Synergistic effect of rSAG1 and rGRA2 antigens formulated in PLGA microspheres in eliciting immune protection against *Toxoplasma gondii*

Running Head: Protection against *T. gondii* by rSAG1/rGRA2-PLGA

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Abstract

There is still no human vaccine against *Toxoplasma gondii* (*T. gondii*), as one of the most successful parasites. In present study, we designed a subunit vaccine composed of recombinant SAG1 (rSAG1) and recombinant GRA2 (rGRA2) proteins. In order to improve the induced immune responses, rSAG1 and rGRA2 were adsorbed on Poly (DL-lactide-co-glycolide) (PLGA) microspheres (MS) prepared by double emulsion solvent evaporation method. BALB/c mice were subcutaneously vaccinated by rSAG1-adsorbed PLGA MS (rSAG1-PLGA), rGRA2-adsorbed PLGA MS (rGRA2-PLGA), and the mixture of both formulations (rSAG1/rGRA2-PLGA), twice with a 3-week interval. PLGA MS characteristics, protein release, cellular and humoral immune responses, and protection against acute toxoplasmosis were evaluated. All vaccinated mice induced significantly partial protection and longer survival times associated with higher IFN- γ /IL-10 ratio and higher amount of *Toxoplasma*-specific IgG antibodies compared to control groups. Interestingly, the synergistic effect of rSAG1 and rGRA2 in eliciting more potent cellular and humoral responses and consequently higher protection in comparison to single antigen was confirmed.

This study introduces the mixture of rSAG1 and rGRA2 (derived from different stages of *Toxoplasma* life-cycle) formulated in PLGA MS as a promising candidate in vaccine development against *T. gondii*.

Keywords: PLGA microspheres; Adsorption; Vaccine; *Toxoplasma gondii*; rSAG1; rGRA2;

1. Introduction

T. gondii is an obligate intracellular protozoan parasite with an extremely wide geographic and host range that is estimated to infect about one third of world's human population (Dubey, 2008) (Montoya and Liesenfeld, 2004). Although toxoplasmosis in immune competent individuals is generally associated with no or mild symptoms, it still remains a major health concern in pregnancy as a consequence of parasite transmission to the fetus. Moreover, primary or reactivated toxoplasmosis in immune-compromised patients such as those with AIDS, cancer, neoplastic diseases, or organ transplantation might cause serious life-threatening medical problems such as toxoplasmic encephalitis (Pifer and Yarovinsky, 2011, Tenter, et al., 2000).

Although current drugs can successfully limit parasite replication and acute infection, they are unable to prevent tissue cyst formation or eradicate them. Hence, the development of a preventive vaccine is of high priority to decrease remarkable burden of *T. gondii* (Garcia, et al., 2014).

Among various *T. gondii* antigens involved in eliciting immunity responses, surface antigen 1 (SAG1, previously named P30) has attracted many attentions in vaccine development (Jongert, et al., 2009, Wang and Yin, 2014, Zhang, et al., 2013). SAG1, the prototype member of SRS (SAG1 related sequences) family, is a major and stage-specific antigen expressed in *Toxoplasma* tachyzoite. Moreover, SAG1 is a key ligand for parasite attachment to host cell during invasion (Velge-Roussel, et al., 2001) which induces a strong and protective immune responses both in human and experimental animal models (Godard, et al., 1990) (Dziadek, et al., 2011). Furthermore, SAG1 sequence is highly conserved among virulent strains of *T. gondii*, a prerequisite for a vaccine candidate antigen. We have recently reported the expression and purification of rSAG1 in *E.coli*. The correct folding, antigenicity and immunogenicity of rSAG1

were assessed by different physicochemical assays i.e. reducing and non-reducing gel electrophoresis, Western blotting, ELISA and reversed-phase HPLC (Allahyari, et al., 2015).

Dense granule excretory-secretory (GRA) proteins are known as major components of both cyst wall containing bradyzoites and parasitophorous vacuole (PV), which modify PV environment, thus make possible the intracellular survival and parasite replication. GRA proteins also induce protective immune responses and have been considered as attractive vaccine candidates (Ching, et al., 2016, Dziadek, et al., 2011, Nam, 2009). We previously demonstrated that rGRA2 formulated in MPL adjuvant (toll like receptor 4 (TLR4) ligand induced Th1-associated cytokines and *Toxoplasma*-specific IgG. Consequently, GRA2-MPL vaccination conferred partial protection against challenge by *T. gondii* through significant reduction of brain cysts in CBA/J mice (Golkar, et al., 2007)

Despite all progress in vaccine research against toxoplasmosis, subunit vaccines still deal with their poor immunogenicity which maybe overcome by using suitable and efficient delivery vehicles. PLGA particles as a FDA-approved vaccine delivery platform has attracted much attention regarding their remarkable features including proved safety profile, biodegradability and biocompatibility, eliciting long-lasting Th1 immunity, the absence of any adverse effect on dendritic cell function and etc. Furthermore, the advantages of using PLGA particles as a delivery system has been successfully proved in diseases caused by intracellular parasites such as hepatitis B (Jaganathan, et al., 2004, Thomas, et al., 2011), malaria (Carcaboso, et al., 2004, Moon, et al., 2012, Rosas, et al., 2001), leishmaniasis (Santos, et al., 2012, Santos, et al., 2013, Tafaghodi, et al., 2010), tuberculosis (Kirby, et al., 2008, Wang, et al., 2009), and toxoplasmosis (Chuang, et al., 2013, Chuang, et al., 2013).

In the present study, we evaluated the synergistic effect of rSAG1 (Allahyari, et al., 2015) and rGRA2 (Golkar, et al., 2007) adsorbed on PLGA MS as vaccine delivery system, in eliciting immune responses and protection against acute toxoplasmosis infection in BALB/c mice.

2. Methods

2.1. Materials and chemicals

Poly (d,l-lactide-co-glycolide) polymer (PLGA), Resomer[®]RG503 (50:50, lactide:glycolide ratio) (viscosity 0.32 - 0.44 dl/g) (Montoya and Liesenfeld, 2004) was obtained from Boehringer Ingelheim, Germany. PVA [poly vinyl alcohol; molecular weight (MW) 30,000 –70,000 Da, 88% hydrolyzed] was purchased from Sigma Chemical Company, Germany. The materials applied for SDS-PAGE gel electrophoresis were supplied from Roche Applied Sciences (Germany). Protein molecular weight marker was obtained from Fermentas, Lithuania. Dichloromethane (DCM) analytical grade) was supplied from Merck, Germany. Cell culture reagents including HEPES, L-glutamine, penicillin-streptomycin solution (10,000 IU/ml), concanavalin A (conA) were obtained from Sigma (Germany). RPMI-1640 and Fetal Calf Sera (FCS) were purchased from Gibco (Life Technologies GmbH, Germany). All solutions were prepared by Milli-Q[™] ultrapure (Milli-Q System, Millipore, France). All other chemicals were of analytical reagent grade.

2.2. Mice and Parasite

Female BALB/c mice, (6–8 weeks old) were obtained from Pasteur Institute of Iran, housed in plastic cages with free access to tap water and standard rodent pellets under a constant 12:12 h light–dark cycle. All animal experiments including handling, maintenance and blood sample

collection were approved by Institutional Animal Care and Research Advisory Committee of Pasteur Institute of Iran.

Tachyzoites of *T. gondii* RH strain were harvested from peritoneal fluid of infected Swiss mice through Percoll purification according to Wu et al. (Wu, et al., 2012), washed with phosphate buffered saline (PBS) and stored at -70 °C. For challenge study, freshly prepared tachyzoites of RH strain were used.

2.3. Preparation of soluble *T. gondii* antigen (STAg)

RH tachyzoites were sonicated (1 min burst, 1 min cooling, 150 W) using an ultrasonic probe sonicator (Ultrasonic Processor UP 400S Sonotrode, hielscher, Ultrasound Technology, Amplitude 70%, 0.5 Cycle). The crude extract was centrifuged at 2000 g for 30 min and the protein concentration of soluble STAg was determined by using Bio-Rad DC protein assay. Aliquots of STAg were stored at -70 °C until used.

2.4. Purification and characterization of rSAG1 and rGRA2 proteins

rSAG1 and rGRA2 proteins were expressed and purified according to our previous studies (Allahyari, et al., 2015, Golkar, et al., 2007) (Golkar, et al., 2005). Briefly, rSAG1 (corresponding to amino acids 49 to 311 of native protein) and rGRA2 (corresponding to amino acids 21 to 185 of native protein) were expressed in *Escherichia coli* BL21pLysS strain upon induction by IPTG. The cell pellet was disrupted by sonication and inclusion bodies were isolated. After denaturation of inclusion body, rSAG1 purification was carried out via a single step immobilized Ni-NTA affinity chromatography. Refolding of denatured rSAG1 was performed in the presence of reduced/oxidized glutathione (Allahyari, et al., 2015). Besides,

rGRA2 was purified via a single native immobilized Ni-NTA affinity chromatography (Golkar, et al., 2007)..

Immunoblotting of rSAG1 was performed by pooled sera from pregnant women with acute toxoplasmosis infection to prove its antigenicity. Moreover, to examine rSAG1 immunogenicity, a rabbit was injected three times with rSAG1 in Freund's adjuvant. Then, Western blot analysis of RH tachyzoites and rSAG1 probed with serum from the immunized rabbit clearly showed the recognition of specific bands related to recombinant and native SAG1 (Allahyari, et al., 2015). Also, immunogenicity of rGRA2 was verified by Western blot technique as mentioned in our previous paper (Golkar, et al., 2007).

The concentration of bacterial endotoxin in purified rproteins was measured by limulus amoebocyte lysate assay using LAL chromogenic endotoxin quantitation kit (Pierce®, Thermo scientific, USA).

2.5. Preparation of blank PLGA microspheres

Blank PLGA MS were prepared using double emulsion solvent evaporation technique at room temperature according to the reported methods (Kazzaz, et al., 2006, Salvador, et al., 2012) with slight modifications. Briefly, water in oil (w/o) emulsion was prepared by adding 400 µl of PBS to PLGA 50:50 dissolved in 4% (w/v) DCM using probe sonicator (Ultrasonic Processor UP 400S Sonotrode, hielscher, Ultrasound Technology) in amplitude of 70% and 0.5 Cycle for 30 s. Then, the obtained emulsion was added to 40 ml of a 2% (w/v) PVA aqueous solution as surfactant and emulsified by an Ultra Turrax® T- 25 turbine homogenizer (IKA, Germany), 10-mm probe at 15000 rpm for 3 min in an ice bath. DCM was allowed to evaporate using rotary evaporator (BUCHI, Germany) at room temperature. The resultant MS were collected using an ultra-centrifuge (Eppendorf, Germany) at 20000 rpm for 20 min and washed three times with 50

ml of Milli-Q™ ultra pure water to remove excess emulsifier. Blank PLGA MS were lyophilized by a Freezone 2.5L bench top lyophilizer (Labconco, Country). Various batches of PLGA MS were prepared and mixed to provide sufficient quantity of homogenous microspheres. The MS were characterized for zeta-average size, polydispersity index (PDI), zeta potential, and surface morphology.

2.6. Adsorption of rSAG1 and rGRA2 on PLGA microspheres

rSAG1-PLGA and rGRA2-PLGA were prepared by incubating a suspension containing 100 mg of freeze-dried blank PLGA MS with 1 mg of rSAG1 or rGRA2 in 10 ml of PBS (pH 7.4) individually with 1 mg of rSAG1 or rGRA2 at 4 °C overnight. The suspensions were then mixed on a rocking shaker (model FMS2 multi shaker FINEPCR) at 4 °C overnight. Thereafter, rSAG1-PLGA and rGRA2-PLGA were collected by centrifugation at 14000 g for 10 min, followed by two washing steps with Milli-Q™ water to remove unbounded rSAG1 or rGRA2. Finally, rSAG1-PLGA or rGRA2-PLGA MS were lyophilized as mentioned above.

2.7. Process yield determination

Process yield for blank PLGA MS in ten experiments, and rprotein-PLGA (rSAG1-PLGA, rGRA2-PLGA) in five experiments were measured according to equation (1) and (2), respectively:

$$(1) \text{ Process Yield \%} = \frac{\text{Weight of obtained PLGA MS}}{\text{Weight of PLGA}} \times 100$$

$$(2) \text{ Process Yield \%} = \frac{\text{Weight of obtained rSAG1- or rGRA2-PLGA}}{\text{Weight of applied PLGA MS} + \text{weight of rSAG1 or rGRA2}} \times 100$$

*Microspheres was abbreviated to MS.

2.8. Adsorption efficiency (AE) of rSAG1 and rGRA2 on PLGA MS

Adsorption efficiency (AE) of both MS was calculated by direct method in three experiments. A micro-bicinchoninic acid (Micro-BCA) protein assay kit (Pierce Biotechnology; Rockford, ILUSA) was used to determine rSAG1 and rGRA2 concentrations on PLGA MS. Ten milligrams of lyophilized rSAG1-PLGA or rGRA2-PLGA were hydrolyzed in 1 ml PBS buffer containing 0.1 M NaOH and 2% (w/v) SDS under vigorous shaking at room temperature during 3 hours. The samples were centrifuged at 8000 g for 10 min and rSAG1 and rGRA2 concentrations in supernatant were estimated against a series of BSA concentrations as protein standard. To minimize the adverse effect of sodium hydroxide and SDS samples on protein assay method, the samples were diluted 1:10 before protein quantification, and blank PLGA MS were used as control.

Adsorption efficiency was evaluated according to following equation in triplicate:

$$\text{Adsorption efficiency \%} = \frac{\text{Amount of rSAG1 or rGRA2 on PLGA}}{\text{Total amount of rSAG1 or rGRA2 used for adsorption}} \times 100$$

2.9. Particle size, zeta potential and surface morphology

The particle size (Z-average size), and PDI of blank PLGA MS, rSAG1-PLGA, and rGRA2-PLGA were determined by dynamic light scattering using the photon correlation spectroscopy (PCS) technique. The surface charge of MS was estimated by analysis of the Zeta potential (laser-Doppler electrophoresis). All measurements were performed at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, UK) and performed in triplicate.

The surface morphology and topology of the blank MS was assessed by scanning electron microscopy (SEM) (Model S-4160, Hitachi, Japan). A uniform layer of MS was fixed onto a double adhesive aluminum stub and coated with gold-palladium under vacuum.

2.10. *In Vitro* release studies of rSAG1-PLGA and rGRA2-PLGA microspheres

Release profiles of freeze-dried rSAG1-PLGA and rGRA2-PLGA were investigated in release medium containing PBS (pH 7.4), and 0.02% sodium azide, as a bacteriostatic agent. The rSAG1 and rGRA2 release studies were carried out by incubating 50 mg of each MS in 1 ml of release medium) individually at 37 °C and 100 rpm in a shaker incubator (Bioshaker BR-42FL, TAITEC, Japan). At predetermined times (1, 2, 3, 4, 6, 8, 12, 24 and 48 h, followed by weekly sampling up to 8 weeks) the samples (60 µl) were taken and centrifuged at 14000 g for 10 min. The supernatants of duplicate samples were stored frozen at -20 °C, until protein content was measured by the Micro BCA Assay. Release studies were performed in triplicate and results expressed as cumulative release percent. Regression analysis was done and the slopes of both release profiles were compared to each other.

2.11. Vaccination studies

2.11.1. Vaccination schedules

BALB/c mice were randomly divided into five groups (12 mice/group) and subcutaneously (s.c) immunized in the right-hind footpad.

The first and second groups (G1, G2) were vaccinated with rSAG1-PLGA and rGRA2-PLGA (20 µg of each rprotein), respectively. The third group (G3) was vaccinated by rSAG1/rGRA2-PLGA (10 µg of each rprotein). Control Groups received blank PLGA MS (G4) and PBS (G5), respectively (Table 1). After three weeks, second immunization was carried out by the same regimen as mentioned above later. Blood samples were taken from the eye vein of each mice, three weeks after second immunization and sera were kept at -20°C in order to assess humoral immunity. Spleen removal of four mice in each group was done to investigate cellular immunity. The eight remained mice in each group were infected by RH tachyzoites to evaluate survival time.

Table 1. Vaccine formulations in different mice groups

Amount of injected rproteins	Immunization groups	No.
rSAG1 (20 µg)	rSAG1-PLGA	G1
rGRA2 (20 µg)	rGRA2-PLGA	G2
rSAG1 (10 µg), rGRA2 (10 µg)	rSAG1-PLGA and rGRA2-PLGA	G3
-	Blank PLGA	G4
-	PBS	G5

2.11.2. Cytokine assays

The spleen cells from four mice in each group were removed aseptically three weeks after second immunization and single cell suspensions were obtained by squeezing the whole organs in red blood cell lysis buffer. Spleen cells of mice were individually suspended in RPMI-1640 5% (supplemented with FCS 5%, L-glutamine 1%, HEPES 1%, penicillin-streptomycin (10,000 U/ml) and beta mercaptoethanol 50 µM) and seeded in 96-well tissue culture plates at 3×10^5 cells per well. The cells were stimulated with rSAG1 or rGRA2 (2 µg/ml) or both of them, and STAg (20 µg/ml). ConA (5 µg/ml) and cell culture medium were applied as positive and negative controls, respectively. Cell culture plates were maintained at 37 °C in 5% CO₂ humidified atmosphere. Cell-free supernatants were harvested 72 and 96 h later and the concentrations of IFN-γ and IL-10 were determined by corresponding sandwich ELISA kit (eBioscience, San Diego, CA), according to manufacturer's instructions. All tests were performed in triplicate. The

limit of detection of IFN- γ and IL-10 were 15 pg/ml and 30 pg/ml, respectively. The results were shown as mean $\pm$ SD.

2.11.3. Determination of *Toxoplasma*-specific total IgG, IgG1, and IgG2a antibodies

Specific antibodies against rSAG1 and rGRA2 were detected in individual serum samples by enzyme-linked immunosorbent assay (ELISA). Hence, sera from all mice in five vaccination groups were collected to investigate the induction of total IgG, IgG1, and IgG2a antibodies, three weeks after the second immunization. Briefly, the 96 flat-bottom wells of microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with the mixture of rSAG1 (1 μ g/ml) and rGRA2 (2 μ g/ml) in 50 mM sodium carbonate buffer (pH 9.6). The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T, pH 7.4) and non-specific binding sites were blocked with blocking buffer (2% BSA in PBS-T) for 2 h at 37 °C. Then, one hundred microliters of each sample sera diluted 1/100 in blocking buffer was added and incubated for 2 h at 37 °C. After subsequent washings, bounded IgG were detected by incubation for 1 h at 37 °C with 100 μ l of anti-mouse HRP conjugated IgG (BD Pharmingen, USA) (1/2,000), or Biotinylated anti-mouse IgG1 light chain (1:10000) or biotinylated anti-mouse IgG2a (1:500) antibodies (BD Pharmingen, USA). Streptavidin-peroxidase (Sigma) was added (0.75 μ g/ml) at 37 °C for 30 min. Finally, the enzymatic activity was revealed using O-phenylenediamine (OPD) (Sigma). After 15 min of incubation at room temperature, the reaction was stopped by adding 100 μ l of sulfuric acid (1 M), and the absorbance was measured at 492 nm. A sample was considered positive, if the mean OD value of duplicated wells was equal to or higher than defined cut off (the mean OD value of unvaccinated mice sera plus three standard deviation (SD)).

2.11.4. Challenge study

To evaluate immunoprotection induced by different vaccine formulations, the remained eight mice in each group were intraperitoneally (i.p.) infected with 1×10^3 tachyzoites of *T. gondii* virulent RH strain, three weeks after second immunization. Mice were daily monitored (every 12 h), and their survival times were recorded.

2.11.5. Statistics

Statistics were carried out using GraphPad Prism 5.0 software (Inc 2007, USA). The mean of each variable (total IgG, IgG1, IgG2a, IFN- γ , IL-10) was compared among different groups via one-way ANOVA followed by Tukey multiple comparison test. The slopes of release profiles of both rSAG1-PLGA and rGRA2-PLGA were compared via linear regression.

Survival analysis was performed using Kaplan-Meier method and the comparisons of survival curves were based on log-rank (Mantel-Cox) test. The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Production of rSAG1 and rGRA2 proteins

According to our previous studies, rGRA2 and rSAG1 were expressed and purified. The antigenicity and immunogenicity of both recombinant proteins (rproteins) were confirmed as described in our previous studies (Allahyari, et al., 2015, Golkar, et al., 2007). Endotoxin concentration in purified rproteins was less than 0.05 EU/ μ g by limulus amoebocyte lysate assay.

3.2. Characterization of PLGA microspheres

All prepared PLGA MS were characterized in terms of zeta average size, PDI, zeta potential, adsorption efficiency and preparation yield. The results, summarized in Table 2, indicated that blank PLGA MS had the average size of 438 ± 11 nm with the size distribution of 0.12 ± 0.01 and were negatively charged with the zeta potential of -5.56 ± 0.68 mV. On the other hand, particle size analysis by PCS demonstrated slightly polydisperse particles ($PDI < 0.2$) with the average size of about 486-490 nm for both rSAG1-PLGA and rGRA2-PLGA. The observed zeta potential of rSAG1-PLGA and rGRA2-PLGA, -1.00 ± 0.33 mV and -1.05 ± 0.16 mV, respectively, were higher than blank MS. With regard to the isoelectric points (pI) of rSAG1 and rGRA2, 7.9 and 8.6, respectively, both rprotein-PLGA had positive charges in adsorption buffer (pH 7.4). This probably resulted in the increased zeta potential of both rprotein-PLGA, compared to the blank PLGA MS. The differences between zeta potential of blank MS and rSAG1-PLGA or rGRA2-PLGA were statistically significant by one way Anova ($p < 0.05$), which clearly proved that rSAG1 and rGRA2 were adsorbed on the surface of blank PLGA. Morphology of blank PLGA was visualized using SEM as shown in Fig. 1. The size consistency of blank PLGA MS was confirmed by SEM, which was in accordance with the measured amount of PDI in blank MS (< 0.12).

Fig. 1. Scanning electron microscopy of blank Poly lactide-co-glycolide (50:50, Resomer®RG503) microspheres prepared by double emulsion solvent evaporation technique.

The adsorption efficacy of rSAG1 and rGRA2 on blank PLGA MS were 69.7 ± 3.56 % and 71.4 ± 3.6 %, respectively. Adsorption of rSAG1 and rGRA2 on blank PLGA MS probably was affected by a complex of driving interactions including hydrophobicity and surface charge.

Table 2. Characterization of blank PLGA, rSAG1-PLGA, and rGRA2-PLGA. Results are represented as mean $\pm$ SD corresponding independent experiments as mentioned.

Formulation	Size (nm)	PDI	Zeta potential (mV)	Adsorption efficiency (%)	Yield (%)
Blank PLGA	438 ± 11	0.12 ± 0.01	-5.56 ± 0.68	-	86.8 ± 3.56
rSAG1-PLGA	486 ± 9.9	0.14 ± 0.02	-1.00 ± 0.33	69.73 ± 3.05	87.4 ± 2.7
rGRA2- PLGA	490 ± 9.6	0.15 ± 0.02	-1.05 ± 0.16	71.4 ± 3.6	88.4 ± 2.07

Poly dispersity index was abbreviated to PDI

3.3. *In Vitro* release profiles of rSAG1 and rGRA2 adsorbed on PLGA microspheres

Release of rSAG1 and rGRA2 from PLGA MS were studied in PBS (pH 7.4) containing 0.02% sodium azide during 4 weeks. The release profiles of rSAG1 and rGRA2 were similar and represented a tri-staged protein release pattern including initial burst release, lag phase and zero-order release (Fig. 2). During the first hour of release study, about 18% and 14% of rSAG1 and rGRA2 were released, respectively. In initial burst release that lasted four hours, about 22% and

19% of rSAG1 and rGRA2 were released. A lag phase was appeared between 4 and 36 h and during this period, approximately 30% of both adsorbed rproteins were released. Then, both rproteins showed a zero-order release pattern that was associated with the sustained release of rSAG1 and rGRA2 till the third week of release study (Fig. 2). Presumably, the complete release of SAG1 and rGRA2 was coupled with PLGA MS degradation. A statistical analysis indicated that the rprotein release rates (i.e. slope) were not significantly different ($p>0.05$) between rSAG1-PLGA and rGRA2-PLGA .

Fig. 2. *In vitro* release profiles of rSAG1 and rGRA2 proteins from PLGA microspheres. Release studies were carried out in three independent experiments. Blank PLGA MS, rSAG1, and rGRA2 proteins were used as negative and positive controls, respectively. Each point is represented as mean $\pm$ SD. In order to prevent over lapping of error bars in graph, only means were represented in rGRA2 profile.

3.4. Cellular immune responses

In order to investigate the cellular immune response induced by vaccination regimens, the production of IFN- γ and IL-10 cytokines as two key indicators of Th1 and Th2 immune responses were assessed three weeks after second immunization. The splenocytes of vaccinated mice with rSAG1-PLGA, rGRA2-PLGA, and rSAG1/rGRA2-PLGA (i.e. G1, G2, and G3 groups, respectively) produced high levels of IFN- γ . The measured amounts of IFN- γ were in G1 322.9 ± 156 pg/ml, 242.9 ± 111 pg/ml (against rSAG1 and STAg, respectively), in G2 446 ± 90 pg/ml and 328.2 ± 40 pg/ml (against rGRA2 and STAg, respectively), and in G3 689.5 ± 55 pg/ml and 388.1 ± 24 pg/ml (against rGRA2+rSAG1 and STAg, respectively) (Fig. 3A). The observed differences in IFN- γ induction were significant in G1 ($p < 0.01$), and in G2 and G3 ($p < 0.001$) compared to control groups G4 and G5 (Fig. 3A). Moreover, the level of IFN- γ in G3 was significantly higher than G1 ($p < 0.001$) and G2 ($p < 0.01$) after re-stimulation of splenocytes with corresponding rproteins (rSAG1, GRA2, and rSAG1/rGRA2) and STAg, suggesting the synergistic effect of rSAG1/rGRA2 in eliciting more potent cellular immune response compared to single rprotein.

On the other hand, the amounts of IL-10 were measured in the supernatant of spleen cell culture in G1 (98.83 ± 4.5 pg/ml, 83.27 ± 9.4 pg/ml against rSAG1 and STAg), G2 (103.8 ± 2.9 pg/ml, 79.6 ± 8.3 pg/ml against rGRA2 and STAg), and G3 (105.5 ± 5 pg/ml, 94.3 ± 18.4 pg/ml against rSAG1/rGRA2 and STAg) (Fig. 3B). No significant difference was found in the induction of IL-10 among vaccinated groups compared to controls. As shown in Fig. 3C, further analysis of induced cytokines demonstrated that IFN- γ /IL-10 ratio was significantly higher ($p < 0.001$) in G2 and G3 compared to control groups (G4, G5). However, IFN- γ /IL-10 ratio in G1 was less than G2 and G3 and this ratio in G1 showed a significant difference compared to G4

and G5 ($p<0.01$). Besides, IFN- γ /IL-10 ratio in mice vaccinated with rSAG1/rGRA2-PLGA (G3) was significantly higher than mice receiving rSAG1-PLGA (G1) ($p<0.001$) and rGRA2-PLGA (G2) ($p<0.05$), demonstrating the synergistic effect of rSAG1/rGRA2-PLGA in eliciting stronger humoral immune responses.

(A)

(B)

Fig. 3. Determination of IFN- γ and IL-10 production by splenocytes of vaccinated mice. Spleen cells cultured in triplicate for 5 days in the presence of rproteins (2 μ g/ml), soluble *T. gondii* antigen (STAg, 20 μ g/ml), ConA (as positive control) and RPMI (as negative control). IFN- γ (A) and IL-10 (B) IFN- γ /IL-10 ratio (C) were measured in the five groups. Each bar is represented as mean $\pm$ SD for mice in each group. ***p<0.001 and **p<0.01.

IgG1 and IgG2a responses

To investigate humoral immunity and also to assess the profile (Th1 or Th2) of humoral responses elicited by different vaccine formulations, sera were collected from mice three weeks after second immunization, and *Toxoplasma*-specific total IgG, IgG1 and IgG2a antibodies were analyzed individually by indirect ELISA.

As shown in Fig. 4A, all three formulations induced significant antibody responses in vaccinated mice. Total IgG antibody titers in G1, G2, and G3 groups were observed to be significantly ($p<0.001$) higher than control groups (G4,G5). Noticeably, mice in G3, vaccinated with rSAG1/rGRA2-PLGA produced significantly higher levels ($p<0.01$) of total IgG than G1 and G2 groups vaccinated with rSAG1-PLGA and rGRA2-PLGA, respectively.

The magnitude of IgG1 and IgG2a responses was evaluated as indicators of Th1 and Th2 immunity, respectively. As represented in Fig. 4B, mice in G1, G2, and G3 groups induced both IgG1 and IgG2a in significantly higher levels ($p<0.001$) compared to control groups (G4,G5). In addition, the significant predominance of *Toxoplasma*-specific IgG2a over IgG1 antibodies in each group (G1, G2, and G3) was observed ($p<0.05$). In addition, IgG2a/IgG2 ratio in sera of mice vaccinated with rSAG1/rGRA2-PLGA was not significantly different from mice receiving rSAG1-PLGA or rGRA2-PLGA (Fig. 4C). Moreover, the higher amounts of IgG2a in G3 compared to G1 ($p<0.05$) and G2 ($p<0.01$) receiving rSAG1-PLGA or rGRA2-PLGA demonstrated synergistic effect of rSAG1/rGRA2-PLGA.

(A)

(B)

(C)

Fig. 4. Analysis of *Toxoplasma*-specific total IgG antibody (A), IgG1 and IgG2a subclasses (B), and IgG2a/IgG1 ratio (C) in sera of vaccinated mice. Sera were collected three weeks after second immunization and analyzed individually. Results are represented as mean $\pm$ SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

3.5. Protection against *T. gondii* challenge infection

To assess the protective effect of rSAG1-PLGA, rGRA2-PLGA, rSAG1/rGRA2 vaccination against toxoplasmosis, the survival times of vaccinated mice were evaluated after intraperitoneally (i.p.) challenge infection with 1×10^3 tachyzoites of highly lethal RH strain of *T. gondii*, three weeks after second immunization. The Kaplan-Meier method was used to compare survival time among different groups.

Although all mice died almost between 8 and 21 days, all vaccinated mice (G1,G2,G3) demonstrated a significantly ($p < 0.0001$) longer survival time compared to control groups (G4,G5) vaccinated by blank PLGA and PBS, respectively (Fig. 5).

Interestingly, higher immunoprotection ($p < 0.05$) was shown in mice vaccinated with rSAG1/rGRA2-PLGA (G3) compared to rSAG1-PLGA (G1) and rGRA2-PLGA (G2). In addition, there was no significant difference between the survival time of vaccinated mice in G1 and G2 groups.

Fig.5. Kaplan-Meier plot comparing survival time of vaccinated BALB/c mice after challenging with 1×10^3 tachyzoite of *T. gondii* RH strain, three weeks after the second immunization. Each group contained eight mice. **** $p < 0.0001$ and * $p < 0.05$. p values are based on log-rank (Mantel-Cox) test.

4. Discussion

Though, the most protective and cost-effective approach to decline *Toxoplasma* global burden, and equally toxoplasmosis consequences would be vaccination, an effective vaccine is not commercially available yet. Many studies are required to enhance the efficacy of experimental vaccines and to pave the way for clinical examination in human.

The majority of vaccine researches are based on *Toxoplasma* rproteins to induce high protection against infection (Dziadek, et al., 2011, Jongert, et al., 2009). Due to the complexity of *T.gonii* life cycle (Dziadek, et al., 2011, Wagner, et al., 2015, Zhou, et al., 2007), and the stage-limited protection by stage-specific antigens (Alexander, et al., 1996) (Zhou, et al., 2007), the exploitation of more than one antigen is essential for the development of an efficient vaccine. Hence in this study we designed a subunit vaccine composed of rSAG1 and rGRA2 expressed in different life-cycle stages in order to elicit more potent immune responses.

On the other hand, in spite of outstanding advantages of subunit vaccines, they still suffer from poor immunogenicity (Sloat, et al., 2010), which makes the application of safe and efficient adjuvant and/or delivery system inevitable in new vaccine design strategy to mimic pathogen properties such as size, hydrophobicity, shape and to achieve desired immune responses (Bachmann and Jennings, 2010).

In this study, we formulated rSAG1 and rGRA2 individually into PLGA MS as an adjuvant and vaccine delivery platform. PLGA micro/nano particles (MPs/NPs) present safe, biodegradable, biocompatible, and promising vaccine delivery system. They protect loaded

peptides/proteins from proteolytic degradation (Mahboubian, et al., 2010), can efficiently promote antigen targeting towards dendritic cells (DCs) (Hamdy, et al., 2011), and induce potent cellular immune response (T O'Hagan, et al., 1998). The DC-targeting feature of PLGA particles can be occurred without any specific recognition and only due to particle size similarity to pathogens that immune system has evolved to combat them through years (Hamdy, et al., 2011). It has been established that PLGA particles have no adverse effect on DC function like maturation, migration, cytokine secretion, and co-stimulatory properties (Waeckerle-Men and Groettrup, 2005). In the present study, we prepared rSAG1-PLGA and rGRA2-PLGA by individual adsorption of each rprotein on the surface of blank PLGA MS according to previously described method by Kazzaz et al. with a few modifications (Kazzaz, et al., 2006). The application of protein adsorption method instead of encapsulation solves many problems regarding protein degradation and aggregation, that may affect protein integrity and immunogenicity (Singh, et al., 2004). The encapsulated proteins are exposed to a range of potentially harsh conditions occurring during particle preparation such as water-oil interfaces, organic solvents, and damaging mechanical, thermal, and chemical stresses (Saini, et al., 2011, van de Weert, et al., 2000). Besides, protein adsorption method facilitates PLGA particle sterilization by gamma irradiation, which can be done before protein loading and other GMP requirements for scale-up (Brito and O'Hagan, 2014, Jain, et al., 2011, Kumari, et al., 2010, O'Hagan, et al., 2006).

Generally, the uptake of antigens by antigen presenting cells (APCs) relies on several particle-associated properties including size, shape, surface charge, hydrophobicity, as well as receptor interactions (Bachmann and Jennings, 2010). Among them, particle size is a critical attribute, which plays a key role in eliciting efficient uptake of particle by APCs and making possible their

access to appropriate DC subsets in lymphoid organs (Bachmann and Jennings, 2010). Indeed, it seems that PLGA NPs smaller than 500 nm can elicit more potent cytotoxic T lymphocyte (CTL) responses than MPs larger than 2 μ m (Allahyari and Mohit, 2015, Hamdy, et al., 2011). While, larger particles (500–2000 nm) were mainly associated with DCs at the injection site, small particles (20–200 nm) were found in lymph node(LN)-resident DC and macrophages, suggesting their free drainage to the LN (Manolova, et al., 2008). However, DCs take up larger particles (200-500 nm) and carry them to LN during 24 hours after administration (van Riet, et al., 2014). Hence, it is concluded that particle trafficking happens in a size-dependent manner. According to the mean diameter of rSAG1-PLGA and rGRA2-PLGA (486 ± 9.9 and 490 ± 9.6 nm, respectively), these vaccine formulations could be efficiently taken up by DCs, trafficked to the LN, and could trigger DC maturation. (Bachmann and Jennings, 2010). The larger size of rSAG1-PLGA and rGRA2-PLGA compared to blank MS can be attributed to the lyophilization of blank MS before protein adsorption process and to some extent protein adsorption on the surface of blank MS. Though, rGRA2-PLGA were slightly smaller than rSAG1-PLGA, there was no significant difference in their size values. The size similarity of both rprotein-PLGA can be referred to the identical preparation method, the same applied polymer, and also comparable size of both rproteins. In addition, the higher adsorption efficacy of rGRA2 might be attributed to its higher pI (8.6 vs. 7.9) making rGRA2 more positive in pH 7.4, and higher hydrophobicity than rSAG1 (32 kDa), which resulted in the stronger interaction of rGRA2 with PLGA MS.

Furthermore, we showed by scanning electronic microscopy that the prepared PLGA particles were spherical in shape and did not appeared to be aggregated. As mentioned earlier, particle shape is another feature influencing cellular uptake by DCs (Bachmann and Jennings, 2010, van Riet, et al., 2014).

PLGA particulate vaccine can provide sustained protein release, which is critical to elicit potent immune responses. It is considered that the impact of PLGA particulate vaccine on immune responses relies on the kinetics of protein release (Demento, et al., 2012). Overall, protein release happens through a combination of bulk and surface diffusion as well as the erosion of the PLGA particles, which can be modulated through many PLGA attributes (Fredenberg, et al., 2011, Wischke and Schwendeman, 2008). A major advantage of PLGA copolymer is the possibility to manipulate its physico-chemical characteristics in favor of the adjustment of particle degradation (Hamdy, et al., 2011), which makes PLGA as a unique vaccine delivery vehicle.

In the present study, the release profile of rSAG1 and rGRA2 from PLGA particles, roughly showed a classic tri-phasic release pattern (Fredenberg, et al., 2011) including initial burst (phase I), slow release phase (lag time), followed by a faster release (phase III) (Makino, et al., 2000, Zolnik and Burgess, 2007), which has been also observed by Chuang et al. (Chuang, et al., 2013) (Chuang, et al., 2013). They demonstrated a 56-day and 35-day release period of rSAG1 and rSAG1/rSAG2 encapsulated in PLGA particles, respectively. The shorter release time of adsorbed rSAG1-PLGA and rGRA2-PLGA in our study than encapsulated rSAG1 and rSAG1/rSAG2 could be justified by using protein adsorption approach instead of protein encapsulation, which can enhance release rate of protein. Also, the similarity of both release profiles probably comes from the identity of MS nature (PLGA) and the resemblance of both rproteins in physicochemical properties, which drive their adsorption/release behavior. It is assumed that phase I release happens due to weakly bounded proteins to particle surface (Fredenberg, et al., 2011). In other words, the initial burst release is attributed to protein desorption from outer layer where protein-protein are mostly bounded by electrostatic forces.

While slow release (phase II) is associated with the desorption of proteins that have directly interacted to PLGA particles surface by stronger hydrophobic forces mainly (Saini, et al., 2011). Finally, in the phase III, a sustained release of rSAG1 and rGRA2 occurs in a zero-order manner (Makino, et al., 2000).

T. gondii is an intracellular parasite, thus immune responses mediated by Th1 CD4⁺ and CD8⁺ cytotoxic T cells cause host resistance to acute or chronic *T. gondii* infection (Denkers and Gazzinelli, 1998) (Hou, et al., 2011, Pifer and Yarovinsky, 2011). Here, the analysis of immune responses after vaccination was performed by the assessment of cellular as well as humoral immunity as evidenced by cytokine assays and *Toxoplasma*-specific IgG antibodies.

It was well established that IFN- γ produced by natural killer (NK) and T cells acts as the major regulator of cell mediated immunity against *T. gondii* in human and mice (Hou, et al., 2011, Pifer and Yarovinsky, 2011) and plays an important role in coordinating innate and adaptive immunity (Wagner, et al., 2015). IFN- γ -mediated CTL response restrains the proliferation and spreading of *T. gondii* through the growth inhibition of actively dividing tachyzoites (acute phase) and the limitation of bradyzoites reactivation (Suzuki, et al., 1988). It was found out that all vaccinated mice could induce significantly higher amounts of IFN- γ compared to control groups and relatively low levels of IL-10.

To have better assessment of induced cellular immunity (by Th1 and Th2 profile), further analysis through IFN- γ /IL-10 ratio was performed and showed that IFN- γ /IL-10 ratio was significantly higher in G3 compared to G1 and G2, suggesting that rSAG1/rGRA2-PLGA can synergistically induce stronger Th1 immune responses than rSAG1-PLGA or rGRA2-PLGA.

In other words, the level of IFN- γ , the pivotal protective cytokine, promotes Th1-directed adaptive immunity, determines the fate of *Toxoplasma* infection (Yarovinsky, 2014) (Pifer and

Yarovinsky, 2011) and controls parasite replication (Sturge and Yarovinsky, 2014, Suzuki, et al., 2011).

Although, one of the negative regulatory consequences of IL-10 is to suppress macrophage killing activity mediated by IFN- γ (Sibley, et al., 1993), it is worth mentioning that IL-10 reduces the severe inflammatory effect aroused by IFN- γ by down regulating Th1 response at the early acute phase of toxoplasmosis (Neyer, et al., 1997) and plays a vital role in balancing immune responses (Suzuki, et al., 2000).

Furthermore, we previously demonstrated that rGRA2 vaccination produced high amounts of IFN- γ and IL-2 in vaccinated CBA/j mice, which resulted in significant protection against brain cyst production (Golkar, et al., 2007). The capability of rSAG1 (Petersen, et al., 1998) (Chuang, et al., 2013, Dziadek, et al., 2011), rGRA2 (Ching, et al., 2016, Zenner, et al., 1999), and rSAG1/rGRA2 fusion protein (Zhou, et al., 2007) in eliciting high level of IFN- γ and other Th1-related immune responses against *T. gondii* has been shown in many studies.

Apart from cellular immunity, the contribution of antibodies to defense against *T. gondii* has been established through research on B cell-deficient (μ MT) mice. *Toxoplasma*-specific antibodies can inhibit tachyzoite adhesion to host cell receptor and enhance intracellular killing of antibody-coated parasite (Dziadek, et al., 2011, Kang, et al., 2000, Sayles, et al., 2000).

In present study, all vaccinated mice produced strong *Toxoplasma*-specific total IgG, IgG1, and IgG2a antibodies compared to control groups, which was in accordance with other studies (Ching, et al., 2016, Dziadek, et al., 2011, Golkar, et al., 2007). Moreover, significantly higher titers of IgG antibodies were observed in mice vaccinated with rSAG1/rGRA2-PLGA compared to mice receiving single rprotein, confirming the synergistic effect of rSAG1/rGRA2-PLGA in eliciting strong humoral responses. However, there was not any significant difference in

IgG2a/IgG1 ratio among these groups. In spite of roughly predominance of IgG2a over IgG1 in all vaccinated mice, the IgG2a/IgG1 ratios were slightly more than one in vaccinated groups. This observation indicated that mixed Th1/Th2 immune responses have been roughly induced after vaccination. Our findings were in agreement with others (Ching, et al., 2016, Dziadek, et al., 2011, Martin, et al., 2004), and in contrast with some researches which reported considerable predominance of IgG2a over IgG1 (Golkar, et al., 2007, Zhou, et al., 2007). Similarly, Chuang et al. (Chuang, et al., 2013, Chuang, et al., 2013) applied PLGA in vaccine formulations and showed that mice immunized with SAG1-PLGA and SAG1/SAG2 PLGA elicited potent Th1-directed immunity against *T. gondii*, confirming the role of PLGA as suitable delivery. However, their studies have some fundamental differences with ours. They encapsulated SAG1, and SAG1/SAG2 into PLGA particles, while we adsorbed rSAG1 and rGRA2 on the surface of PLGA microspheres to overcome problems regarding protein degradation occurring during protein encapsulation and to provide sufficient amount of high-quality proteins required for appropriate stimulation of antigen presenting cells (APCs) (Saini, et al., 2011). We immunized mice subcutaneously and challenged them intraperitoneally, while Chuang *et al.* (Chuang, et al., 2013) did vice versa. Importantly, the size of rprotein-loaded PLGA in their study was in micrometer range, while we prepared PLGA particles in nanometric size (less than 500 nm). As discussed earlier, particles with size less than 500 nm are more potent in inducing cell mediated immunity.

The efficacy of PLGA particles as vaccine delivery system has been shown in development of vaccine against other intracellular parasite such as *Leishmania* (Santos, et al., 2012, Santos, et al., 2013), *Plasmodium vivax* (Moon, et al., 2012) (Carcaboso, et al., 2004), *Mycobacterium tuberculosis* (Kirby, et al., 2008). In addition to PLGA particles as vaccine delivery vehicle

against *T.gondii*, the ability of porous maltodextrin-based with lipid core nanoparticles (DGNP) loaded by total extract of *T. gondii* antigen has been evaluated in design of nasal vaccine against acute and chronic toxoplasmosis in CBA/j (Dimier-Poisson, et al., 2015). The results showed mixed Th1/Th17 responses associated with high protection against toxoplasmosis regarding survival and parasite burden.

As the ultimate goal of vaccination is to raise protection against toxoplasmosis (Dziadek, et al., 2011), we explored the protective effect of different vaccine formulations in proportion of their survival time after challenge by RH tachyzoite. All formulations prolonged survival time of vaccinated mice compared to control groups, though all mice finally succumbed to death. Moreover, the level of protection was dependent on vaccine formulation, so that rSAG1/rGRA2-PLGA provided significantly longer survival time i.e. higher protection in comparison with rSAG1-PLGA or rGRA2-PLGA alone. While, there was no significant difference between survival time of rSAG1- or rGRA2 vaccinated mice. Our findings were in accordance with a recent study that showed vaccination of BALB/c with rGRA2 or rGRA5 caused an increase in survival time upon challenge against virulent *T. gondii* RH strain. They demonstrated that rGRA2 and rGRA5 subunit vaccine induced high level of IFN- γ and IL-2, and IgG antibodies with IgG2a/IgG1 ratio less than one. Their results suggested a mixed Th1/Th2 immunity with the predominance of Th1-mediated immune responses conferring immunoprotection (Ching, et al., 2016). Also, Zhou et al. showed that vaccination of BALB/c mice by rSAG1/rGRA2 fusion protein expressed in yeast raised the survival time of mice from 6 to 15 days after challenge with *T. gondii* RH strain and resulted in partial protection (Zhou, et al., 2007). Besides, we demonstrated in our previous study (Golkar, et al., 2007), that rGRA2 formulated in MPL (Monophosphoryl lipid A could reduce brain cysts formation significantly in the immunized

CBA/J mice either alone or mixed with rGRA6, thereby protecting against chronic *T. gondii* infection.

The advantage of bivalent over monovalent vaccines against toxoplasmosis has been shown in studies using rSAG1-rGRA2 (Zhou, et al., 2007), DNA pSAG1/SAG3 (Cong, et al., 2013), and pGRA4 and pSAG1mut (Mévélec, et al., 2005) vaccines, though a few researches came to contradictory conclusions (Golkar, et al., 2007, Martin, et al., 2004).

Taken together, our results indicated that the adsorption of rSAG1 and rGRA2 on PLGA MS produced stable vaccine formulations with protective effect against *T. gondii* infection in BALB/c. Moreover, the efficient role of PLGA MS as delivery/adjuvant platform and the synergistic effect of rSAG1/rGRA2-PLGA in conferring stronger protection were proved. We believe that the production of high amount of IFN- γ and *Toxoplasma*-specific IgG antibodies by vaccinated mice can justify the longer survival time upon infection by virulent tachyzoites of *T. gondii*. Despite the considerable predominance of IFN- γ over IL-10 in vaccinated mice, the comparable titers of IgG2a and IgG1 were observed, suggesting a mixed Th1/Th2 responses. Interestingly, the strong and prolonged antibody induction confirmed that rprotein adsorption on PLGA and consequently rprotein release did not affect their integrity and immunogenicity.

For future studies, we think vaccine formulations comprising several immunogenic antigens, adsorbed on PLGA MS, and strong Th1 adjuvant(s) e.g. a combination of TLR ligands might be a further advancement in providing an effective vaccine against *T. gondii*.

Our future studies are being designed to evaluate the efficiency of other antigens mixture to get more potent immune response and consequently protection against *T. gondii* infection.

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Legend to figures:

Fig. 1. Scanning electron microscopy of blank Poly lactide-co-glycolide (50:50, Resomer®RG503) microspheres prepared by double emulsion solvent evaporation technique.

Fig. 2. *In vitro* release profiles of rSAG1 and rGRA2 proteins from PLGA microspheres. Release studies were carried out in three independent experiments. Blank PLGA MS, rSAG1, and rGRA2 proteins were used as negative and positive controls, respectively. Each point is represented as mean $\pm$ SD. In order to prevent overlapping of error bars in graph, only means were represented in rGRA2 profile.

Fig. 3. Determination of IFN- γ and IL-10 production by splenocytes of vaccinated mice. Spleen cells cultured in triplicate for 5 days in the presence of rproteins (2 μ g/ml), soluble *T. gondii* antigen (STAg, 20 μ g/ml), ConA (as positive control) and RPMI (as negative control). IFN- γ (A) and IL-10 (B) IFN- γ /IL-10 ratio (C) were measured in the five groups. Each bar is represented as mean $\pm$ SD for mice in each group. *** p <0.001 and ** p <0.01.

Fig. 4. Analysis of *Toxoplasma*-specific total IgG antibody (A), IgG1 and IgG2a subclasses (B), and IgG2a/IgG1 ratio (C) in sera of vaccinated mice. Sera were collected three weeks after second immunization and analyzed individually. Results are represented as mean $\pm$ SD. *** p <0.001, ** p <0.01, and * p <0.05.

Fig.5. Kaplan-Meier plot comparing survival time of vaccinated BALB/c mice after challenging with 1×10^3 tachyzoite of *T. gondii* RH strain, three weeks after the second immunization. Each group contained eight mice. **** p <0.0001 and * p <0.05. p values are based on log-rank (Mantel-Cox) test.