Development of Novel Vaccines against *Toxoplasma gondii* Infection

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Abbreviations

AIDS: Acquired immunodeficiency syndrome

AMA: Apical membrane antigen

APC: Antigen presenting cell

BCG: Bacille Calmette-Guerin

CMV: Cytomegalovirus

ConA: Concavanalin A

CSP: Circumsporozoite protein

CTL: Cytotoxic T lymphocyte

DC: Dendritic cell

DHFR-TS: Dihydrofolate reductase-thymidylate synthase

ELISA: Enzyme-linked immunosorbent assay

ESA: Excretory-secretory antigen

FBS: Fetal bovine serum

GFP: Green fluorescent protein

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GRA: Dense granule antigen

GST: Glutathione *S*-transferase

HIV: Human immunodeficiency virus

HRP: Horseradish peroxidase

IFAT: Immunofluorescence antibody test

IFN-γ: Interferon gamma

IgG: Immunoglobulin G

IL: Interleukin

i.m.: intramuscular

i.p.: intraperitoneal injection

IPTG: Isopropyl-β-D-thiogalactopyranoside

kDa: Kilodalton

KMP-11: Kinetoplastid membrane protein-11

LC: Langerhans cell

McAb: Monoclonal antibody

MEM: Minimum essential medium

MIC: Microneme antigen

ORF: Open reading frame

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PRF: Profilin

RBC: Red blood cells

ROP: Rhoptry antigen

SAG: Surface antigen

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TgGRA4: *Toxoplasma gondii* dense granule antigen 4

TgPRF: *Toxoplasma gondii* profilin protein

TgSAG1: *Toxoplasma gondii* surface antigen 1

TLA: *Toxoplasma gondii* lysate antigen

TLR: Toll-like receptor

General introduction

1. *Toxoplasma gondii* and toxoplasmosis

Toxoplasma gondii is an obligate intracellular protozoan parasite that belongs to the phylum Apicomplexa, subclass coccidian (Cornelissen et al., 1984; Tenter et al., 2000). *T. gondii* infects most species of domestic animals, birds and humans in most part of the world (Dubey, 2000; Lehmann et al., 2003; Kravetz et al., 2005). It has been found that nearly one-third of the human are infected with *T. gondii*. Although *T. gondii* infection is usually asymptomatic in immunocompetent individuals, it is life-threatening in those immunocopromised due to AIDS or receiving immunosuppressive therapy due to malignancies or post-transplantation (Montoya et al., 2004; Sensini et al., 2006).

T. gondii was first identified over 100 years ago in the tissues of birds and mammals. In 1908, Nicolle and Manceaux reported the first description of the asexual tachyzoite forms in the North African rodent (Nicolle and Manceaux, 1908). The cat was identified as the definitive host by several groups working independently including Frenkel in 1970 (Frenkel et al., 1970). There are three infectious stages of *T. gondii* for the hosts: tachyzoites, bradyzoites (in tissue cysts), and sporozoites (in oocysts). Sexual stages of the parasite occur within gut epithelial cells of the cat, and the products of gamete fusion, the oocysts, which are shed in the feces. Once in contact with the atmosphere, the oocysts sporulate and become infective to other definitive or intermediate hosts. In the intermediate host, after infection of intestinal epithelial cells, the infective stages (oocysts or bradyzoites) transform into tachyzoites, which display rapid multiplication by endodyogeny within an intracellular parasitophorous vacuole. When the cells become packed with tachyzoites, the host cell plasma membrane ruptures and

parasites are released into the extracellular milieu. The free tachyzoites can then infect virtually any nucleated cell they encounter, and they continue intracellular replication, spreading throughout host tissues. If not controlled by the immune system, tachyzoites are highly virulent and cause a generalized toxoplasmosis which is always fatal (Frenkel and Escajadillo, 1987; Frenkel, 1988). Indeed, many studies show that normally avirulent strains of *T. gondii* are highly virulent in T-lymphocyte-deficient animals (Gazzinelli et al., 1993). Therefore, induction of T-cell-mediated immune responses and resistance to the tachyzoite stage is a key step in the *T. gondii* life cycle, determining the survival of the intermediate host and the parasite itself (Gazzinelli et al., 1991; Gazzinelli et al., 1993).

T. gondii can cause several clinical syndromes including encephalitis, chorioretinitis and congenital infection. Although infection of immunocompetent humans is usually asymptomatic, toxoplasmosis still presents as serious medical problem. Infection during pregnancy can result in neurologic and ocular complications in the fetus. *T. gondii* infection is widespread in humans and prevalence varies with geography and increase in age. The most serious problem in AIDS is the recrudescence of latent infection, often resulting in a fatal encephalitis (Denkers and Gazzinelli, 1998). In domestic animals, especially pigs, sheep and goats, the parasite can cause abortions, stillbirth and neonatal loss resulting in significant losses to the livestock industry (Buxton, 1998; Dubey, 2000). In addition, the tissue cysts of *T. gondii* in meat of infected livestock are an important source of infection for humans (Wyss et al., 2000; Dautu et al., 2007).

The diagnosis of congenital toxoplasmosis relies mainly on detecting specific antibodies in patient serum and on the isolation of *T. gondii* DNA from amniotic fluid and many serological screening methods can detect IgG and IgM that are specific for *T. gondii* (Liesenfeld, 2001; Roberts et al., 2001). On the other hand, more emphasis on prevention is needed because

currently available antibiotic therapy seems to have little effect on mother-to-child transmission, and whether treatment affects the clinical manifestations in the newborn with congenital toxoplasmosis is still under debate (Thïbaut et al., 2007). Thus, a vaccine against *T. gondii* would be valuable for preventing both human and animal infections.

2. Vaccines for toxoplasmosis

Toxoplasmosis can be treated with the combination of pyrimethamine plus sulfadiazine. However, there is a significant occurrence of adverse reactions to this therapy, especially in human immunodeficiency virus (HIV)–infected patients, which often results in discontinuation of therapy and relapse of disease (Veenstra et al., 1997). It was previously mentioned that a primary infection with *T. gondii* could give the host a protective immunity against re-infection (Desolme et al., 2000). Therefore, these considerations are compelling arguments for the development of a vaccine against toxoplasmosis. A live vaccine for sheep based on the attenuated S48 strain has been licensed in Europe and New Zealand (Buxton et al., 1991); however, concern that it might revert to a pathogenic strain makes it a poor vaccine candidate for humans. Thus, the development of an effective vaccine against *T. gondii* infection would be of great value to both human and animals other than sheep.

In recent years, some *T. gondii* proteins were investigated as vaccine candidates. Several researchers have shown that mice and rats immunized with the whole *T. gondii* tachyzoite extracts or specific native antigens from surface membrane or excretory-secretory antigens (ESA) conferred protection against tissue cyst or tachyzoite challenge as assessed by reduction in a number of brain cysts (Araujo and Remington, 1984; Duguesne et al., 1990; Darcy et al., 1992; Lunden et al., 1993; Debard et al., 1996; Zenner et al., 1999; Velge-Roussel et al., 2000). Therefore, significant progress has been made in the

identification of vaccine candidates which can induce protective immunity against *T. gondii* infection. A lot of work has been focused on surface antigens of tachyzoites, including SAG1 (30 kDa), SAG2 (22 kDa) and SAG3 (43 kDa). Among these surface antigens, SAG1 is one of the most promising vaccine candidates.

Interesting work has also been done on ESA of *T. gondii* and these ESA (GRA1, GRA4 and GRA7) have been shown to play an important role in the stimulation of the protective immune system in mice (Chardes et al., 1990; Cesbron-Delauw and Capron, 1993) and it was shown that they are expressed by both stages (tachyzoite and bradyzoite stages). Among these secretory antigens that have been characterized, the dense granule antigen 4 (GRA4) is considered to be a strong candidate for vaccine development. The GRA4 has been shown to induce both humoral and systemic immune responses following oral infection with *T. gondii* (Desolme et al., 2000). Vaccination with either the recombinant GRA4 protein or a plasmid encoding the GRA4 gene provided partial protection against toxoplasmosis in mice (Martin et al., 2004). In addition, the protective efficacy of the GRA4 DNA vaccine has been improved by co-inoculation of a plasmid encoding a colony-stimulating factor (GM-CSF) (Mevelec et al., 2005).

Some other secretory antigens such as microneme proteins and rhoptry proteins have been implicated as vaccine candidate. The micronemal proteins MIC1, MIC2, MIC3 and MIC4 were recently shown to be suitable vaccine candidates to induce protective immunity in mice (Ismael et al., 2003; Bhopale, 2003; Beghetto et al., 2005; Lourenco et al., 2006). Rhoptry proteins are secreted by the rhoptry and expressed in tachyzoite, bradyzoite and cyst stages of *T. gondii*, which are involved in invasion of host cells, making it a promising candidate antigen for development of subunit vaccine or genetically engineered vaccine (Nigro et al., 2001; Mishima et al., 2002). It has been shown that vaccination with ROP2 can

increase the host survival rates after a lethal infection (Chen et al., 2001; Echeverria et al., 2006).

Recently many research works are focusing on developing recombinant vaccines against *T. gondii* infection. Recombinant vaccines are those in which desired antigens of the specific genes are inserted into a vector, usually a virus, that has a very low virulence. The vector expressing the antigen may be used as the vaccine, or the antigen may be purified and injected as a subunit vaccine. Advantages of recombinant vaccines are that the vector can be chosen to be not only safe but also easy to grow and store, reducing production cost. On the other hand, DNA-based vaccination is also one of the most promising strategies for the development of new generation effective vaccines against intracellular parasite. DNA vaccines are based on the direct inoculation of purified DNA in order to raise immune responses. Administration of a plasmid encoding the gene or genes for an antigenic portion results in the expression of the antigen and leads to the induction of antigen-specific immunity (Mor and Plasmid, 1998). It is known that the protection against *T. gondii* infection is associated with a type 1 response, the main effectors of which are T lymphocytes (Gazzinelli et al., 1991) and gamma interferon (IFN-γ) (Suzuki et al., 1988). Both DNA vaccine and recombinant viruses have been shown to be extremely efficient at inducing immune responses mediated by T-lymphocytes. This cellular immunity has recently been demonstrated to be of key importance for protection against malaria and toxoplasmosis (Rocha et al., 2004). Therefore, it is a promising vaccination strategy to develop a live vector vaccine and DNA vaccine against toxoplasmosis.

3. Aims of the present study

In the present study, the TgSAG1, TgGRA4 and TgPRF genes form *T. gondii* were chosen as the vaccine candidates to develop recombinant vaccine against *T. gondii* infection in mice

model. The aims of the present study are as follows: to evaluate the feasibility of the heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing GRA4 (Chapter 1); to construct a DNA vaccine with a gene encoding profilin of *Toxoplasma gondii* and evaluate its protective effects (Chapter 2); to construct a recombinant *Neospora caninum* vector expressing TgSAG1, and evaluate its protective effects (Chapter 3).

Chapter 1

A heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing TgGRA4, induced protective immunity against *Toxoplasma gondii* infection in mice

1-1. Introduction

Toxoplasma gondii is an obligate intracellular parasite that infects a wide variety of warm-blooded animals and humans. For humans, although *T. gondii* rarely afflicts most healthy individuals, it may cause abortion and congenital diseases in pregnant women and foetuses and lethal encephalitis in immunocompromised individuals (Kami and Louis, 2004; Rorman et al., 2006). *T. gondii* also causes abortion and congenital diseases in sheep and goats, with great economic losses (Haumont et al., 2000; Dubey, 2004). It was previously mentioned (Desolme et al., 2000) that a primary infection with *T. gondii* could give the host a protective immunity against re-infection and therefore, the development of effective vaccines is considered to be a realistic goal.

Attenuated live vaccine is currently available for the control of *T. gondii* infection in sheep, in which the parasites are unable to cause chronic infection (Buxton, 1993). However, it is not suitable for use in humans because of the risk of reversion to a pathogenic form (Ogra et al., 1991). The drawbacks of a live vaccine could be circumvented via the development of subunit vaccines. In this context, the search for novel antigens involved in protective immunity has mainly focused on surface antigens (TgSAGs), dense granule antigens

(TgGRAs), rhoptry antigens (TgROPs), and microneme antigens (TgMICs). Among the antigens that have been characterized, the dense granule antigen 4 (TgGRA4) is considered to be a strong candidate for vaccine development. The TgGRA4 has been shown to induce both humoral and systemic immune responses following oral infection with *T. gondii* (Chardes et al., 1990). Vaccination with either the recombinant TgGRA4 protein or a plasmid encoding the TgGRA4 gene provided partial protection against toxoplasmosis in mice (Desolme et al., 2000; Martin et al., 2004). In addition, the protective efficacy of the TgGRA4 DNA vaccine has been improved by co-inoculation of a plasmid encoding a colony-stimulating factor (GM-CSF) (Desolme et al., 2000; Mevelec et al., 2005).

Currently, several vaccination strategies have been reported to greatly enhance the immunogenicity of plasmid-delivered antigens (Shedlock and Weiner, 2000; Indresh et al., 2003). One of the most promising strategies is the sequential delivery generically referred to as a heterologous prime-boost vaccination regime (Ramshaw and Ramsay, 2000; Moore et al., 2004). A heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing the same antigen, has been shown to be effective to control *Plasmodium* ssp. in both animal models and humans (Vuola et al., 2005; Miao et al., 2006). To date, no report has described the heterologous prime-boost vaccination regime against *T. gondii* infection.

The aim of the present chapter is to evaluate the feasibility of the heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing TgGRA4, against *T. gondii*. My results demonstrate that the heterologous prime-boost vaccination regime can elicit both humoral and cellular immune responses and provide complete protection against acute and chronic *T. gondii* infections in mice.

1-2. Materials and methods

Parasite culture. *T. gondii* tachyzoites of RH and PLK/GFP (PLK expressing green fluorescence protein gene) strains were maintained in Vero cells grown in Eagle's minimum essential medium (MEM) supplemented with 8% fetal bovine serum (FBS) at 37°C in a 5% CO_2 air environment. For the purification of tachyzoites, the parasites were scraped from the flask and then passed through a 27G needle and, subsequently, a 5.0 μm filter (Millipore, USA). The parasites were then washed in phosphated-buffered saline (PBS) and stored at -30°C until use.

Cloning and expression of the TgGRA4 gene. The purified *T. gondii* tachyzoites (1 x 10^8) of the RH strain were lysed in 0.1 M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate (SDS), 0.1 M NaCl, and 10 mM EDTA and then treated with proteinase K (100 μg/ml) at 55°C for 2 hrs. The genomic DNA was extracted by phenol/chloroform followed by ethanol precipitation. The DNA pellets were dissolved in a TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and used as a template DNA for PCR. The DNA fragment encoding GRA4 was amplified by PCR using oligonucleotide primers with introduced *Eco*RI sites, 5'-ACGAATTCTACAATGCAGGGCACT-3' and 5'-ACGAATTCTCTTTGCGCATTCTTT-3'. The PCR product was digested with *Eco*RI and then cloned into the *Eco*RI site of the bacterial expression vector, pGEX-4T-3 (Promega, USA). The resulting plasmid was designated as pGEX/TgGRA4 after checking by sequencing. The TgGRA4 gene was expressed as a glutathione *S*-transferase (GST) fusion protein (rTgGRA4) in *E. coli* (BL21 strain) according to the manufacturer's instructions.

Production of anti-TgGRA4 mouse serum. One hundred micrograms of the rTgGRA4 were injected intraperitoneally into mice (ddY, 6-week-old, female; SCL, Japan) with Freund's complete adjuvant (Difco Laboratories, USA). On days 14 and 28

post-immunization, the same antigen was intraperitoneally injected with Freund's incomplete adjuvant (Difco Laboratories, USA). The anti-rTgGRA4 sera were collected 10 days after the last immunization.

Construction of a DNA vaccine expressing TgGRA4. The entire TgGRA4 gene was obtained from pGEX/TgGRA4 after digestion with *Eco*RI and ligated into the *Eco*RI site of a eukaryotic expression vector pcDNA3.1 (Invitrogen, USA) containing the CMV promoter. The resulting plasmid was designated as pcDNA3.1/TgGRA4 (pGRA4). The control plasmid pcDNA3.1/GFP (pGFP) was constructed by getting the GFP gene from pCX-EGFP (kindly provided by Dr. Miyazaki). The plasmids were purified by using a column chromatography kit (QIAGEN, Germany) according to the instructions of the manufacturer, dissolved in a TE buffer, and stored at -20°C. Vero cells were transfected with 2 μg of pGRA4 by using the cellfectin reagent (Invitrogen, USA) and harvesting the transfected cells after 48 hrs. They were then checked by IFAT (Xuan et al., 1995) and Western blot analysis (Boldbaataar et al., 2001) with anti-rTgGRA4 mouse serum as the primary antibody.

Construction of a recombinant vaccinia virus expressing TgGRA4. The recombinant vaccinia virus (VV) LC16mO strain, which expresses TgGRA4 or GFP, was constructed as follows (VV/GRA4 or VV/GFP). The TgGRA4 gene was obtained from pGEX/TgGRA4 after digestion with *Eco*RI and blunted with the Klenow Fragment (Takara, Japan) and cloned into the *Sal*I site of the vaccinia virus transfer vector, pAK8 (Nishikawa et al., 2001). For the GFP, plasmid pCX-EGFP was cut with *Eco*RI, and the fragment (732 bp) containing the GFP gene was blunted with the Klenow fragment and cloned into the *Sal*I site of the vaccinia virus transfer vector, pAK8. Rabbit kidney (RK13) cells infected with a vaccinia virus (LC16mO) were transfected with the recombinant transfer vectors by using the cellfectin reagent (Invitrogen, USA). Thymidine kinase-negative (TK-) viruses were isolated by plaque assay on

143TK- cells in the presence of 5-bromo-2'-deoxyuridine at a concentration of 100 μg/ml. The *in vitro* expression of TgGRA4 was checked by IFAT and Western blot analysis using anti-rTgGRA4 mouse serum as the primary antibody.

Animals and immunizations. Female inbred six-week-old C57BL/6 (CLEA, Japan) mice were used for immunization (n=10, Table 1). The gene gun vaccination was performed as described previously (Saito et al., 2001). The Helios Gene Gun System (Nippon Bio-Rad Laboratories, Japan) was used in accordance with the company's manual. Briefly, plasmid pGRA4 or pGFP was affixed onto gold particles (1.0 μm diameter) at a rate of 2 μg DNA per 1 mg of gold by the addition of 1 M $CaCl_2$ in the presence of 0.05 M spermidine. Then, a gene gun vaccination was performed on the shaved ventral skin of the mice using the handheld He-pulse gun at discharge pressures (400 psi), and each mouse received two shots (approximately 2 μg of DNA per mouse). Mice were immunized three times at three-week intervals. The recombinant vaccinia virus (VV/GRA4 or VV/GFP, 10^6 PFU virus/mouse) immunization was performed two weeks after the last gene gun vaccination.

Measurement of humoral responses. The total anti-rTgGRA4 antibodies (IgG) in the sera from the sixth week post-immunization were measured by ELISA with the rTgGRA4 as the antigen. Briefly, 96-well microtitre plates (Nunc, Denmark) were coated with 250 ng of rTgGRA4. Mouse sera were diluted 1:100 with PBS containing 3% skim milk and applied to the wells, followed by goat anti-mouse IgG-HRP (Bethyl Laboratories, USA) conjugate as a secondary antibody. After incubation in a substrate solution, the absorbance was measured at 415 nm.

***In vitro* spleen cell proliferation.** Three mice from each group were sacrificed just before challenge, and their spleens were removed and single-cell suspensions obtained by teasing the spleens apart. The erythrocytes in the spleen cell suspension were revolved by

RBC lysing buffer (Sigma, USA), and the remaining cells were washed and suspended in an RPMI 1640 medium (Sigma, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The cell suspension was adjusted to 5 x 10^6 cells/ml and 100 μl per well were seeded in triplicate in flat-bottomed 96-well microtiter plates. One hundred microlitres of antigen (rTgGRA4 with a final concentration of 10 μg/ml) or 5 μg/ml concavanalin A (Con A) had previously been added to the plates. The plates were incubated for 72 hrs in 5% CO_2 at 37°C. Supernatants were then stored at -70°C for cytokine quantification.

Measurement of cytokine production. Cell-free supernatants were harvested as described above and assayed for interleukin-4 (IL-4) and gamma interferon (IFN-γ) activity at 72 hrs. The IL-4 and IFN-γ concentrations were evaluated using a commercial ELISA kit according to the manufacturer's instructions (BioSource, USA). The cytokine concentrations were determined by reference to standard curves constructed with known amounts of mouse recombinant IL-4 and IFN-γ.

Challenge infection. Three weeks after the boosting, mice were challenged intraperitoneally with 20,000 *T. gondii* tachyzoites of PLK/GFP, and the survival rates of mice were monitored every day. Forty-five days after challenge, whole brains from the surviving mice were harvested and homogenized in 2 ml PBS. Brain cysts and free parasites were counted as follows: 10 μl of brain suspension was placed on a glass slide and mounted with a cover slip. Since the parasites used for challenge are carring GFP gene, the fluorescent parasites can be recognized and counted easily under the fluorescent microscope (Nikon microphot FXA, Japan) at ×40 magnification. The total number of cysts and free parasites are calculated in the whole brain.

Statistical analysis. The statistical significance of the differences for the IFN-γ production and brain free parasites loads between groups was calculated with one-way analysis of variance by using S-Plus 6 software (Insightful Co., USA). The p values less than 0.05 ($p < 0.05$) were considered significant.

1-3. Results

***In vitro* expression of TgGRA4.** The expression of TgGRA4 in mammalian cells was investigated. Vero cells were transfected with pGRA4 by using the cellfectin reagent, and, after 48 hrs, the transfected cells were checked by IFAT and Western blot analysis with anti-rTgGRA4 mouse serum as the primary antibody. RK13 cells were infected with VV/GRA4, and, after 48 hrs, the cells were harvested. IFAT and Western blot analysis with anti-rTgGRA4 mouse serum as the primary antibody was performed with both sets of transfected cells. The specific fluorescence was seen on both Vero cells transfected with pGRA4 and RK13 cells infected with VV/GRA4 (Fig. 1). In the Western blot analysis, a specific band with a molecular mass of 40 kDa, which is similar to that of the native protein in *Toxoplasma* lysed antigen (TLA), was detected in both Vero cells transfected with pGRA4 and RK13 cells infected with VV/GRA4 (Fig. 2). In addition, the TgGRA4 expressed in Vero and RK13 cells was reacted with *T. gondii*-infected mice sera (data not shown).

Humoral immune responses induced by heterologous prime-boost vaccination regimes. In order to evaluate the vaccine efficacy of TgGRA4, C57BL/6 mice were vaccinated with pGRA4 priming using a gene gun followed by recombinant vaccinia virus VV/TgGRA4 boosting. Sera from immunized mice were collected prior to challenge and analyzed by ELISA for specific anti rTgGRA4 responses. A strong IgG antibody response was found in all the groups of immunized mice from the sixth week after the first immunization,

and the response was found to increase until challenge (Fig. 3). To examine whether a Th1 and/or Th2 was elicited in immunized mice, the distribution of IgG sub-types was analyzed against rTgGRA4. A predominance of anti-*T. gondii* IgG1 over IgG2a was observed in sera from the mice with three vaccination regimes: pGRA4 + pGRA4, pGRA4 + VV/GRA4, and VV/GRA4 + pGRA4, suggesting that vaccination with the three vaccine regimes elicited a humoral Th2-dominant response (Fig. 4).

Cellular immune responses induced by heterologous prime-boost vaccination regimes. To examine the cellular immune responses elicited in mice with the prime-boost vaccination regimes, I quantified the production of the cytokines released from splenocytes of immunized mice that were restimulated with rTgGRA4 *in vitro*. The production of IFN-γ was observed in the spleen cell cultures from vaccinated mice (Fig. 5). The statistical significance of the difference for the IFN-γ production between group means was calculated using one-way analysis of variance with S-Plus 6 software, the p value was equal to 0.0025, which is highly significant, therefore I conclude that the vaccination regimes effected the production level of IFN-γ. In addition, the production level of IFN-γ in the mice vaccinated with DNA priming followed by VV/GRA4 boosting was significantly higher than those in control group, and pGRA4 + pGRA4 vaccinated group (Fig. 5, $p<0.05$). There was no detectable IL-4 production in the spleen cell cultures from mice with all heterologous prime-boost vaccination regimes.

Protective efficacy of heterologous prime-boost vaccination regimes in mice. To evaluate the protective effect of heterologous prime-boost vaccination regimes, all mice were challenged by intraperitoneal injection with 20,000 *T. gondii* tachyzoites of the PLK/GFP strain after three weeks of boosting. The mice vaccinated with pGRA4 priming using a gene gun and boosting with VV/GRA4 (pGRA + VV/GRA4) were completely protected from the *T.*

gondii challenge infection (Fig. 6). On the other hand, the mice vaccinated with pGRA4 + pGRA4 and VV/GRA4 + pGRA4 regimes were moderately protected from the *T. gondii* challenge infection (Fig. 6). In contrast, the mice vaccinated with VV/GRA4 + VV/GRA4 and the control vectors had a very low level of protection (Fig. 6). To evaluate the inhibitory effect on *T. gondii* cyst development in the chronic phase of infection, the surviving mice were sacrificed 45 days after infection, and the numbers of free parasites and cysts in the brain was determined microscopically. In the two mice surviving in the control DNA+VV group, the cyst numbers were 400 and 600, respectively, and, the cyst number in the only one mouse surviving in the VV+VV vaccinated group was 400. In contrast, the formation of cysts was inhibited in the mice vaccinated with pGRA4 + VV/GRA4, pGRA4 + pGRA4, and VV/GRA4 + pGRA4 regimes (Table 2). The number of free parasites in mice vaccinated with the pGRA4 + VV/GRA4 regime was the lowest loads as compared to other groups, and it was significantly lower than all the other groups tested except VV/GRA4+pGRA4 group (Fig. 7, $p< 0.05$).

1-4. Discussion

In the present chapter, I have demonstrated that the heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing TgGRA4 of *T. gondii*, could induce strong specific humoral and cellular immune responses in mice. The immunity acquired during the vaccination is capable of protecting the mice from *T. gondii* challenge infection with lethal dose. To my knowledge, this is the first report showing that the heterologous prime-boost vaccination regime is useful for controlling acute and chronic *T. gondii* infections in mice.

The *in vitro* expression of TgGRA4 in eukaryotic cells by either a DNA vaccine or a

vaccinia virus was confirmed with anti-rGRA4 sera and *T. gondii*-infected mouse sera. TgGRA4 expressed in eukaryotic cells has been shown to have a similar molecular mass to that of native TgGRA4 from *T. gondii*. These results are consistent with previous reports (Desolme et al., 2000; Mevelec et al., 2005). The C57BL/6 mice vaccinated with DNA vaccine pGRA4 priming and followed by vaccinia virus VV/GRA4 boosting (pGRA4 + VV/GRA4 regime) produced a strong IgG antibody response against rTgGRA4 (Fig. 3). In addition, the mice vaccinated with pGRA4 + pGRA4 and VV/GRA4 + pGRA4 regimes also produced a comparable IgG antibody response. However, the mice vaccinated with VV/GRA4 + VV/GRA4 induced detectable but not significant IgG antibody response. These results indicate that VV/GRA4 alone might not be effective to induce humoral immunity in C57BL/6 mice.

It is well known that the primary obstacle in developing vaccines for the control of *T. gondii* infection is the ability to induce strong and long-lasting cell-mediated immunity associated with IFN-γ (Kobayashi et al., 1999; Yap and Sher, 1999). In this chapter, the mice vaccinated with pGRA4 + VV/GRA4 showed higher IFN-γsecretion than mice vaccinated with pGRA4 + pGRA4 and VV/GRA4 + pGRA4 regimes (Fig. 5). These results indicate that the pGRA4 + VV/GRA4 regime is the most effective method to produce IFN-γ secretion. Interestingly, the mice vaccinated with VV/GRA4 + VV/GRA4 also showed significantly higher IFN-γ secretion than mice vaccinated with empty vectors, although this regime has been shown to be ineffective to induce an antibody response, as mentioned above.

The survival rate of vaccinated mice against a lethal *T. gondii* challenge infection is thought to be a most direct parameter for evaluating a vaccine candidate. In this chapter, all the mice vaccinated with pGRA4 + VV/GRA4 regime survived the lethal-dose challenge infection with *T. gondii* (Fig. 6). In addition, I observed a partial protective effect from the

mice vaccinated with pGRA4 + pGRA4 and VV/GRA4 + pGRA4 regimes. These results were correlated with the levels of IFN-γ produced described above. The results of IFN-γ production and the survival rate suggest that the order of the immunization is very important. The regime with DNA vaccine priming followed by vaccinia virus boosting was better than that with vaccinia virus priming followed by DNA vaccine boosting or homologous prime-boost regimes. This observation was consistent with previous reports (Irvine et al., 1997; Moore et al., 2004; Vuola et al., 2005). To evaluate the vaccine effect on cyst development in the chronic stage, the cysts or free tachyzoites in brains from surviving mice were investigated. The cysts or free tachyzoites were significantly reduced in brains from mice vaccinated with the pGRA4 + VV/GRA4 regime. Taken together, these results indicate that the heterologous prime-boost vaccination regime with DNA vaccine priming followed by vaccinia virus boosting, both expressing TgGRA4, is the most effective method to improve the protective ability of TgGRA4 for the control of acute infection as well as chronic infection in mice.

Previous studies have shown that a DNA vaccine with the TgGRA4 gene could induce Th1-dominant immunity with a higher level of IgG2a or IgG2b than IgG1 (Desolme et al., 2000; Mevelec et al., 2005). In the present chapter, the mice vaccinated with the heterologous prime-boost regime using a DNA vaccine followed by a vaccinia virus, both expressing GRA4, had Th2-dominant immunity with a higher level of IgG1 than IgG2a (Fig. 4). The conflict between previous and present results might be due to the use of a gene gun in the latter. It is known that gene gun vaccination triggers Th2-dominant immunity (Weiss et al., 2002; Kristina et al., 2003). In addition, the heterologous prime-boost regime with TgGRA4 induced a high level of IFN-γ production. These results suggest the induction of humoral and cellular immunity plays a crucial role for the control murine toxoplasmosis.

1-5. Summary

The *T. gondii* dense granule antigen 4 (TgGRA4) is known as an immundominant antigen of *T. gondii* and, therefore, is considered as a vaccine candidate. For further evaluation of its vaccine effect, a recombinant plasmid and vaccinia virus, both expressing TgGRA4, were constructed, and a heterologous prime-boost vaccination regime was performed in a mouse model. The mice immunized with the heterologous prime-boost vaccination regime showed a high level of specific antibody response against TgGRA4 and a significantly high level of IFN-γ production, and survived completely against a subsequent challenge infection with a lethal dose of *T. gondii*. In addition, the formation of cysts was inhibited in the mice vaccinated with the heterologous prime-boost vaccination regime. These results demonstrate that the heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing TgGRA4, could induce both humoral and cellular immune responses and provide effective protection against lethal acute and chronic *T. gondii* infections in mice.

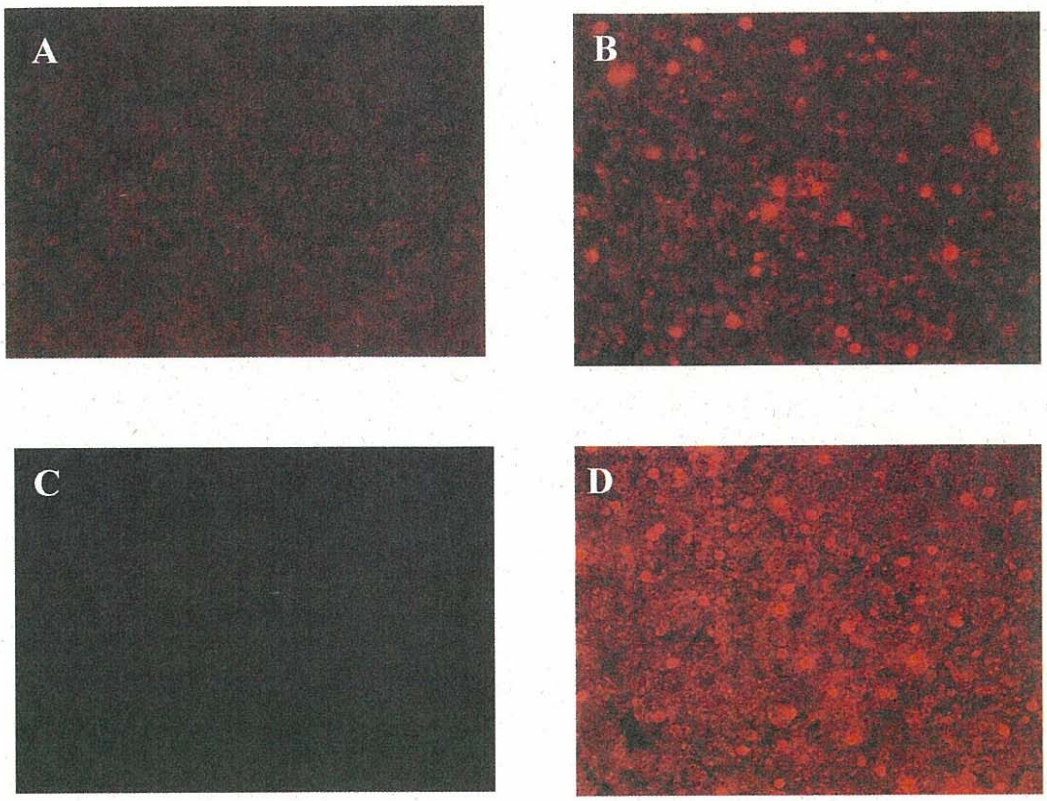

Fig. 1. The expression of TgGRA4 in vitro was confirmed by IFAT with mouse anti-rTgGRA4 serum followed by Alexa Flour-594-conjugated secondary antibodies. A, Vero cells transfected with pcDNA; B, Vero cells transfected with pcDNA/GRA4; C, RK13 cells infected by VV/GFP; D, RK13 cells infected with VV/GRA4.

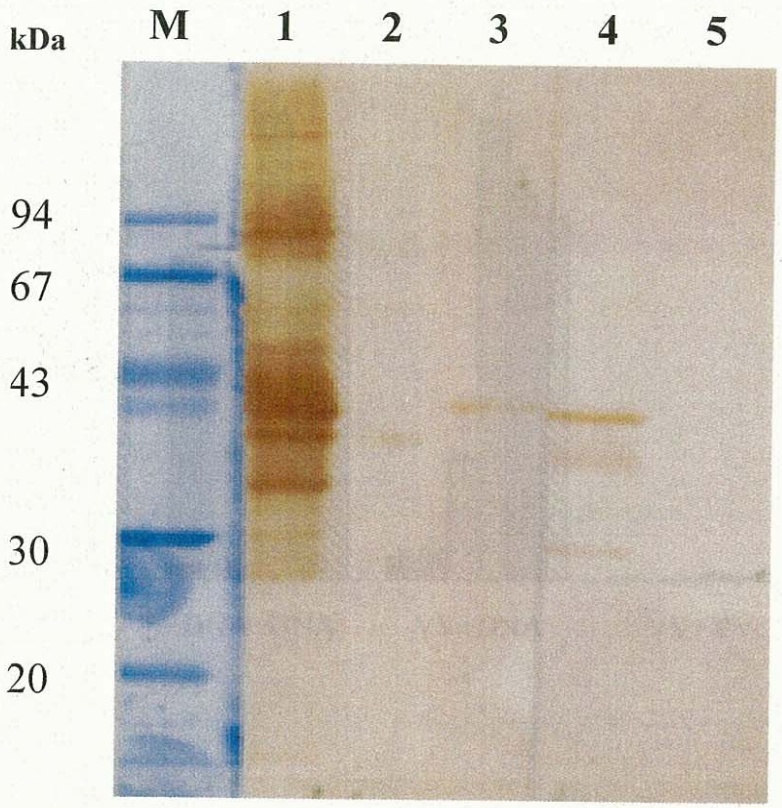

Fig. 2. Western blot analysis of the *in vitro* expression of TgGRA4 using mouse anti-rTgGRA4 serum. M, Molecular weight; lane 1, VV/GRA4-infected RK13 cells; lane 2, VV/GFP-infected RK13 cells; lane 3, *T. gondii* tachyzoites; lane 4, pGRA4-transfected Vero cells; lane 5, with pGFP-transfected Vero cells.

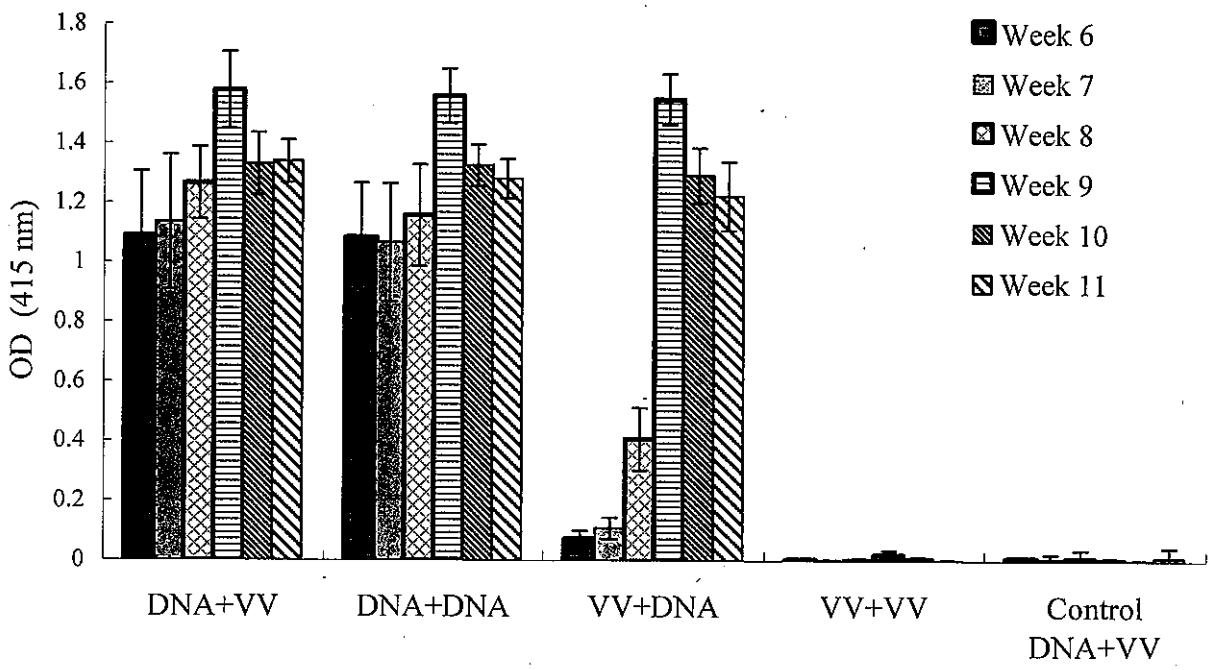

Fig. 3. Specific anti-TgGRA4 IgG antibody response induced in mice vaccinated with prime-boost regimes. Serum samples were taken from the 6th week after the first immunization and analyzed by ELISA for the detection of IgG to rGRA4. DNA, pGRA4; VV, VV/GRA4; control DNA, pGFP; control VV, VV/GFP.

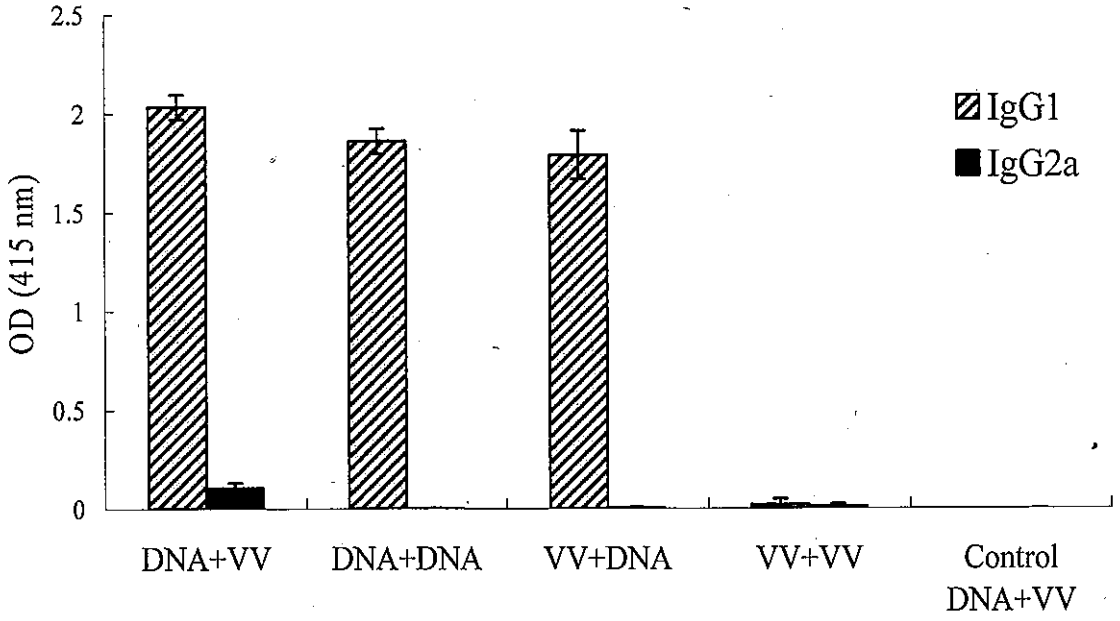

Fig. 4. Specific anti-TgGRA4 IgG1 and IgG2a antibody responses induced in mice vaccinated with prime-boost regimes. Serum was taken from the 11th week after the first immunization and analyzed by ELISA for detection of IgG1 or IgG2a to rTgGRA4. DNA, pGRA4; VV, VV/GRA4; control DNA, pGFP; control VV, VV/GFP.

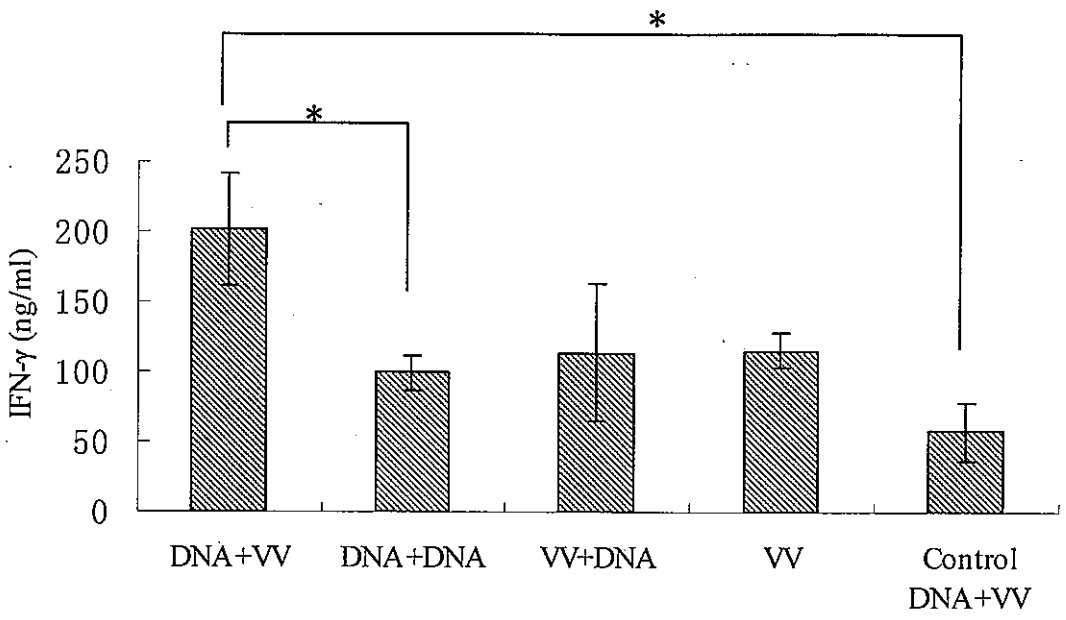

Fig. 5. IFN-γ production in splenocytes taken from mice vaccinated with prime-boost regimes. The supernatants were assayed for the presence of IFN-γ after stimulating with rTgGRA4. Each bar represents the IFN-γ production of splenocytes from three mice. The differences of IFN-γ production between experimental groups were analyzed by one-way analysis of variance and that a p value of less than 0.05 was considered significant (*, $p < 0.05$). DNA, pGRA4; VV, VV/GRA4; control DNA, pGFP; control VV, VV/GFP.

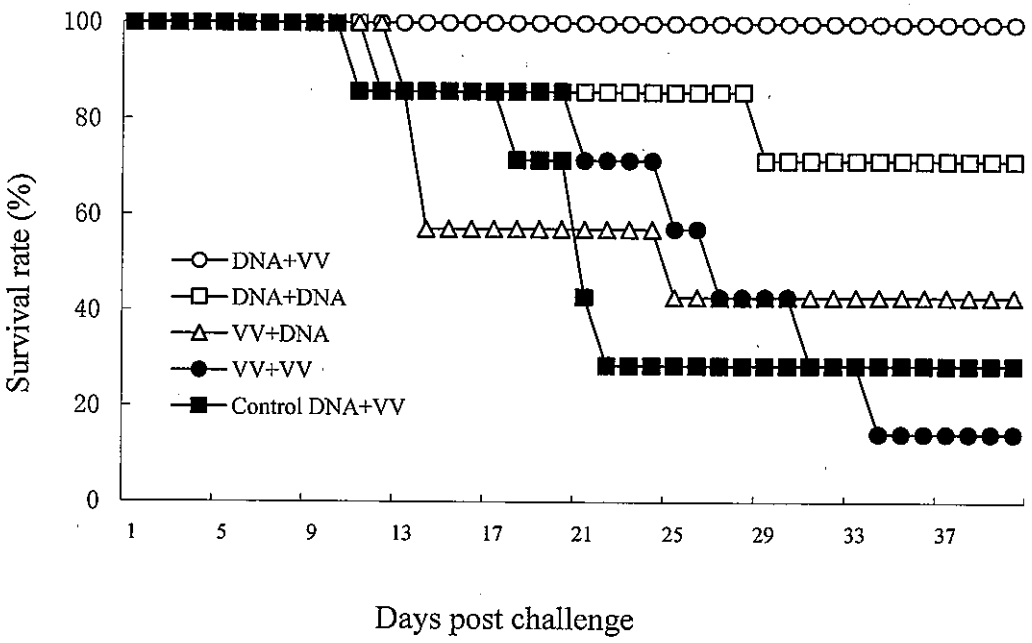

Fig. 6. Protective effects of prime-boost regimes against *T. gondii* infection in C57BL/6 mice. The mice vaccinated with prime-boost regimes were challenged with 20,000 PLK/GFP tachyzoites 3 weeks after the boosting (n = 7). DNA, pGRA4; VV, VV/GRA4; control DNA, pGFP; control VV, VV/GFP.

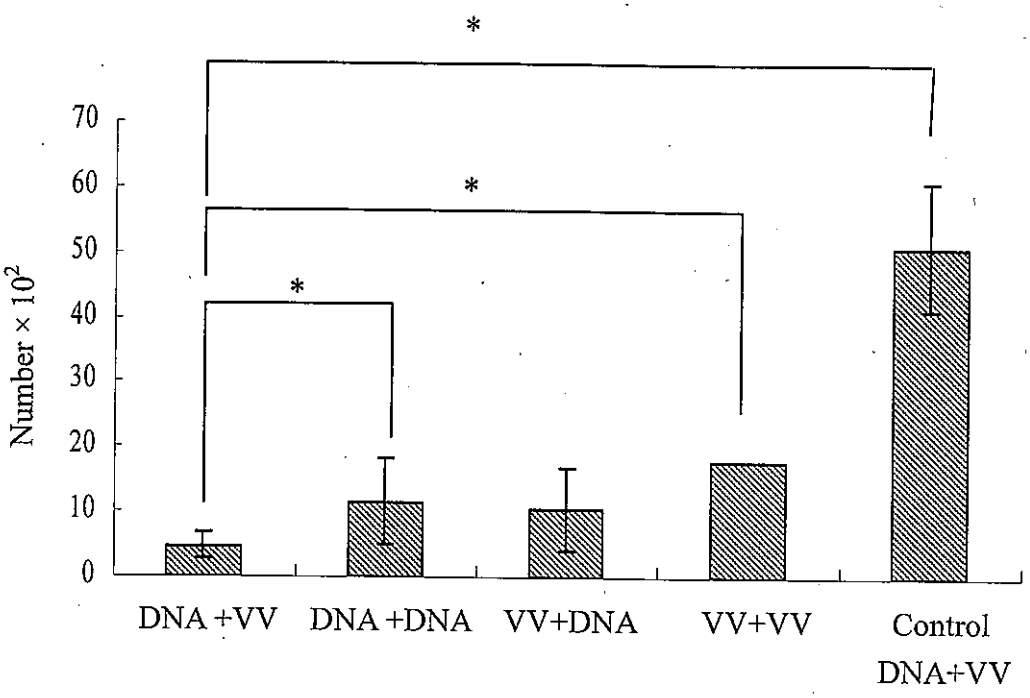

Fig. 7. The number of free parasites in the brain of surviving mice. Free parasites were determined in the surviving mice brain one month post-challenge infection (*, $p<0.05$). DNA, pGRA4; VV, VV/GRA4; control DNA, pGFP; control VV, VV/GFP.

Table 1. The immunization regimes.

Group	1st	2nd	3rd	Boost
Control DNA+VV	pGFP	pGFP	pGFP	VV/GFP
DNA+DNA	pGRA4	pGRA4	pGRA4	pGRA4
DNA+VV	pGRA4	pGRA4	pGRA4	VV/GRA4
VV+DNA	VV/GRA4	pGRA4	pGRA4	pGRA4
VV+VV	VV/GRA4	—	—	VV/GRA4

Table 2. The cyst numbers in the whole brain of survival mice.

Group	DNA+VV	DNA+DNA	VV+DNA	VV+VV	Control DNA+VV
Survival mice number	7	5	3	1	2
Cyst number/mice	0	0	0	400	400[a] 600[a]

(a, There are two mice survival in control DNA+VV group, the cyst number is 400 and 600, respectively.)

Chapter 2

Immunization with a DNA plasmid encoding theTgPRF against *Toxoplasma gondii* infection in mice

2-1. Introduction

Protective immunity against *T. gondii* mainly depends on T lymphocytes and is mediated by production of gamma interferon (IFN-γ) (Gazzinelli et al., 1991; Parker et al., 1991). DNA vaccination appears to activate all pathways of immunity especially cytotoxic T-cell responses, which have been difficult to induce with protein vaccines (Stevenson and Rosenberg, 2001). The ability of plasmid DNA encoding specific antigen to induce both $CD4^+$ and $CD8^+$ T cells suggests that this approach will be of particular usefulness for protection against diseases that require cell-mediated immunity such as intracellular protozoan infections with *Leishmania* species (Mendez et al., 2001), *Plasmodium* (Belperron et al., 1999) or *T. gondii* (Desolme et al., 2000; Vercammen et al., 2000; Saito et al., 2001) parasites.

In recent years, some *T. gondii* proteins were investigated as candidates for vaccine, most of the works are focused on surface antigens (TgSAG1, TgSAG2 and TgSAG3), dense granule antigens (TgGRA1, TgGRA4 and TgGRA7), rhoptry antigens (TgROP1 and TgROP2), and microneme antigens (TgMIC2, TgMIC4, TgM2AP and TgAMA1) (Debard et al., 1996; Desolme et al., 2000; Vercammen et al., 2000; Dautu et al., 2007). Vaccination with stage-specific antigens leads to stage-limited protection (Vercammen et al., 2000), and subunit vaccines are weakly immunogenic. To circumvent these limitations, the combination of

antigenic regions of *T. gondii* antigen protein and vector vaccines were performed to develop the effective vaccine against *Toxoplasma* infection (Debard et al., 1996; Vercammen et al., 2000; Dautu et al., 2007).

However, the immunogenicity of DNA vaccines remain to be enhanced because the immune responses induced by DNA vaccines are often weak (Alexander et al., 1996). By the use of molecular adjuvants, such as interleukin-12 (IL-12), granulocyte-macrophage colony-stimulating factor (GM-CSF), the immune responses may be enhanced and modulated (Donnelly et al., 2005; Zhang et al., 2007). Recently the inflammatory profilin protein (TgPRF) from *T. gondii* was indentified, which can induce a potent IL-12 response in murine dendritic cells (DCs) that is dependent on myeloid differentiation factor 88 (Mevelec et al., 2005; Yarovinsky et al., 2005; Denkers and Striepen, 2008). TgPRF activates DCs through Toll-like receptor (TLR) 11 and it is the first chemically defined ligand for this receptor. Moreover, TLR11 is required *in vivo* for parasite-induced IL-12 production and optimal resistance to infection, thereby establishing a role for the receptor in host recognition of protozoan pathogens (Mevelec et al., 2005; Yarovinsky et al., 2005). It has been demonstrated that profilin is an immunodominant protein in the $CD4^+$ T cell response to a soluble extract of the tachyzoite stage of the parasite as well as to live *T. gondii* infection, although it recognized as a minor component in the parasite (Mevelec et al., 2005; Yarovinsky et al., 2005). Therefore, TgPRF is thought to be a good vaccine candidate to control *T. gondii* infection.

In this chapter, a recombinant mammalian expression plasmid pcDNA3.1, expressing TgPRF (pTgPRF) is successfully constructed and delivered by gene gun into the ventral skin, over which Langerhans cells (LC)/DC are abundantly distributed, to investigate the protective effect of TgPRF against *T. gondii* infection in mice.

2-2. Materials and methods

Parasite culture. *T. gondii* tachyzoites of RH and PLK/GFP (the parasite PLK strain expressing green fluorescence protein gene) strains were maintained in Vero cells grown in Eagle's minimum essential medium (MEM) supplemented with 8% foetal bovine serum (FBS) at 37°C in a 5% CO_2 air environment. For the purification of tachyzoites, the parasites were scraped from the flask and then passed through a 27G needle and, subsequently, a 5.0 μm filter (Millipore, USA). The parasites were then washed in phosphated-buffered saline (PBS) and stored at -30°C until use.

Cloning and expression of the TgPRF gene. The purified *T. gondii* tachyzoites (1×10^8) of the RH strain were lysed in 0.1 M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate (SDS), 0.1 M NaCl, and 10 mM EDTA and then treated with proteinase K (100 μg/ml) at 55°C for 2 hrs. The genomic DNA was extracted by phenol/chloroform followed by ethanol precipitation. The DNA pellets were dissolved in a TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and used as a template DNA for PCR. The DNA fragment encoding TgPRF was amplified by PCR using oligonucleotide primers with introduced *Eco*RI and *Xho*I sites in the 5'-terminal of the primers : forward primer, 5'- TTGAATTCAGTATGT CCGACCCTGTTGTC-3'; reverse primer, 5'- TTCTCGAGTTAGTACCCAGACTGAAGATA CTC-3'. The PCR product was digested with *Eco*RI and *Xho*I, then cloned into the *Eco*RI and *Xho*I sites of the bacterial expression vector, pGEX-4T-1 (Promega, USA). The resulting plasmid was designated as pGEX/TgPRF after sequence confirmation by sequencing. The TgPRF gene was expressed as a glutathione *S*-transferase (GST) fusion protein (rTgPRF) in *E. coli* (BL21 strain). The expressed recombinant protein (rTgPRF) was purified according to the manufacturer's instructions.

Production of anti-rTgPRF mouse serum. One hundred micrograms of the rTgPRF were

injected intraperitoneally into mice (ddY, 6-week-old, female, SCL, Japan) with Freund's complete adjuvant (Difco Laboratories, USA). On days 14 and 28 post-immunization, the same antigen was intraperitoneally injected with Freund's incomplete adjuvant (Difco Laboratories, USA). The anti-rTgPRF sera were collected 10 days after the last immunization.

Construction of a DNA vaccine expressing TgPRF. The entire TgPRF gene was obtained from pGEX/TgPFR after digestion with *Eco*RI and *Xho*I, then ligated into the *Eco*RI and *Xho*I sites of a eukaryotic expression vector pcDNA3.1 (Invitrogen, USA) containing the CMV promoter. The resulting plasmid was designated as pcDNA3.1/TgPRF (pTgPRF). The control plasmid pcDNA3.1/GFP (pGFP) was constructed by inserting the GFP gene from pCX-EGFP (kindly provided by Dr. Miyazaki) into the vector pcDNA3.1. The plasmids were purified by using a column chromatography kit (QIAGEN, Germany) according to the instructions of the manufacturer, dissolved in a TE buffer, and stored at -20°C. Vero cells were transfected with 2 μg of pTgPRF or pGFP by using the cellfectin reagent (Invitrogen, USA). Transfected cell were harvested after 48 hrs and then checked by IFAT (Xuan et al., 1995) and Western blot analysis (Boldbaataar et al., 2001) with anti-rTgPRF mouse serum as the primary antibody for confirmation of the expression of TgPRF in Vero cells.

Animals and immunizations. Female six-week-old BALB/c mice (CLEA, Japan) were used for immunization (n=10, Table 1). The gene gun vaccination was performed as described previously (Saito et al., 2001). The Helios Gene Gun System (Nippon Bio-Rad Laboratories, Japan) was used in accordance with the company's manual. Briefly, plasmid pTgPRF or pGFP was affixed onto gold particles (1.0 μm diameter) at a rate of 2 μg DNA per 1 mg of gold by the addition of 1 M $CaCl_2$ in the presence of 0.05 M spermidine. Then, a gene gun vaccination was performed on the shaved ventral skin of the mice using the handheld He-pulse gun at discharge pressures (400 psi), and each mouse received two shots (approximately 2 μg of

DNA per mouse). Mice were immunized three times at three-week intervals.

Measurement of humoral responses. The total anti-rTgPRF antibodies (IgG) in the sera from the sixth week post-immunization were measured by ELISA with the rTgPRF as the coated antigen. Briefly, 96-well microtitre plates (Nunc, Denmark) were coated with 250 ng of rTgPRF. Mouse sera were diluted 1:100 with PBS containing 3% skim milk and applied to the wells, followed by goat anti-mouse IgG-HRP (Bethyl Laboratories, USA) conjugate as a secondary antibody. After incubation in a substrate solution, the absorbance was measured at 415 nm.

Challenge infection. Three weeks after the boosting, mice were challenged intraperitoneally with 10,000 *T. gondii* tachyzoites of PLK/GFP, and the survival rates of mice were monitored every day.

2-3. Results

***In vitro* expression of TgPRF.** The expression of TgPRF in mammalian cells was investigated. Vero cells were transfected with pTgPRF by using the cellfectin reagent, and, after 48 hrs, the transfected cells were checked by IFAT with anti-rTgPRF mouse serum as the primary antibody, the specific fluorescence was seen on Vero cells transfected with pTgPRF (Fig. 8). In the Western blot analysis, a specific band with a molecular mass of 25 kDa, which is similar to that of the native protein in *Toxoplasma* lysed antigen (TLA), was detected in Vero cells transfected with pTgPRF (Fig. 9). In addition, a specific band was detected also from the supernatants of the pTgPRF transfected Vero cells, suggesting that TgPRF might be a secretory protein.

Humoral immune responses induced by DNA vaccine encoding TgPRF. In order to evaluate the vaccine efficacy of TgPRF, the BALB/c mice were vaccinated with pTgPRF by a

gene gun. Sera from immunized mice were collected prior to challenge and analyzed by ELISA for specific anti-rTgPRF responses. A strong IgG antibody response was detected in the pTgPRF-immunized group from the sixth week after the first immunization, and that response was increased gradually until challenge (Fig. 10). To examine whether a Th1 and/or Th2 was elicited in immunized mice, the distribution of IgG sub-types was analyzed against rTgPRF. The similar level of anti-*T. gondii* IgG1 and IgG2a was observed in sera from the pTgPRF-immunized mice, suggesting that vaccination set a balance between Th1 and Th2 immune responses (Fig. 11).

Protective efficacy of DNA vaccine encoding TgPRF. To evaluate the protective efficacy of DNA vaccine encoding TgPRF, all mice were challenged by intraperitoneal injection with 10,000 *T. gondii* tachyzoites of the PLK/GFP strain after two weeks of the last immunization. The mice vaccinated with pTgPRF were partially (33.33%, 2/6) protected from the *T. gondii* challenge infection (Fig. 12). In contrast, there is no protection against the challenge for the mice vaccinated with pGFP, and all the mice of this group died after twenty days of challenge (Fig. 12).

2-4. Discussion

In this chapter, TgPRF was targeted as a vaccine candidate. A mammalian expression plasmid pTgPRF which expresses profilin of *T. gondii* was successfully constructed and immunized to the mice by gene gun. This DNA immunization induced strong specific antibody responses in mice and partially protected the mice against *T. gondii* challenge infection with lethal dose, indicating that TgPRF could be used as a vaccine candidate.

Profilins are mostly known for their function in actin filament polymerization across eukaryotes, and they are important in cytoskeleton assembly and myosin-based motility

(Witke, 2004; Yarovinsky et al., 2006). Recently TgPRF was identified as a parasite molecule with IL-12-inducing activity on mouse splenic DC and it was found to activate DC through mouse TLR-11 (Yarovinsky et al., 2005; Jockusch et al., 2007). Therefore, TgPRF acted as a "danger" molecule, alerting the host innate immune system to the presence of infection. Furthermore, infection of mice with TgPRF-negative parasite fails to elicit serum IL-12, a cytokine necessary for the protective IFN-γ response (Yarovinsky et al., 2005; Denkers and Striepen, 2008). On the other hand, TgPRF is indispensable for gliding motility, host cell invasion, and active egress from the host cells (Denkers and Striepen, 2008). The mice immunized with pTgPRF produced specific antibody resposes to TgPRF. The antibody induced by the immunization with TgPRF would also affected *T. gondii* invasion into the host cell. Therefore, the mice immunized with pTgPRF could be protected against the challenge of lethal dose of *T. gondii*. The production of IL-12 and IFN-γ in pTgPRF-immunized animals will be examined in the future experiment to evaluate the induction of cellular immune responses.

In order to obtain the efficacious immunogenicity of the expressed antigen, the best route was selected to deliver plasmid DNA by gene gun. It is well known that the skin is the most accessible organ of the body and an ideal target for gene therapy, compared with the musculature (Peachman et al., 2003; Plattner et al., 2008). As a barrier to prevent external agents, including pathogens, from entering the body, skin has a complex and efficient immune surveillance system, which includes LC and DC. By contrast, muscle contains few if any antigen presenting cells (APC), although some professional APC may be recruited to the muscle by local irritation following injection (Foldvari et al., 2006; Plattner et al., 2008). By targeting the body's natural defense system, skin–DNA immunization attempts to produce an efficient immune response. Nucleic acid vaccines provide recombinant DNA for protein

expression in a variety of cells, including keratinocytes, LC, and DC, which are located in the two main areas of the skin, the epidermis and the dermis (Peachman et al., 2003; Foldvari et al., 2006). It is reported that intradermal gene vaccination by gene gun directly transfects DC with the recombinant DNA, which can later be detected in draining lymph nodes where antigens are presented to T cells (Tighe et al., 1998; Foldvari et al., 2006). Therefore, direct transfection of professional APC such as LC and DC by gene gun would be the most effective event in the gene gun vaccination. In this chapter, a single delivery of 2 μg of plasmid DNA by gene gun induced high level specific antibody response in pTgPRF-immunized mice. It is reported that gene gun vaccination with DNA was highly reproducible and reliable in the induction of specific cytotoxic T lymphocyte (CTL) and the production of IFN-γ and antibodies compared with musculature injection vaccination (Saito et al., 2001). The results are also in agreement with others regarding gene gun vaccination requiring smaller amounts of DNA, which can induce a high level of immune responses (Leitner et al., 1997; Belperron et al., 1999).

However, the immunogenicity of DNA vaccines remain to be enhanced because the immune responses induced by DNA vaccines are often weak (Vercammen et al., 2000). IL-12 and the granulocyte-macrophage colony-stimulating factor (GM-CSF) are chosen to be used as genetic adjuvants to enhance the immunogenicity and protective efficacy of anti-toxoplasmosis (Donnelly et al., 2005; Zhang et al., 2007). Since *T. gondii* PRF is not only associated with the parasite gliding and invasion, but also can be recognized by TLR-11 on DC to induce the IL-12 production, TgPRF would be a good genetic adjuvant candidate and also a vaccine candidate to improve the effect of DNA vaccine.

2-5. Summary

TgPRF has recently been identified as a potent ligand recognized by TLR-11, and it is indispensable for gliding and host cell invasion. In this chapter, TgPRF was targeted as a vaccine candidate, and a mammalian plasmid pTgPRF, expressing TgPRF was successfully constructed and immunized into the mice by gene gun. This DNA immunization induced strong specific antibody responses in mice and partially protected the mice from *T. gondii* challenge infection with lethal dose, suggesting that TgPRF would be used as a vaccine candidate in the future.

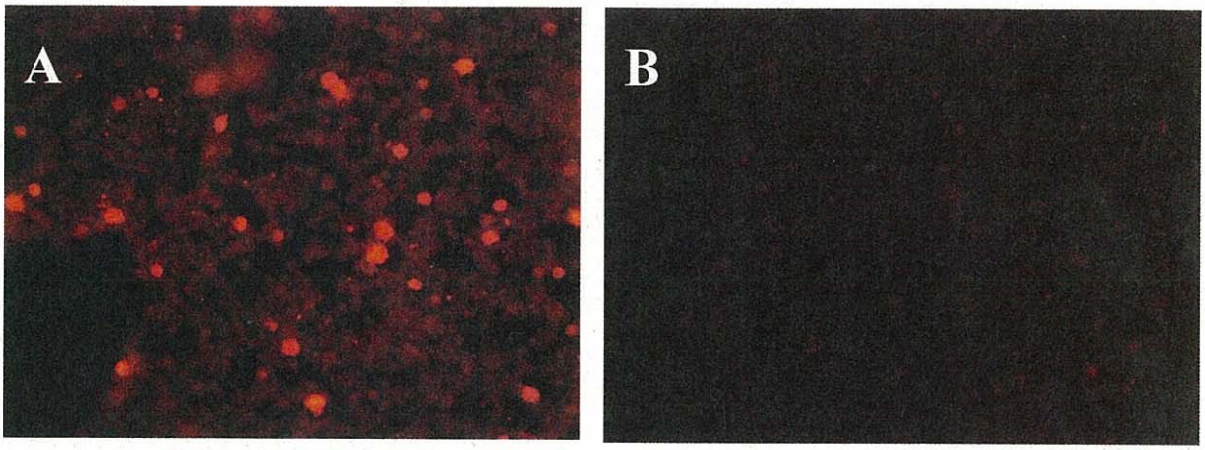

Fig. 8. The expression of TgPRF *in vitro* was confirmed by IFAT with mouse anti-rTgPRF serum followed by Alexa Flour-594-conjugated secondary antibodies. A, Vero cells transfected with pTgPRF; B, Vero cells transfected with pGFP.

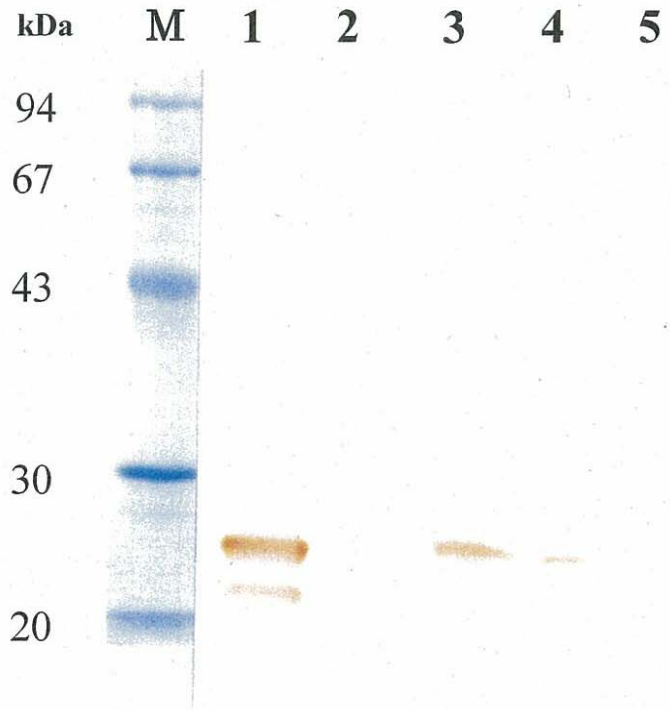

Fig. 9. The expression of TgPRF *in vitro* was confirmed by Westerblot analysis with anti-rTgPRF. M, Molecular weight; lane 1, *T. gondii* tachyzoites; lane 2, pGFP-transfected Vero cells; lane 3, pTgPRF-transfected Vero cells; lane 4. culture medium of Vero cells transfected with pTgPRF; lane 5, culture medium of Vero cells transfected with pGFP.

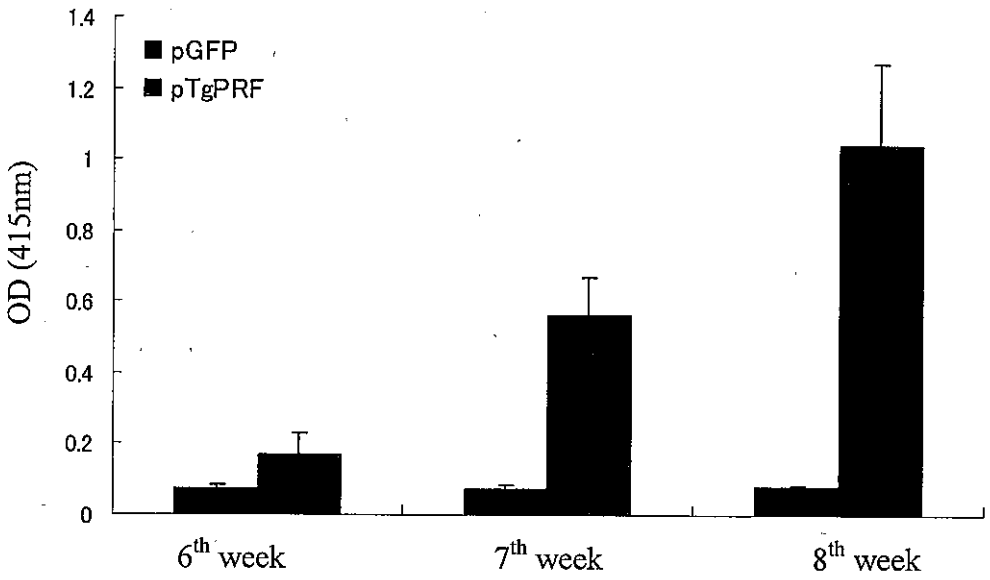

Fig. 10. Specific anti-TgPRF IgG humoral response induced in mice vaccinated with pPRF plasmid DNA were analyzed by ELISA. Samples from mice immunized with pGFP plasmid DNA was analyzed as control. Serum samples were collected from the sixth week after the first immunization.

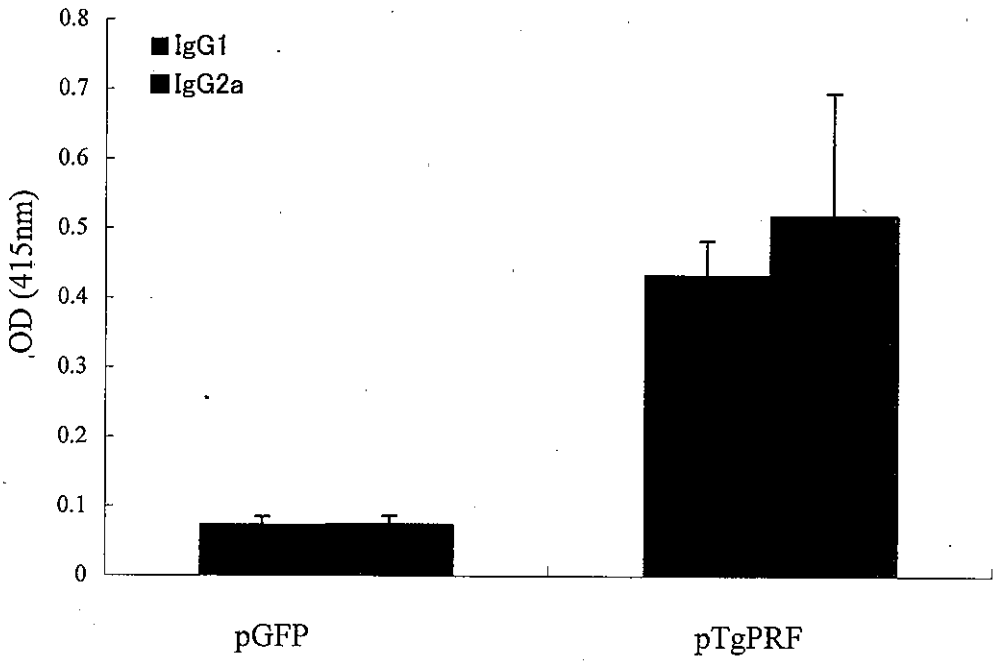

Fig. 11. Specific anti-TgPRF IgG1 and IgG2a antibody responses induced in mice vaccinated with pTgPRF plasmid DNA were analyzed by ELISA. Samples from mice immunized with pGFP plasmid DNA was analyzed as control. Serum samples were collected on the 8th week after the first immunization.

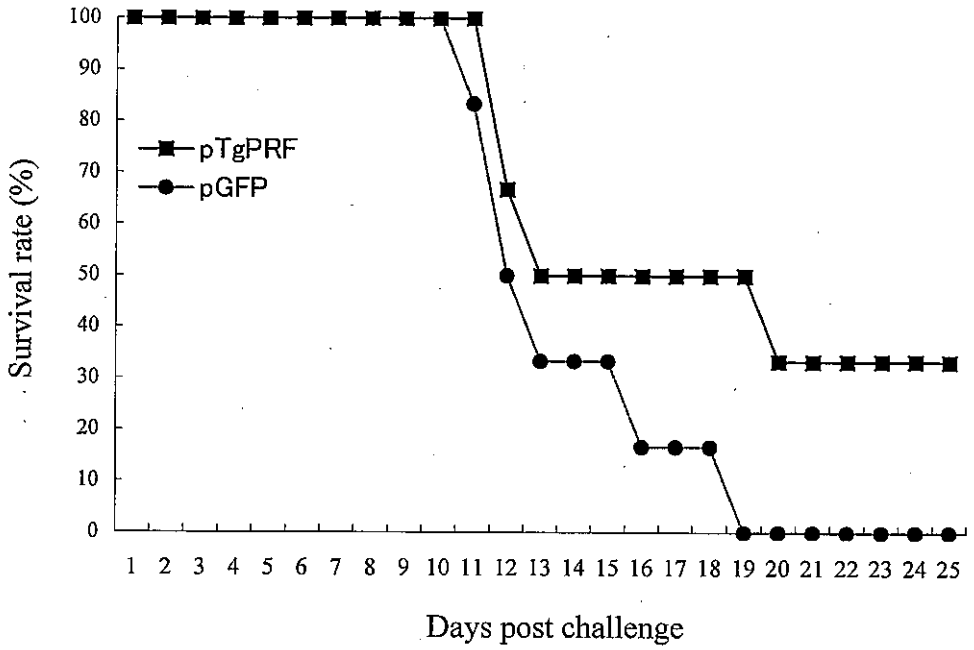

Fig. 12. Protective effects of DNA vaccine encoding TgPRF against *T. gondii* infection in BALB/c mice. All the mice were challenged with 10,000 PLK/GFP tachyzoites 2 weeks after the last immunization.

Chapter 3

Construction of recombinant *Neospora caninum* expressing TgSAG1 and evaluation of its protective effects against *Toxoplasma gondii* infection in mice

3-1. Introduction

Live vaccine vehicles can offer a potent approach for inducing protective immunity against pathogenic microorganisms. These genetically engineered vehicles provide a method for delivering heterologous vaccine antigens into the animals in a form that mimics the production of specific antibody by the carrier, exploiting its particular presentation capabilities. A variety of different attenuated viruses, bacteria and protozoa, have been utilized successfully as vaccine delivery systems in several experimental models, and a number of these constructs have been tested in clinical trials (Stover, 1994). *Neospora caninum* is an intracellular, apicomplexan parasite which is known to have highly similar biological and morphological characteristics with *Toxoplasma gondii* (Ellis et al., 1994; Dubey et al., 2007). It has been shown that a cellular and humoral responses were induced following experimental infection by *N. caninum* tachyzoites or oocysts in both non-pregnant and pregnant cattle (Lunden et al., 1998; Marez et al., 1999). Especially, T cell immune response could be induced by *N. caninum* in infected host, which is at least partially mediated by interleukin-12 (IL-12) and interferon gamma (IFN-γ) (Khan et al., 1997; Andrianarvio et al., 2001). Likewise, host resistance to *T. gondii* is highly dependent on early production of IL-12 as well as IFN-γ

produced by both $CD4^+$ and $CD8^+$ T cells. The immunity produced by *Neosproa* raised interest in its use as vaccine vector against toxoplasmosis, a disease whose control is prominently dependent on T cell activity.

In recent years, the search for novel antigens involved in protective immunity has mainly focused on surface antigens (TgSAGs), dense granule antigens (TgGRAs), rhoptry antigens (TgROPs), and microneme antigens (TgMICs). Among these vaccine candidates, the main surface antigen 1 (TgSAG1) is the best characterized. TgSAG1, which is highly conserved in *T. gondii* strains (Windeck et al., 1996), and was shown to induce both humoral and cellular immune responses (Lunden, 1995; Nielsen et al., 1999). Consequently, a number of studies utilising DNA vaccination with TgSAG1 antigen have been shown to protect mice against *T. gondii* infection (Angus et al., 2000; Mevelec et al., 2005; Xue et al., 2008).

In this chapter, TgSAG1 gene was transferred into *N. caninum* to investigate its protective efficiency against *T. gondii* infection in mice. The mice inoculated with recombinant *N. caninum* expressing TgSAG1, induced TgSAG1-specific Th1-dominant immune responses and protected the mice from a lethal challenge infection of *T. gondii*. These results indicated that the *N. caninum* could be used as live vaccine vehicle to induce strong cellular responses against a heterologous antigen.

3-2. Materials and methods

Parasite culture. *N. caninum* tachyzoites of NC-1 strain and *T. gondii* tachyzoites of RH strain were maintained in Vero cells grown in Eagle's minimum essential medium (MEM) supplemented with 8% fetal bovine serum (FBS) at 37℃ in a 5% CO_2 air environment. For the purification of tachyzoites, the parasites were scraped from the flask and then passed through a 27G needle and, subsequently, a 5.0 μm filter (Millipore, USA). The parasites were

then washed in phosphated-buffered saline (PBS) and stored at -30℃ until use.

Cloning and expression of the TgSGA1 gene. The purified *T. gondii* tachyzoites (1 x 10^8) of the RH strain were lysed in 0.1 M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate (SDS), 0.1 M NaCl, and 10 mM EDTA and then treated with proteinase K (100 μg/ml) at 55℃ for 2 hrs. The genomic DNA was extracted by phenol/chloroform followed by ethanol precipitation. The DNA pellets were dissolved in a TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and used as a template DNA for PCR amplification, which used the primers 5′-ACGAATTCGACG AGT ATG TTT-3′ and 5′-ACGAAT TCA ACG GTG ATC-3′ together with PCR buffer, dNTP and Taq polymerase. The amplified TgSAG1 gene was inserted into the *Eco*RI site of the plasmid pGEX-4T-3. The resulting plasmid was designated as pGEX-4T-3/TgSAG1 after checking by sequencing.

Construction of transfer vector pDMG/TgSAG1. The transfer vector plasmid pDMG for expression of TgSAG1 in *N. caninum* was constructed as follows. The 5′ flanking region of the dense granule protein 1 (GRA1) gene and the 3′ flanking region of the GRA2 gene were PCR amplified from the plasmid GRAGFP (kindly provided by Dr. J.C. Boothroyd, Stanford University) by using primers for GRA1-5′UTR (5′-ACG TCG ACC GAA GGC TGT AGT ACT-3′ and 5′-ACA AGC TTC TTG CTT GAT TTC TTC-3′) and for GRA2-3′UTR (5′-ACG GAT CCA AGA CTA CGA CGA AAG-3′ and 5′-ACT CTA GAG TCG ACT GGA ACT ACG-3′), respectively. The PCR products of GRA1-5′UTR and GRA2-3′UTR were digested with *Sal*I–*Hin*dIII and *Bam*HI–*Xba*I, respectively, and cloned into the corresponding sites of pBluescript KS+; pKS/GRA5′-GRA3′ was obtained. The GFP coding sequence was PCR amplified from plasmid GRAGFP by using specific primers (5′-ACG AAT TCC AAG ATG CAT AAA GGA-3′ and 5′-ACG AAT TCT TAT TTG TAT AGT TCA-3). The resulting PCR product was digested with *Eco*RI, and cloned into *Eco*RI site between GRA1-5′UTR and

GRA2-3′UTR of pKS/GRA5′-GRA3′, and then the pKS/GRA-GFP was obtained. The *SalI–XbaI* fragment (GRA5′-GFP-GRA3′) from pKS/GRA-GFP was blunt ended by Klenow fragment and inserted into blunt ended *XbaI* site of pDHFR-TSc3 (kindly provided by Dr. D.S. Roos, University of Pennsylvania), and hence, pDHFR-GFP was obtained. The *SalI–XbaI* fragment (GRA5′-multi-cloning sites (MCS)-GRA3′) from pKS/GRA5′-GRA3′ was blunt ended by Klenow fragment and inserted into the blunt ended *SpeI* site of pDHFR-GFP. The plasmid with the DHFR-GRA5′-MCS-GRA3′-GRA5′-GFP-GRA3′ fragment was designated as pDMG (Fig. 8). The TgSAG1 gene was obtained from pGEX-4T-3/TgSAG1 after digestion with *Eco*RI, blunt-ended with the Klenow Fragment, and then was cloned at the *Eco*RV site of the pDMG vector. The structure of recombinant plasmids pDMG/TgSAG1 was checked by restriction enzyme analysis (Fig. 8).

Transfection and selection of *N. caninum* expressing TgSAG1. Electroporation of *N. caninum* tachyzoites was performed as described (Sibley et al., 1994). Briefly, the freshly harvested *N. caninum* tachyzoites were resuspended ($3\sim6\times10^7$/ml) in "intracellular" electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4 (pH 7.6), 25 mM HEPES (pH 7.6), 2 mM EDTA, 5 mM $MgCl_2$, 2 mM ATP, 5 mM glutathione) and transfer 300 μl parasites (around 10^7) to a sterile 2-mm-gap (green) electroporation cuvette. Then 5~50 μg plasmid DNA was added to resuspend in 100 μl electroporation buffer and mix gently by pipetting. Electroporate parasites using Gene Pulser II (BioRad, Japan) with the following setting: 2,000 V-25 μF-50 Ω. After transfection, *N. caninum* tachyzoites were allowed to infect Vero cells in a drug-free culture medium for 18 hrs to permit the phenotypic expression of the dihydrofolate reductase (DHFR)-thymidylate synthase (TS) gene and GFP selectable markers, at which time pyrimethamine was added at a final concentration of 1 μM. Polyclonal transfected pyrimethamine-resistant tachyzoite cultures were subjected to plaque

purification. The cultures were passaged three times in the medium containing 1% agarose and a single plaque was obtained and expanded with growing in flasks for further analysis.

Immunoflourescence analysis. The parasites were scraped from the T25 flask (Nunc, Denmark) and passed through a 27G needle and, subsequently, a 5.0 μm filter (Millipore, USA), then centrifuged at 2, 000 rpm for 10 minutes, and washed with PBS one time. Then the parasites were suspended in PBS, and dropped 10 μl (approximately 2×10^5/well) for each well on the 12-well slide (Matsunami, Japan). The slide was air dried and fixed in acetone. TgSAG1 detection were performed using McAb against TgSAG1 (Advanced Immuno Chemical Inc, USA) as primary antibodies diluted 1:250 in PBS containing 3% BSA, respectively. Goat anti-mouse Alexa Flour-594-conjugated antibodies (Molecular Probes, USA), diluted 1:500 in PBS containing 3% BSA were used as secondary antibodies. Coverslips and glass slides were mounted with Mowiol (Calbiochem, Germany) and observed using an epifluorescence microscope (Microphot FXA; Nikon Inc., Japan).

Western blot analysis. The lysate of the parasites was resuspended in SDS gel-loading buffer (50 mM Tris–HCl (pH 6.8), 50 mM 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) under reducing conditions. Samples were heated at 95°C for 5 min and were then subjected to SDS-polyacrylamide gel electrophoresis. Protein bands in the gel were electrically transferred to a nitrocellulose transfer membrane (Millipore, USA). The membrane was blocked with PBS containing 3% skim milk and then incubated with McAb against TgSAG1 (Advanced Immuno Chemical Inc, USA) at 37°C for 60 min. The membrane was washed three times with PBS containing 0.1% Tween20 for 5 min and then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (Bethyl Laboratories, USA) diluted 1:4,000 with PBS containing 3% skim milk at 37 °C for 60 min. The membrane was washed three times with PBS for 5 min and then were placed into a substrate solution

containing 0.5 mg/ml diaminobenzidine and 0.005% H_2O_2 to visualize the specific antigen bands.

Vaccination and challenge infection. Three groups of BALB/c mice (n=9) were inoculated with 1×10^5 (in 0.5 ml minimum essential eagle's medium (MEM) per mouse, i.p.) tachyzoites of the live Nc/TgSAG1, Nc/GFP and four weeks later boosted with 5×10^5 tachyzoites, respectively. Another one group was inoculated only MEM (0.5 ml) as control. Three weeks after the last immunization all the mice were challenged with a lethal dose of bradyzoites of *T. gondii* Beverley strain (500 bradyzoites /mouse, i.p.).

Measurement of humoral responses. The total anti-TgSAG1 antibodies (IgG) in the sera from the sixth week post-immunization were measured by ELISA with the recombinant TgSAG1 (rTgSAG1) as the antigen. Briefly, 96-well microtiter plates (Nunc, Denmark) were coated with 250 ng of rTgSAG1. Mouse sera were diluted 1:100 with PBS containing 3% skim milk and applied to the wells, followed by goat anti-mouse IgG-HRP (Bethyl Laboratories, USA) conjugate as a secondary antibody. After incubation in a substrate solution, the absorbance was measured at 415 nm.

***In vitro* spleen cell proliferation.** Three mice from each group were sacrificed just before challenge, and their spleens were removed and pressed. Single-cell suspensions were obtained by teasing the spleens apart. The erythrocytes in the spleen cell suspension were revolved by lysis, and the remaining cells were washed and suspended in an RPMI 1640 medium (Sigma, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The cell suspension adjusted to 5×10^6 cells/ml in a final volume of 100 µl was seeded in triplicate in flat-bottomed 96-well microtiter plates. One hundred microlitres of antigen (rTgSAG1) with a final concentration of 10 µg/ml or 5 µg/ml Concavanalin A had previously been added to the plates. The plates were incubated for 72 hrs in 5% CO_2 at 37℃. Supernatants were then

stored at -70℃ for cytokine quantification.

Measurement of cytokine production. Cell-free supernatants were harvested as described above and assayed for interleukin-4 (IL-4) and interferon gamma (IFN-γ) activity at 72 hrs. The IL-4 and IFN-γ concentrations were evaluated using a commercial ELISA kit according to the manufacturer's instructions (BioSource, USA). The cytokine concentrations were determined by reference to standard curves constructed with known amounts of mouse recombinant IL-4 and IFN-γ.

Statistical analysis. The statistical significance of the differences for the IFN-γ and IL-4 production between groups were calculated with one-way analysis of variance by using S-Plus 6 software (Insightful Co., USA). The *p* values less than 0.05 ($p< 0.05$) were considered significant.

3-3. Results

Generation of recombinant *N. caninium* expressing TgSAG1. The recombinant *N. caninium* expressing TgSAG1 was generated by using a transfer vector plasmid pDMG/TgSAG1. In order to construct and purify the recombinant *N. caninum*, a modified transfer vector was constructed, which contains a promoter from the 5′ flanking region of the dense granule protein 1 (GRA1) gene, the GFP gene as a color marker, and DHFR-TS gene as a pyrimethamine resistant marker. The resulting vector pDMG/TgSAG1 is shown in Fig. 13. Electroporation was used to transfer the plasmid construct pDMG/TgSAG1 into *N. caninum* tachyzoites and a recombinant parasite was selected by adding pyrimethamine, the plaque-purified, and designated as Nc/TgSAG1.

Analysis of TgSAG1 expressed in recombinant *N. caninium*. In order to confirm the expression of TgSAG1 in recombinant *N. caninium*, immunoflourescence analysis and Western

blot analyses were performed by using McAb against TgSAG1 (Advanced Immuno Chemical Inc, USA). First, the expression of TgSAG1 in the extracellular tachyzoites was examined with IFAT. The specific fluorescence was mainly detected on the cell surface of recombinant Nc/TgSAG1 tachyzoites (Fig. 14). In the Western blot analysis, a specific band with a molecular mass of 30 kDa, which was identical to that of natural TgSAG1 expressed in *T. gondii*, was detected in Nc/TgSAG1 tachyzoites (Fig. 15). These results indicated that TgSAG1 was successfully expressed in Nc/TgSAG1 tachyzoites.

Immunization with Nc/TgSAG1 induced a specific anti-TgSAG1 immune responses. To determine whether the Nc/TgSAG1 can induce a specific immunity, BALB/c mice were inoculated with 1×10^5 (i.p.) tachyzoites of the live Nc/TgSAG1, and the control mice were inoculated with Nc/GFP tachyzoites. Four weeks after the primary immunization the mice were boosted with 5×10^5 tachyzoites, respectively. One more group was inoculated with only MEM (0.5 ml) as negative control. Sera from immunized mice were collected prior to challenge and analyzed by ELISA for specific anti-rTgSAG1 responses. A strong IgG antibody response was observed in the Nc/TgSAG1-immunized group on the fourth week and increased from the fifth week after the first immunization, and the response was found to increase until challenge (Fig. 16). To examine whether a Th1 and/or Th2 was elicited in immunized mice, the presence of IgG sub-types to TgSAG1 was analyzed. A predominance of IgG2a against TgSAG1 was observed in sera from the mice immunized with Nc/TgSAG1, this effect was not detected in the sera collected from mice immunized with Nc/GFP tachyzoites and medium only, suggesting that vaccination with Nc/TgSAG1 elicited a Th1-dominant immune response (Fig. 17).

Immunization with Nc/TgSAG1 induced cellular immune responses. To further confirm if the cellular immune responses were elicited by the immunization, the production of the cytokines released from splenocytes were measured in immunized mice that were restimulated

with rTgSAG1 protein *in vitro* and the supernatants were subjected to assay for the production of IFN-γ and IL-4. There was no detectable IL-4 among the various groups (limit of the detection, 15 ng/ml). The quantity of antigen specific IFN-γ released from splenocytes derived from mice immunized Nc/TgSAG1 tachyzoites showed a significantly higher levels (Fig. 18, $p< 0.05$) compared to the level of Nc/GFP group and medium only group. These results suggested that the mice immunized with recombinant Nc/TgSAG1 tacyzoites elicited a strong cellular immune responses.

Protection effects of Nc/TgSAG1 against challenge infection. To determine the protective effect of Nc/TgSAG1 immunization, BALB/c mice were challenged with a lethal dose of bradyzoites of *T. gondii* Beverley strain (500 bradyzoites /mouse, i.p.) three weeks after the last immunization. The Nc/TgSAG1-immunized group showed 83.33% (5/6) protection compared to the other two control groups (Fig. 19). The mice immunized with medium only started to die from the fifth day after challenge, and all the mice died within ten days. The Nc/GFP-immunized group showed some cross-protection, 16.67% (1/6) mice were survived from the lethal challenge infection.

3-4. Discussion

In this chapter, I demonstrated that *N. caninium* can be effectively used as recombinant carrier for expression of heterologous antigen. This is the first time to report using live *N. caninium* tachyzoites as a vaccine vector. The mice inoculated with recombinant *N. caninum* expressing TgSAG1, induced TgSAG1-specific Th1-dominant immune responses and protected the mice from a lethal challenge infection of *T. gondii*. Several prokaryotic or viral vaccine carriers have been proposed so far, including *Salmonella* (Cong et al., 2005; Qu et al., 2008), BCG (Supply et al., 1999; Wang et al., 2007), and vaccinia virus (Roque et al., 2004).

Here I explored the possibility to use the *N. caninium* as eukaryotic vaccine carrier for the delivery of the heterologous *Toxoplasma* antigen TgSAG1. It has been shown that cellular and humoral responses were induced following experimental infection by *N. caninum* tachyzoites or oocysts in both non-pregnant and pregnant cattle (Lunden et al., 1998; Marez et al., 1999). Moreover, there are highly similar biological and morphological characteristics between *N. caninum* and *T. gondii* (Ellis et al., 1994; Mugridge et al., 1999; Zhang et al., 2007). It has also been reported that mice which are infected intraperitoneally with *N. caninum* were protected against a lethal challenge from *T. gondii* (Kasper and Khan, 1998). Therefore, *Neospora* was chosen as a carrier, which was expected to show its capability to induce strong immune responses in infected animals, which may also prime specific cellular immunity to the heterologous antigen expressed in *Neospora*. Moreover, a promoter from the 5′ flanking region of the dense granule protein 1 (GRA1) gene of *T. gondii* was added to the plasmid and then transform to *Neospora*, which allows expression of the heterologous antigen in an immunogenic form that may mimic the natural expression of the antigen in the *N. caninum* vector. Thus, recombinant proteins derived from *Toxoplasma*, that are expressed in *Neospora* should adopt a similar folding and post-translational modification pattern as the native protein.

Here, I choose mice model to investigate the protective effect, because it has been reported that mice were highly resistant to infection with large numbers (10^6 or more) of *N. caninum* tachyzoites, but sensitive to infection of virulent *T. gondii* such as Beverley strain (Tanaka et al., 2000). It showed a significant protection (83.33%) against a lethal dose of *Toxoplasma* challenge in Nc/TgSAG1-vaccinated mice. Since a favorable outcome of parasitic infections depends on the critical balance established by the immune system (Haumont et al., 2000), I decided to confirm the humoral and cellular responses triggered by immunization. Concerning

the humoral response, the Nc/TgSAG1-immunized mice had remarkable levels of antigen-specific IgG2a and IgG1 that are characteristics of Th1 and Th2 immunity, respectively. High ratio of IgG2a to IgG1 was observed in the sera of the Nc/TgSAG1-immunized mice, suggesting that the response was oriented toward a Th1-type response. On the other hand, the strong adjuvant effect of *Neospora* was anticipated, since this intracellular protozoan parasite elicits a strong IL-12 and IFN-γ cytokine response in the host (Khan et al., 1997; Andrianarivo et al., 2001). As a result, large amounts of IFN-γ were produced in the supernatants of restimulated splenocyte cultures from mice immunized with Nc/TgSAG1. Here I explored the antigen specific IFN-γ production restimulated by rTgSAG1. It has been reported that *N. caninum* protects against *T. gondii* infection by the induction of $CD8^+$ T cells that are immunoreactive to both parasites (Kasper and Khan, 1998), the *N. caninum* vector itself can also induce IFN-γ against itself. Nevertheless, most importantly, live protozoan vectors are able to induce systemic immune response associated with Th1-type cytokine contribution (Charest et al., 2000; Ramirez et al., 2001). Thus, both the antibody response and the cytokine production pattern were indicative of a predominantly Th1 response to the Nc/TgSAG1 immunization.

In recent years, significant progress has been made in the identification of *T. gondii* proteins as vaccine candidates, which can induce the long-term protective immunity against *T. gondii* infection. Among those vaccine candidates, the main surface antigen 1 (TgSAG1) is the best characterized. TgSAG1, highly conserved in *T. gondii* strains, has been shown to induce both humoral and cellular immune responses (Angus et al., 2000; Mevelec et al., 2005; Nielsen et al., 1999; Liu et al., 2006). Similar results were also obtained in this chapter. High level of TgSGA1-specific IgG2a and IFN-γ were found in animals immunized with Nc/TgSAG1 tachyzoites. Moreover, mice immunized with recombinant parasites could

efficiently resist the challenge of bradyzoites of *T. gondii* breverley strain. TgSAG1 is known to be a specific antigen to the tachyzoite stage. However, it could also show protection against the challenge of bradyzoites of *T. gondii* in this chapter. Therefore, vaccination with recombinant Nc/TgSAG1 has potential for protection against both tachyzoite and bradyzoite stages of *T. gondii* infection. In conclusion, the results of this chapter suggest that *Neospora* could be used as a new delivery system for vaccine carrier because of its potent adjuvant effect and ease of its genetic manipulation. The mice immunized with Nc/TgSAG1 induced TgSAG1-specific Th1-dominant immune responses and protected the mice from a lethal challenge of *T. gondii*. On the other hand, as a pathogen, *N. caninum* can infect a wide range of mammals, although human infection has not yet been described. Therefore, the virulent gene knocked out or attenuated *N. caninum* strain, which has been lost their virulence will be useful for developing a safe and effective vaccine against *Toxoplasma* infection.

3-5. Summary

The surface antigen 1 of *Toxoplasma gondii* (TgSAG1) is a major immunodominant antigen and it is considered to be a good candidate for the development of an effective recombinant vaccine against toxoplasmosis. *Neospora caninum* is an apicomplexan parasite closely related with *T. gondii,* however, its virulence is much lower than that of *T. gondii* in mice. In this chapter, the recombinant *N. caninum* expressing TgSAG1 gene (Nc/TgSAG1) was constructed by using pyrimethamine-resistant DHFR-TS and GFP genes as double selection markers. The expression of TgSAG1 by the recombinant *N. caninum* was confirmed by IFAT and Western blot analysis. The mice immunized with Nc/TgSAG1 induced TgSAG1-specific Th1-dominant immune responses, as shown by high level of IgG2a

antibody and splenocyte gamma interferon (IFN-γ) in the immunized animals. Immunization with recombinant Nc/TgSAG1 tachyzoites protected the mice from a lethal challenge infection of *T. gondii*. These results indicate that the *N. caninum* vector carrying TgSAG1 antigen may provide a new vehicle for production of a live recombinant vaccine against toxoplasmosis in animals.

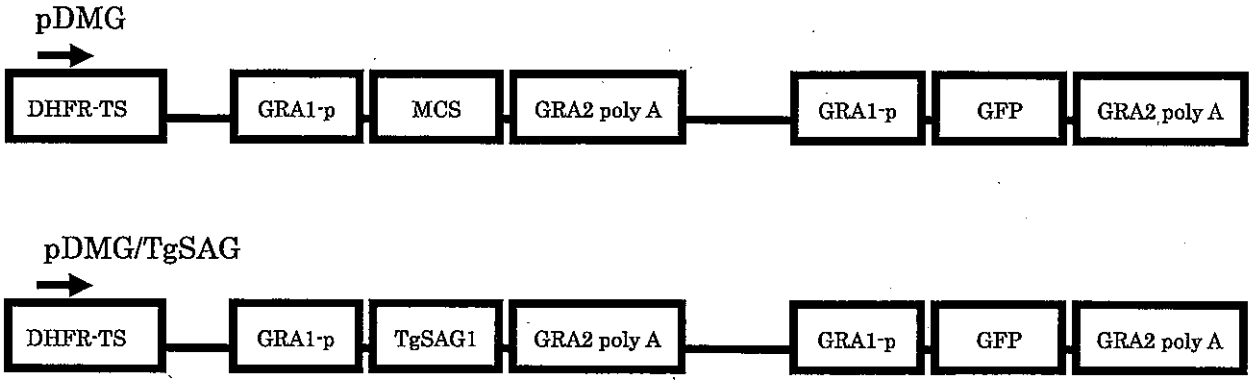

Fig. 13. Schematic representation of the DNA constructs pDMG and pDMG/TgSAG1 designed to express TgSAG1 in *N. caninum*. Pyrimethamine-resistant DHFR-TS gene and green fluorescent protein (GFP) gene were used as selection markers. The 5′ flanking region of the dense granule protein 1 gene (GRA1-p) and the 3′ flanking region of the GRA2 gene (GRA2 poly A) of *T. gondii* were introduced into the pDMG. TgSAG1 gene was inserted into the multiple cloning sites (MCS) of plasmid pDMG.

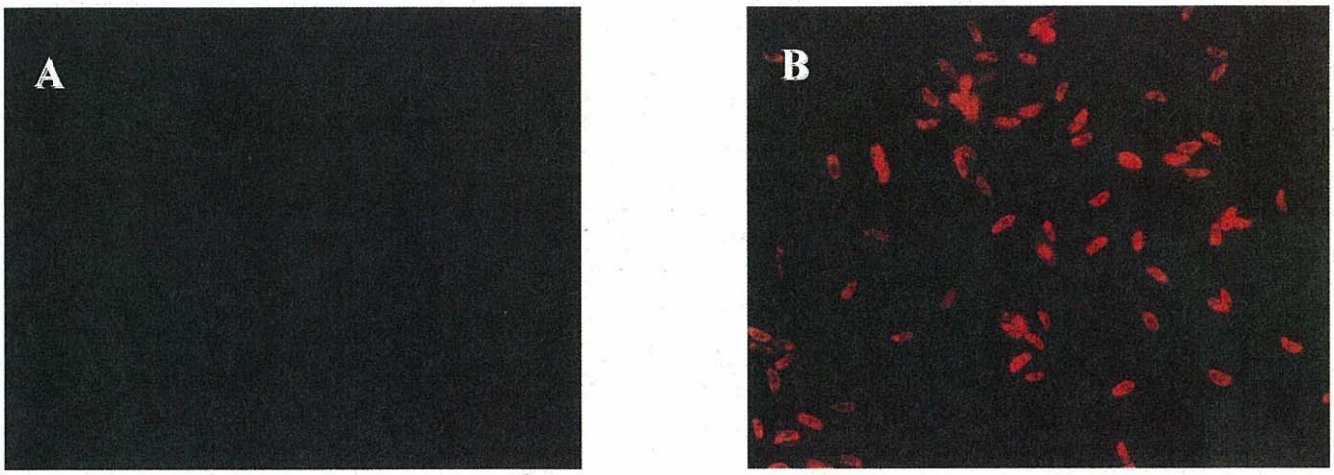

Fig. 14. Expressions of TgSAG1 by the recombinant *N. caninum* were confirmed by IFAT with McAb against TgSAG1 as primary antibody and Alexa Flour-594-conjugated as secondary antibody on Nc/GFP (A) or Nc/TgSAG1 (B) tachyzoites.

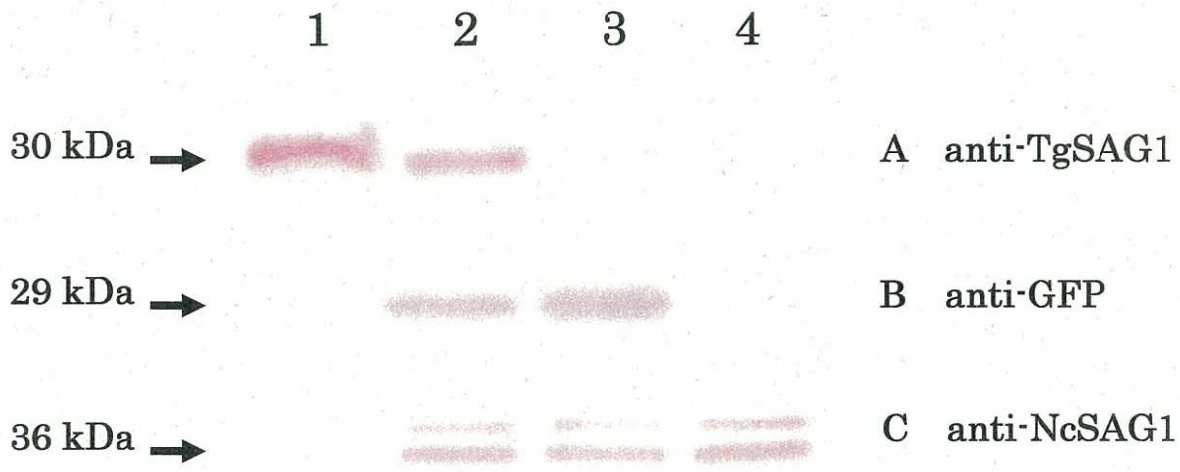

Fig. 15. Expression of TgSAG1 by the recombinant *N. caninum* was confirmed by Western-blot analysis. Purified tachyzoites lysates were run on SDS-PAGE, transferred to nitrocellulose membranes as following: lane 1, *T. gondii* lysate; lane 2, Nc/TgSAG1 lysate; lane 3, Nc/GFP lysate; lane 4, *N. caninum* lysate, and were probed with anti-TgSAG1 McAb (A), anti-GFP antibody (B) and anti-NcSAG1 antibody (C).

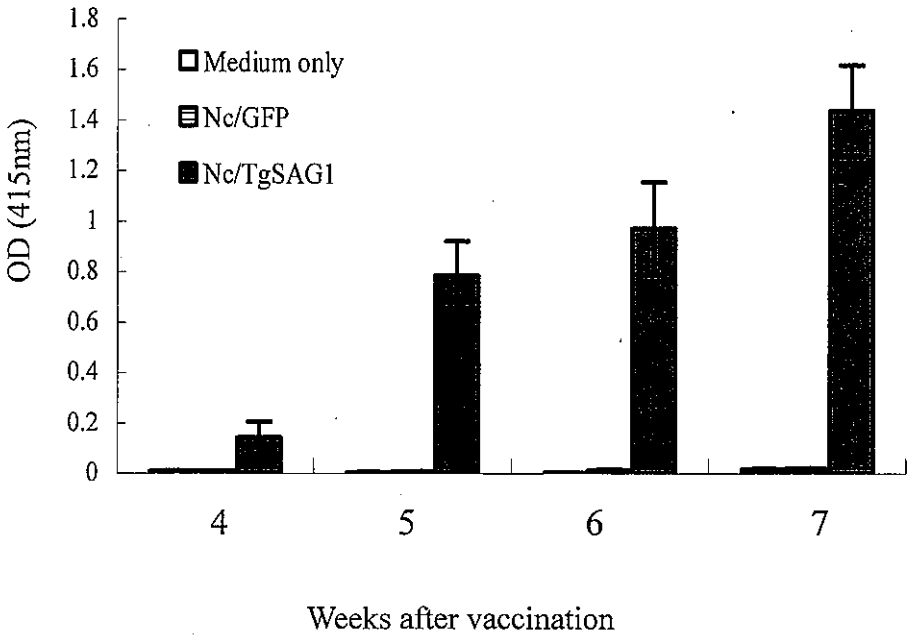

Fig. 16. Total IgG antibody specific response to TgSAG1 in mice was tested by ELISA. The serum samples were taken from the 4th week after the first immunization and analyzed by ELISA with the rTgSAG1 as coated antigen.

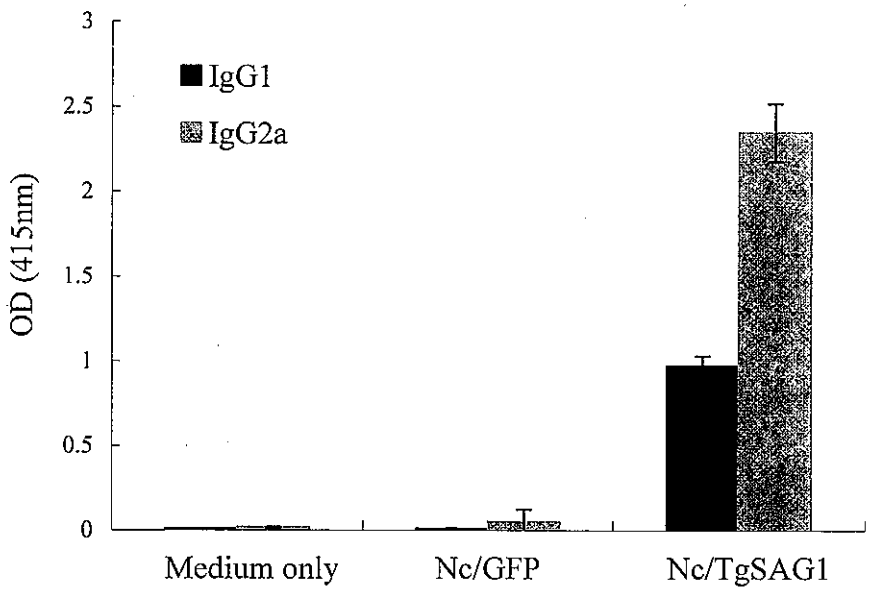

Fig. 17. To evaluate whether the T helper cells involvement resulting from the recombinant Nc/TgSAG1 tachyzoites vaccination was Th1 or Th2 type, the levels of IgG1 and IgG2a antibodies to TgSAG1 were analyzed by ELISA on the 7th week.

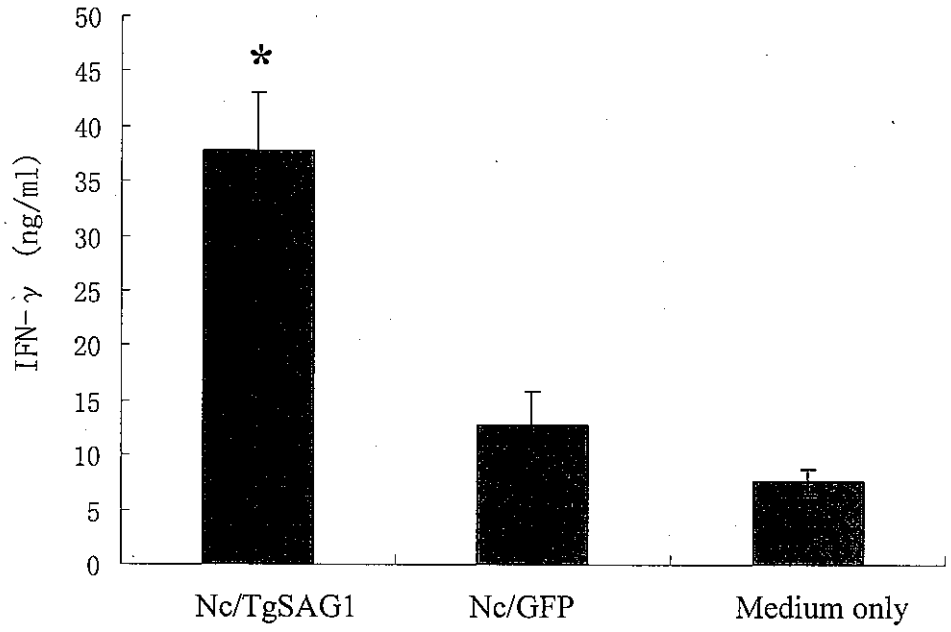

Fig. 18. IFN-γ production in splenocytes taken from vaccinated mice. The supernatants were assayed for the presence of IFN-γ after stimulating with rTgSAG1. Each bar represents the IFN-γ production of splenocytes from three mice. The differences of IFN-γ production between experimental groups were analyzed by one-way analysis of variance using S-Plus 6 software and a p value of less than 0.05 was considered significant (*, $p < 0.05$).

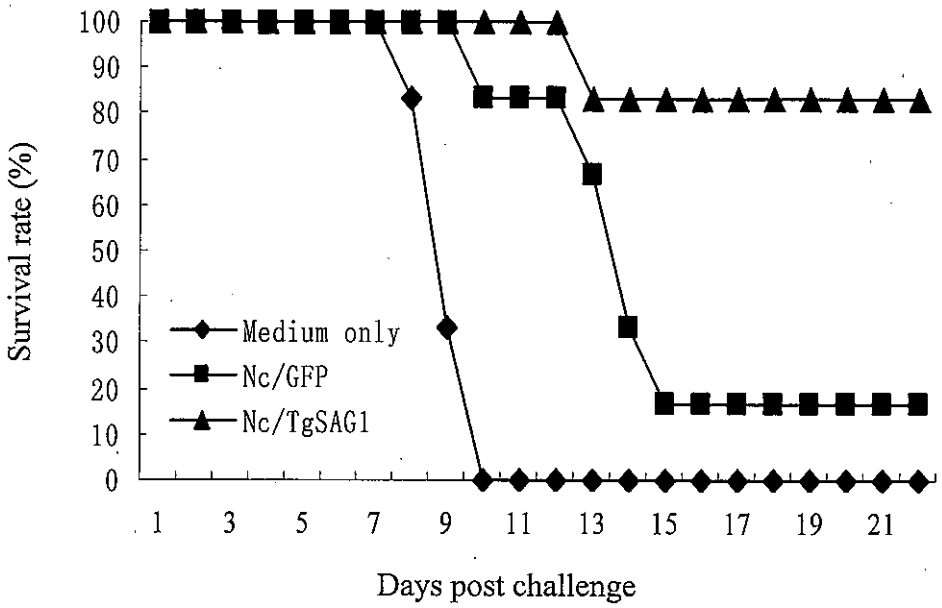

Fig. 19. The protective effects of recombinant Nc/TgSAG1 in mice. Mice vaccinated with Nc/TgSAG1 (n=6), Nc/GFP (n=6) and medium (n=6) were infected with a lethal dose of bradyzoites of *T. gondii* Beverley strain (500/mouse).

General discussion

Toxoplasma gondii infects all warm-blooded animals, including humans, and it is estimated that one-third people worldwide are infected with *T. gondii* (Dunn et al., 1999; Petersen et al., 2001). Toxoplasmosis is of major medical and veterinary importance, being a cause of congenital disease and abortion in humans and in domestic animals (Buxto, 1998; Gilbert et al., 2001). In addition, it has gained importance due to *Toxoplasma* encephalitis in AIDS patients (Luft and Remington, 1992; Wallon et al., 2004). Moreover, *T. gondii* tissue cysts in the meat of infected animals are the main source of infection in humans (Buffolano et al., 1996; Cook et al., 2000; Dubey, 2000). Treatment of this disease is difficult due to toxic effects of available drugs, and reinfection occurs rapidly. Therefore, development of effective vaccine is an attractive alternative against toxoplasmosis. Thus, in this study TgSAG1, TgGRA4 and TgPRF were chosen as vaccine candidates. The recombinant DNA vaccines, which express TgGRA4 and TgPRF, recombinant vaccinia virus which express TgGRA4 and the recombinant *N. caninum,* which express TgSAG1, were all constructed and applied to mice model to evaluate their vaccination effects against *T. gondii* infection.

Vaccine development is based on the observation that primary exposure to *T. gondii* results in complete resistance to secondary challenge (Araujo, 1994). A vaccine based on the live attenuated S48 strain was developed for veterinary uses (Ferguson and Hutchison, 1987). However, this vaccine is expensive, causes side effects and has a short shelf life. Furthermore, this vaccine may revert to a pathogenic strain and therefore it is not suitable for human use (Ferguson and Hutchison, 1987). Several studies have shown that mice and rats immunized with whole *T. gondii* tachyzoite extracts or specific native antigens such as TgSAG1,

TgGRA2, TgGRA5, or excretory-secretory antigens conferred protection against tissue cyst or tachyzoite challenge as assessed by reduction in the number of brain cysts (Araujo and Remington, 1984; Duguesne et al., 1990; Darcy et al., 1992; Brinkmann et al., 1993; Lunden et al., 1993; Zenner et al., 1999; Velge-Roussel et al., 2000). According to these observations, researchers have utilized recombinant antigens, which are expressed in *E. coli* to immunize mice or rats against *T. gondii*, and give a partial protection against tissue cyst or tachyzoite challenge (Letscher-Bru et al., 1998; Petersen et al., 1998). But the native protein purification from *T. gondii* is difficult and the preparation of the recombinant protein is somehow costly and time consuming. And sometimes it is difficult to induced immune response by using recombinant proteins. Recently, DNA-based vaccination is one of the most promising strategies for the development of new generation effective vaccines against intracellular parasites (Dupre et al., 1997; Sepulveda et al., 2000). Therefore, in this study, the recombinant vaccines by using plasmid vector (Chapter 1 and Charter 2), viral vector (Chapter 1) and protozoan vector (Chapter 3) to express the target genes were constructed.

In Chapter 1 and Chapter 2, the recombinant plasmid pTgGRA4 and pTgPRF were constructed. DNA vaccine has been shown to be a powerful method for the induction of specific humoral and cellular immune responses. It is a novel method involving the injection of the naked DNA plasmid into the host, whose cells express the encoded proteins (Donnelly et al., 1997; Kowalczyk and Ertl, 1999). Mice immunized with plasmid encoding the TgSAG1 gene showed 80–100% protection against *T. gondii* challenge (Nielsen et al., 1999). Evidences have also shown that there is an increase in survival days of infected mice immunized with plasmids encoding TgGRA4 (Desolmeet al., 2000) or TgGRA1, TgGRA7, TgMIC2 and TgROP2 (Supply et al., 1999; Vercammen et al., 2000; Dautu et al., 2007). The route to deliver plasmid and the immunization strategy are important factors that affect the

immunization. So firstly the best route to deliver plasmid was chosen by using gene gun, which can deliver plasmid into the ventral skin, over which Langerhans cells (LC)/DC is abundantly distributed. It has been reported that gene gun vaccination with DNA, which only needs small amount of DNA, was highly reproducible and reliable in the induction of specific CTL and the production of IFN-γ and humoral antibodies compared with i.m. vaccination (Leitner et al., 1997; Yoshida et al., 2000). Secondly a heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing the same antigen was applied to the immunization (Chapter 1). Prime-boost vaccines including DNA can elicit immune responses that differ in magnitude, quality, and balance of cellular and humoral responses from those elicited by single components and thus provide further enhancement for DNA immunizations (Vuola et al., 2005; Miao et al., 2006). As recombinant vaccinia virus is known to be very potent vector for gene delivery, modified vaccinia virus has been used as a vector for the delivery of various antigens in animals and humans (Sharpe et al., 2001; McConkey et al., 2003), and recent vaccination studies with recombinant vaccinia virus in mice have shown to induce both $CD4^+$ and $CD8^+$ T-cell responses (Hodge et al., 2003). Both recombinant herpes virus and recombinant vaccinia virus carrying the ROP2 gene are able to induce protective immunity against the *T. gondii* infection (Roque-Resendiz et al., 2004). Therefore, in Chapter 1, the C57BL/6 mice vaccinated with DNA vaccine pGRA4 priming and followed by vaccinia virus VV/GRA4 boosting (pGRA4 + VV/GRA4 regime) induced both a strong IgG antibody response against rTgGRA4, and a high level of IFN-γ production. These results demonstrate that the heterologous prime-boost vaccination regime using DNA and a vaccinia virus, could induce both humoral and cellular immune responses, and provide effective protection against lethal acute and chronic *T. gondii* infections in mice.

Not only the recombinant viruses but also the recombinant protozoa can be used as live vectored vaccine. It has reported that recombinant *Toxoplasma* itself has the potential to serve as an efficient vaccine carrier for cutaneous leishmaniasis. A protective role for *Leishmania* antigen kinetoplastid membrane protein-11 (KMP-11) when given in such a vaccine formulation was established (Ramirez et al., 2001). And *T gondii* ts-4 strain constructs expressing plasmodium CSP also induce specific immune responses against this malaria protein that resembles those triggered by the *T. gondii* carrier against its own antigens (Charest et al., 2000). As we know that *N. caninum* is an intracellular, apicomplexan parasite, which has highly similar biological and morphological characteristics with *T. gondii* (Ellis et al., 1994; Dubey et al., 2007). Similar to the situation with *T. gondii*, it has been shown that a cellular and humoral response was induced following experimental infection by *N. caninum* tachyzoites or oocysts in both non-pregnant and pregnant cattle (Lunden et al., 1998; Marez et al., 1999). Especially, T cell immune response could be induced by *N. caninum* in infected host, which is at least partially mediated by interleukin-12 (IL-12) and interferon gamma (IFN-γ) (Khan et al., 1997; Andrianarivo et al., 2001). Therefore in Chapter 3, a recombinant *N. canimum* was constructed to express TgSAG1. The mice immunized with Nc/TgSAG1 induced TgSAG1-specific Th1-dominant immune responses, which is detected by high level of IgG2a antibody and splenocyte IFN-γ in the immunized animals. Immunization with recombinant Nc/TgSAG1 tachyzoites protected the mice from a lethal challenge infection of *T. gondii*. These results indicate that the *N. caninum* vector carrying TgSAG1 antigen may provide a new live vector for production of a live recombinant vaccine against toxoplasmosis in animals. Live vaccines are more likely to mimic what is happening in natural infection and parasite antigens will be presented to the immune system in the correct context. But as a pathogen, *N. caninum* can infect a wide range of mammals, although infections in human have

not yet been described. Therefore, the virulent gene knocked out or attenuated *N. caninum* strain, which has lost their virulence will be useful for developing a new effective vaccine against *Toxoplasma*. Some researchers have tried to develop attenuated strains of *N. caninum* by creating temperature sensitive mutants (Lindsay et al., 1999) or treating tachyzoites with gamma irradiation (Ramamoorthy et al., 2006) and through prolonged culture *in vitro* (Bartley et al., 2006). Since *Neospora* is a genetically modifiable parasite, it might be possible to make virulent gene knocked out or attenuated strain to be used as safe vaccine carrier for developing an effective and safe vaccine against toxoplasmosis.

Based on the results of this study, TgGRA4, TgPRF and TgSAG1 were shown to have protective effects against *T. gondii* infection in mice model. Although it is difficult to make comparisons between studies that utilized different strains of inbred mice, different strains of *T. gondii* for challenge infection or different measures of protection, the data indicate that TgGRA4, TgSAG1 and TgPRF were good candidates for developing the vaccine against toxoplasmosis. On the other hand, the life cycle of *T. gondii* has three infectious stages: tachyzoite, bradyzoite and sporozoite. It is unfortunate that vaccination with stage-specific antigens can only lead to stage-limited protection (Alexander et al., 1996). Higher rates of survival and fewer brain cysts were observed in mice immunized with plasmid DNA containing sequences for TgGRA1, TgGRA7, and TgROP2 (Vercammen et al., 2000). In the latter study, the co-injection of mice with DNA encoding GRA4 and GM-CSF enhanced protective effects (Mevelec et al., 2005). Moreover, the immunogenicity of DNA vaccines remains to be enhanced because the immune responses induced by DNA vaccines are often weak (Donnelly et al., 2005). By the use of molecular adjuvants, the immune responses may be enhanced and modulated. So IL-12 and GM-CSF are chosen to be act as a genetic adjuvant to enhance the immunogenicity and protective efficacy of anti-toxoplasmosis (Mevelec et al.,

2005; Donnelly et al., 2005). Thus in the future, multi-antigenic DNA vaccine or the combination of the molecular adjuvant regions with the different stage specific immunodominat antigen should be concerned to develop the vectored vaccines against toxoplasmosis.

General summary

In order to develop novel vaccines against toxoplasmosis, recombinant vectored vaccines were constructed with different antigens and different vaccination strategies in this study.

As described in Chapter 1, the mice vaccinated with DNA plasmid encoding TgGRA4 (pGRA4) priming and followed by vaccinia virus VV/GRA4 boosting (pGRA4 + VV/GRA4 regime) induced both a strong IgG antibody humoral response against rTgGRA4 and a high level of IFN-γ production and provided an effective protection against lethal acute and chronic *T. gondii* infections in mice.

In Chapter 2, a DNA plasmid encoding TgPRF, which can induce a potent IL-12 through TLR11, was successfully constructed and immunized to the mice by gene gun. This DNA immunization induced strong specific antibody immune responses in mice and protected the mice from *T. gondii* challenge infection with lethal dose, suggesting that TgPRF would be another vaccine candidate for vaccine development against toxoplasmosis in the future.

In Chapter 3, *N. caninum* was used as a live vector to express TgSAG1 and the mice inoculated with the recombinant *N. caninum* Nc/TgSAG1 induced TgSAG1-specific Th1-dominant immune responses. Furthermore, vaccination with Nc/TgSAG1 protected the mice from a lethal challenge infection of *T. gondii*.

In conclusion, three novel vaccines constructed based on TgGRA4, TgSAG1, and TgPRF were developed in the present study. Vaccination with these three vaccines can all induce strong homural immune response as well as cellular immune response in two of the vaccinations. Moreover, the immunized mice could be protected from *T. gondii* challenge infection with lethal dose. Thus, these vaccines provide a potential alternative for the control of *Toxoplasma* infection.

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