

GRA1 protein vaccine confers better immune response compared to codon-optimized GRA1 DNA vaccine

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Abstract

The present study evaluates immunogenicity and protection potency of a codon-optimized GRA1 DNA vaccine, wild type GRA1 DNA vaccine and an adjuvanted recombinant GRA1 protein vaccine candidate in BALB/c mice against lethal toxoplasmosis. Of the three GRA1 vaccines tested, the recombinant GRA1 protein vaccine results reveal significant increase in immune response and prolonged survival against acute toxoplasmosis compared to DNA vaccinations. Immune response and protection conferred by codon-optimized GRA1 DNA vaccine was slightly better than wild type GRA1 DNA vaccine.

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1. Introduction

Toxoplasma gondii is one of the most successful protozoan parasites because it has a very broad host range, infecting all warm-blooded animals, including humans [1] and causes serious clinical presentations. There is no 100% effective drug to treat all clinical presentations of *T. gondii*. Available drugs have many side effects and reactivation may occur any time. The development of a vaccine, which can prevent the consequences of acute infection, is therefore, an attractive

alternative. After classification in category B bioterrorism agents as a food and water safety threat, the demand for a protective vaccine has increased [2].

Vaccine strategies against toxoplasmosis aim to induce Th1 response and IFN- γ production because immune protection against *T. gondii* in mice is primarily correlated with Th1 cell mediated immunity [3,4] and IFN- γ secretion [5]. Many attempts have been made to create an immunogenic protective vaccine against toxoplasmosis using mutant *T. gondii* strains, purified proteins, and DNA vaccines since 1990s [6]. The accumulated results suggest that a protective vaccine against *T. gondii* infection is feasible. Recombinant protein vaccines and DNA vaccines using Surface Antigen 1 (SAG1), SAG2, Dense Granule Antigen 1 (GRA1), GRA4, GRA7, Rhoptry Protein 2 (ROP2), Microneme Protein 3 (MIC3) and Heat

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Shock Protein 70 (HSP70) elicit partial, full, or occasionally no protection in animals depending on the virulence of the *T. gondii* strains [7–30]. Of all the vaccine candidates against toxoplasmosis, GRA1 appears to be an immunogenic promising vaccine candidate [8,26–28,31]. Existing GRA1 vaccination trials used recombinant protein or DNA vaccines. Although immune response and protection potency have been investigated in detail about GRA1 DNA vaccination [26,28], the cellular immune response and protection potency of an adjuvanted recombinant GRA1 protein vaccine haven't been evaluated in mice yet [8,27,31]. Taken together, the experimental studies using various GRA1-derived vaccines are encouraging, however much work remains to put together the pieces of the puzzle, i.e. highlight the complexity of the immune response and the unpredictable relationships among various parameters such as vaccine strategy to be used against toxoplasmosis, protection and cellular immune response elicited by recombinant protein vaccine or parasite strain used to challenge the mice. In order to comprehensively evaluate these variables, the present study compares cellular immune response and protection potency of a recombinant GRA1 protein vaccine adjuvanted with a strong Th1 inducer Provacx [32,33], a wild type GRA1 DNA vaccine and a GRA1 DNA vaccine codon-optimized for protein expression in mammalian cells. Codon usage optimization is widely accepted as a means of increasing the level of *in vivo* antigen expression from DNA vaccines and improving the cellular and humoral immune responses against the expressed antigens from diverse microorganisms [34–47]. The codon-optimized GRA1 DNA vaccine used in this study is the first example of a codon-optimized gene being used as DNA vaccine candidate against *T. gondii*.

2. Materials and methods

Unless noted otherwise, all reagents were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA).

2.1. Mice

Female 6 week-old BALB/c mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). Mice were housed and fed under standard, suitable conformist conditions. The experimental plan was performed under the instructions and approval of the Institutional Animal Care and Use Committee of UCI for animal ethics.

2.2. Parasites and preparation of *T. gondii* antigen

T. gondii RH strain tachyzoites were used to challenge the immunized mice and to prepare *T. gondii* antigen. RH strain tachyzoites were maintained in confluent monolayers of human foreskin fibroblasts (HFF) cell culture grown in Dulbeccos modified Eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Hyclone,

Logan, UT), 2 mM L-glutamine and 50 mg gentamycin per ml (Invitrogen–Gibco, Carlsbad, CA). *T. gondii* antigen (TAG) was prepared with the RH strain tachyzoites obtained from HFF cell culture as described [12].

2.3. Computationally optimized DNA assembly (CODA) of the GRA1 gene

The GRA1 protein sequence from amino acid positions 25–190, excluding the signal peptide, was obtained from GENBANK database (accession number M26007), and submitted to CODA at the Computational Biology Research (CBR) Laboratory of the UC Irvine Institute for Genomics and Bioinformatics. The CODA algorithm generated a list of 36 linearly overlapping and abutting oligonucleotides, 39–64 nucleotides (nts) in length, optimized for self-assembly and expression of the codon-optimized GRA1 gene (co-GRA1) in mammalian cells. These oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA).

2.3.1. Assembly of co-GRA1 intermediate DNA fragments

The full-length co-GRA1 DNA sequence was divided into three partially overlapping intermediate DNA fragments, each of which was subdivided into 12 short overlapping and abutting DNA oligonucleotides (intermediate fragment 0:237 bp; intermediate fragment 1:196 bp; intermediate fragment 2:196 bp). The co-GRA1 gene was assembled in two steps in which the DNA oligonucleotides were assembled first to form the three intermediate DNA fragments and then the intermediated fragments were mixed to form the full-length gene. For the assembly of each of the three intermediate DNA fragments of GRA1, the constituent DNA oligonucleotide set was added to a primer extension reaction at a final concentration of 0.1 μ M along with an excess (0.5 μ M) of leader and trailer primer oligonucleotides (the most 5'- and 3'-distant DNA oligonucleotides). The reactions included 2.5 U of *Pfu*Ultra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA), 300 μ M dNTPs, and 1 \times *Pfu*Ultra reaction buffer. The primer extension and PCR amplification reactions were performed in an Eppendorf Mastercycler (Hamburg, Germany) using the following calculated-control protocol: 5 min initial denaturation step at 95 °C, followed by 25 cycles of 20 s at 95 °C, 30 s at 62 °C, and 1 min at 72 °C, and an extension of 5 min at 72 °C. The PCR products were visualized by agarose gel electrophoresis.

2.3.2. TOPO PCR cloning

PCR product of each intermediate DNA fragment of the GRA1 gene was cloned into a pCRII-Blunt-TOPO vector according to the manufacturer's recommended protocol using One Shot electrocompetent Top 10 *Escherichia coli* cells (Invitrogen, Carlsbad, CA). The resulting plasmids, which contained intermediate DNA fragments of the correct size were visualized by agarose gel electrophoresis and purified for DNA sequencing using a plasmid mini purification kit

according to the manufacturer's protocol (Aurum, Bio-Rad, Hercules, CA).

2.3.3. PCR assembly of full length co-*GRA1*

One of each sequence-verified intermediate DNA fragment of the *GRA1* gene was isolated from the pCR-Blunt II TOPO vector by PCR amplification using the most 5'- and 3'-distant oligonucleotides for each intermediate DNA fragment. The amplification reactions included an excess of 0.25 μ M of leader and trailers, 2.5U of *Pfu*Ultra High-Fidelity DNA polymerase (Stratagene), 150 μ M dNTPs, and 1 \times *Pfu*Ultra reaction buffer. The PCR amplification reactions were performed using the following calculated-control protocol: 5 min initial denaturation step at 95 °C, followed by 25 cycles of 20 s at 95 °C, 1 min at 68 °C, and 1 min at 72 °C, and a final extension of 5 min at 72 °C. Subsequently, 1 μ l of each intermediate DNA fragment PCR product was combined with 1 μ M *GRA1* gene leader (5'- most distant oligonucleotide of 45 nts and 1 μ M gene trailer (the 3'- most distant oligonucleotide of 44 nts) in another primer extension, using the same PCR amplification reaction defined above to generate the full-length co-*GRA1* gene (531 bp). The PCR product visualization, TOPO PCR cloning and plasmid purification for DNA sequencing of the full-length co-*GRA1* gene were performed as described above.

2.4. Cloning of wt-*GRA1* and co-*GRA1* into a mammalian expression vector

A cytomegalovirus (CMV) promoter containing mammalian expression vector (10 μ g; 5.1 kb; kanamycin resistant; Gene Therapy Systems, San Diego, CA) was linearized by *Bam*HI as described [48]. The following primers were designed to generate a linear acceptor vector with sequences suitable for cloning by homologous recombination, 5'-CCATCACCATCACCATCACCATCACGTCGAC-3' (forward primer, 33 nt), and 3'-TGATCTTTTATTAGCCAGAA-GTGATCTGGATCC-5' (reverse primer, 33 nt). During the PCR reaction an N-terminal polyhistidine tag was introduced to CMV vector immediately after the start codon, which was fused in frame with the ORF for purification and detection purposes. The linear acceptor vector was amplified as described [48]. The resulting linear CMV vector was used for *in vivo* recombination cloning.

2.4.1. PCR amplification of wt-*GRA1* and co-*GRA1*

Wt-*GRA1* and co-*GRA1* were isolated from their recombinant plasmids with primers designed to incorporate adapter termini into the PCR product to facilitate directional cloning by homologous recombination ('HiRec') into the CMV vector. The primers for the wild type *GRA1* were 5'-CCATCACCATCACCATCACCATCACGTCGACGCCG-AAGGCGGCGACAACCA-3' (forward primer, 53 nt; overlapping sequences, underlined) and 3'-TGATCTTTTATTAGCCAGAAAGTGATCTGGATCCCTCTCTCTCTCTCTCTCTG-TTAGGA-5' (reverse primer, 56 nt; overlapping sequences,

underlined). The primers for co-*GRA1* were 5'-CACCATCACCATCACCATCACCATCACCATCACGTCGACGCGAGAGGG-AGGCGACAACCA-3' (forward primer, 53 nt; overlapping sequences, underlined) and 3'-TGATCTTTTATTAGCCAGAAAGTGATCTGGATCCCTCCCTCTCGCCGGTC-AGGA-5' (reverse primer, 56 nt; overlapping sequences, underlined). Co-*GRA1* and wt-*GRA1* genes were isolated from their template plasmids using the designed primers (0.5 μ M each), 2U of *Taq*DNA polymerase (Fisher Scientific), 150 μ M dNTPs and 1 \times *Taq*DNA polymerase reaction buffer with the following calculated PCR protocol; 5 min initial denaturation step at 95 °C, followed by 30 cycles of 0.5 min at 95 °C, 0.5 min at 50 °C, and 3.5 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR products were visualized by agarose gel electrophoresis, purified using a PCR purification kit (Qiagen) according to the manufacturer's protocol, and quantified by spectrophotometry.

2.4.2. *In vivo* recombination cloning method

In vivo recombination cloning was performed as described [48]. Briefly, the linear CMV vector, purified PCR product, and DH5 α competent cells were mixed and heat shocked. Plasmids from overnight culture were purified using plasmid mini purification kit according to the manufacturer's protocol (Aurum, Bio-Rad) and visualized by agarose gel electrophoresis. The resulting plasmids containing wt-*GRA1* and co-*GRA1* were named CMV/wt-*GRA1* and CMV/co-*GRA1*, respectively.

2.5. *In vitro* transfection of human embryonic kidney cells

Human embryonic kidney (HEK) 293 mammalian cells were obtained from the American Type Culture Collection (ATCC number: CRL-1573, Manassas, VA). HEK 293 cells were maintained in Dulbecco's modified eagle medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU of penicillin per ml, and 100 g of streptomycin per ml (Invitrogen-Gibco) at 5% CO₂ and 37 °C. Then HEK 293 cells were transfected with CMV/wt-*GRA1* and CMV/co-*GRA1* using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). HEK 293 cells transfected in four-well chamber slides were observed using monoclonal anti-polyhistidine antibody and immunofluorescence microscopy to determine the level of protein expression. In addition, the same HEK 293 cells, which were transfected in 10 cm-diameter culture dishes with the same protocol, were lysed to compare the protein expression using monoclonal anti-polyhistidine antibody and Western blotting.

2.6. Immunofluorescence

HEK 293 cells transfected with CMV/wt-*GRA1* or CMV/co-*GRA1* plasmid in four-well chamber slides fixed

with methanol and acetone and incubated with 1% BSA (diluted in $1 \times$ PBS) (Sigma–Aldrich, St. Louis, MO). The slides were then probed with the monoclonal anti-polyhistidine antibody (Sigma) with a dilution of 1:200 for 1 h at room temperature, washed and then probed with anti-mouse IgG antibody conjugated with FITC produced in sheep (clone, His-1; Sigma) with a dilution of 1:500 for 1 h at room temperature. Thereafter, slides were washed and visualized using an immunofluorescence microscope (Zeiss Axiomat, Thornwood, NY).

2.7. Sodium dodecyl sulfate-polyacrylamide gels and Western blotting

HEK 293 cells, transfected with CMV/wt-GRA1 and CMV/co-GRA1 obtained from 10 cm-diameter culture dishes, were scraped from the dishes and counted with a hemocytometer. Equal amount of cells (approximately 6×10^6 cell per each sample) were washed thrice with $1 \times$ PBS followed by several cycles of freezing and thawing. The lysate of cells were centrifuged at $10,000 \times g$ for 10 min. The amount of protein in the supernatant was determined using Coomassie Plus Protein Assay Reagent kit according to the manufacturer's protocol (Pierce, Rockford, Ill, USA). Serially diluted bovine serum albumin (BSA) was used as the reference. To compare the expression levels of CMV/wt-GRA1 and CMV/co-GRA1 in HEK 293 cell lysate, an equal amount of protein (approximately, 500 μ g protein per each sample) was separated by 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). To detect the immunoreactivity of the vaccine-induced antibodies, TAG was separated with 12% SDS-PAGE gel. The separated protein was transferred to a polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-P, Millipore, Billerica, MA) and the membranes, which contained HEK 293 cell lysate, were probed with a 1:1000 dilution of the monoclonal anti-polyhistidine antibody (Sigma). To detect the immunoreactivity, the TAG-containing membranes were probed with 1:100 dilutions of four different serum sample pools, collected from three groups of vaccinated mice and control groups. Then the membranes were probed with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) antibody (Bio-Rad). Thereafter, the blot was developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro-BT (Fisher Scientific) diluted in alkaline phosphatase-developing buffer (0.1 M Na_2CO_3 , pH 9.5, 0.1 M NaCl, 5 mM MgCl_2).

2.8. Protein expression and purification

E. coli RosettaTM 2(DE3) pLysS chemically competent cells (Novagen, San Diego, CA) were transformed with pUETΔGRA1 [49] and grown in 1 l LB supplemented with 100 μ g/ml ampicillin and 50 μ g/ml chloramphenicol with vigorous shaking at 37 °C up to an optical density of 0.4, calculated at 600 nm. Then, the cells were induced with

isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM with vigorous shaking at 20 °C overnight. The cells were centrifuged at $5000 \times g$ and the pellet was resuspended with 50 ml pre-chilled loading buffer (50 mM Tris-Cl pH: 7.5, 0.3 M NaCl and 0.5 mM CaCl_2). Then, the cells were disrupted with an M-110L microfluidizer processor (Microfluidics Corporation, Newton, MA) at a low temperature under internal pressure of 18,000 psi. The processed sample was centrifuged at $30,000 \times g$ for 1 h at 4 °C. The clarified supernatant was filtered through 0.45 μ m filter (Nalgene, Rochester, NY). Purification of the protein was performed by ÄKTA FPLC, a liquid chromatography system, which is controlled by UNICORNTM software, using a 5 ml HiTrap Chelating HP column (Amersham Biosciences, Pittsburgh, PA). Clarified supernatant was applied to the HiTrap column with loading buffer. Then, the column was washed with 100 mM imidazole containing 50 mM Tris-Cl, pH 7.5, 0.3 M NaCl, and 0.5 mM CaCl_2 buffer overnight at 4 °C. The recombinant GRA1 (rGRA1) protein was eluted by raising the imidazole concentration to 500 mM. The protein fractions were detected by UV280, confirmed by 12% SDS-PAGE, pooled, and concentrated with an Ultrafree-MC Centrifugal Filter Units (Millipore) at 4 °C. The protein was further purified by FPLC on a Superdex 200 column to remove excess endotoxin. The subsequent protein fractions were pooled, concentrated and quantitated. The molecular size of purified GRA1 protein was determined by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Applied Biosystems, Foster City, CA) using a sinapinic acid matrix. Three purified protein samples from three different preparations were analyzed.

2.9. Depletion and determination of endotoxin from purified protein

Detoxi-gel affinity column, which contains immobilized Polymyxin B, was used to remove endotoxin from the purified GRA1 protein sample according to the manufacturer's protocol (Pierce). The amount of endotoxin in the eluted sample was determined with a Limulus Ameobocyte Lysate Gel-Clot test using Pyrotell single test vials according to the manufacturer protocol (Associates of Cape Cod, East Falmouth, MA). The concentration of endotoxin-depleted, purified rGRA1 samples were calculated as described above and stored at -80 °C.

2.10. Vaccination and challenge

Three groups of female, 6 week-old, BALB/c mice (eight per each group) were vaccinated twice at three weeks interval with CMV/wt-GRA1, CMV/co-GRA1, and rGRA1 protein with PROVAXTM adjuvant (rGRA1-Provax). The plasmids were purified using an endo-free plasmid purification kit (EndoFree, Qiagen). During DNA vaccinations, 100 μ g plasmid was injected into both tibialis anterior muscle of anesthetized mice.

The rGRA1-Provax vaccine was formulated using rGRA1 protein and Provax adjuvant. Provax was prepared as described using Tween-80, pluronic L121 and squalane [50]. To evaluate the efficacy of rGRA1-Provax vaccine five mice were vaccinated intraperitoneally with 33 µg of only rGRA1 protein as a control group.

Negative controls were composed of three groups of animals (eight per each) vaccinated with 100 µg empty vector and 33 µl of stabilized Provax emulsion. A group of naïve mice was also used as a control to evaluate the GRA1 vaccinations during the measurement of antigen specific proliferative response and cytokine determination. Tail bleeds were performed three weeks after each vaccination. Eight mice from each group were challenged intraperitoneally with 1×10^5 viable *T. gondii* RH strain tachyzoites nine weeks after the prime vaccination.

2.11. Measurement of humoral antibody response using protein microarray analysis

Protein microarray chips were prepared as described [48,51]. Briefly, 15 µl purified rGRA1 (2.5 mg/ml) printed onto nitrocellulose-coated FAST™ glass slides (Schleicher & Schuell, Keene, NH) using an OmniGrid 100 microarray printer (Genomics Solutions, Ann Arbor, MI). The slides were probed with mouse serum samples diluted in Protein Array Blocking Buffer (PABB, Schleicher & Schuell) which were obtained three weeks after the first and second vaccinations. Proteome slides were washed with $1 \times$ TBS-T buffer (0.5% Tween-20, 20 mM Tris-HCl, 0.5 M NaCl) and complexed antibodies were visualized after incubating the slides with Cy3-conjugated IgG antibody (Jackson ImmunoResearch, West Grove, PA). The proteome slides were washed with $1 \times$ TBS-T and $1 \times$ TBS buffers before scanning in a ScanArray 4000 laser confocal scanner (GSI Lumonics, Billerica, MA). The fluorescence intensity (FI) of each spot was quantified using QuantArray software (GSI Lumonics). Samples were considered positive if the FI of the 1/50-diluted vaccinated animal serum sample exceeded the FI + 5 S.D. of the 1/50-diluted serum sample of the animal injected with the corresponding negative control for the particular group of vaccination.

2.12. Determination of IgG subclass antibody response using protein microarray analysis

Analysis of serum anti-rGRA1 IgG1 and IgG2a subclass antibodies in vaccinated mouse serum samples were performed by protein microarray analysis as described above. After the slides were probed with 1/50-diluted mouse serum samples, the slides were incubated with Cy3-conjugated IgG1 and IgG2a antibody (Jackson ImmunoResearch) at 1/200 dilution for 2 h. Fluorescence intensities were analyzed using ScanArray 4000 laser confocal scanner and QuantArray software (GSI Lumonics) as described above.

2.13. Measurement of antigen-specific proliferative immune response

Nine weeks after the prime vaccination, spleens were surgically removed from euthanized mice (three mice per group). Single-cell suspensions of splenocytes were prepared as described [28]. Aliquots of 5×10^5 viable splenocytes in 100 µl growth medium [$1 \times$ RPMI 1640 supplemented with 10% FCS, 55 µM 2-mercaptoethanol (Invitrogen-Gibco), 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acid, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Cellgro)] were plated in triplicate into each well of 96 well round bottom plate. Before the stimulation of spleen cells, endotoxin-depleted purified rGRA1 was incubated with polymyxin B to a final concentration of 50 µg/ml for 1/2 h. Cells were stimulated with 0.6–50 µg/ml purified rGRA1 protein or stimulated with a concentration of 15 µg/ml unrelated vaccinia virus recombinant H3L (rH3L) control protein. As a positive control in all experiments, cells were incubated with Concanavalin A at a final concentration of 10 µg/ml. As a negative control, only 200 µl growth medium was used. Cultures of cells in a final volume of 200 µl in each well were incubated 72 h at 5% CO₂ and 37 °C and then pulsed with 1 µCi of ³[H]-thymidine (Perkin Elmer Life and Analytical Sciences, Boston, MA) per well, diluted in 50 µl growth medium, during the last 16 h. Cells were harvested using a cell harvester (TOMTEC, manual harvester 96, Mac IIM, Hamden, CT) and glass filter paper (Wallac, Turku, Finland). The radioactivity was quantified by liquid scintillation counting (BETA plate liquid scintillation counter (Wallac). The results are expressed as the stimulation index (SI), which is calculated as the mean counts per minute (cpm) of cells stimulated with rGRA1 protein divided by the mean cpm of controls.

2.14. Cytokine determination

Splenocytes were prepared as described above. Cell-free supernatant was collected and assayed for IL-4 activity at 24 and 72 h and gamma interferon (IFN-γ) activity at 72 h. The concentration of IL-4 and IFN-γ were determined using ELISA kits (eBioscience, San Diego, CA) according to the manufacturer protocol. The sensitivity limit for the ELISA was deduced from the standard curves after serial dilution of the recombinant mouse IFN-γ and recombinant mouse IL-4 standards provided by the kit. Values higher than 100 pg/ml and 10 pg/ml, calculated from the results with naïve mice, were considered positive for IFN-γ and IL-4, respectively.

2.15. Statistical analysis

Data obtained during the study were processed using Prism 3.03 (GraphPad, San Diego, CA). A two-tailed unpaired *t*-test or one-way analysis of variance with 95% confidence interval was used to determine the significance between the vaccination groups. Kaplan–Meier survival

curves were constructed to illustrate protection from lethal toxoplasmosis. Results of humoral immune response, cellular immune response and survival time were expressed as mean \pm standard deviation (S.D.).

3. Results

3.1. PCR assembly of co-*GRA1* and *in vivo* recombination cloning

Wild type *GRA1*, without the signal peptide, is 498 bp in length and has a 52% G/C content. To optimize the *GRA1* gene for expression in mammalian cells, 19 codons were excluded, six codons were reduced in frequency, and 23.1% of the codons were changed (Table 1). The final co-*GRA1* gene has a 63% G/C content. The codon usage of *Homo sapiens* was used to measure the codon adaptation index (CAI) of wt-*GRA1* and co-*GRA1* codons. The CAI of wt-*GRA1* is 0.697 and the CAI of co-*GRA1* is 0.917.

The self-assembly of the co-*GRA1* gene was performed with two PCR and TOPO cloning reactions. The primer extension and PCR amplification products of the overlapping CODA-designed DNA oligonucleotides to produce the 237 bp, and two 196 bp, product intermediate gene fragments are shown in Fig. 1, lanes 2–4, respectively. The 531 bp, full-length, co-*GRA1* primer extension and PCR product of these three overlapping intermediate gene fragments is shown in Fig. 1, lane 1. Then, wt-*GRA1* and co-*GRA1* were isolated from pUET Δ *GRA1* and pCR-Blunt II TOPO vectors, respectively and rapidly cloned into the CMV vector. The resulting kanamycin-resistant *E. coli* cell clones contained either the CMV/wt-*GRA1* plasmid with the directionally inserted in-frame wt-*GRA1* gene or the CMV/co-*GRA1* plasmid with the co-*GRA1* gene.

3.2. *In vitro* transfection studies

To determine the *in vitro* protein expression levels of co-*GRA1* and wt-*GRA1* in mammalian cells, human embry-

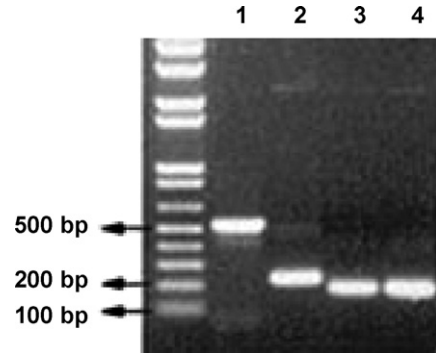


Fig. 1. Two-step PCR assembly of co-*GRA1*: 531 bp full-length co-*GRA1* gene (lane 1); 196 bp and 196 bp, 237 bp intermediate gene fragment 0 (lane 2); 196 bp intermediate gene fragment 1 (lane 3); 196 bp intermediate gene fragment 3 (lane 4). DNA ladder is from Invitrogen.

onic kidney (HEK) 293 cells were transfected with the CMV/wt-*GRA1* and CMV/co-*GRA1* plasmids. A CMV vector containing green fluorescent protein (CMV/GFP) was used as a positive control and an empty CMV vector, as a negative control (data not shown). Expression levels of co-*GRA1* and wt-*GRA1* were determined by immunofluorescence and Western blotting using anti-polyhistidine antibody. The results in Fig. 2 demonstrate that the expression level of r*GRA1* in HEK 293 cells is clearly higher in the HEK 293 cells transfected with the CMV/co-*GRA1* compared to HEK 293 cells transfected with the CMV/wt-*GRA1*.

3.3. Protein expression, purification, and characterization

The expression of r*GRA1* was induced with 1 mM IPTG when the growing cells reached an optical density of 0.4 at 600 nm. The cells were harvested after 16 h, lysed, and processed. The r*GRA1* protein was purified in two steps, first on a HiTrap Ni²⁺ Chelating HP column and then on a Superdex 200 column. The protein purity was ascertained by SDS-PAGE and the results are shown in Fig. 3. The purification protocol yielded 2.5 mg of purified r*GRA1* from 1 l of cell

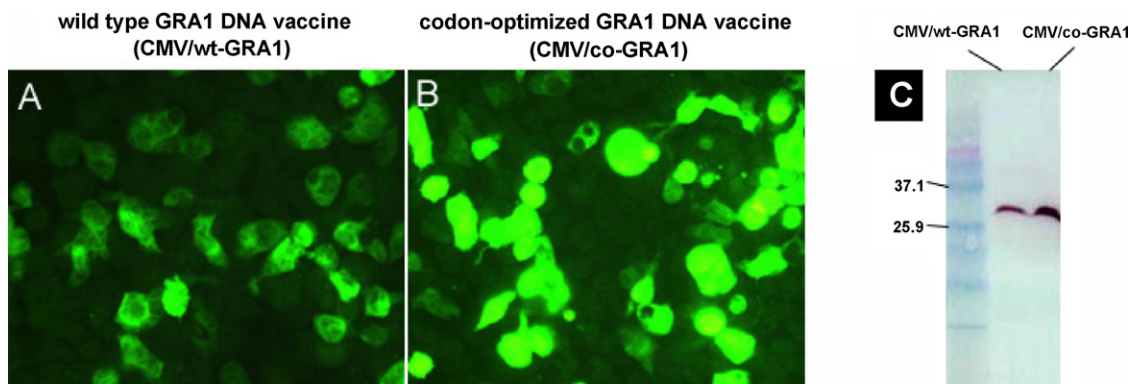


Fig. 2. *In vitro* transfection study results: immunofluorescence microscopy reveals that r*GRA1* protein expression during *in vitro* expression is clearly higher with (B) CMV/co-*GRA1* transfected HEK 293 cells compared to (A) CMV/wt-*GRA1* transfected HEK 293 cells. (C) In the Western blot analysis of r*GRA1* protein expression levels of the lysed HEK 293 cells, CMV/co-*GRA1* expressed approximately 40% more protein than CMV/wt-*GRA1* in HEK 293 cells as measured by ImageJ software. The protein ladder is from Invitrogen.

Table 1
Comparison of wt-GRA1 and co-GRA1 gene sequences

wt-GRA1 ^a	GCC	GAA	GGC	GGC	GAC	AAC	CAG	TCG	AGC	GCC	GTC	TCA	GAT	CGG	GCG	TCT	CTC	TTT	GGT	TTG
co-GRA1	GCT	GAG	GGA	GGA	GAC	AAC	CAG	AGC	TCT	GCT	GTG	TCC	GAT	CGC	GCC	TCT	CTG	TTC	GGC	CTG
Diff(19)	..T	..G	..A	..A	AGC	TCT	..T	..G	..CC	..CG	..C	..C	C..
	A	E	G	G	D	N	Q	S	S	A	V	S	D	R	A	S	L	F	G	L
wt-GRA1	CTG	AGT	GGA	GGG	ACA	GGG	CAG	GGA	TTA	GGA	ATC	GGA	GAA	TCT	GTA	GAT	TTG	GAG	ATG	ATG
co-GRA1	CTG	TCT	GGC	GGC	ACC	GGC	CAA	GGC	CTC	GGA	ATC	GGC	GAG	TCT	GTG	GAC	CTG	GAG	ATG	ATG
Diff(15)	...	TC.	..C	..C	..C	..C	..A	..C	C.CC	..GG	..C	C..
	L	S	G	G	T	G	Q	G	L	G	I	G	E	S	V	D	L	E	M	M
wt-GRA1	GGG	AAC	ACG	TAT	CGT	GTG	GAG	AGA	CCC	ACA	GGC	AAC	CCG	GAC	TTG	CTC	AAG	ATC	GCC	ATT
co-GRA1	GGC	AAC	ACC	TAT	CGC	GTC	GAA	CGC	CCC	ACT	GGC	AAC	CCC	GAC	CTG	CTG	AAG	ATC	GCC	ATC
Diff(13)	.G.C.G.	.T.	.AA	.G.C.C.	..CT.TC
	G	N	T	Y	R	V	E	R	P	T	G	N	P	D	L	L	K	I	A	I
wt-GRA1	AAA	GCT	TCA	GAT	GGA	TCG	TAC	AGC	GAA	GTC	GGC	AAT	GTT	AAC	GTG	GAG	GAG	GTG	ATT	GAT
co-GRA1	AAG	GCC	AGC	GAC	GGC	AGC	TAC	AGC	GAG	GTG	GGC	AAC	GTG	AAC	GTC	GAG	GAG	GTG	ATC	GAC
Diff(17)	..G	..C	AGC	..C	..C	AGCG	..GC	..GCC	..C
	K	A	S	D	G	S	Y	S	E	V	G	N	V	N	V	E	E	V	I	D
wt-GRA1	ACT	ATG	AAA	AGC	ATG	CAG	AGG	GAC	GAG	GAC	ATT	TTC	CTT	CGT	GCG	TTG	AAC	AAA	GGC	GAA
co-GRA1	ACC	ATG	AAG	AGC	ATG	CAG	CGC	GAC	GAG	GAC	ATC	TTT	CTG	CGG	GCT	CTG	AAC	AAG	GGC	GAG
Diff(12)	..CG	C.CC	..T	..G	..G	..T	C..GG
	T	M	K	S	M	Q	R	D	E	D	I	F	L	R	A	L	N	K	G	E
wt-GRA1	ACA	GTA	GAG	GAA	GCG	ATC	GAA	GAC	GTG	GCT	CAA	GCA	GAA	GGG	CTT	AAT	TCG	GAG	CAA	ACC
co-GRA1	ACC	GTG	GAG	GAA	GCC	ATC	GAG	GAC	GTC	GCC	CAG	GCC	GAG	GGG	CTC	AAC	AGC	GAG	CAG	ACC
Diff(15)	..C	..GCGC	..C	..G	..C	..GC	..C	AGCG	...
	T	V	E	E	A	I	E	D	V	A	Q	A	E	G	L	N	S	E	Q	T
wt-GRA1	CTG	CAA	CTG	GAA	GAT	GCA	GTG	AGC	GCG	GTG	GCG	TCT	GTT	GTT	CAA	GAC	GAG	ATG	AAG	GTG
co-GRA1	CTC	CAG	CTG	GAA	GAT	GCC	GTG	AGC	GCC	GTG	GCC	AGC	GTG	GTG	CAG	GAC	GAG	ATG	AAG	GTG
Diff(11)	..C	..GCCC	AGC	..G	..G	..G
	L	Q	L	E	D	A	V	S	A	V	A	S	V	V	Q	D	E	M	K	V
wt-GRA1	ATC	GAC	GAT	GTG	CAG	CAG	CTT	GAA	AAG	GAC	AAA	CAA	CAG	CTT	AAG	GAT	GAC	ATT	GGG	TTC
co-GRA1	ATC	GAC	GAC	GTG	CAG	CAG	CTG	GAA	AAG	GAC	AAA	CAG	CAA	CTC	AAG	GAC	GAC	ATC	GGC	TTC
Diff(8)CGG	..A	..CCC	..C	...
	I	D	D	V	Q	Q	L	E	K	D	K	Q	Q	L	K	D	D	I	G	F
wt-GRA1	CTA	ACA	GGA	GAG	AGA	GAG														
co-GRA1	CTG	ACC	GGC	GAG	CGC	GAG														
Diff(5)	..G	..C	..C	...	C.C	...														
	L	T	G	E	R	E														

^a The first row displays the wt-GRA1 gene sequence; the second row, the CODA-optimized GRA1 gene sequence; and the third row, the modified bases in each codon. Although the codons are changed, the amino acid sequence of the GRA1 protein remains the same, as shown in the fourth row.

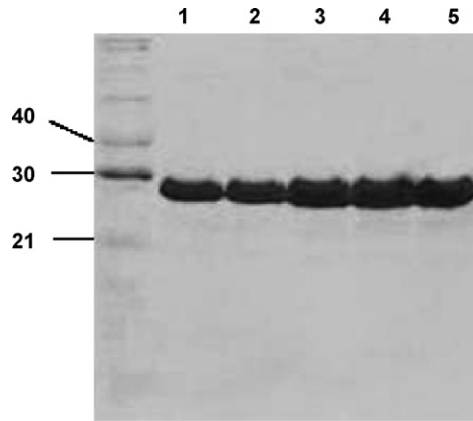


Fig. 3. SDS-PAGE analysis of purified rGRA1 loaded into each well from concentrations of 100 through 300 μ g (lanes 1 through 5). The protein ladder is from QuantPro (Expression Technologies, San Diego, CA).

culture. On SDS PAGE and on Western blots using by anti-polyhistidine monoclonal antibody, the purified rGRA1 had an apparent molecular mass of 26 kD. The calculated molecular mass of the rGRA1, expressed from the 669 bp ORF, was only 24,085. To resolve the discrepancy, purified rGRA1 from three separate batches were analyzed by MALDI-TOF Mass spectroscopy (MS) and each showed a peak at 24,120.

3.4. Humoral immune response to GRA1 vaccines

The rGRA1 protein has been reported to strongly bind *T. gondii*-specific antibodies in human sera [52]. Several types of experiments were carried out to determine the humoral immune response to each type of vaccine candidate. In the first experiment, total *T. gondii* parasite lysate was probed with a pool of serum samples obtained from each group of vaccinated BALB/c mice. Each type of vaccine induced a strong antibody response against a 24 kD protein in *T. gondii* lysate, as detected by Western blots shown in Fig. 4. No antibody response was detected in the control group, in which mice were vaccinated with Provacx or an empty vector.

In the second set of experiments, serum samples from each group of vaccinated mice were tested for the presence of antiGRA1-specific IgG antibodies. The negative controls consisted of sera pools of mice vaccinated with Provacx for the rGRA1-Provacx group or empty vector for the DNA vaccine groups. In all vaccination groups, the amount of IgG antibodies significantly increased after the booster vaccination ($P < 0.01$). Fig. 5 summarizes the fluorescence intensity data collected for each group of vaccinated mice, after the prime and the boost vaccinations. Among all vaccination groups, the highest humoral immune response was achieved after boosting with rGRA1-Provacx. The CMV/co-GRA1 vaccine yielded a somewhat higher antibody response than the CMV/wt-GRA1-DNA vaccine.

In the final evaluation of the humoral immune response, the frequency of the IgG1/IgG2a polarization was determined as a preliminary indication of whether one or more of the

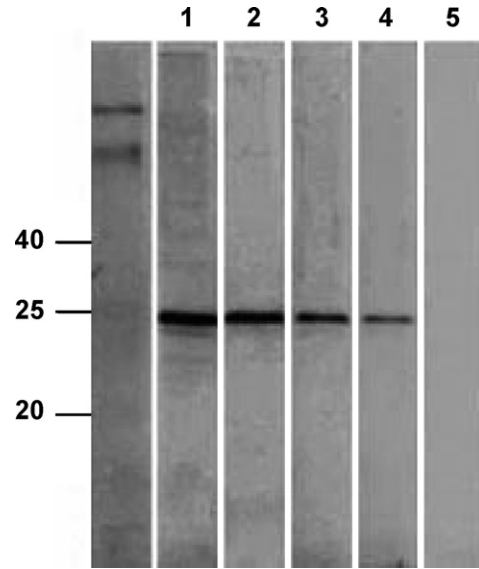


Fig. 4. Probe of native *T. gondii* lysate using pooled sera of mice vaccinated with rGRA1-Provacx (lane 1), CMV/co-GRA1 (lane 2), CMV/wt-GRA1 (lane 3) only rGRA1 (lane 4), control groups vaccinated with Provacx and empty vector (lane 5). The protein ladder is from Invitrogen.

GRA1 vaccines induced a Th1 or Th2 response. The results are shown in Fig. 6. Of eight mice vaccinated with rGRA1-Provacx, six showed a strong IgG1 response as well as a weaker IgG2a response; two mice experienced a nearly equivalent, but weak IgG1 and IgG2a response (Fig. 6B). In the DNA vaccination groups, four displayed a stronger IgG2a response; three showed a stronger IgG1 response, and one had a comparable IgG1 and IgG2a response (Fig. 6C and D). Overall, the GRA1 DNA vaccinations induced a similar pattern of Th1 and Th2 polarity, with a slight bias towards a Th1 response. Furthermore, to evaluate the contribution of the adjuvant to the observed immune responses, five mice were vaccinated with only rGRA1 protein. Similar to a previous study [31], there was a weak IgG1 and IgG2a response slightly biasing towards IgG1 response to the rGRA1 protein in the

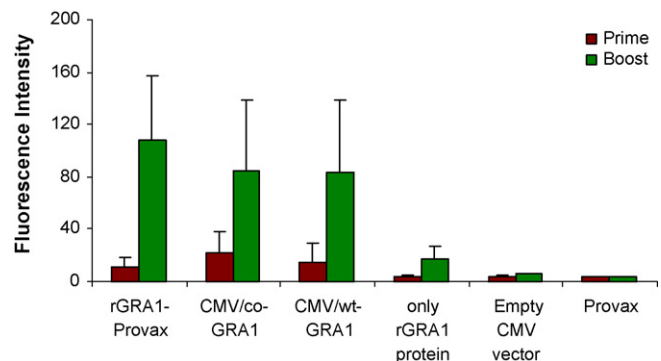


Fig. 5. IgG antibody response against GRA1 vaccines. Serum samples in each vaccine group of eight mice were collected three weeks after priming and three weeks after boosting. The brown bars represent the IgG response after the initial vaccination and the green bars represent the response after the boosting.

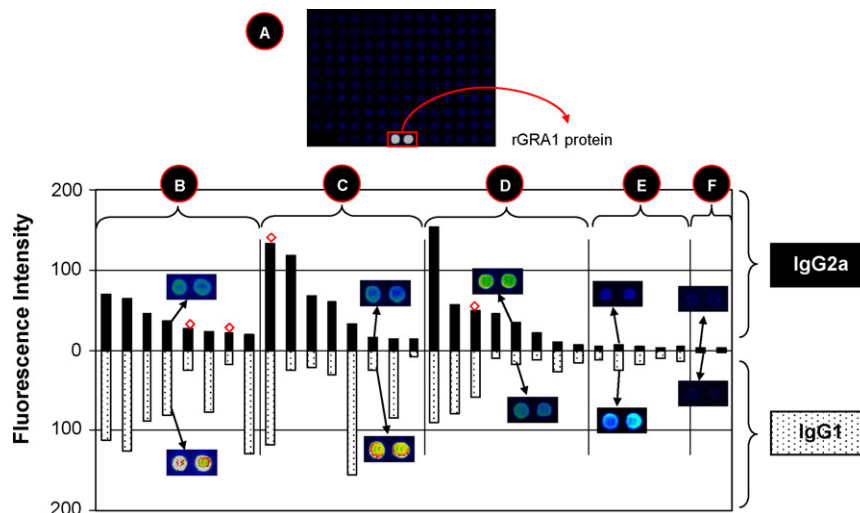


Fig. 6. GRA1 microarray analysis of IgG1 and IgG2a subclass antibodies. (A) Representative protein microarray slide compare the illuminated antibody response to rGRA1 protein printed spots. The spots were printed in duplicate with 68 different proteins from vaccinia virus and human papilloma virus as well as empty vector controls. In (B) through (F), the intensity values of serum samples collected from each mouse, vaccinated with one of the GRA1 vaccine formulations or with a control, are displayed. The solid black bars represent the IgG2a response and the dotted bars, the IgG1 response. Representative illuminated spots in duplicate are shown for each vaccination and control group. The groups have been vaccinated with the following: (B) rGRA1-Provax; (C) CMV/co-GRA1; (D) CMV/wt-GRA1; (E) only rGRA1 protein; and (F) empty vector and Provac adjuvant. The symbol (\diamond) represents the individual serum samples, which show nearly equivalent IgG1 and IgG2a responses.

absence of an adjuvant (Fig. 6E). Thus, Provac adjuvant significantly improved the protective IgG2a response to rGRA1 protein ($P=0.0029$). All controls, containing empty vector and Provac, adjuvant, had no detectable immune response to either IgG1 or IgG2a (Fig. 6F).

3.5. Cell-mediated immune response to rGRA1 vaccines

Splenocytes from individual immunized mice were stimulated *in vitro* with purified rGRA1 protein and with the mitogen, Con A, as well as with unrelated vaccinia virus rH3L protein as controls. Incorporation of [3 H]-thymidine was used to measure lymphocyte proliferation achieved by rGRA1 vaccines. As Table 2 reports, in the lymphoproliferation assays, the T-cell stimulation index (SI) was significantly higher with the mice vaccinated with rGRA1-Provax protein vaccine compared to the CMV/co-GRA1 vaccinated mice and CMV/wt-GRA1 vaccinated mice ($P=0.0007$). How-

ever, there was no statistical difference in lymphoproliferative response in mice vaccinated with either the CMV/wt-GRA1 or the CMV/co-GRA1 DNA vaccines ($P=0.1$). In the controls, the mean cpm, 2607 ± 349 , of the non-stimulated cells from GRA1-vaccinated groups was similar to the mean cpm of 2586 ± 406 from naïve mice spleen cells stimulated with rGRA1 and to the mean cpm of 2866 ± 403 GRA1-vaccinated mice spleen cells stimulated with unrelated vaccinia virus rH3L protein. Mice vaccinated with only rGRA1 protein showed a slightly higher SI compared to control mice (data not shown). For all groups, stimulation with Con A displayed counts above 50,000 cpm. In addition, spleen cells from all experimental and control groups proliferated to comparable levels in response to Con A and showed no response to vaccinia virus rH3L protein.

As a measure of the Th1 or Th2 immune response, the production of IFN- γ and IL-4 in spleen cells of vaccinated mice and control groups were determined by ELISA (Table 2). After stimulating with rGRA1, the production of IFN- γ at 72 h was significantly higher in mice vaccinated with the rGRA1-Provax vaccine compared to mice vaccinated with either DNA vaccine ($P=0.0083$). The IFN- γ levels were only slightly higher in the mice vaccinated with CMV/co-GRA1, compared to mice vaccinated with CMV/wt-GRA1 ($P=0.5$). The IFN- γ levels in mice vaccinated with Provac adjuvant was 89 ± 12 pg/ml and were below the cut-off value. The splenocytes from mice vaccinated with empty vector showed nonspecific IFN- γ production at low levels of 243 ± 48 pg/ml. IL-4 levels were assayed in cell culture supernatants at two time points after stimulation with rGRA1. IL-4 levels were not detected in any animal group at 24 h (data not shown). In contrast, a significant increase in IL-

Table 2

Lymphoproliferation and cytokine production of BALB/c mice vaccinated with rGRA1-Provax, CMV/co-GRA1 and CMV/wt-GRA1

Vaccine candidates	Stimulation index ^a	Cytokine production (pg/ml) ^b	
		IFN- γ	IL-4
rGRA1-Provax	4.7 ± 0.2	1502 ± 301	28 ± 0
CMV/co-GRA1	3.4 ± 0.6	565 ± 22	16 ± 4
CMV/wt-GRA1	2.8 ± 0.4	481 ± 25	15 ± 2

^a As described in methods, the stimulation index is the ratio of the mean counts per minute (cpm) of spleen cells stimulated with varying concentrations of rGRA1 to the mean cpm of controls.

^b The culture supernatants were assayed for cytokine production. Values for IFN- γ and IL-4 were obtained from 72 h culture.

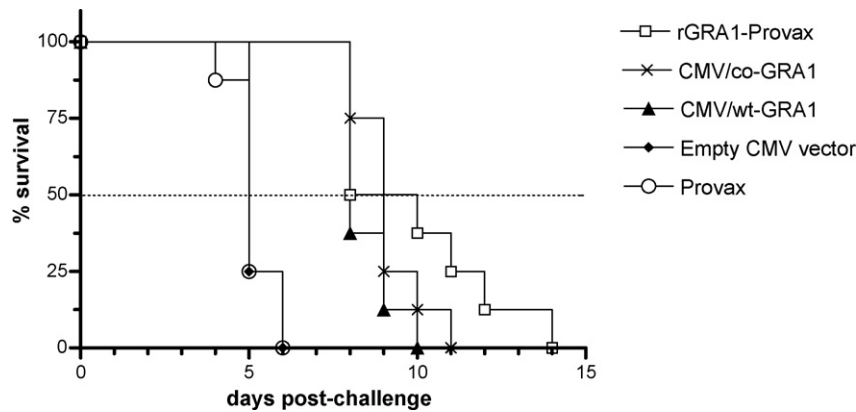


Fig. 7. Survival profiles of vaccinated BALB/c mice against lethal toxoplasmosis. All mice were challenged with 1×10^5 *T. gondii* RH strain tachyzoites nine weeks after the prime vaccination. *T. gondii* tachyzoites were administered to mice vaccinated with rGRA1-Provax (■), CMV/co-GRA1 (×), CMV/wt-GRA1 (▲), and the control groups, empty vector (◆) or Provax (○).

4 was detected after 72 h of stimulation in mice vaccinated with rGRA1-Provax compared to naïve animals or the control groups vaccinated only with Provax ($P < 0.0001$). The IL-4 levels produced by mice vaccinated with either DNA vaccine were only slightly higher than mice vaccinated with naïve mice or empty vector. IL-4 production in control groups of mice vaccinated with empty vector and Provax adjuvant was 12 ± 2 and 13 ± 2 pg/ml, respectively. Mice vaccinated with only rGRA1 protein achieved cytokine levels slightly above cut-off value (data not shown). IL-4 and IFN- γ levels produced by spleen cells obtained from GRA1 vaccinations after stimulation with unrelated vaccinia virus rH3L protein was below the cut-off value (data not shown). Taken together, all vaccines induced higher IFN- γ levels compared to IL-4 levels, with the rGRA1-Provax vaccine producing the highest IFN- γ levels among all groups.

3.6. Protection against lethal toxoplasmosis

Protection against lethal toxoplasmosis was compared in vaccinated BALB/c mice using *T. gondii* RH strain. Animals were intraperitoneally injected with 1×10^5 RH strain tachyzoites nine weeks after the prime vaccination. After challenging in the control groups, mice vaccinated with only Provax or empty CMV vector survived 5.1 ± 0.6 and 5.3 ± 0.5 days, respectively. Survival prolonged significantly in groups vaccinated with rGRA1-Provax, CMV/co-GRA1, and CMV/wt-GRA1 compared to controls ($P < 0.0001$). Mice vaccinated with rGRA1-Provax survived approximately 9.8 ± 2.3 days, which was slightly higher than the mice vaccinated with CMV/wt-GRA1 (8.5 ± 0.8 days) and CMV/co-GRA1 (9.1 ± 1 days) ($P = 0.2$). Nine days after challenging, 50% of mice vaccinated with rGRA1-Provax were alive compared to 25% of CMV/co-GRA1 and 12.5% CMV/wt-GRA1 vaccinated mice. Eleven days after challenging, among three vaccination groups, 25% of mice vaccinated with rGRA1-Provax were alive and none of the CMV/co-GRA1 and CMV/wt-GRA1 DNA vaccinated mice

was alive. Survival prolonged approximately 1.6 times in CMV/wt-GRA1 vaccinated group, 1.7 times in CMV/co-GRA1 vaccinated group and 1.9 times in rGRA1-Provax vaccinated group compared to controls. Overall, the rGRA1-Provax vaccination prolonged survival in 12.5% of mice up to 14 days, almost doubled the survival time compared to controls and thus, afforded the most protection among all vaccination groups. The results are summarized in Fig. 7.

4. Discussion

Although vaccine research against toxoplasmosis shows promise, much effort is yet required to develop a safe and effective *T. gondii* vaccine with long-lasting effects. To date, the only commercial vaccine, which has been developed using live tachyzoites from the S48 *T. gondii* strain, is used for protection against toxoplasmosis in sheep [53]. The vaccine is not widely used as a consequence of reactivation of the live tachyzoites to the pathogenic form, short shelf life, and high cost [6]. To circumvent the negative features, current research is focused on developing safe vaccines from DNA segments or from antigenic protein(s) of *T. gondii*.

GRA1 is considered to be a promising vaccine candidate because GRA1 DNA vaccines elicit immune responses as well as afford protection in rodent and livestock models [8,26–28]. Purified rGRA1 protein vaccines have also found to be immunogenic, however protection studies in animal models haven't been conducted yet [8,27,31]. Thus, the present study focuses on the immunogenicity and protection potency of three types of GRA1-derived vaccines candidates. The GRA1 vaccines in this study include a wild type GRA1 DNA vaccine (CMV/wt-GRA1), a GRA1 DNA vaccine that has been codon optimized for expression in mammalian cells (CMV/co-GRA1), and a GRA1 recombinant protein vaccine adjuvanted with Provax (rGRA1-Provax).

DNA vaccination strategy has become popular in 1990s. Intramuscular injection of DNA vaccines mostly induce dom-

inant Th1 type immune response as shown by the induction of IgG2a antibody response, increased levels of IFN- γ and small quantities of IL-4 production [54]. One of the limiting factors in inducing strong immune response after the administration of wild type DNA vaccines is the low (picogram to nanogram) quantities of protein expression *in vivo* [54]. Another factor for suboptimal protein expression occurs when the codon usage of the target gene in a DNA vaccine is different from the mammalian cells. The presence of rare codons in cloned genes affects mRNA and plasmid stability as well as protein expression level. The excessive presence of rare codons can result in ribosome stalling, slow translation, and translation errors. In some cases, rare codons inhibit protein synthesis and cell growth [55]. Several approaches have been used successfully to compound these problems. One of them is to use cytomegalovirus (CMV) promoter in DNA vaccines to increase gene expression in host cells [54]. The other common strategy is to remove rare codons by codon optimization to increase the protein expression. For this purpose, in the present study, a CMV promoter containing plasmid and codon optimization strategies were used to increase the efficacy of wild type GRA1 DNA vaccination. To optimize the codon usage of wild type GRA1 for expression in mammalian systems, the novel algorithm for codon optimization, termed CODA was used. CODA includes codon substitutions to facilitate the error-free self-assembly of the gene from DNA oligonucleotides in a few simple steps [56]. In the present study, after the assembly of co-GRA1 and construction of CMV/co-GRA1 plasmid, a comparison of CMV/co-GRA1 and CMV/wt-GRA1 protein expression levels in HEK 293 cells demonstrated that CMV/co-GRA1 expressed rGRA1 protein clearly higher than CMV/wt-GRA1.

Immune protection against *T. gondii* in mice is primarily correlated with IFN- γ level [3–5]. IgG2a production from B cells is under the influence of Th1 response or low IFN- γ secretion [4] thus, a vaccine candidate against toxoplasmosis has to primarily increase IgG2a and especially IFN- γ levels. In the present study, both types of DNA vaccines elicited a significant humoral and cellular immune response as well as increased survival rates in mice infected with the lethal RH tachyzoites compared to controls. After prime and boost vaccinations, CMV/co-GRA1 vaccine yielded a higher IgG antibody response than the CMV/wt-GRA1 vaccine. During the evaluation of subgroup IgG antibody response, 50% of both DNA vaccines have polarized towards Th1 response. In the previous GRA1 DNA vaccination studies, the VR1020 plasmid encoding GRA1 achieved similar results [26,28]; however, the same plasmid mostly produced an IgG1/Th2 response [8]. During the evaluation of antigen specific lymphocyte proliferation and IFN- γ production of spleen cells, stimulated with endotoxin depleted rGRA1 protein, CMV/wt-GRA1 and CMV/co-GRA1 DNA vaccinations showed significant increase compared to controls which were similar in the previous studies using the *T. gondii* lysate antigens for the stimulation of spleen cells [26,28]. In addition, an increase in IL-4 levels was obtained from

the spleen cells stimulated 72 h compared to controls. An increase in IL-4 levels, which was not detected in previous studies, explains the correlation between the IgG1 response and IL-4 response obtained from DNA vaccinations. The reason of the IgG1/Th2 and IgG2a/Th1 response variation between vaccinated animals observed in the present study and previous DNA vaccination studies is not yet known; however, the site of antigenic protein expression (i.e., myocytes versus dendritic cells/skin cells), differential stimulation of innate immune system, or the immune system of animal used in the experiments may be responsible for this variation [54].

During the evaluation of protection potency, CMV/co-GRA1 vaccine was slightly better than CMV/wt-GRA1 vaccine, which was comparable to immune responses elicited by the vaccines. Nine days after challenging 25% of CMV/co-GRA1 and 12.5% CMV/wt-GRA1 vaccinated mice were alive and survival was prolonged in BALB/c mice 1.6 and 1.7 times, respectively compared to controls that survived approximately five days after challenging. In two consecutive studies, VR1020 plasmid encoding rGRA1 protein was evaluated in C3H, BALB/c and C57BL/6 mice with non-standardized doses of *T. gondii* IPB-G and 76 K strain cysts [26,28]. In the BALB/c mice group, protection after challenging with 200 and 50 cysts of *T. gondii* IPB-G strain was 0% and 70% whereas protection in controls mice was 0% and 80%, respectively. In the C3H mice group protection after challenging with 50 cysts of IPB-G strain and 50 cysts of 76 K strain was 70% and 90% whereas protection in the control groups were 10% and 50%, respectively [28]. In the following study, two groups of four C3H mice were challenged with 40 cysts of IPB-G strain and protection was 75% and 100% in the vaccinated groups and 0% and 25% in the control groups, respectively [26].

In the literature, similar to the present study, protection was determined using lethal doses of RH strain tachyzoites and among these, the best survival was conferred by SAG1 encoding plasmid in C3H and BALB/c mice groups in which the protection was 100% and 80% compared to 30% and 20% protection in control groups, respectively [23]. Other studies using ROP2 or SAG1 single gene DNA vaccinations did not increase survival significantly [12,18]; however, ROP2-SAG1 cocktail vaccine and DNA vaccine encoding *T. gondii* genomic library increased survival up to four times compared to controls [12,13]. Overall, the elicited immune response, as determined by IgG1, IgG2a, IFN- γ and IL-4 levels, as well as the protection potency was somewhat higher for the CMV/co-GRA1 DNA vaccine than for the CMV/wt-GRA1 DNA vaccine and mammalian codon-optimized GRA1 DNA vaccine showed immunogenicity and protection comparable to previous vaccination studies. However, immunogenicity and protection potency of codon-optimized GRA1 DNA vaccine did not increase, similar to the results obtained from *in vitro* studies. The reason might be that the 40% increase achieved by codon-optimized GRA1 DNA vaccine *in vitro* may not be enough to significantly increase the immune response and protection because the low (only picogram to

nanogram) quantities of protein expression *in vivo* is the most important limiting factor of DNA vaccines in inducing strong immune response [54]. As a result, codon-optimization of GRA1 marginally improved the properties of the GRA1 DNA vaccine.

The rGRA1 protein vaccine was prepared from recombinant GRA1 purified to homogeneity, resulting in an overall yield of 2.5 mg rGRA1 protein per liter of cultured *E. coli* cells. Although rGRA1 protein has a calculated molecular mass of 24,085, the recombinant protein migrates as a 26 kD protein on SDS-PAGE, an anomaly also observed in other laboratories. To resolve the discrepancy, the molecular mass of rGRA1 protein was determined by MALDI-TOF mass spectroscopy to be 24,120. GRA1 is located in the electron-dense secretory organelles, which continuously release their content into the parasitophorous vacuole (PV) during the intracellular development of *T. gondii*. GRA1 functions as a calcium-binding protein and is found in a soluble form in the lumen and network of the PV during invasion of the host cell [57,58]. Calmodulin protein is an EF-hand Ca^{2+} binding protein [59,60]. Calmodulin protein demonstrates altered migration on SDS PAGE gels upon Ca^{2+} binding [61–63]. Recombinant and naïve GRA1 protein has two EF-hand Ca^{2+} binding sites also [57,58]. Thus, the Ca^{2+} binding property of rGRA1 protein might be causing the discrepancy similar to the one detected in calmodulin protein.

The immunogenicity of rGRA1 protein was evaluated in previous studies, however protection potency and cellular immune response of an adjuvanted rGRA1 protein vaccine was not evaluated comprehensively [8,27,31]. To formulate the protein vaccine for the present research, purified and endotoxin depleted rGRA1 protein was adjuvanted to Provac, which is known to be a strong inducer of Th1 cytokine and antigen-specific CD8 lymphocyte production. Indeed, Provac is prepared by mixing previously known strong adjuvants squalane and pluronic L121 to Tween-80 [32,33,64]. Adjuvants have been reported to increase the immunogenicity of protein vaccines [64]. A similar observation was made in the experiments reported herein. Importantly, the Provac adjuvant significantly enhanced the humoral and cellular immune response to the rGRA1 protein vaccine. As shown in results, the highest IgG antibody response was achieved after boosting with rGRA1-Provac among all GRA1-derived vaccinations. rGRA1-Provac induced a strong IgG1 response and a weaker, but still prominent, IgG2a response. The rGRA1-Provac vaccination increased IFN- γ production approximately three times more than the GRA1 DNA vaccines. Consistent with IgG1 response, small quantities of IL-4 levels were detected after 72 h stimulation of spleen cells with endotoxin depleted rGRA1 protein. Overall, the Provac adjuvant significantly enhanced the protective IgG2a and IFN- γ response to the rGRA1 protein. Consistent with the strong immune response, the rGRA1-Provac vaccine nearly doubled the survival rate in mice against lethal *T. gondii* infection. Vaccination studies using purified recombinant proteins of *T. gondii* are few compared to DNA

vaccinations. Recombinant SAG1 protein adjuvanted with alum increased survival 44% compared to 20% in the control group after challenge with lethal doses of RH strain tachyzoites [24] and contrarily, in another vaccination trial, the mortality in mice after vaccination with adjuvanted SAG1 protein was significantly higher than the controls [30].

Thus, the present study comprehensively evaluates immunogenicity and protection potency of an rGRA1 protein vaccine for the first time and compares the results with that of the two GRA1 DNA vaccines. Overall, the rGRA1-Provac vaccine appears to be somewhat better in all measured immune responses, particularly the IFN- γ level which is primarily correlated with immune protection against *T. gondii* in mice [3,4]. As a result of this strong immune response, the protection potency of the rGRA1-Provac vaccine in the BALB/c mice against the RH strain *T. gondii* infection was also better than the two GRA1 DNA vaccines. The reason for the increased immunogenicity and protection of the recombinant GRA1 protein vaccine is not yet clear and requires additional experiments to determine whether the outcome would be different by varying the protein concentration or the type of the adjuvant. Consequently, commercially available successful recombinant protein vaccines against Hepatitis B Virus and Human papillomavirus and the immunogenicity of the adjuvanted rGRA1 protein vaccine shown in the present study, address the importance of developing recombinant protein vaccine strategies against toxoplasmosis.

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References

- [1] Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet* 2004;363:1965–76.
- [2] National Institute of Allergy and Infectious Diseases Biodefense Research Agenda for Category B and C Priority Pathogens, June 2003 [Online]. <http://www3.niaid.nih.gov/Biodefense/Research/categorybandc.pdf>. Assessed 1 April 2006.
- [3] Denkers EY. From cells to signaling cascades: manipulation of innate immunity by *Toxoplasma gondii*. *FEMS Immunol Med Microbiol* 2003;39:193–203.
- [4] Pier GB. Vaccines and vaccination. In: Pier GB, Lyczak JB, Wetzler LM, editors. *Immunology, infection, and immunity*. Washington, D.C: American Society for Microbiology; 2004. p. 497–528.
- [5] Denkers EY, Gazzinelli RT. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev* 1998;11:569–88.

- [6] Bhopale GM. Development of a vaccine for toxoplasmosis: current status. *Mic Infect* 2003;5:457–62.
- [7] Angus CW, Klivington-Evans D, Dubey JP, Kovacs JA. Immunization with a DNA plasmid encoding the SAG1 (P30) protein of *Toxoplasma gondii* is immunogenic and protective in rodents. *J Infect Dis* 2000;181:317–24.
- [8] Bivas-Benita M, Laloup M, Versteheyne S, Dewit J, De Braekeleer J, Jongert E, et al. Generation of *Toxoplasma gondii* GRA1 protein and DNA vaccine loaded chitosan particles: preparation, characterization, and preliminary *in vivo* studies. *Int J Pharm* 2003;266:17–27.
- [9] Bonenfant C, Dimier-Poisson I, Velge-Roussel F, Buzoni-Gatel D, Del Giudice G, Rappuoli R, et al. Intranasal immunization with SAG1 non-toxic mutant heat labile enterotoxins protects mice against *Toxoplasma gondii*. *Infect Immun* 2001;69:1605–12.
- [10] Couper KN, Nielsen HV, Petersen E, Roberts F, Roberts CW, Alexander J. DNA vaccination with the immunodominant tachyzoite surface antigen (SAG-1) protects against adult acquired *Toxoplasma gondii* infection but does not prevent maternofetal transmission. *Vaccine* 2003;21:2813–20.
- [11] Desolme B, Mevelec MN, Buzoni-Gatel D, Bout D. Induction of protective immunity against toxoplasmosis in mice by DNA immunization with a plasmid encoding *Toxoplasma gondii* GRA4 gene. *Vaccine* 2000;18:2512–21.
- [12] Fachado A, Rodriguez A, Angel SO. Protective effect of a naked DNA vaccine cocktail against lethal toxoplasmosis in mice. *Vaccine* 2003;21:1327–35.
- [13] Fachado A, Rodriguez A, Molina J, Silverio JC, Marino AP, Pinto LM, et al. Long-term protective immune response elicited by vaccination with an expression genomic library of *Toxoplasma gondii*. *Infect Immun* 2003;71:5407–11.
- [14] Haumont ML, Delhaye L, Garcia M, Jurado P, Mazza V, Daminet V, et al. Protective immunity against congenital toxoplasmosis with recombinant SAG1 protein in a Guinea pig model. *Infect Immun* 2000;68:4948–53.
- [15] Ismael AB, Sekkai D, Collin C, Bout D, Mevelec MN. The MIC3 gene of *Toxoplasma gondii* is a novel potent vaccine candidate against toxoplasmosis. *Infect Immun* 2003;71:6222–8.
- [16] Letscher-Bru V, Villard O, Risse B, Zauke M, Klein JP, Kien TT. Protective effect of vaccination with a combination of recombinant surface antigen 1 and interleukin 12 against toxoplasmosis in mice. *Infect Immun* 1998;66:4503–6.
- [17] Letscher-Bru V, Pfaff AW, Abou-Bacar A, Filisetti D, Antoni E, Villard O, et al. Vaccination with *Toxoplasma gondii* SAG-1 protein is protective against congenital toxoplasmosis in BALB/c mice but not in CBA/J mice. *Infect Immun* 2003;71:6615–9.
- [18] Leyva R, Herion P, Saavedra R. Genetic immunization with plasmid DNA coding for the ROP2 protein of *Toxoplasma gondii*. *Parasitol Res* 2001;87:70–9.
- [19] Martin V, Supanitsky A, Echeverria PC, Litwin S, Tanos T, De Roodt AR, et al. Recombinant GRA4 or ROP2 protein combined with alum or the GRA4 gene provides partial protection in chronic murine models of toxoplasmosis. *Clin Diagn Lab Immunol* 2004;11:704–10.
- [20] Mevelec MN, Bout D, Desolme B, Marchand H, Magne R, Bruneel O, et al. Evaluation of protective effect of DNA vaccination with genes encoding antigens GRA4 and SAG1 associated with GM-CSF plasmid, against acute, chronic and congenital toxoplasmosis in mice. *Vaccine* 2005;23:4489–99.
- [21] Mishima M, Xuan X, Shioda A, Omata Y, Fujisaki K, Nagasawa H, et al. Modified protection against *Toxoplasma gondii* lethal infection and brain cyst formation by vaccination with SAG2 and SRS1. *J Vet Med Sci* 2001;63:433–8.
- [22] Mohamed RM, Aosai F, Chen M, Mun HS, Norose K, Belal US, et al. Induction of protective immunity by DNA vaccination with *Toxoplasma gondii* HSP70, HSP30 and SAG1 genes. *Vaccine* 2003;21:2852–61.
- [23] Nielsen HV, Laemoller SL, Christiansen L, Buus S, Fomsgaard A, Petersen E. Complete protection against lethal *Toxoplasma gondii* infection in mice immunized with a plasmid encoding the SAG1 gene. *Infect Immun* 1999;67:6358–63.
- [24] Petersen E, Henrik VN, Christiansen L, Spenter J. Immunization with *E. coli* produced recombinant *T. gondii* SAG1 with alum as adjuvant protect mice against lethal infection with *Toxoplasma gondii*. *Vaccine* 1998;16:1283–9.
- [25] Roque-Resendiz JL, Rosales R, Herion P. MVA ROP2 vaccinia virus recombinant as a vaccine candidate for toxoplasmosis. *Parasitology* 2004;128:397–405.
- [26] Scorza T, D'Souza S, Laloup M, Dewit J, De Braekeleer J, Verschuere H, et al. A GRA1 DNA vaccine primes cytolytic CD8.T cells to control acute *Toxoplasma gondii* infection. *Infect Immun* 2003;71:309–16.
- [27] Supply P, Sutton P, Coughlan SN, Bilo K, Saman E, Trees AJ, et al. Immunogenicity of recombinant BCG producing the GRA1 antigen from *Toxoplasma gondii*. *Vaccine* 1999;17:705–14.
- [28] Vercammen M, Scorza T, Huygen K, De Braekeleer J, Diet R, Jacobs D, et al. DNA vaccination with genes encoding *Toxoplasma gondii* antigens GRA1, GRA7 and ROP2 induces partially protective immunity against lethal challenge in mice. *Infect Immun* 2000;68:38–45.
- [29] Yang CD, Chang GN, Chao D. Protective immunity against *Toxoplasma gondii* in mice induced by a chimeric protein rSAG1/2. *Parasitol Res* 2004;92:58–64.
- [30] Kasper LH, Currie KM, Bradley MS. An unexpected response to vaccination with a purified major membrane tachyzoite antigen (P30) of *Toxoplasma gondii*. *J Immunol* 1985;134:3426–31.
- [31] Kato M, Claveria FG, Maki Y, Tanaka T, Suzuki N, Nagasawa H. *Toxoplasma gondii* antigens GRA1 (p24) and SAG1 (p30): a comparison of their stimulatory influence on T-cell activation and cytokine expression in *in vitro* cultures. *Pathobiology* 2005;72:160–4.
- [32] Rajanathanan P, Attard GS, Sheikh NA, Morrow WJ. Novel aggregate structure adjuvants modulate lymphocyte proliferation and Th1 and Th2 cytokine profiles in ovalbumin immunized mice. *Vaccine* 1999;18:140–52.
- [33] Sheikh NA, Attard GS, van Rooijen N, Rajanathanan P, Hariharan K, Yang YW, et al. Differential requirements for CTL generation by novel immunostimulants: APC tropism, use of the TAP-independent processing pathway, and dependency on CD80/CD86 costimulation. *Vaccine* 2003;21:3775–88.
- [34] Andre S, Seed B, Eberle J, Schraut W, Bultmann A, Haas J. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J Virol* 1998;72:1497–503.
- [35] Casimiro DR, Tang A, Perry HC, Long RS, Chen M, Heidecker GJ, et al. Vaccine-induced immune responses in rodents and nonhuman primates by use of a humanized human immunodeficiency virus type 1 pol gene. *J Virol* 2002;76:185–94.
- [36] Deml L, Bojak A, Steck S, Graf M, Wild J, Schirmbeck R, et al. Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the human immunodeficiency virus type 1 Gag protein. *J Virol* 2001;75:10991–1001.
- [37] Disbrow GL, Sunitha I, Baker CC, Hanover J, Schlegel R. Codon optimization of the HPV-16 E5 gene enhances protein expression. *Virology* 2003;311:105–14.
- [38] Hamdan FF, Mousa A, Ribeiro P. Codon optimization improves heterologous expression of a *Schistosoma mansoni* cDNA in HEK293 cells. *Parasitol Res* 2002;88:583–6.
- [39] Mossadegh N, Gissmann L, Muller M, Zentgraf H, Alonso A, Tomakidi P. Codon optimization of the human papillomavirus 11 (HPV 11) L1 gene leads to increased gene expression and formation of virus-like particles in mammalian epithelial cells. *Virology* 2004;326:57–66.
- [40] Nagata T, Uchijima M, Yoshida A, Kawashima M, Koide Y. Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. *Biochem Biophys Res Commun* 1999;261:445–51.
- [41] Narum DL, Kumar S, Rogers WO, Fuhrmann SR, Liang H, Oakley M, et al. Codon optimization of gene fragments encoding *Plasmodium fal-*

- ciparum* merzoite proteins enhances DNA vaccine protein expression and immunogenicity in mice. *Infect Immun* 2001;69:7250–3.
- [42] Ramakrishna L, Anand KK, Mohankumar KM, Ranga U. Codon optimization of the tat antigen of human immunodeficiency virus type 1 generates strong immune responses in mice following genetic immunization. *J Virol* 2004;78:9174–89.
- [43] Stratford R, Douce G, Zhang-Barber L, Fairweather N, Eskola J, Dougan G. Influence of codon usage on the immunogenicity of a DNA vaccine against tetanus. *Vaccine* 2000;19:810–5.
- [44] Strugnell RA, Drew D, Mercieca J, DiNatale S, Firez N, Dunstan SJ, et al. DNA vaccines for bacterial infections. *Immunol Cell Biol* 1997;75:364–9.
- [45] Uchijima M, Yoshida A, Nagata T, Koide Y. Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. *J Immunol* 1998;161:5594–9.
- [46] Vinner L, Nielsen HV, Bryder K, Corbet S, Nielsen C, Fomsgaard A. Gene gun DNA vaccination with Rev-independent synthetic HIV-1 gp160 envelope gene using mammalian codons. *Vaccine* 1999;17:2166–75.
- [47] zur Megede J, Chen MC, Doe B, Schaefer M, Greer CE, Selby M, et al. Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene. *J Virol* 2000;74:2628–35.
- [48] Davies DH, Liang X, Hernandez JE, Randall A, Hirst S, Mu Y, et al. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc Natl Acad Sci USA* 2005;102:547–52.
- [49] Hiszczyńska-Sawicka E, Brillowska-Dabrowska A, Dabrowski S, Pietkiewicz H, Myjak P, Kur J. High yield expression and single-step purification of *Toxoplasma gondii* SAG1, GRA1, and GRA7 antigens in *Escherichia coli*. *Protein Expr Purif* 2003;27:150–7.
- [50] Hariharan K, Hanna N. Development and application of PROVAX adjuvant formulation for subunit cancer vaccines. *Adv Drug Deliv Rev* 1998;32:187–97.
- [51] Davies DH, McCausland MM, Valdez C, Huynh D, Hernandez JE, Mu Y, et al. Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. *J Virol* 2005;79:11724–33.
- [52] Pietkiewicz H, Hiszczyńska-Sawicka E, Kur J, Petersen E, Nielsen HV, Stankiewicz M, et al. Usefulness of *Toxoplasma gondii*-specific recombinant antigens in serodiagnosis of human toxoplasmosis. *J Clin Microbiol* 2004;42:1779–81.
- [53] Buxton D, Innes EA. A commercial vaccine for ovine toxoplasmosis. *Parasitology* 1995;110:11–6.
- [54] Kenney RT, Edelman R. DNA vaccines. In: Levine MM, Kaper JB, Rappuoli R, Liu MA, Good MF, editors. *New generation vaccines*. 3rd ed. New York: Marcel Dekker; 2004. p. 368–70.
- [55] Zhou Z, Schnake P, Xiao L, Lal AA. Enhanced expression of a recombinant malaria candidate vaccine in *Escherichia coli* by codon optimization. *Protein Expr Purif* 2004;34:87–94.
- [56] Lathrop RH, Sazhin A, Sun Y, Steffen N, Irani SS. A multi-queue branch-and-bound algorithm for anytime optimal search with biological applications. *Genome Inform Ser Workshop Genome Inform* 2001;12:73–82.
- [57] Cesbron-Delauw MF, Guy B, Torpier GR, Pierce J, Lenzen G, Cesbron JY, et al. Molecular characterization of a 23 kD major antigen secreted by *Toxoplasma gondii*. *Proc Natl Acad Sci USA* 1989;86:7537–41.
- [58] Cesbron-Delauw MF. Dense-granule organelles of *Toxoplasma gondii*. *Parasitol Today* 1994;10:293–6.
- [59] Bhattacharya S, Bunick CG, Chazin WJ. Target selectivity in EF-hand calcium binding proteins. *Biochim Biophys Acta* 2004;1742:69–79.
- [60] Kawasaki H, Nakayama S, Kretsinger RH. Classification and evolution of EF-hand proteins. *Biometals* 1998;11:277–95.
- [61] Bandyopadhyay S, Ghosh SK. Goat testis calmodulin: purification and physicochemical characterization. *J Protein Chem* 1990;9:603–11.
- [62] Tojyo Y, Matsumoto Y. Identification of calmodulin in new-born rat calvaria. *Biochem Int* 1986;13:155–61.
- [63] Seeber F, Beuerle B, Schmidt HH. Cloning and functional expression of the calmodulin gene from *Toxoplasma gondii*. *Mol Biochem Parasitol* 1999;99:295–9.
- [64] Vogel FR, Hem SL. Immunologic adjuvants. In: Plotkin SA, Orenstein WA, editors. *Vaccines*. 4th ed. Pennsylvania: Saunders Elsevier Inc; 2004. p. 69–79.