Induction of protective immune responses by a multiantigenic DNA vaccine encoding GRA7 and ROP1 of *Toxoplasma gondii*

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ABSTRACT

*Toxoplasma gondii* is distributed worldwide and infects most species of warm-blooded animals, including humans. The heavy incidence and severe or lethal damage caused by *T. gondii* infection clearly indicates the need for the development of a vaccine. To evaluate the protective efficacy of multi-antigenic DNA vaccine expressing GRA7 and ROP1 of *T. gondii* with/without a plasmid encoding murine IL-12 (pIL12), we constructed DNA vaccines using the eukaryotic plasmids pGRA7, pROP1 and pGRA7-ROP1. Mice immunized with pGRA7, pROP1 or pGRA7-ROP1 showed significantly increased serum IgG2a titers, production of IFN-γ, IL-10 and TNF-α, *in vitro* T cell proliferation, survival days, as well as decreased cyst burdens in the brain compared to mice immunized with either the empty plasmid, pIL12, or vector+pIL12. Moreover, mice immunized with the multi-antigenic DNA vaccine pGRA7-ROP1 had higher IgG2a titer, production of IFN-γ and TNF-α, survival days, and cyst reduction rate as compared with those vaccinated with either pGRA7 or pROP1 alone. Furthermore, mice immunized with either a pGRA7-ROP1+pIL12 or a single-gene vaccine combined with pIL12 showed greater Th1 immune response and protective efficacy as compared with the single-gene vaccinated groups. Our data suggest that the multi-DNA antigen pGRA7-ROP1 was more effective in stimulating host protective immune responses than separately-injected single-antigens and that IL-12 serves as a good DNA adjuvant.
Keywords: *Toxoplasma gondii*, Double-antigenic GRA7-ROP1 DNA vaccine, Protective immunity, Th1 immune response
Toxoplasma gondii is an obligate intracellular protozoan that infects one-third of the world’s population. Although 80-90% individuals with primary infection are asymptomatic, toxoplasmosis is a significant cause of morbidity and mortality in immunocompromised and congenitally-infected individuals (6). In veterinary medicine, T. gondii infection has economic importance due to abortion and neonatal loss in livestock (mainly sheep and goats) or as a source of transmission to humans (8). Thus, a vaccine against T. gondii would be valuable for reducing the high incidence, and for preventing both fetal infection and reactivation in immunocompromised individuals. The development of a vaccine might also reduce economic losses in the livestock industry.

The live vaccine has a short shelf life, and there is a risk of the vaccine reversing to the pathogenic phenotype. DNA vaccines have become a major focus because they promote specific expression of an encoded vaccine antigen by host cells and have the ability to deliver multivalent vaccines to a host in a single dose. Additionally, DNA vaccines can also elicit potent, long-lasting humoral and cell-mediated immunity (1). The family of vaccine candidate antigens includes T. gondii membrane-associated surface antigen SAG1 (10, 18) and SAG2 (4); excreted-secreted dense-granule proteins GRA1 (8, 9), GRA2 (11), GRA4, GRA6 (8) and GRA7 (3, 8, 9, 16); rhoptry proteins ROP1 (3, 10) and ROP2 (9, 18); and micronemal proteins MIC1, MIC2, MIC3 (14), and MIC6 (13). GRA proteins are potent antigens that trigger strong T and B cell responses upon infection and GRA7 is expressed by all infectious stages of T. gondii (6, 8). ROP1 is released at the
time of invasion into the forming parasitophorous vacuole, and is related to the T. gondii penetrating
enhancing factor (2).

DNA cocktail vaccinations have been reported to enhance protection against toxoplasmosis in a
mouse model as compared to single-gene vaccines (4, 5, 12). In this study, we selected T. gondii
GRA7 and ROP1 as targets for DNA antigenicity generation because they are known to be
important during invasion and penetration into host cells (2, 6, 8). In addition, they are potent
stimulators of humoral and cellular immune responses (3, 8, 9, 10). However, no evaluation of the
protective efficacy of multi-antigenic DNA vaccine expressing GRA7 and ROP1 of T. gondii
with/without a plasmid encoding murine IL-12 (pIL12) has been published. To evaluate protective
immunity we constructed DNA vaccines expressing the GRA7 and ROP1 antigens (pGRA7 and
pROP1) and a fusion of both (pGRA7-ROP1). We then examined their expression in eukaryotic
cells, and investigated the immunogenicity and protective efficacy of these DNA vaccines with or
without co-administration of pIL12 as a genetic adjuvant to protect BALB/c mice against
toxoplasmosis.
MATERIALS AND METHODS

Animals and Toxoplasma gondii strains.

Female BALB/c mice were purchased from DaeHan BioLink Co. (Chungcheongbuk-do, Korea). All mice were maintained under specific-pathogen-free conditions and were 6-8 weeks of age when first immunized. Animal studies were carried out under the authority of the Chungnam National University Animal Ethics Committee (Application number 2010-2-33). Two T. gondii strains were used. The tachyzoites of virulent RH strain were used for plasmid construction and preparation of soluble tachyzoite antigen (STAg), and the cyst-forming Me49 strain was used to infecting orally mice to evaluate survival days and brain cyst numbers.

Cell culture.

Human retinal pigment epithelial (ARPE-19; ATCC, Rockville, MD) were maintained in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Life Technologies, Carlsbad, CA) and nutrient mixture F12 (DMEM/F12) containing 10% heat-inactivated FBS, 0.348% sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell cultures were maintained at 37 °C and 5% CO2, and the medium was changed every 3-4 days.

Isolation of T. gondii tachyzoites and cysts.
Tachyzoites of *T. gondii* RH strain were infected into ARPE-19 cells (parasite:cell ratio=5:1) and incubated at 37°C, 5% CO₂ for 2–3 days. Following spontaneous host cell rupture, lysed tachyzoites and host cellular debris were centrifuged at 900 g for 10 min using Percoll (Sigma Chemical Co., St Louis, MO, USA) to pellet parasites. The final pellet was suspended in cold PBS, and the suspension was passed through a 5.0 μm pore filter (Millipore, Bedford, MA). Purified tachyzoites were used in all experiments.

The cysts of *T. gondii* Me49 strain was obtained from the brains of infected mice. Each brain was suspended in PBS to final volume of 5 ml, and homogenized by serial passages through each of 18-gauge syringe needles. Mean cyst numbers in the brain were determined triplicate by counting 10 µl samples of the homogenate under microscope at 200× magnification.

**Preparation of soluble tachyzoite lysate antigens (STAg).**

Purified tachyzoites were centrifuged at 5000 g for 3 min and disrupted by three cycles of freezing at -20°C and thawing at 4°C. Finally, the lysate was sonicated on ice at 60W/s and centrifuged for 40 min at 100,000 g. The supernatants were pooled and sterile filtered (Gelman Sciences, Ann Arbor, Michigan) and the protein concentration was determined via the Bradford method, using bovine serum albumin as the standard. STAg was stored in aliquots at -70°C until use.
Construction of DNA vaccine plasmid.

The mammalian expression vector pCMV-Tag2B was used as a DNA vaccine vector. To construct the GRA7-ROP1 fusion expression plasmid, the GRA7 gene (786 bp, no stop codon, aa residues 17–225) and ROP1 gene (1,023 bp, aa residues 22–381) were amplified by PCR from cDNA of *T. gondii* (RH strain), using the following primers: (GRA7, forward: 5’- CGCGGATCCGCGGCGGCTTTGCCCCAGTT-3’ and reverse: 5’- AATCTGCAGAGGCACCTCTTGCTCGAGTG-3’, BamHI and PstI, respectively, recognition sites underlined; ROP1, forward: 5’-CCCAAGCTTGCCGCCCTTTCGAGCCACAA-3’ and reverse: 5’- CCGCTCGAGGCCCTCCTCGCCATTAGTTC-3’, HindIII and XhoI, respectively, recognition sites underlined). PCR products were cloned into the pGEM-T easy vector (Promega Corporation, Madison, WI), digested with the appropriate restriction enzyme, and purified from agarose gels.

GRA7 and ROP1 gene fragments were inserted into the mammalian expression vector pCMV-Tag2B, generating pCMV-Tag2B-GRA7 (pGRA7) and pCMV-Tag2B-ROP1 (pROP1). The BamHI/PstI fragment encoding GRA7 was excised and cloned into the BamHI/PstI sites of the pROP1 vector to produce pCMV-Tag2B-GRA7-ROP1 plasmid (pGRA7-ROP1). All recombinant plasmids were propagated in *Escherichia coli* DH5α and confirmed by restriction analysis and PCR sequencing (Solgent, Daejeon, Korea). The murine IL-12 expression plasmid containing the p35 and
p40 sequences designated pUMVC3-mIL-12 (pIL12) was provided by Dr. Alexander Rakhmilevich (University of Wisconsin-Madison, USA).

**Plasmid extraction and purification.**

Large-scale plasmid DNA was prepared using the Endotoxin-Free Mega Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany), and concentrations were determined by A_{260/280} measurement. The OD 260/280 ratios were 1.8-2.0, indicating no major protein contamination. Plasmid DNA was dissolved (1 mg/ml) in sterile endotoxin-free PBS and stored at -20 °C until use.

**Expression of the compound gene in vitro and in vivo.**

ARPE-19 cells were transfected with pGRA7, pROP1, pGRA7-ROP1, or pCMV-Tag2B (control), using Lipofectamine LTX and PLUS Reagents (Invitrogen Life Technologies, Carlsbad, CA). Six-well culture plates were seeded with ARPE-19 cells (2×10^5 cells/well) and then cultured to 50-80% confluence. DNA (2.5 µg) in 500 µl DMEM and 2 µl PLUS reagent mixed gently and incubated at RT for 5 min. Lipofectamin LTX was mixed with diluted DNA and incubated at RT for 30 min. DNA-lipid complexes were added to cells that had been rinsed with serum-free medium.
After incubation for 6 h at 37°C in a 5% CO\textsubscript{2} incubator, medium was changed with containing 10% FBS. Following further 48 h incubation, cells were washed with PBS and harvested.

To examine the level of \textit{in vivo} expression, total RNA were isolated from spleens of DNA vaccinated mice 4 week post-inoculation and were analyzed by multiplex reverse transcription polymerase chain reaction (M-RT-PCR) with GRA7 and ROP1 specific primers. PCR reactions were carried out in 50 \(\mu\text{l}\) of total volume. Each PCR mixture contained 0.25 \(\mu\text{l}\) of Takara Ex Taq (5 U/\(\mu\text{l}\)), 5 \(\mu\text{l}\) of 10x Ex Taq buffer, 4 \(\mu\text{l}\) of dNTP mixture (2.5 mM each), 2 \(\mu\text{l}\) of each primer (10 pmol/\(\mu\text{l}\)) and 3 \(\mu\text{l}\) of cDNA. All PCR reactions were performed in MyCycler (Bio-Rad Laboratories, CA). Denaturation of DNA (94°C for 30 s) was followed by 30 cycles of amplification (98°C for 10 s, 60°C for 30 s and 72°C for 2 min) and ended by a 10 min extension at 72°C. Amplified products were electrophoresed in 1.2% agarose gel and visualised by ethidium bromide staining. Also, these PCR products were confirmed by sequencing.

SDS-PAGE and western blot analysis.

After 48 h, pellets of transfected cells were suspended in 150 \(\mu\text{l}\) SDS-PAGE sample buffer, and then sonicated and heated at 100°C for 5 min. Lysates were separated on a 12% polyacrylamide gel, transferred to polyvinylidene difluoride membranes, and then blocked with Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBST) and 5% skim milk.
Membranes were washed three times with TBST, and then incubated with diluted mouse anti-FLAG primary antibody (Sigma-Aldrich, St. Louis, MO) for 2 h at room temp. Unreacted antibody was washed out with TBST, followed by incubating with goat anti-mouse IgG-peroxidase conjugate (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:10,000 in 5% skim milk/TBST. Peroxidase activity was detected using the enhanced chemiluminescence (ECL) Western blot detection system (Amersham-Pharmacia, Freiburg, Germany).

**Construction of bacterial expression plasmid and purification of recombinant protein.**

To construct pGEX-GRA7 and pGEX-ROP1 expression plasmid, the coding sequence of the *T. gondii* GRA7 and ROP1 genes (GenBank accession no. DQ459443.2 and M71274.1, respectively) were amplified by PCR (using Pfu-X DNA Polymerase, Solgent, Daejeon, Korea) from cDNA of *T. gondii* RH strain with a pair of oligonucleotide primers (GRA7, forward primer: 5’-CGCGGATCCATGGCCCGACACGCAATTTT-3’; GRA7, reverse primer: 5’-TCCCCGGGCTACTGGCCCCATCTCCC-3’; ROP1, forward primer: 5’-CGCGGATCCATGGAGCAAAGGCTGCCAA-3’; ROP1, reverse primer: 5’-GGCCTCGAGTTATTGCGATCCCATCCATCC-3’) and recognition sites (GRA7, BamHI and SmaI; ROP1, BamHI and XhoI) underlined. The PCR products were digested with the appropriate restriction enzyme and cloned into the BamHI/Smal or BamHI/XhoI sites of the pGEX-4T-1
expression vector containing a N-terminal glutathioneS-transferase (GST). The resulting plasmids were named pGEX-GRA7 and pGEX-ROP1.

Bacterially-expressed recombinant *T. gondii* soluble proteins were purified with Pierce GST Spin Purification Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s instructions. Purified GST fusion proteins were dialyzed against a dialysis buffer (PBS containing 2 mM EDTA and 1 mM DTT) and protein concentration was determined by Bradford assay method using bovine serum albumin (BSA) as the standard and stored at -70°C until use.

**DNA immunization and experimental design.**

Nine groups (23 mice per group) were vaccinated twice at two-week intervals with empty vector, pIL12, empty vector+pIL12, pGRA7, pROP1, pGRA7-ROP1, pGRA7+pIL12, pROP1+pIL12, or pGRA7-ROP1+pIL12. Mice were inoculated with an injection of 50 µg plasmid DNA (in 50 µl sterile endotoxin-free PBS) into the tibialis anterior muscles of both hind legs (100 µg/per mouse) using a G26 gauge needle. Blood and spleens were collected to assess serum IgG, *in vitro* T cell proliferation, and cytokine levels at 0, 2, 4, and 8 weeks after immunization. Four weeks after the final immunization, mice were challenged with Me49 strains to determine survival days of mice and parasite burdens in the brain.
Evaluation of survival days and parasite burdens in the brain.

Four weeks after the final immunization, six mice were challenged with lethal dose (1,500 cysts per mouse) of *T. gondii* Me49 strain according to previous reports (10), and mortality was monitored daily for 4 weeks. In addition, five mice per group were orally challenged with a nonlethal dose of strain Me49 (20 cysts per mouse) four weeks after final immunization. Four weeks after the challenge, mice were euthanized and the brain cyst were counted as indicated above. All samples were counted in triplicate. Cyst reduction rate to vector control was calculated with the formula: 

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\frac{1 - \frac{\text{brain cyst numbers of vaccinated mice}}{\text{brain cyst number of vector control}}}{1} \times 100.
\]

Determination of *T. gondii*-specific IgG and IgG subclass titers.

*T. gondii*-specific serum IgG, IgG1, and IgG2a antibody levels were determined by ELISA. The 96-well plates were coated with 10 µg/ml of STAg, purified GST, recombinant GRA7 and/or ROP1 (5 µg/ml) in 50 mM carbonate buffer (pH 9.6) (100 µl per well) at 4°C overnight. Plates were washed twice with PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T20), and blocked with PBS containing 1% BSA (PBS–1% BSA) for 2 h at RT. Sera were diluted in PBS–0.1% BSA and incubated for 2 h. After washing with PBS-T20, plates were incubated with HRP-conjugated anti-mouse IgG (diluted 1:8000 in PBS–1% BSA), IgG1 and IgG2a (1:2000) for 2 h. After washing with PBS-T20, 200 µl of substrate solution (10 mg of O-phenylenediamine and 10 µl of 30% H₂O₂ in 25
ml of 0.1 M citrate–phosphate buffer, pH 5.0) was added. Plates were then incubated in the dark for 30 min, and the reaction stopped by addition of 3N HCl (50 µl). The optical density (OD) was then measured by an ELISA reader at 490 nm.

We also determined the IgG2a titers against recombinant *T. gondii* GRA7 and ROP1 by endpoint dilution. Each serum sample was performed conventional 2-fold serial dilutions starting at 1:100, and then determined the OD levels using the same ELISA methods as described above. The mean OD + 5 standard deviations from 10 uninfected healthy mouse sera was used as the cutoff between anti-*T. gondii* IgG2a antibody-positive and -negative results, which made significant differences of IgG2a titers between uninfected mouse sera and *T. gondii*-infected mouse sera. The endpoint titer was the highest serum dilution that yielded an OD greater than the value that defined the cutoff. For convenience, titers are expressed as reciprocal values.

**In vitro T cell proliferation assay**

*In vitro* T cell proliferation was measured using a chemiluminescent BrdU ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). Spleens from immunized mice were collected under aseptic conditions, in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA). Erythrocytes were removed by lysis and the remaining cells washed and suspended in RPMI 1640 medium supplemented with 10% FBS, HEPES (10 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), β-mercaptoethanol (50 mM), gentamycin (50 mg/ml), penicillin (100 U/ml), and streptomycin
15 (100 mg/ml). Cells were then seeded in triplicate in flat bottomed 96-well microtiter plates (Corning Incorporated, Corning, NY) at 5×10⁶ cell/ml in 100 µl complete medium; thereafter, 10 µg/ml of STAg was added. The plates were incubated at 37 °C in 5% CO₂. After 72 h, BrdU labeling solution (10 µl) was added to each well and incubated for 2 h. The plates were centrifuged and the labeling medium was removed. The cells were dried, FixDenat (200 µl) was added, and then the plates were incubated for 30 min at RT. After removing the FixDenat solution, 100 µl of anti-BrdU-POD working solution was added followed by incubation for 90 min. After removing antibody conjugate, the wells were rinsed three times with PBS (pH 7.4). After 30 min incubation with 100 µl substrate solution, the absorbance of the samples was evaluated by an ELISA reader at 370 nm with a 492 nm reference.

Spleen cell cultures and cytokine quantification.

Four weeks after the last vaccination, 3 mice per group were euthanised, and their spleens were isolated. Single-cell suspensions of splenocytes were stimulated by incubation with purified GST antigen alone or in combination with recombinant GRA7 and ROP1 antigens (5 µg/ml). Cell-free supernatants were harvested and assayed for IL-4 activity at 24 h, for IL-10 activity at 72 h, for IFN-γ activity at 96 h, and for TNF-α activity at 48 h. The IL-4, IL-10, IFN-γ or TNF-α concentrations were evaluated using commercial ELISA kit according to the manufacturer’s instructions (R&D...
Systems, Minneapolis, MN). Cytokine concentrations were determined by reference to standard curves constructed with known amounts of mouse recombinant IL-4, IL-10, IFN-γ or TNF-α. The sensitivity limits for the assays were 2 pg/ml for IL-4, 4.0 pg/ml for IL-10, 2 pg/ml for IFN-γ and 1.88 pg/ml for TNF-α.

Statistical analysis.

Results are expressed as the mean ± SD for each group. Statistical evaluation of the differences in survival rates was checked by Kaplan-Meier test, and then a Log-Rank test was done. Statistical differences in parasite burdens, antibody titers, in vitro T cell proliferation and cytokine levels were determined using the Kruskal Wallis test. Differences among the various groups were considered significant when $P < 0.05$. 
RESULTS

Expression of recombinant *T. gondii* ROP1 and GRA7 in ARPE-19 cells *in vitro* and in BALB/c mice *in vivo*.

We constructed the recombinant plasmids pGRA7, pROP1 and pGRA7-ROP1, and confirmed that they expressed the *T. gondii* GRA7 and/or ROP1 proteins *in vitro* by western blot (Fig. 1A). The lysate of ARPE-19 cells transfected with pGRA7, pROP1 and pGRA7-ROP1 showed specific expression of GRA7 and ROP1 proteins recognized by anti-Flag antibody (pGRA7, 27 kDa; pROP1, 43 kDa and pGRA7-ROP1, 66 kDa) as expected, based on their secretory protein property. In addition, we also confirmed that pGRA7, pROP1 and pGRA7-ROP1 were localized to the cytoplasm of transfected cells (Fig. 2). To identify the expression of recombinant plasmid *in vivo*, the presence of GRA7 or/and ROP1 from spleen of vaccinated mice were analyzed using M-RT-PCR method. Fig. 1B shows that expressions of GRA7 and ROP1 were not detected in the vaccinated mice with only vector, pIL12 or vector+pIL12 DNA, whereas expressions of GRA7 (645 bp) or/and ROP1 (1098 bp) were detected in the pGRA7 and/or pROP1 injected mice with M-RT-PCR. In case of pGRA7-ROP1, the sequence containing fused region (1749 bp) were amplified by PCR using GRA7 forward and ROP1 reverse primer (Fig. 1C). These RT-PCR products
were further sequenced to assure their identity. Thus, it was proven that the pGRA7, pROP1 and pGRA7-ROP1 were transfected and expressed their own products in the immunized mice.

T. gondii specific IgG2a titers were higher in pGRA7-ROP1-immunized mice than those of pGRA7 or pROP1-immunized mice.

As shown in Fig. 3, T. gondii IgG titers increased with time in mice immunized with pGRA7 and/or pROP1, with or without pIL12. In particular, high IgG titers were detected in mice immunized with pGRA7-ROP1 combined with pIL12 at 4 weeks after first immunization (P<0.01 versus the other groups). However, there were no significant differences in the IgG titers of mice immunized with the single-gene vaccines (pGRA7 and pROP1) with or without pIL12 and pGRA7-ROP1, nor were there any significant differences between mice immunized with pGRA7 and/or pROP1 throughout the study period. Mice immunized with empty vector, pIL12, or vector+pIL12 did not produce IgG against STAg.

In order to determine whether a Th1 or Th2 response was elicited in immunized mice, T. gondii specific IgG1 and IgG2a titers of immunized mice sera (eight weeks after first immunization) were analyzed using STAg (Fig. 4). IgG2a titers were significantly higher compare to IgG1 titers in mice immunized with pGRA7, pROP1, and pGRA7-ROP1 (IgG2a OD>0.5, IgG1 OD<0.2), however,
there were no significant differences in mice immunized with vector, pIL12 and vector+pIL12 control group. Furthermore, the preponderance of IgG2a over IgG1 was markedly higher in mice immunized with either a single-gene vaccine combined with pIL12 or the multiple-gene vaccine compared to single-gene immunized mice ($P<0.05$).

To measure the IgG2a titer of $T. gondii$ antigen specific antibody in immunized mice, we purified bacterially expressed-recombinant $T. gondii$ GRA7 and ROP1 protein (Fig. 5) and the presence of specific antibodies against each recombinant protein in the sera were examined by endpoint dilution method. There were a good correlation ($r^2=0.76$) between the OD (antibody titers) and the serum dilation factor of $T. gondii$-infected mouse sera, and calculated endpoint titers were determined by using the straight line through the OD-dilution points to find the serum dilution at which the OD would equal the cutoff value. In endpoint titration of specific IgG2a antibody against $T. gondii$ GRA7 and ROP1, mice immunized with pGRA7 and/or pROP1 produced high levels of specific IgG2a antibodies against rGRA7 and rROP1 (Table 1). Moreover, mice immunized with a single-gene and pIL12 showed higher endpoint titration compared with a single-gene vaccinated mice. These results indicated that pGRA7 and/or pROP1 immunized mice induced a specific antibodies against $T. gondii$ GRA7 and/or ROP1, and induced predominant Th1 type immune response.
pGRA7-ROP1-immunized mice had further increased IFN-γ and TNF-α production than those of pGRA7 or pROP1-immunized mice.

To determine whether single- or multiple-gene immunization augments the Th1 or Th2 cytokine response, purified GRA7- and ROP1-treated culture supernatants of splenocytes were obtained from immunized mice four weeks following the final immunization. As shown in Table 2, IL-10, IFN-γ, and TNF-α, but not IL-4, levels in mice immunized with single- or multiple-gene vaccines were significantly higher than those of mice immunized with empty vector, pIL12, or vector+pIL12. Moreover, IFN-γ and TNF-α levels of mice immunized with pROP1 or pGRA7-ROP1 were further increased by pIL12 co-administration. However, IL-4 levels in all of immunized mice didn’t show significant differences.

In vitro splenocyte proliferation was significantly higher in pGRA7 and/or pROP1-immunized mice.

As shown in Fig. 6, in vitro splenocyte proliferation was significantly higher in mice immunized with pGRA7 and/or pROP1 with or without pIL12 from two weeks after immunization as compared with mice immunized with empty vector, pIL12, or vector+pIL12 (P<0.05). However, there were no significant differences between single-gene immunizations with or without pIL12 co-delivery and multiple-gene immunized mice. Co-administration of pGRA7-ROP1 and pIL12 at four and eight...
weeks after immunization augmented spleen cell proliferation when compared with spleen cells from mice immunized with pGRA7-ROP1 alone or the single-gene with pIL12 ($P<0.05$).

pGRA7-ROP1-immunized mice showed enhanced protection against *T. gondii* challenge than those of pGRA7 or pROP1-immunized mice.

Survival days after challenge with lethal dose of *T. gondii* cysts are shown in Fig. 7. Mice immunized with empty vector, pIL12, or vector+pIL12 died from 8 days after *T. gondii* infection, and all mice were dead until 18 postinfection. Significantly longer survival days were obtained in single-gene or multiple-gene immunized mice compared with mice immunized with empty vector, pIL12, or vector+pIL12 ($P<0.01$). Especially, pGRA7-ROP1- and pGRA7-ROP1+pIL12-immunized mice were survived significantly longer than those of single-gene immunized mice, and the survival rates of them were 33.3% and 50.0% at 4 weeks after infection, respectively.

Four weeks after oral challenge with *T. gondii* cysts, the number of cysts in the brain was determined microscopically, and the cyst reduction rates to vector control were higher in the multiple-gene immunized mice than those of single-gene immunized mice (Fig. 8). The reduction rates of single- or multiple-gene immunized mice were further increased with pIL12 co-delivery, and the highest reduced rate were observed in pGRA7-ROP1+IL12 immunized mice.
DNA vaccination can protect animals and human beings against pathogenic microorganisms, particularly intracellular parasites (1). In the present study, BALB/c mice immunized intramuscularly with pGRA7, pROP1 or pGRA7-ROP1 produced specific antibodies against *T. gondii* GRA7 and/or ROP1, and protective immunity was induced. Moreover, immunization with the multi-antigenic gene plasmid pGRA7-ROP1 induced a greater tendency toward Th1-type immune responses than did immunization with the single-antigen gene plasmids pGRA7 or pROP1. This type of immune response was further enhanced by co-delivery of pIL12 as a genetic adjuvant.

It has been demonstrated that a Th1-biased response is required for effective protection in naturally-occurring *T. gondii* infection (6). To determine the T-helper response of immunized mice, we evaluated the IgG subclass induced by DNA immunization. The ratio of IgG2a to IgG1 titers against STAg, characteristic of Th1-type response, was markedly higher in pGRA7-ROP1 immunization than in single-gene vaccination, which was also confirmed by IgG2a endpoint titration against *T. gondii* recombinant GRA7 and/or ROP1. Furthermore, immunization with a single-gene vaccine with pIL12 augmented the predominance of IgG2a over IgG1 induced by single-gene vaccine alone. Our data confirmed previous reports that seroconversion can readily be obtained by DNA vaccination (16), and the preponderance of anti-*Toxoplasma* IgG2a over IgG1 was greater in multiple-gene than in single-gene vaccination (18). It is well-known that IFN-γ is the central cytokine...
responsible for resistance against *T. gondii* during both the early and late stages of infection (6). We also detected both Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokines in splenocytes from pGRA7, pROP1 or pGRA7-ROP1-immunized mice. In this study, IFN-γ production was increased about 8-12 folds in single- and multiple-gene immunized mice, however IL-4 productions was not changed significantly after GRA7 and/or ROP1 immunization. Our results show that immunization with pGRA7, pROP1 or pGRA7-ROP1 elicits a Th1 immune response, and multiple-gene vaccine induced greater Th1 immune responses than those of single-gene vaccine. Also this phenomenon of single-gene vaccine is further enhanced by the addition of pIL12. These results were similar with previous data that immunization with a plasmid encoding the ROP1 genes has enhanced the production of IFN-γ *in vitro* and ROP1-specific IgG2a antibody in sheep (10).

Generally, cell-mediated immunity, and especially the T cell immune response, plays a key role in protection against *T. gondii* infection. And it was reported that multi-antigenic gene plasmids are more protective against *T. gondii* challenge than single-antigen gene plasmids (4, 5, 12). In contrast, a significant suppression of immune responses was shown when the plasmids were pooled in a cocktail and injected into a single site (15). In this study, specific T cell responses, GRA7 or ROP1 specific lymphoproliferation based on BrdU assay, were observed in spleen cell cultures from mice immunized with either pGRA7 or pROP1 with or without pIL12, but not in mice immunized with empty vector, pIL12 or vector+pIL12. Also pGRA7- and/or pROP1- vaccinated mice produced
significantly higher levels of IFN-γ, and these mice also had significantly higher reduction rate of parasite burden in the brain and longer survival days as compared the empty vector-, pIL12- or vector+pIL12-vaccinated mice. These data indicate that the pGRA7, pROP1 and pGRA7-ROP1 DNA vaccines, either alone or in combination with pIL12, may provide an appropriate epitope for presentation on MHC-I (17), and evoke cellular immune response to control the multiplication and spreading of *T. gondii* infection (7, 11). These results were similar with the previous study (9) that the combination of GRA1 and GRA7 reduced brain cyst burden up to 89% in C3H/HeN mice after challenged with 76K. However, Vercummen et al. (16) reported that BALB/c mice vaccinated with genes encoding *T. gondii* ROP2, GRA1 and GRA7 led to mortality rates at least 80% after challenge with IPB-G strain, so no protection was observed in BALB/c-vaccinated mice. These results were somewhat similar with and somewhat different from ours. The mortality rate of single-gene vaccinated mice (80-100%) was similar with ours (survival rate 0-16%), however they did not evaluate the protective effects of muti-antigenic vaccine and adjuvant, also used different parasite strain.

From this study, we show that DNA vaccine expressing the *T. gondii* GRA7 or ROP1 antigens (pGRA7 or pROP1) and a fusion of both (pGRA7-ROP1) induced the specific humoral and cellular immune responses in BALB/c mice, and immunization with either a multi-antigenic DNA vaccine pGRA7-ROP1 or a single-gene vaccine combined with pIL12 induces greater Th-1 type immune
responses and protective efficacy against *T. gondii* infection as compared with individual antigen.

Nevertheless, further studies are needed to develop more effective multi-antigenic DNA vaccines by varying the components thereof.
ACKNOWLEDGEMENTS

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GRA4 and SAG1 associated with GM-CSF plasmid, against acute, chronic and congenital toxoplasmosis in mice. Vaccine 23:4489–4499.


TABLE 1. IgG2a endpoint titers determined by ELISA against *Toxoplasma gondii* recombinant GRA7 and/or ROP1 protein

<table>
<thead>
<tr>
<th>Group</th>
<th>Endpoint titer against recombinant <em>T. gondii</em> antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rGRA7</td>
</tr>
<tr>
<td>pGRA7</td>
<td>8,533</td>
</tr>
<tr>
<td>pROP1</td>
<td>0</td>
</tr>
<tr>
<td>pGRA7-ROP1</td>
<td>14,933</td>
</tr>
<tr>
<td>pGRA7+pIL12</td>
<td>17,066</td>
</tr>
<tr>
<td>pROP1+pIL12</td>
<td>0</td>
</tr>
<tr>
<td>pGRA7-ROP1+pIL12</td>
<td>17,066</td>
</tr>
</tbody>
</table>

Data represent the average endpoint titer of 3 mice per group. Three independent experiments were performed and data from one representative were shown. All mice seroconverted to the antigens they were vaccinated. Mice vaccinated with the empty vector, pIL12, vector+pIL12 did not develop antibodies to the recombinant antigens.
TABLE 2. Cytokine concentrations of culture supernatants of the splenocytes from mice immunized with empty vector, pIL12, pGRA7 and/or pROP1 by ELISA

<table>
<thead>
<tr>
<th>Groups</th>
<th>GST antigen-stimulated (pg/ml)</th>
<th>GRA7 and ROP1 antigen-stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-4</td>
<td>IL-10</td>
</tr>
<tr>
<td>vector</td>
<td>34±4</td>
<td>36±14</td>
</tr>
<tr>
<td>pIL12</td>
<td>35±7</td>
<td>36±8</td>
</tr>
<tr>
<td>vector+pIL12</td>
<td>33±15</td>
<td>34±13</td>
</tr>
<tr>
<td>pGRA7</td>
<td>38±12</td>
<td>68±16</td>
</tr>
<tr>
<td>pROP1</td>
<td>35±2</td>
<td>77±13</td>
</tr>
<tr>
<td>pGRA7-ROP1</td>
<td>36±3</td>
<td>94±11*</td>
</tr>
<tr>
<td>pGRA7+pIL12</td>
<td>34±7</td>
<td>86±11*</td>
</tr>
<tr>
<td>pROP1+pIL12</td>
<td>36±7</td>
<td>77±23</td>
</tr>
<tr>
<td>pGRA7-ROP1+pIL12</td>
<td>35±3</td>
<td>101±21*</td>
</tr>
</tbody>
</table>

a) Values for IL-4, IL-10, IFN-γ and TNF-α are for 24h, 72h, 96h and 48 h, respectively. Data represent the mean±SD of 3 mice per group. Splenocytes from mice were harvested 4 weeks after the last immunization. Single-cell suspensions of splenocytes were stimulated by incubation with purified GST antigen alone or in combination with recombinant GRA7 and ROP1 antigens (5 μg/ml). Three independent experiments were performed and data from one representative experiment were shown.

* P<0.05, as compared with mice immunized with empty vector.
**FIG. 1.** Detection of plasmid expression *in vitro* and *in vivo*. (A) Western blot analysis of ARPE-19 cells transfected with recombinant plasmid. Lysates of ARPE-19 cells transfected with empty vector, pGRA7, pROP1 or pGRA7-ROP1 recombinant plasmid DNA were electrophoresed in a 12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes and reacted with an anti-flag primary antibody. (B) *In vivo* expression of pGRA7, pROP1 and pGRA7-ROP1. Total RNA were isolated from spleens of DNA vaccinated mice (lane 1, vector; lane 2, pIL12; lane 3, vector+pIL12; lane 4, pGRA7; lane 5, pROP1; lane 6, pGRA7-ROP1; lane 7, pGRA7+pIL12; lane 8, pROP1+pIL12; lane 9, pGRA7-ROP1+pIL12) 4 week postinoculation and were analyzed by multiplex reverse transcription polymerase chain reaction (M-RT-PCR) using both GRA7 and ROP1 primers. (C) *In vivo* expression of pGRA7-ROP1 in pGRA7-ROP1 (lane 1) and pGRA7-ROP1+pIL12 (lane 2) immunized mice by RT-PCR using GRA7 forward and ROP1 reverse primer. Positions of molecular size standards are indicated on the left of the panel.

**FIG. 2.** Vector, pGRA7, pROP1 and pGRA7-ROP1 were localized to the cytoplasm of transfected cells. ARPE-19 cells were transfected with vector (A), pGRA7 (B), pROP1 (C) or, pGRA7-ROP1
(D) Cells were fixed 48 h after transfection and anti-flag antibody (red) was used to evaluate expression of protein levels and nuclear DNA was stained with DAPI (shown in blue).

FIG. 3. Detection of total IgG level in the vaccinated mice sera by ELISA. Anti-*T. gondii* IgG titers in sera (diluted 1:100) of mice immunized with empty vector, pIL12, pGRA7 and/or pROP1. Immune sera were collected at each time point and IgG titers were determined by ELISA. Results were expressed as mean ± SD (3 mice per group). Three independent experiments were performed and data from one representative experiment were shown.

FIG. 4. Detection of IgG1 and IgG2a levels in the vaccinated mice sera by ELISA. Anti-*T. gondii* IgG1 and IgG2a titers in the sera (diluted 1:100) of mice immunized with empty vector, pIL12, pGRA7 and/or pROP1. Immune sera were collected at 8 weeks after first immunization and determined by ELISA. Results were expressed as mean ± SD (3 mice per group). Three independent experiments were performed and data from one representative experiment were shown.
FIG. 5. Expression and purification of recombinant proteins. Proteins were submitted to SDS–PAGE and revealed by Coomassie blue staining. (A) Lane 1, pGEX-4T-1 transformed *E. coli*; lane 2, IPTG-induced pGEX-4T-1 transformed *E. coli* soluble fraction; lane 3, purified GST protein (26 kDa).

(B) Lane 1, pGEX-GRA7 transformed *E. coli*; lane 2, IPTG-induced pGEX-GRA7 transformed *E. coli* soluble fraction; lane 3, purified GRA7 protein (53 kDa). (C) Lane 1, pGEX-ROP1 transformed *E. coli*; lane 2, IPTG-induced pGEX-ROP1 transformed *E. coli* soluble fraction; lane 3, purified ROP1 protein (69 kDa). (D) Western blot analysis of purified recombinant proteins with anti-GST primary Ab. Lane 1, GST; lane 2, GRA7; lane 3, ROP1. M, protein molecular weight marker.

FIG. 6. *In vitro* proliferation of splenocytes from mice after stimulation with the soluble tachyzoite antigen of *T. gondii* (STAg). Splenocytes were cultured with 10 μg/mL STAg for 72 h and proliferation was measured using a chemiluminescent BrdU ELISA kit. Absorbance was evaluated by an ELISA reader at 370 nm with a 492 nm reference. Results are expressed as mean ± SD (3 mice per group). Three independent experiments were performed and data from one representative experiment were shown.
FIG. 7. Survival curves of mice immunized with empty vector, pIL12, pGRA7, and/or pROP1 after challenge with 1,500 cysts of *T. gondii* Me49 strain of orally four weeks after the final immunization (6 mice per group). Two independent experiments were performed; data from one representative experiment were shown.

FIG. 8. Reduction rate to vector control of *T. gondii* cysts numbers in the brain of mice immunized with empty vector, pIL12, pGRA7 and/or pROP1. Four weeks after the last immunization, mice were orally challenged with a nonlethal dose of the *T. gondii* Me49 strain (20 cysts per mouse) and evaluated 4 weeks after infection. Cyst reduction rate to vector control was calculated with the formula: \[1-(\text{brain cyst numbers of vaccinated mice/brain cyst number of vector control})] \times 100.\] Data were represented as mean ± SD. Three independent experiments were performed and data from one representative experiment were shown. ** As compared with mice immunized with empty vector (*P*<0.01). * As compared with mice immunized with single-gene vaccines (pGRA7 or pROP1) only (*P*<0.05).
FIG. 1.
FIG. 2
FIG. 3.

Absorbance (490 nm)

Weeks after Immunization

0 W 2 W 4 W 8 W

0.0 0.5 1.0 1.5 2.0

Absorbance (490 nm)
FIG. 4.

Absorbance (490 nm)

- vector
- pIL12
- vector+pIL12
- pGRA7
- pROM1
- pGRA7-ROM1
- pGRA7-pIL12
- pROM1-pIL12
- pGRA7-ROM1-pIL12
- pGRA7-pROM1-pIL12
- IgG1
- IgG2a

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
FIG. 5. – please convert into white and black
FIG. 6.
FIG. 7.
FIG. 8.

Cyst reduction rate to vector control (%)

- vector
- pIL12
- vector+pIL12
- pGRA7
- pGR
- A7
- pROP1
- pGR
- A7-ROP1
- pGRA7+pIL12
- pROP1+pIL12
- pGRA7-ROPI+pIL12

Legend:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001

Graph showing the cyst reduction rates for different vector combinations compared to a control vector.