GST and GST proteins were adjusted to concentrations of 0.1 μM with coating buffer, added to the wells of ELISA plates, and incubated overnight at 4 °C. Recombinant antigen of TgGRA7 was also used as a reference antigen [Terkawi et al., 2013]. The plates were washed twice with washing buffer and then blocked with 3% 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 HRP-conjugated goat anti-mouse total IgG, IgG1 or IgG2c antibody (Bethyl Laboratories), 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 was added to each well.

After the samples were incubated at room temperature in the dark for 1 h, the absorbance at 415 nm was determined with an ELISA reader. The ELISA results for TgPrx1-specific antibody were determined by measuring the mean optical density of GST protein subtracted from that of TgPrx1-GST antigen. The formulations for the coating and washing buffers and the substrate solution were as described in the DAS-ELISA experiment.

Preparation and culture of murine peritoneal macrophages

Four days after C57BL/6J mice were injected intraperitoneally with 2 mL of 4.05% BBL™ Brewer modified thioglycolate medium (Becton Dickinson, Sparks, MD, USA), their peritoneal macrophages were collected with two peritoneal lavages of 5 mL of cold PBS. The harvested cells were centrifuged at 1,300 × *g* for 10 min and suspended in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 8% FBS. Red blood cells were removed with lysis buffer (0.83% NH4Cl, 0.01 M Tris-HCl [pH 7.2]) and then washed away with medium. The macrophage suspension was added to a 96-well microplate at 3×10^5 cells/well and incubated at 37°C in a 5% CO_2 incubator for 4 h, allowing the cells to settle to the bottom. The wells were washed with FBS-free DMEM to remove the floating cells, and incubated for 20 h after the addition of the indicated stimulants, including positive and negative controls. To confirm the effects of the resident lipopolysaccharide (LPS) in the protein samples, polymixin B (Sigma) was used.

Culturing and stimulation of RAW 264.7 cell lines

The mouse macrophage RAW 264.7 was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. NF-kB secreted alkaline phosphatase reporter cell line generated from RAW 264.7 (NF-kB/SEAP cells) was obtained from Novus biological inc. (Littleton, CO, USA). The cells were cultured in DMEM supplemented with 10% (v/v) FBS and 100

U/mL penicillin, 100 mg/mL streptomycin (Life Technologies, Darmstadt, Germany) for both cell lines while 0.5 mg/mL G418 (Geneticin, Roche, Mannheim, Germany) was added for NF-kB/SEAP cells for drug based-selection and then cells were cultivated at 37°C in a 5% CO_2 incubator. For induction experiments, cells were seeded in 96-well plate ($2x10^5$ in 100 μL/well) and incubated at 37o C for 4 hrs to allow adherence of the cells to the bottom. The proteins of TgPrx1-GST and GST at final concentrations 10 nM and 100 nM, and LPS at a final concentration 10 ng/mL as a positive control and only medium as a negative control were added to the cells, then the plates were incubated again at 37oC for 48 hrs. The stimulants including recombinant proteins, LPS and the medium were added to the cells with and without polymixin B at a final concentration 20 μg/mL to validate the test against the effect of resident endotoxin. Optimum condition including the concentrations for recombinant proteins, LPS and polymixin B, incubation period and RAW cell density were adjusted according to several preliminary experiments.

Preparation of TLA

Toxoplasma gondii lysate antigens were prepared from tachyzoites of PLK and RH strains as previously described [Fatoohi et al., 2002], with slight modifications. Briefly, the purified tachyzoites were washed with PBS and adjusted to 1×10^8 tachyzoites/mL of PBS. The parasites were destroyed by sonication on ice followed with exposure to three freezing–thawing cycles. The crude extract was harvested after centrifugation at 10,000 × *g* for 10 min and filtration with a 0.45-μm low-protein binding Supor® membrane. The concentration was measured using a BCA protein assay kit.

Stimulation of splenocytes for measuring the proliferation assay and cytokines production

Spleens of immunized mice were surgically dissected under aseptic condition after 2 weeks of the last immunization and processed as illustrated previously [Nishikawa et al., 2009], with slight modifications. Spleen of each mouse was crushed between 2 sterile glass slides, then cells were suspended thoroughly in RPMI 1640 medium (Sigma) supplemented with 10 % FBS. Red blood cells were destructed via addition of lysis buffer (0.83% NH4Cl and 0.01 M Tris-HCl, pH 7.2) to the cell suspension then washed with medium. The cells were plated into 96-well microplates at 2.5×10^5/100 μl/well in RPMI 1640 medium. Spleen cells were stimulated with TgPrx1-GST and GST recombinant proteins, TLA, concanavalin A (Con A; Sigma) as positive control and stimulant-free medium as a negative control. The plates were incubated for 48 h at 37°C in 5% CO_2, then 100 μl from the supernatants of cultures were collected and assayed for cytokines. Simultaneously, for estimation of splenocyte proliferation assay, 10 μl of Cell Counting Kit-8 reagent (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was added to each well for the previously stimulated cells. After 2 h of incubation at 37°C in 5% CO_2, optical density was measured using an ELISA reader at 450 nm.

Sandwich ELISA to measure cytokine levels and secreted alkaline phosphatase (SEAP) reporter assay

The supernatant was collected from the cell culture of mouse peritoneal macrophages, RAW 264.7, or spleen cells to measure the levels of cytokines. The cytokines (IL-4, IL-6, IL-10, IL-12p40 and IFN-γ) were measured with commercial ELISAs (Pierce Biotechnology Inc., Rockford, IL, USA), according to the manufacturer's recommendations. The cytokine concentrations were calculated from standard cytokine curves constructed from samples run on the same plate. The culture supernatant from NF-kB/SEAP cells was collected to measure the levels of SEAP by SEAP reporter assay kit (Novus) according to the manufacturer's instructions. The SEAP concentrations

were calculated from SEAP assay standard curve generated by adding a serial dilution of SEAP protein run on the same plate and performed according to the kit assay protocol.

Immunization and infection

To investigate the immunoprophylactic properties of TgPrx1, mice were inoculated subcutaneously with 25 pmol of recombinant TgPrx1-GST or GST protein in PBS, or with PBS alone (each 100 μL) three times at 14-day intervals (Total number = 18 mice per group from 3 independent trials). Fourteen days after the third immunization, the mice were challenged intraperitoneally with 1×10^3 tachyzoites of the *T. gondii* PLK strain. The mouse survival rates were measured for 30 days after challenge. Serum (20 μL) was collected from the mice at 14, 28, and 42 days after immunization, via the tail vein, to measure the specific antibodies directed against TgPrx1 with ELISAs. To confirm the lack of an antibody response in the unvaccinated and uninfected mice, control sera were collected from all the animals on day 2 before immunization. Thirty days after infection, serum and brain samples were collected from all the surviving mice after they were euthanized.

DNA isolation and quantitative PCR analysis

The parasites in the brains of the immunized mice were extracted, purified, and quantified as previously described [Tanaka et al., 2013], 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 [Contini et al.,

2005]. The PCR mixture (25 μl total volume) contained 1 × SYBR Green PCR buffer, 2 mM MgCl2, 200 μM 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, Carlsbad, CA, USA), 0.5 μmol of each primer, and 50 ng of genomic DNA. Amplification was performed with 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 (Applied Biosystems), and the calculated cycle threshold (Ct) values were exported to Microsoft Excel for analysis. A standard curve was established with *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 a known concentration of parasite. 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).

Statistical analysis

The GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) was used. Data are presented as means ± standard deviation. Statistical analyses were performed with the Student's *t* test, one-way or two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for group comparisons. The significance of the differences in 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 used. A P value < 0.05 was considered statistically significant.

2-3. Results

Detection of TgPrx1 antigen and specific antibody during T. gondii *infection*

The peritoneal fluid of mice experimentally infected with *T. gondii* PLK (avirulent type II strain) or RH (virulent type I strain) were examined to detect the release of TgPrx1 antigen into the body fluid during infection (Fig. 5A). Although the release of TgPrx1 into the peritoneal fluid of both the RH- and PLK-infected mice was confirmed, higher level of secreted antigen was detected in the fluid of the RH-infected animals. To determine the efficacy of TgPrx1 for initiating the production of specific antibodies, the sera of mice infected with PLK, collected 4 weeks after infection were tested with an indirect ELISA based on the TgPrx1 antigen and on TgGRA7, a well-known potent diagnostic antigen [Terkawi et al., 2013]. Although TgPrx1-specific antibodies were detected, the levels of the antibodies were significantly lower than the levels of TgGRA7-specific antibodies (Fig. 5B). These results indicate that TgPrx1 is less antigenic than TgGRA7 while they are released into the body fluid of mice after *T. gondii* infection.

Activation of murine macrophages by TgPrx1

To investigate whether the released TgPrx1 stimulated immune cells, murine peritoneal macrophages were stimulated with the recombinant TgPrx1-GST *in vitro* (Fig. 6). TgPrx1-GST treatment triggered IL-6 production while there was no significant difference between the TgPrx1-GST and mock-treated macrophages (Fig. 6A). Furthermore, TgPrx1-GST at concentrations of 100 nM significantly enhanced the production of IL-12p40 compared with that expressed in mock-treated cells (Fig. 6B). On the contrary, anti-inflammatory cytokine IL-10 was not detected in the culture of macrophages treated with TgPrx1-GST while LPS stimulation triggered IL-10

production (Fig. 6C). The levels of IL-6 and IL-12p40 after treatment with TgPrx1 were not altered by the presence of polymixin B. Treatment of the cells with LPS also triggered the expression of these cytokines, but polymixin B reduced their expression. GST and the mock treatment did not trigger any response. These results demonstrate the capacity of TgPrx1 to enhance macrophage responses.

The immunogenicity of TgPrx1 was also estimated by using RAW cells. The TgPrx1-GST induced significant secretion of NF-kB dependent SEAP from NF-kB/SEAP cells (Fig. 7A). The production of SEAP was evident at concentration of 10 nM and the production was clearly observed at the concentration of 100 nM which was comparable to the positive control level induced with LPS. Addition of polymixin B induced obvious reduction in the production of SEAP for LPS-treated cells, but not TgPrx1-GST-treated cells, indicating the efficacy of polymixin B and the genuine stimulation by recombinant protein of TgPrx1. In the same context, TgPrx1-GST enhanced the production of IL-6 from RAW 264.7 cells (Fig. 7B). Although the level of IL-12p40 in RAW 264.7 cells was measured, the level was undetectable even in the positive control samples using LPS. Collectively, recombinant TgPrx1 exhibited a potential effect in promoting the function of murine macrophages via NF-kB signaling pathway.

Immunization of mice with TgPrx1 against T. gondii *infection*

Because the recombinant TgPrx1 protein stimulated the macrophage response, its utility as a vaccine antigen with immune-stimulating activity to control *T. gondii* infection was evaluated. As shown in Fig. 8, mice immunized with TgPrx1-GST produced significant levels of specific antibodies, including IgG1 and IgG2c antibodies. The levels of antibodies developed against TgPrx1 were significantly higher than in the control mice inoculated with PBS or GST. To determine whether immunization with TgPrx1-GST induced the cellular immune response, the proliferation of

spleen cells and the production of IFN-γ and IL-4 were measured following the *in vitro* stimulation of spleen cells isolated from mice after the third immunization (Fig. 9). The proliferation of spleen cells was enhanced in TgPrx1-GST-immunized mice by stimulation with TgPrx1-GST at 10 and 50 μg/ml (Fig. 9A). The production of IFN-γ was higher in the spleen cells from mice immunized with TgPrx1-GST than in the cells from animals immunized with PBS or GST when they were stimulated with TLA or TgPrx1-GST (Fig. 9B). Interestingly, treatment of spleen cells from mice immunized with PBS or GST with TgPrx1-GST triggered IFN-γproduction compared with no treated cells (Fig. 9B). However, the IL-4 production in the spleen cells from mice immunized with TgPrx1-GST and the control mice inoculated with PBS or GST was not significantly enhanced by stimulation with TLA or immunized antigens, except for the treatment of spleen cells from TgPrx1-GST-immunized mice with 10 μg/ml TgPrx1-GST (Fig. 9C). Together, these results indicate that immunization with TgPrx1-GST triggered parasite- and antigen-specific humoral and cell-mediated immune responses in the mice.

The survival rate of mice immunized with TgPrx1-GST was 66.7% that was markedly higher than those of the GST- (38.9%) and PBS-injected (27.8%) mice used as the controls (Fig. 10A). For more confirmation of the protective efficacy of the TgPrx1 in the immunized mice, the brains of the surviving mice in all the groups were collected 30 days after infection to quantify the parasite with quantitative PCR. The number of parasites in the TgPrx1-GST- immunized groups was lower than those in the GST- and PBS-injected groups, although the differences were not statistically significant (Fig. 10B). This result indicates that TgPrx1 can be used as novel vaccine antigens.

2-4. Discussion

To develop a vaccine against *T. gondii*, a vaccine antigen with an appropriate immune-stimulating activity is required. Recently, the use of subunit vaccines against toxoplasmosis has shown promising results [Dziadek et al., 2009, Fereig and Nishikawa, 2016a]. In previous study, the TgPrx3 exerted strong immune response and conferred a protective potential against infection of mice with *T. gondii* PLK strain [Fereig and Nishikawa, 2016b]. In additions, other parasitic peroxiredoxins have also been shown to be potent vaccine antigens, as reported in mouse and nonhuman primate models of cutaneous leishmaniasis induced by *Leishmania major* [Campos-Neto et al., 2001], and in mice, against microfilaria *Brugia malayi* infections [Anand et al., 2008]. In the same context, goats immunized with the recombinant peroxiredoxin of *Fasciola hepatica* displayed a 33.04% reduction in the fluke burden, and less gross and microscopic liver damage than the control group [Mendes et al., 2010]. Therefore, *Toxoplasma* peroxiredoxin 1 might be an excellent candidate for the development of subunit vaccines against *T. gondii* infection.

To investigate the immunogenicity and protective efficacy of TgPrx1, the presence of this protein in the body fluid of infected mice was measured to confirm its biological activity. High level of TgPrx1 was observed in the peritoneal fluid of mice experimentally infected with either RH (high-virulence strain) or PLK (low-virulence strain) during the first week of infection. The amounts of TgPrx1 detected in this study may be attributable to the *in vivo* growth rates of the *T. gondii* strains, with RH having a higher growth rate than PLK [Radke et al., 2001]. These findings suggest that TgPrx1 may be a factor in the pathogenesis of toxoplasmosis during acute infection because its release from infected cells disappears after the acute phase. Therefore, the antigenicity of TgPrx1 was markedly lower than that of TgGRA7, known to be a highly antigenic protein [Terkawi et al., 2013]. Previous reports have shown the ability of Prx1 to eliminate reactive oxygen species, such as hydrogen peroxide [Ding et al., 2004, Akerman and Muller, 2005], that are secreted by certain immune cells [Kono et al., 1996]. They are also crucial in the defense mechanisms against invading

pathogens, suggesting that TgPrx1 protects *T. gondii* from oxidative stress derived from its hosts. In contrast, TgPrx1 may induce hyperinflammation during the acute stage of infection because triggering the production of proinflammatory cytokines, such as IL-6 and IL-12p40, by macrophages. Further research is required to clarify the role of the TgPrx1 in the virulence of *T. gondii*, particularly with the generation of *TgPrx1*-knockout parasites.

Since abundant TgPrx1 is released into the body fluids during *T. gondii* infection, it is speculated that TgPrx1 may play a role in host–parasite interactions. Importantly, TgPrx1 triggered the production of IL-6 and IL-12p40 by isolated macrophages and RAW 264.7 cell lines. From another perspective, this reaction may support the survival of the host animal because macrophages are powerful effector immune cells that act against protozoan diseases, including *T. gondii* infection [Saeij et al., 2007, Rosowski et al., 2011]. Moreover, this data suggest that the production of IL-6 and IL-12p40 from TgPrx1-treated macrophage is NF-kB-dependent. Because previous reports indicate the role of NF-kB in modulating the host resistance against *T. gondii* infection [Harris et al., 2010, Koblansky et al., 2013, Yang et al., 2015], TgPrx1 is considered for a candidate of vaccine antigen. Only one study has demonstrated the role of TgPrx1 as an immunomodulator, and revealed that it primarily activates the alternative macrophage pathway and mainly induces the secretion of the anti-inflammatory cytokine IL-10. However, a reduction in IL-1β, but no significant effect on IL-12p40, has also been reported [Marshall et al., 2011]. This discrepancy might be attributable to the use of different parasite strains, different expression system and concentrations of recombinant proteins, and macrophages of different origins.

Based on previous results and successful trials of *T. gondii*-derived enzymes as vaccine candidates, such as protein disulfide isomerase [Wang et al., 2013], calcium-dependent protein kinase-3 [Zhang et al., 2013b], and glutathione reductase [Hassan et al., 2014] and peroxiredoxin 3 [Fereig and Nishikawa, 2016b], the protective efficacy of TgPrx1 was assessed. Interestingly, mice

immunized with TgPrx1 displayed significant resistance to *T. gondii* infection suggesting the considerable immunoprophylactic potency. This study showed that TgPrx1 as an effective stimulator of macrophages and spleen cells for production of IL-12 and IFN-γ, respectively. Th1 immune cells such as macrophages, CD4+ and CD8+ T cells are predominantly triggered by the IL-12 and result in significant production of IFN-γ, which stimulates effector cells for pathogen killing [Zhu and Paul, 2008]. Moreover, TgPrx1 more potently induced specific IgG1 antibodies than the specific IgG2c antibodies. Thus, the ability of TgPrx1 is to stimulate both humoral and cell-mediated immunity against *T. gondii* infection. These immune responses induced by the naked recombinant TgPrx1 alone consistent with those in several reports revealed successful vaccine antigens against *T. gondii* infection [Cui et al., 2012, Gong et al., 2013, Tao et al., 2013, Tanaka et al., 2014]. Therefore, the future studies will focus on the combination of this protein with a potent adjuvant to improve its protective efficacy against lethal toxoplasmosis.

2-5. Summary

In this chapter, the immunogenicity and prophylactic potential of TgPrx1 was evaluated. The TgPrx1 was detected in the ascitic fluid of mice 6 days postinfection, while specific antibody levels were low in the sera of chronically infected mice. Treatment of murine peritoneal macrophages with recombinant TgPrx1 triggered IL-12p40 and IL-6 production, but not IL-10 production. In response to TgPrx1, activation of NF-kB and IL-6 production were confirmed in mouse macrophage cell line (RAW 264.7). These results suggest the immune-stimulating potentials of TgPrx1. Immunization of mice with recombinant TgPrx1 stimulated specific antibody production (IgG1 and IgG2c). Moreover, spleen cell proliferation and IFN-γ production significantly increased in the TgPrx1- sensitized cells from mice immunized with the same antigen. Immunization with

TgPrx1 also increased mouse survival and decreased cerebral parasite burden against lethal *T. gondii* infection. Thus, the current results suggest that TgPrx1 efficiently induces humoral and cellular immune responses and is useful as a new vaccine antigen against toxoplasmosis.

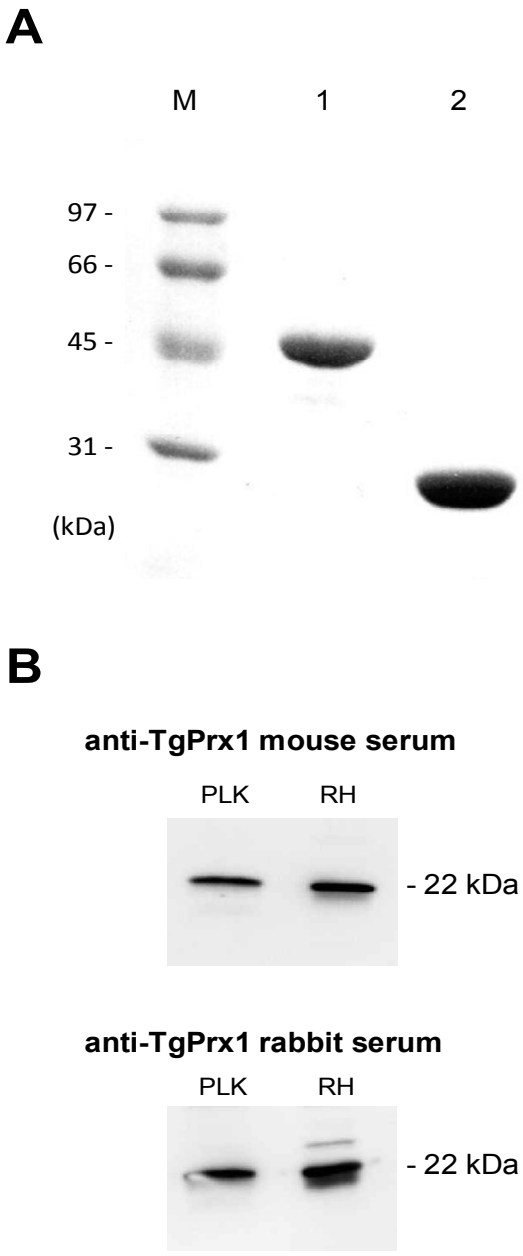

Fig. 4. Expression of recombinant proteins and detection of TgPrx1 using specific antibodies. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant proteins, with Coomassie Blue staining. Lanes: M, molecular mass marker; lane 1, TgPrx1–GST; lane 2, GST. (B) Western blot using lysate of *T. gondii* tachyzoites (RH and PLK strains) using mouse and rabbit anti-TgPrx1 sera.

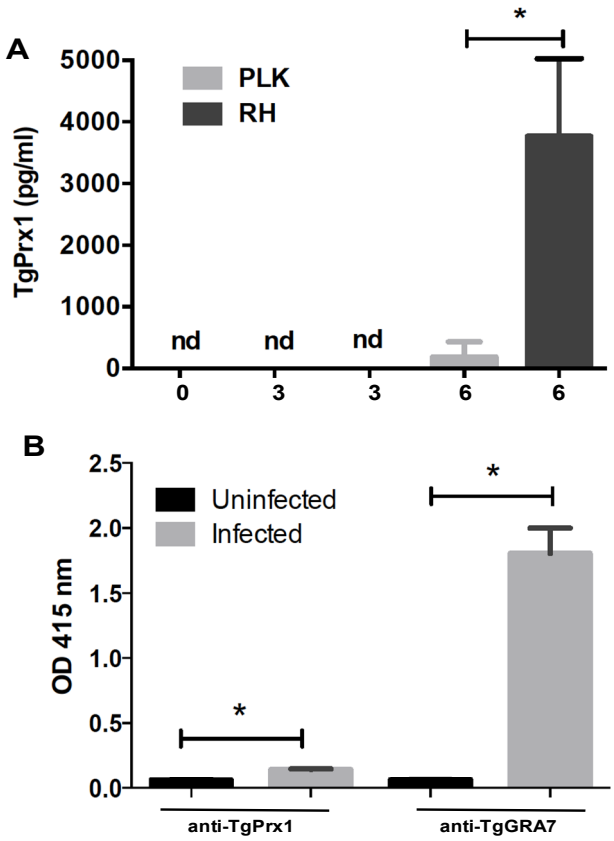

Fig. 5. Antigen and antibody levels of TgPrx1 in body fluids of infected mice. (A) C57BL/6 mice (*n* = 4) were intraperitoneally infected with 10^3 *T. gondii* PLK or RH tachyzoites. TgPrx1 antigen was measured in ascitic fluid collected from experimentally infected mice at 0, 3, and 6 dpi. Each value represents the mean ± standard deviation of quadruplicate samples. nd, not detected. *, statistically significant differences were observed between the two groups with the Student's *t* test ($P < 0.05$). (B) Production of IgG antibodies against TgPrx1 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 TgPrx1-GST 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 ($P < 0.05$).

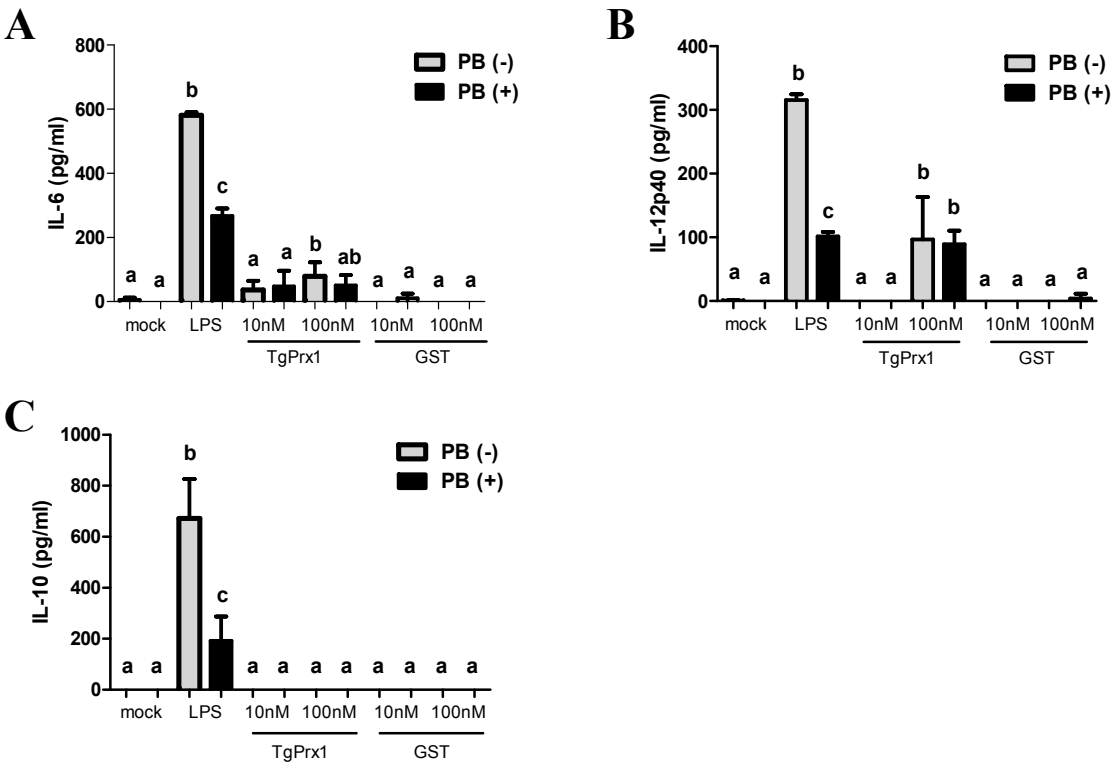

Fig. 6. Production of IL-6, IL-12p40 and IL-10 by murine peritoneal macrophages**.** Murine peritoneal macrophages were treated with 1 ng/mL LPS and recombinant TgPrx1-GST or GST protein for 20 h in the presence or absence of 1 μg/mL polymixin B. The levels of IL-6 (A), IL-12p40 (B) and IL-10 (C) value represents the mean ± standard deviation of quadruple samples. The results are representative of three repeated experiments with similar results. 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, $P < 0.05$).

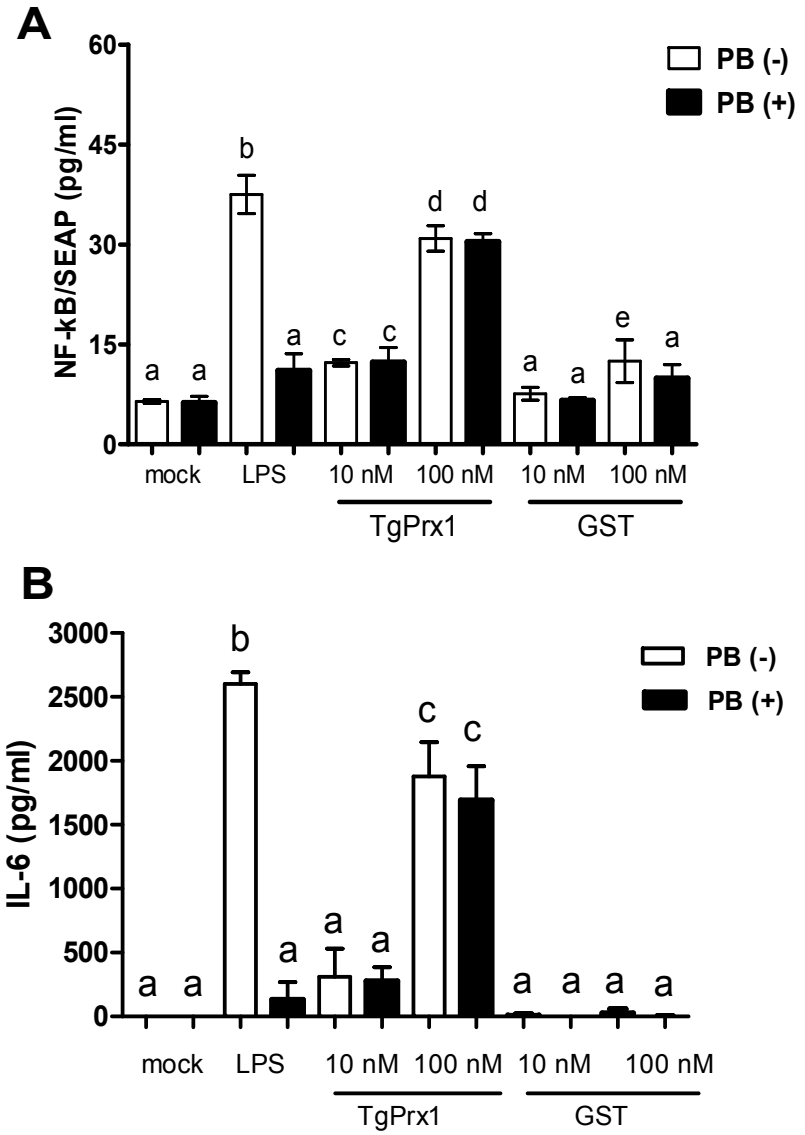

Fig. 7. Effects of recombinant TgPrx1 on RAW cell lines. NF-kB/SEAP cells (A) and RAW 264.7 cells (B) were treated with 1 ng/mL LPS and recombinant TgPrx1-GST or GST protein for 48 h in the presence or absence of 20 μg/mL polymixin B to measure the SEAP and IL-6, respectively. Each value represents the 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, $P < 0.05$).

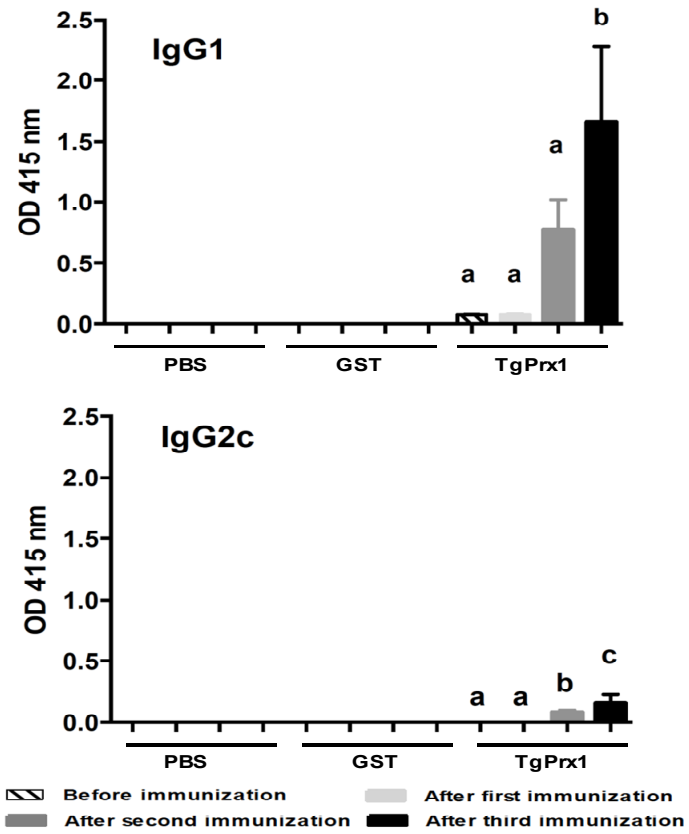

Fig. 8. Production of specific antibodies against TgPrx1. C57BL/6 mice were subcutaneously immunized with 25 pmol of recombinant protein (GST and TgPrx1-GST) or PBS (control group). Sera were collected from all mouse groups 2 days before immunization and tested for specific IgG1 and IgG2c antibodies with indirect ELISAs. The antibody responses of each experimental group were tested against the TgPrx1-GST, and GST. 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 TgPrx1-GST antigen. Each bar represents the mean ± standard deviation for the mice used per group (n = 6) and the results are representative of three 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, $P < 0.05$).

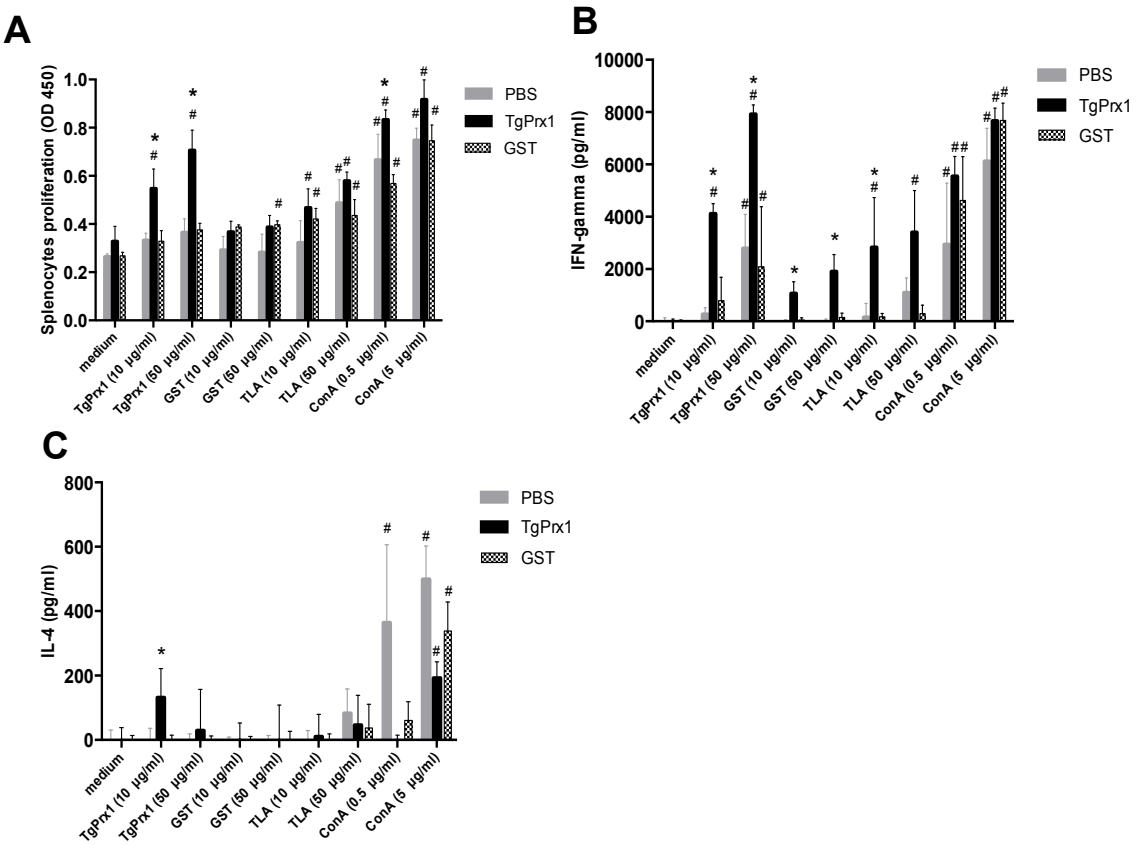

Fig. 9. Proliferation and cytokine production of spleen cells. Single-cell suspensions were prepared from the spleens of individual mice immunized with PBS, TgPrx1-GST, or GST and cultured for 48 h in the presence of Con A, TLA, TgPrx1-GST, GST, or without any stimulator (Medium). (A) Cell proliferation was measured at 48 h. (B) The culture supernatants were assayed for IFN-γ, and IL-4 production with ELISAs. Each bar represents the mean ± standard deviation (PBS; n = 3, TgPrx1 and GST; n = 4). *, statistically significant differences were observed between TgPrx1-GST-immunized group and other groups in same stimulator with two-way ANOVA and Tukey–Kramer *post hoc* analysis, $P < 0.05$. #, statistically significant differences were against medium well in same immunized group with two-way ANOVA and Tukey–Kramer *post hoc* analysis, $P < 0.05$.

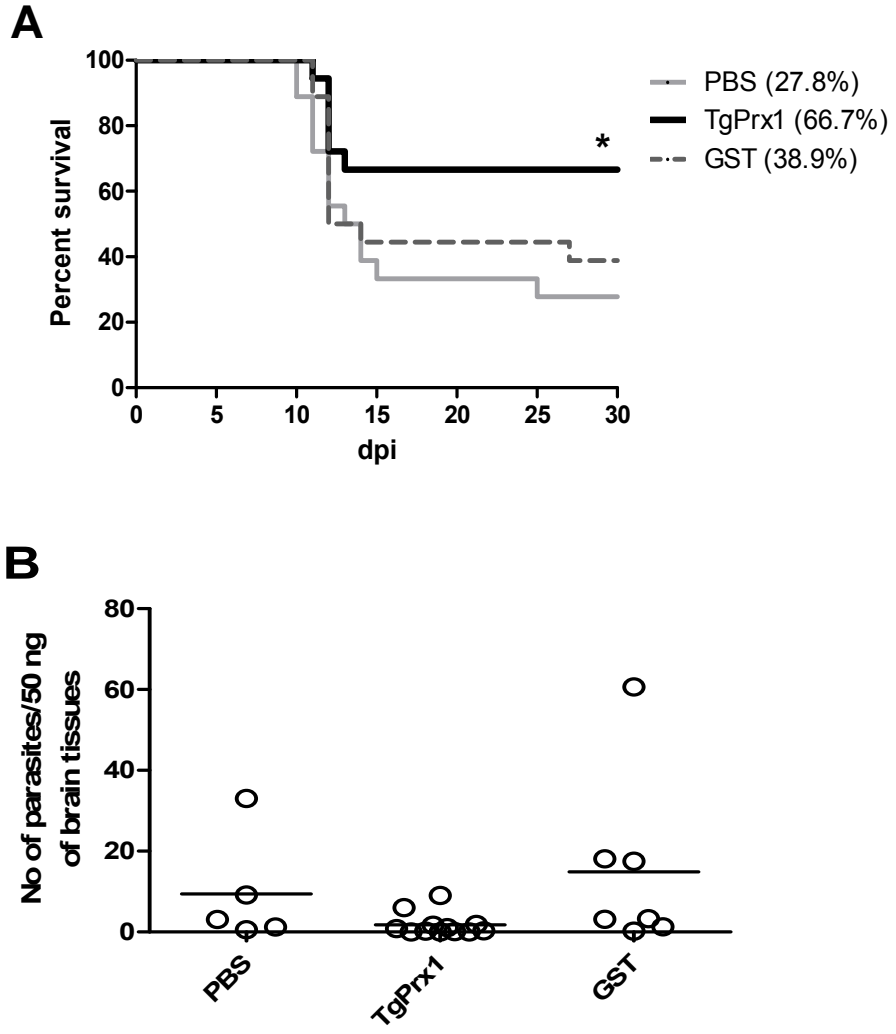

Fig. 10. Survival of mice and parasite numbers in brains of surviving mice. (A) Six mice per group were immunized with TgPrx1-GST, GST, or PBS, and then challenged with *T. gondii.* The survival rates (surviving mice/total mice) are calculated from three pooled independent experiments: PBS, 5/18 (27.8%); TgPrx1, 12/18 (66.7%); GST, 7/18 (38.9%). *, statistically significant differences in the survival rates at 30 days postinfection (dpi) were observed between the PBS-injected group and the recombinant-protein-immunized groups with a χ^2 test ($P < 0.05$). (B) Parasite numbers in the brains of the surviving mice at 30 dpi. Results are from three pooled independent experiments (PBS, $n = 5$; TgPrx1, $n = 12$; GST, $n = 7$). The results were analyzed with one-way ANOVA plus a Tukey–Kramer *post hoc* analysis, but there were no significant differences.

Chapter 3

Evaluating TgPrx3 as a novel vaccine candidate

3-1. 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 [Montoya and Liesenfeld, 2004, Dubey, 2010, Cenci-Goga et al., 2011]. Contracting an infection with this parasite is of great concern for AIDS patients and pregnant women because of the high mortality and fetal losses induced by it, respectively [Weiss and Dubey, 2009]. 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 [Schluter et al., 2014]. 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 [Dubey, 2010, Elmore et al., 2010].

It is difficult to control toxoplasmosis using the drugs currently available because of their toxic effects and limited efficacies [Peters et al., 2007, Rodriguez and Szajnman, 2012]. 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 [Buxton et al., 1993], it cannot be used at large scale because of its inadequacy of protection in other animals and worries about reversion of parasite virulence [Innes et al., 2009b]. 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 [Fereig and Nishikawa, 2016a]. 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 [Chae et al., 1994]. 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 [Ding et al., 2004, Akerman and Muller, 2005]. There have been several studies published on TgPrx1 and TgPrx2, particularly structural and functional analyses [Son et al., 2001, Ding et al., 2004, Akerman and Muller, 2005, Deponte and Becker, 2005, Sautel et al., 2009, Charvat and Arrizabalaga, 2016]. Additionally, immunological investigation of TgPrx1 revealed its strong ability to activate macrophage function and cytokine production [Marshall et al., 2011]. Although TgPrx3 may have an important role in the redox process and in protecting the parasite from cellular damage by free radicals [Hofmann et al., 2002, Woods et al., 2003], the immunomodulatory effect of TgPrx3 remains unknown.

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

3-2. Materials and methods

Ethics statement

Strictly following to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan were applied.

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.

Animals

Seven-week-old female C57BL/6J mice used for experiments of macrophage collection, infections and vaccinations were purchased from Clea Japan.

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 cultured in EMEM (Sigma) supplemented with 8% heat-inactivated FBS (Nichirei Biosciences) and 1% streptomycin–penicillin (Sigma). For tachyzoite purification, the parasites and host cell debris were washed with sterile PBS, and the infected cell monolayer was removed from the plate with a cell scraper (BD Biosciences Inc.). The final cell pellet was resuspended in RPMI 1640 medium (Sigma) and passed through a 27-gauge needle and a filter with a pore size of 5.0 μm (Millipore).

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.). 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), and an ABI PRISM 3100 genetic analyzer (AB Applied Biosystems). Nucleotide sequence of the obtained clone and target sequence from GenBank was checked and analyzed with GENETYX software (GENETYX Co., Tokyo, Japan) (Fig. 11A).

Expression and purification of TgPrx3 recombinant protein

The TgPrx3 recombinant protein was expressed as a GST fusion protein in *Escherichia coli* BL21 (DE3) cells (New England BioLabs Inc.). Protein expression was achieved at 37 °C for 8 h after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (Wako). 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), 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). Proteins for use in cell culture were filtered with a 0.45-μm low-protein binding Supor® membrane (Pall Life Sciences). The endotoxin levels in the TgPrx3-GST and GST preparations were estimated with Limulus Amebocyte Lysate (LAL) reagents (Seikagaku Inc.). The purities of the TgPrx3-GST and GST proteins were assessed to be single bands on SDS-PAGE followed by staining with Coomassie Brilliant Blue R250 (MP Biomedicals Inc.) (Fig.

11B). The protein concentrations were measured with BCA assay (Thermo Fisher Scientific, Inc.).

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 Inc.), the peritoneal macrophages were harvested by lavage with 5 mL of ice-cold PBS, twice. The cells were centrifuged at 1300 × g for 10 min and then suspended in DMEM (Sigma) containing 10% FBS. Red blood cells were removed with lysis buffer (0.83% NH4Cl; 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, LPS as a positive control, or medium alone for 20 h. To confirm the effects of the resident LPS, polymixin B (Sigma) was used.

Indirect 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 3% 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 HRP-conjugated goat anti-mouse IgG1, or IgG2c antibodies (Bethyl Laboratories) 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 Inc.). 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.

Spleen cell proliferation and cytokine responses

Spleens from the immunized mice were aseptically dissected two weeks after the last immunization, and then treated as reported previously [Nishikawa et al., 2009], 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 2.5×10^5/well in 100 μL RPMI 1640 medium. Spleen cells were stimulated with the TgPrx3-GST and GST recombinant proteins, and Con A (Sigma) 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 CCK-8 (Dojindo Laboratories) 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.

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.), 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.

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.

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 [Tanaka et al., 2013], 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 [Contini et al., 2005]. The PCR mixture (25 µL total volume) contained 1 × SYBR Green PCR buffer, 2 mM MgCl2, 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).

Statistical analyses

Data processing and analysis were performed by the GraphPad Prism 5 software (GraphPad Software Inc.). 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 $\chi 2$ 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.

3-3. Results

Protein expression

Recombinant TgPrx3-GST and GST proteins were expressed in highly pure forms (Fig. 11B). 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.

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. 12). 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. 12). However, TgPrx3-GST did not stimulate the IL-10 production (Fig. 12).

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. 13). 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 noticeable 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. 14). Splenocyte proliferation in the TgPrx3-GST-immunized mice was very obvious following stimulation with the same antigen, but not with GST alone (Fig. 14A). 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 Con A (Fig. 14B). 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. 14B). 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. 14C). Overall, these results reveal the enhanced antigen-specific cell-mediated immune responses in mice vaccinated with recombinant TgPrx3.

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 dpi in mice immunized with recombinant TgPrx3-GST (55.6%) was higher than that with GST (38.9%) or PBS (27.8%) (Fig. 15A). 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. 15B). 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.

3-4. Discussion

In this study, an attempt was performed 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 [Campos-Neto et al., 2001], and in microfilaria *Brugia malayi* infections in mice [Anand et al., 2008] as well as in infections with *Fasciola hepatica* in goats [Mendes et al., 2010]. 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 [Campos-Neto et al., 2001, Anand et al., 2008, Mendes et al., 2010]. 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 [Zhang et al., 2013a]. 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 [Wang et al., 2013], calcium dependent protein kinase 3 [Zhang et al., 2013b] and glutathione reductase [Hassan et al., 2014]. 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, its effect against macrophages was investigated. 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 [Morgado et al., 2014]. 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 [Marshall et al., 2011]. This conflict with this study might be due to the functional differences between TgPrx1 and TgPrx3 [Ding et al., 2004, Akerman and Muller, 2005].

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 [Fuhrman and Joiner, 1989, Vercammen et al., 1999, Kang et al., 2000, Johnson and Sayles, 2002]. 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 [Terkawi et al., 2013]. Although TgPrx3-specific antibodies were detected, the levels of the antibodies were significantly lower than those of TgGRA7-specific antibodies (Fig. 16). 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. The 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 developed in this study may exceed its role in humoral immunity because the control of natural *T. gondii* infection is mediated primarily by Th1 cells and cytokines [Denkers and Gazzinelli, 1998, Dupont et al., 2012]. Resistance to toxoplasmosis is largely dependent on IFN-γ released from CD4+ and CD8+ T lymphocytes [Nathan et al., 1983, Suzuki et al., 1988, Gazzinelli et al., 1991]. IFN-γ exerts anti-*Toxoplasma* activity by generating nitric oxide (NO) by inducible NO synthase [Khan et al., 1997, Lüder et al., 2003, Nishikawa et al., 2003], by disrupting the parasitophorous vacuole via immunity-related GTPases and p65 guanylate-binding proteins [Haldar et al., 2013], by inducing

tryptophan starvation via up-regulation of indoleamine 2,3-dioxygenase [Taylor and Feng, 1991], and by upregulating the production of oxygen radicals [Aline et al., 2002] and the activity of P2X7 receptors [Lees et al., 2010]. This study has 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 in this study no any adjuvant (eg. Complete Freund's adjuvant, alum) was used 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, a tendency for lower parasite burdens in the brains of the TgPrx3-immunized mice was noticed 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*.

3-5. Summary

This chapter shows the first immunological characterization of TgPrx3. *In vitro* stimulation of peritoneal macrophages with recombinant TgPrx3 protein fused to GST (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, IFN-γ 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. The current results suggest the possible use of TgPrx3 as a vaccine candidate against *T. gondii* infections.

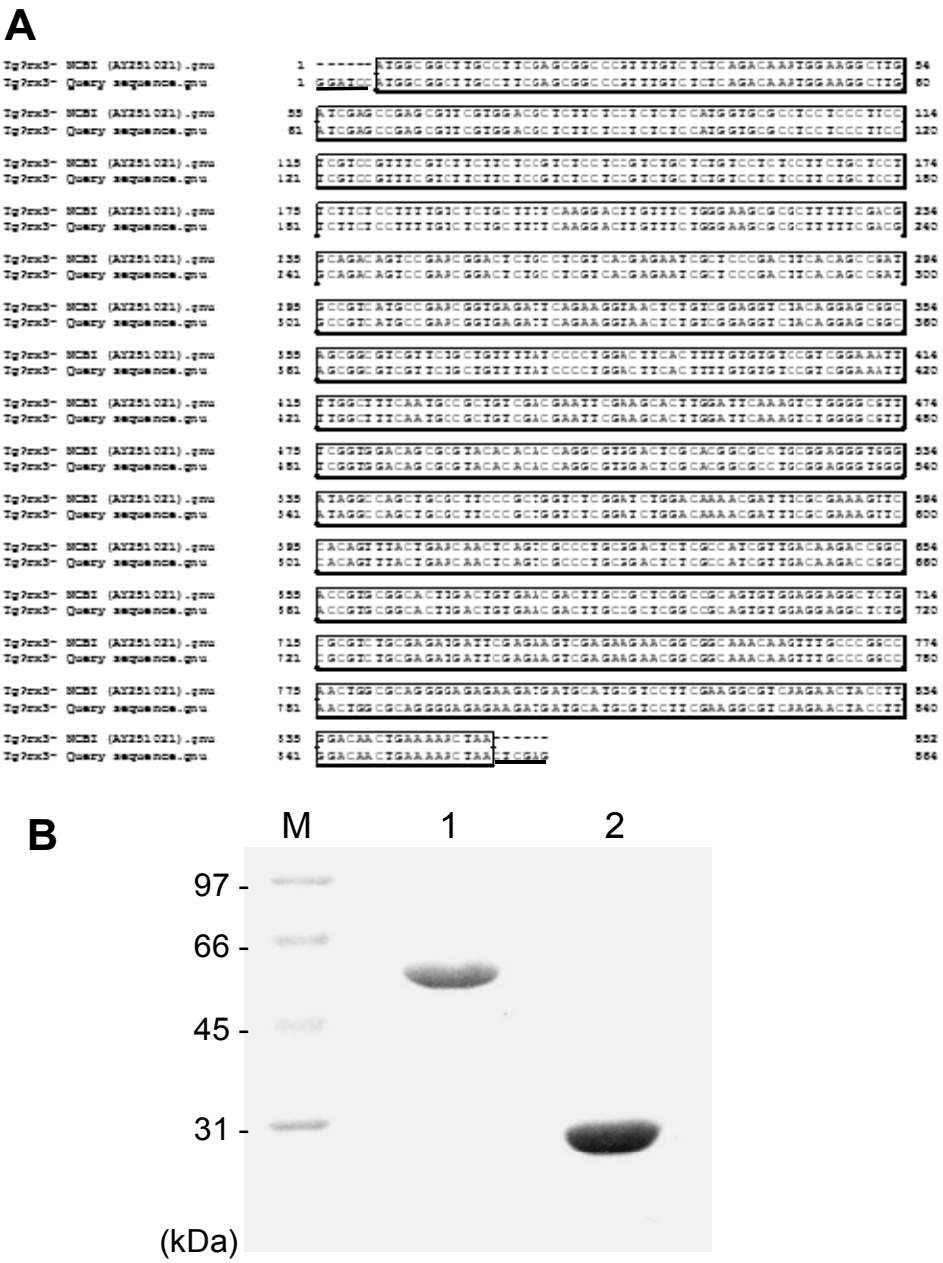

Fig. 11. Panel A. shows the sequence alignment between sequence of the cloned product (query sequence) and those that we used from GeneBank (Accession number: AY251021). The sequence analysis showed 100% identity and also recognition sites for restriction enzymes (underlined sequences); *Bam*HI (GGATCC), and *Xho*I (CTCGAG) at the sites of forward and reverse primers, respectively. 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).

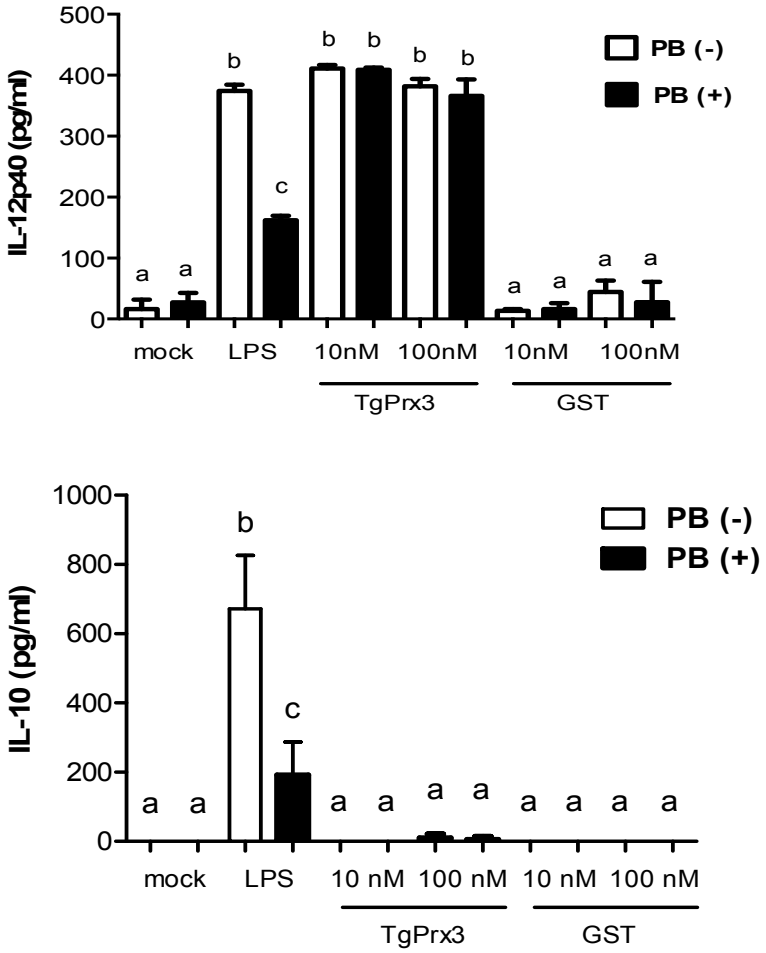

Fig. 12. IL-12p40 (A) and IL-10 (B) production by mouse peritoneal macrophages. Murine peritoneal macrophages were treated with 1 ng/mL of LPS, TgPrx3-GST or GST recombinant proteins for 20 h in the presence or absence of 1 μg/mL polymixin B (PB). The IL-12p40 and IL-10 levels were measured in the culture 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, $P < 0.05$).

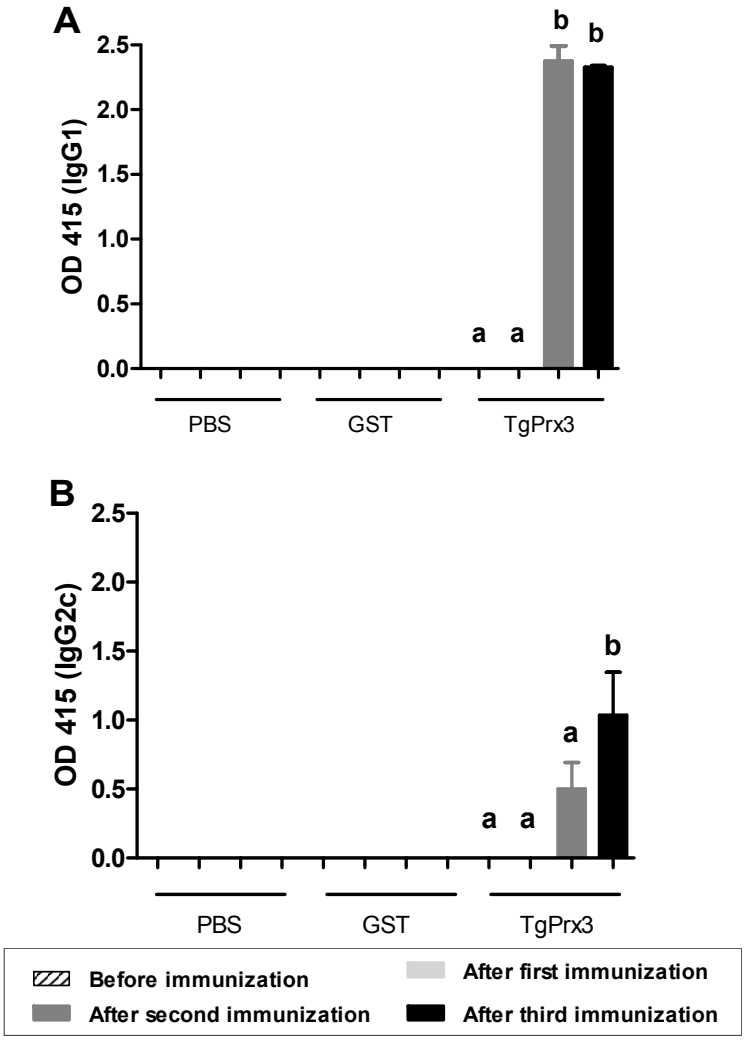

Fig. 13. Antibody production against recombinant TgPrx3. Panel (A) shows IgG1, and panel (B) shows IgG2c responses in the mice against recombinant TgPrx3, GST, and PBS. The specific antibody responses in each experimental group were tested against the TgPrx3 and GST recombinant proteins using serum samples 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, $P < 0.05$).

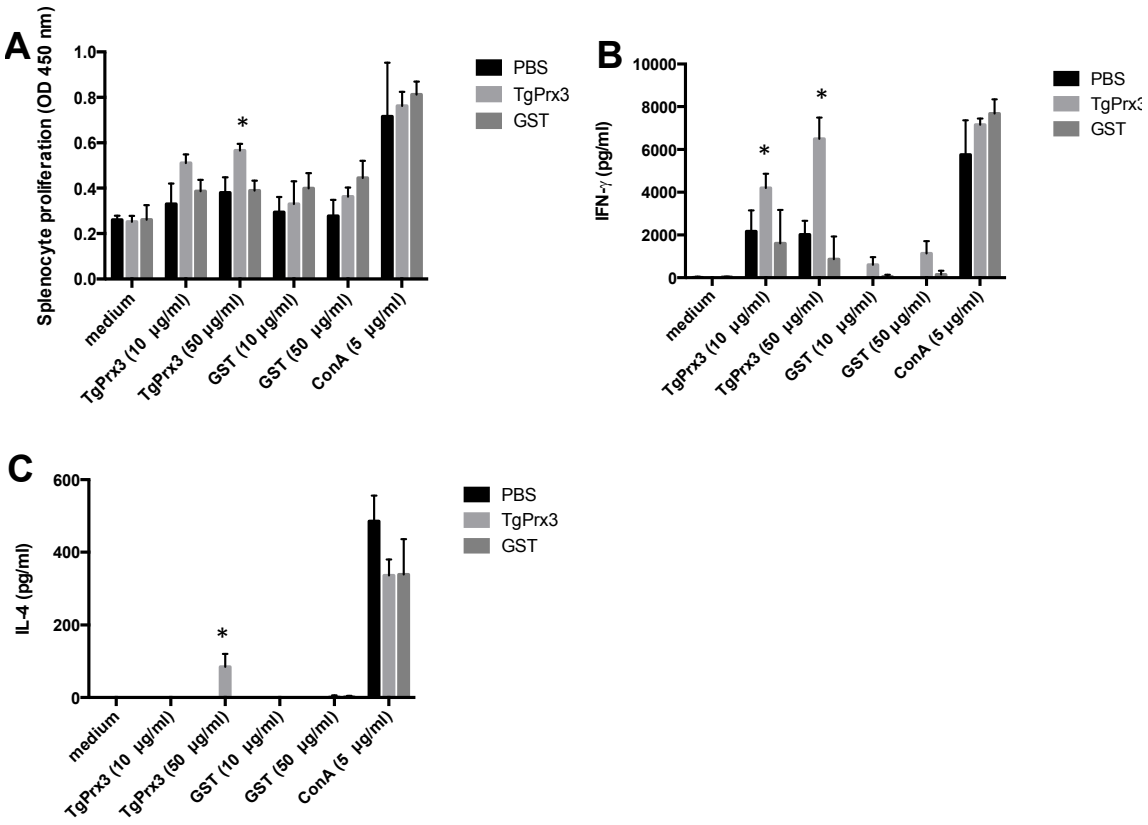

Fig. 14. Proliferation and cytokine production in spleen cells. Monolayers of spleen cells from mice immunized with TgPrx3-GST, GST or PBS were cultured in the presence of TgPrx3-GST or GST recombinant proteins, Con A, or without any stimulator (medium alone). (A) Cell proliferation was measured at 48 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, $P < 0.05$.

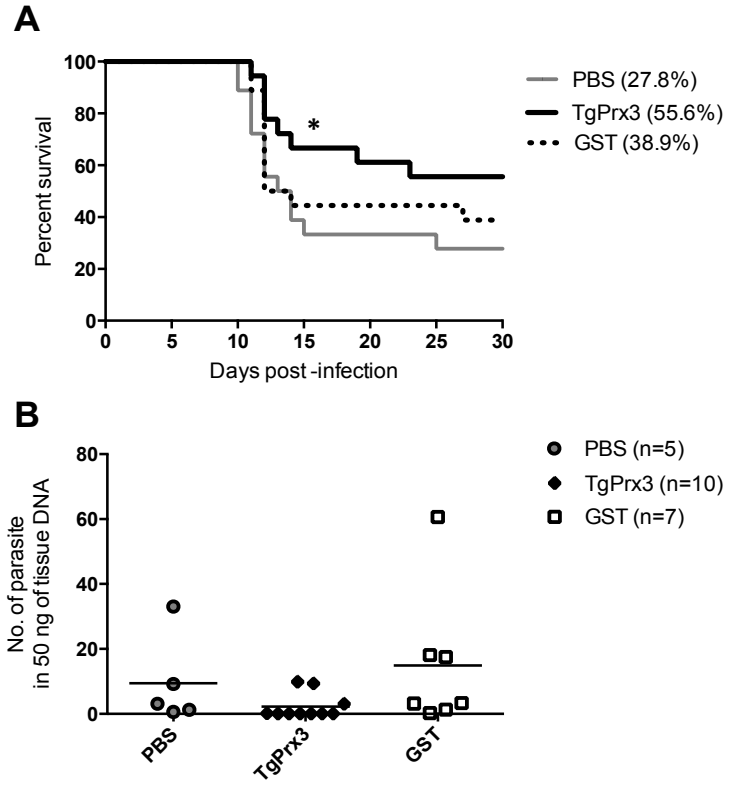

Fig. 15. Mouse survival and parasite numbers in the brains of the surviving mice. (A) Mice immunized with recombinant TgPrx3, GST, or PBS (n = 6 per trial) were challenged with *T. gondii* tachyzoites and then monitored daily until 30 dpi. The survival rates were calculated from three successive independent 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 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. 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.

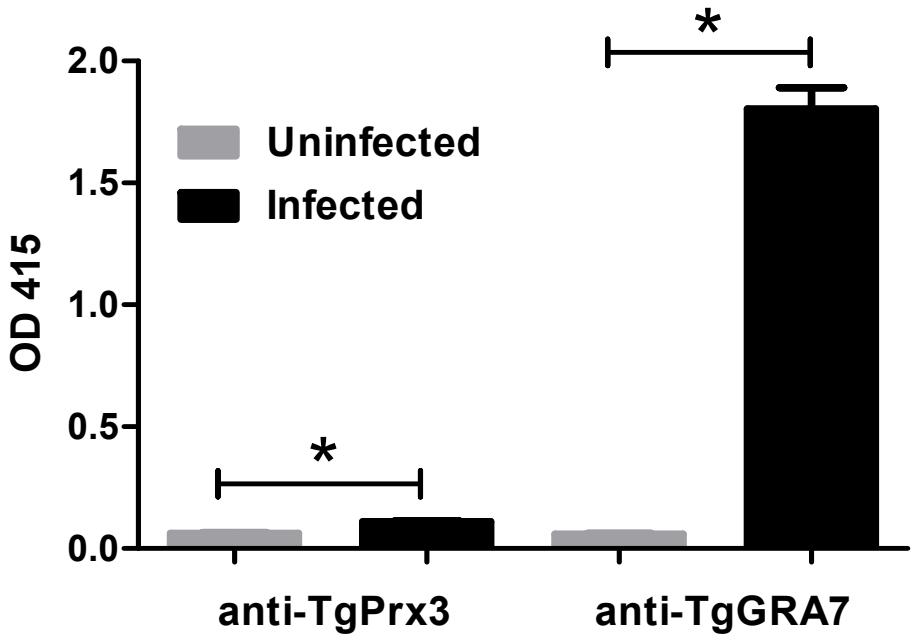

Fig. 16. Production of specific IgG antibodies against TgPrx3 in chronically infected mice. C57BL/6 mice ($n = 5$) were intraperitoneally infected with 10^3 *T. gondii* PLK tachyzoites. Serum samples were collected from the mice 4 weeks after infection and tested with indirect ELISAs, using recombinant 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 ($P < 0.05$).

General discussion

Toxoplasmosis as a foodborne infection caused by the protozoan parasite *T. gondii* is implicated in the high morbidity and mortality in both human and numerous animal species. It is transmitted via ingestion of contaminated water, meat or other foodstuffs with the infective source of the parasite such as cat-shed oocysts or tissue cysts from food animals [Hill and Dubey, 2016].

The main purpose of this study was to establish a model of control strategy against *T. gondii* infection based on development of efficient novel vaccine candidates. The accurate detection of endemic status of *T. gondii* infection is the corner stone for effective control policies, hence, the seroprevalence of specific anti-*T. gondii* antibodies is additionally performed to recognize the current status of *T. gondii* in southern Egypt among different farm animals.

The specific antibodies against *T. gondii* were surveyed in serum samples of sheep, goat, cattle and donkeys from different localities in Egypt, because such animals constitute an essential part of the agricultural sector in Egypt. In particular, the infection of sheep and goat with *T. gondii* is reportedly induces drastic economic losses because the induced abortions and fetal abnormalities [Dubey, 2010]. In chapter 1, the LAT and TgGRA7-based ELISA have been used to demonstrate the anti-*T. gondii* specific antibodies which were widely used in the studies of prevalence and epidemiology of *T. gondii* in different regions of the world and in multiple animal species [Matsuo and Husin, 1996, Kyan et al., 2012, Terkawi et al., 2013, Gu et al., 2015, Ichikawa-Seki et al., 2015]. In the same context, the obtained results in this study validated the field using of detection system based on aforementioned methods as well, and this evidenced in the recorded high kappa value, specificity and sensitivity.

The obtained data provided a comprehensive record for serostatus of anti-*T. gondii* antibodies in Egypt because of high variety of investigated animals and regions. The investigated regions were representative to all the Egyptian localities and environments; Qena and Sohag

governorates are located in the southern region, Giza and Minoufiya from the middle region and Kafr El Sheikh and Matrouh from the northern region. The high seropositive values for anti-*T. gondii* antibodies, which have been reported among the screened animals and regions, suggested the distribution and endemicity of such infection in all over the Egyptian localities and referring to the expected hazards on animals and human health.

Moreover, the risk factor analyses were conducted to detect the effects of animal species, localities and climate on *T. gondii* prevalence in Egypt. Higher seropositive rates were recorded in sheep in relation to other species particularly cattle and donkey. Kafr El Sheikh, in the far northern region of Egypt, exhibited the higher positive sera for anti-*T. gondii* antibodies than other investigated regions, suggesting that it may be attributable to the favorable environmental condition for oocyst development and infectivity, where this locality is characterized by more temperate and humid weather in comparison to other governorates [Dubey, 1998].

After accurate and comprehensive documentation of the seroprevalence of *T. gondii* in Egypt, an attempt to establish a novel control strategy via vaccine development based on potent immune stimulating antigens was conducted. According to my results, assessment of peroxiredoxin proteins derived from *T. gondii* was targeted because of the importance of such molecules in protecting the parasite from the reactive oxygen species and other free radicals which are crucial weapons of the host-immune system against invading pathogens [Ding et al., 2004, Akerman and Muller, 2005]. Two from the three previously identified TgPrxs were successfully expressed as recombinant protein fused with the GST-tag; TgPrx1 and TgPrx3. In the current study, both recombinant protein of TgPrx1 and TgPrx3 were immunologically characterized including the assessment as novel candidates of vaccine antigen. Only one study revealed an aspect of TgPrx1 as immunomodulator [Marshall et al., 2011], while no any reports about that function for TgPrx3. Regarding the study of TgPrx1 in chapter 2, prior to evaluating the protective potential,

immunobiological properties were firstly investigated. The identification of antigen and antibodies to TgPrx1 in the peritoneal fluid and sera of experimentally infected mice with *T. gondii*, respectively, indicated the potency of TgPrx1 and the possible role during *T. gondii* infection. Moreover, the recombinant protein of TgPrx1 exhibited a potent immune stimulating activity against the murine macrophages evidenced in the production of IL-12p40 and IL-6. This result was supported via the ability of TgPrx1 for activating the NF-kB-dependent pathway, which is the crucial pathway for induction of pro-inflammatory cytokines and host resistance against the parasite [Tato et al., 2003, Butcher et al., 2004]. Macrophages are considered the first line of innate immunity and play a key role in the resistance against *T. gondii* infection, which is primarily mediated with IL-12 [Morgado et al., 2014]. The potential of TgPrx1 to stimulate a protective immunity was additionally investigated by evaluating potent markers for humoral and cellular immunity.

The immunization with TgPrx1 induced the development of specific IgG1 and IgG2c. Moreover, in the splenocyte proliferation assay, antigen-specific cell proliferation and IFN-γ production were primarily observed in the TgPrx1-immunized group. Collectively, these results indicated the ability of TgPrx1 to enhance Th1 and Th2-mediated immunity which are considered the two arms for successful development of any effective vaccine candidate [Cui et al., 2012, Gong et al., 2013, Hassan et al., 2014]. As expected, the immunization of mice with TgPrx1 conferred marked protection indicated in a significantly higher survival rate of the infected mice and apparently lower parasite burden in the brain of survived mice of TgPrx1-immunized group than PBS or GST immunized groups

In chapter 3, the immunogenicity and protective efficacy of TgPrx3 was evaluated. Both TgPrx1 and TgPrx3 are 2-cys peroxiredoxins, but they have different localization of the expression, so assorted effects might be expected. The localization of TgPrx3 was identified in the mitochondrion while the TgPrx1 is cytosolic [Ding et al., 2004]. Similar to the effect of TgPrx1,

TgPrx3 is promoted the macrophage function which was recognized in the robust production of IL-12p40. No response was identified regarding IL-10 production from macrophage under the stimulation with TgPrx1 or TgPrx3, suggesting their role in inducing proinflammatory response directed towards the parasite elimination. This effect was similar to those reported for human and *Plasmodium berghei*-derived peroxiredoxins [Furuta et al., 2008, Riddel et al., 2010, Shichita et al., 2012]. However, my results were different from those identified for TgPrx1 [Marshall et al., 2011], suggesting that it may be due to using different parasite strain, origin of macrophages or experimental procedures in case of *T. gondii*. In the same context, the mice immunized with recombinant TgPrx3 exhibited substantial humoral and cellular immune response rather than the control groups. Furthermore, the TgPrx3 conferred partial protection for immunized mice after challenge with lethal dose of PLK strain of *T. gondii*. Although the immune-stimulating responses of TgPrx3 were higher than those of TgPrx1, the protective properties of TgPrx1 were higher compared with TgPrx3. This result indicated that an appropriate level of immune response would be required for successful protection against *T. gondii* infection.

General summary

Because of medical and veterinary importance, in addition to suitability for using as a model for biological, molecular and immunological studies for unicellular and intracellular pathogens, *T. gondii* is considered one of the most frequently studied pathogen of parasitic origin. This study proposed the developing of an effective control strategy for toxoplasmosis via assessing the current situation of the occurrence of *T. gondii* in Egypt and evaluating the recombinant TgPrx1 and TgPrx3 as novel vaccine candidates.

In chapter 1, a successful detection system was used to demonstrate the specific antibodies against *T. gondii* in multiple animal species and in different regions of Egypt. The LAT is a reference test for detection of *T. gondii* infection and TgGRA7-based ELISA is a widely used method for surveying of *T. gondii* in the field. In this study, only the samples with simultaneously positive result for both LAT and TgGRA7-based ELISA were considered positive. Among 652 sera obtained from animals of different localities in Egypt, 174 (25.7%) were identified as positive for anti-*T. gondii* antibodies. The prevalence in different animal species was 38.7%, 28.7%, 23.6% and 22.6% in sheep (111), goats (94), cattle (301) and donkeys (146), respectively. These results indicated the considerable occurrence of *T. gondii* infection in Egypt and implied such infection as a national challenge. Additionally, my epidemiological study revealed that sheep among other investigated animals and Kafr El Sheikh from other surveyed localities displayed the highest prevalence rate.

In chapters 2 and 3, the TgPrx1 and TgPrx3 were evaluated as immunomodulators and novel vaccine candidates. To accomplish the aforementioned goal, the recombinant TgPrx1 and TgPrx3 were expressed as GST-fusion proteins in *E. coli* and the recombinant GST was used as a control protein. The endotoxin was removed from all protein lots before subsequent use in different immunological assays either *in vitro* or *in vivo*. The role of TgPrx1 and TgPrx3 was identified in promoting the macrophages function which was evidenced in production of proinflammatory

cytokine IL-12p40 but not IL-10 which has anti-inflammatory properties. This result suggested the ability of TgPrx1 and TgPrx3 to induce the Th1-mediated immunity which is crucial for parasite restriction and elimination. Consequently, both TgPrx1 and TgPrx3 might possess a protective potential against *T. gondii* infection if they were used as vaccine antigen.

For vaccination study, C57BL/6J mice were subcutaneously injected with recombinant proteins for three times by 14 days as interval, and then challenged with lethal dose of *T. gondii* PLK strain via intraperitoneal route. The development of specific IgG1 and IgG2c antibodies against TgPrx1 or TgPrx3 was efficiently observed after the second boost immunization. Moreover, in the recalling assay using spleen cells of immunized mice, in particular, the stimulation of TgPrx1 or TgPrx3-sentitized cells with the relevant antigen revealed the highest response for splenocyte proliferation and IFN-γ production. Accordingly, these results indicated antigen-specific humoral and cellular mediated immune response for both TgPrx1 and TgPrx3. The immunized mice exhibited higher survival rate and lower parasite burden in the brain. Thus, these findings indicated the induction of resistance in the TgPrx1 and TgPrx3-immunized mice and implied their successes as novel vaccine candidates against *Toxoplasma* infection. A diagram summarized the mechanism of protection induced with immunization with TgPrx1 and TgPrx3 was illustrated in Fig. 17.

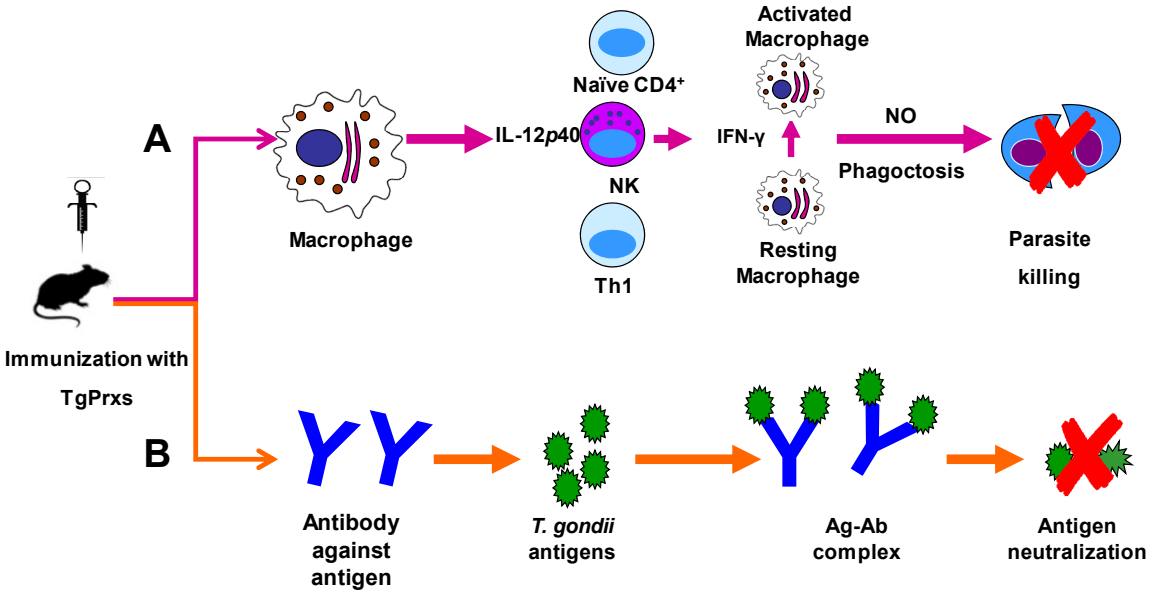

Fig. 17. Diagram showing the mechanism of protection induced with TgPrxs as subunit vaccine.

Japanese summary 和文要約

医学、獣医学的重要性と病原体の生物学的、分子免疫学的解析の有用なモデル生物として、トキソプラズマ原虫は寄生虫学領域で盛んに研究されている。本学位論文の目的は、トキソプラズマ感染およびトキソプラズマ症を効果的に制御する方法の開発を目指したものであり、エジプトにおけるトキソプラズマ感染の疫学調査と新規ワクチン抗原としてトキソプラズマ由来ペルオキシレドキシン（TgPrx1、TgPrx3）の有用性を評価した。

第一章では、エジプト全土における各種家畜動物を対象としトキソプラズマ抗体を指標とした血清疫学調査を実施した。ラテックス凝集試験（LAT）はトキソプラズマ抗体を簡便に検出する標準法であり、トキソプラズマ抗原を用いた ELISA（TgGRA7-ELISA）は所属研究室で開発されたトキソプラズマ抗体を検出する方法で多検体の解析に有効である。本研究では、LAT と TgGRA7-ELISA の両方法により陽性と判定された検体をトキソプラズマ抗体陽性として解析を行った。採取した全動物種 652 検体のうち 174 検体（25.7%）がトキソプラズマ抗体陽性であった。動物種別の陽性率は、ヒツジで 38.7%（111 検体中）、ヤギで 28.7%（94 検体中）、ウシで 23.6%（301 検体中）、ロバで 22.6%（146 検体中）がトキソプラズマ抗体陽性であった。これらの結果は、エジプト全体にトキソプラズマ感染が蔓延していることを示唆している。さらに統計学的解析により、感染率の高い動物種としてヒツジが、感染率の高い地域として Kafr El Sheikh が明らかとなった。

第二章および第三章では、TgPrx1 と TgPrx3 の免疫活性化能と新規ワクチン抗原としての有効性を評価した。使用する抗原はグルタチオン S-トランスフェラーゼ（GST）との融合タンパク質として大腸菌タンパク質発現系で作製し、コントロールタンパク質として GST を使用した。作製した精製抗原はエンドトキシンを除去し、*in vitro* および *in vivo* の免疫学的試験に使用した。TgPrx1 と TgPrx3 はマクロファージの炎症性サイトカイン IL-12p40 の産生を誘導したが、抗炎症性サイトカイン IL-10 の産生は誘導しなかった。この結果は、TgPrx1 と TgPrx3 の作用が原虫感染の防御免疫に重要な Th1 免疫を誘導することを示している。そこで TgPrx1 と TgPrx3 を免疫源として用いたワクチン評価試験を実施した。マウスを 2 週間隔で 3 回、皮下接種による免疫を行い、トキソプラズマの攻撃試験を行った。接種抗原特異的な IgG1 と IgG2c 抗体は、3 回目の免疫後に効果的に誘導された。さらに抗原を免疫したマウスから脾臓細胞を回収し抗原刺激試験を行ったところ、TgPrx1 および TgPrx3 の免疫による抗原特異的な脾臓細胞の増殖とインターフェロン・ガンマの産生が認められた。これらの結果は、TgPrx1 および TgPrx3 の免疫により抗原特異的な液性免疫と細胞性免疫が誘導されることを強く示唆している。TgPrx1 および TgPrx3 を免疫したマウスでは、コントロール群と比較してマウスの生存率の上昇とマウス脳内の原虫数の抑制が認められた。以上のことから、TgPrx1 および TgPrx3 はトキソプラズマ感染を制御する新規ワクチン抗原としての可能性が示された。

本研究の成果により、エジプトにおけるトキソプラズマ感染の実態とその感染を制御す

る予防戦略を提示することができた。本研究の成果をさらに発展させていくことで、トキソプラズマ感染の対策が必要な地域を特定し、そこで使用可能なワクチン開発を進めていくことが期待される。

Dedication

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