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Protection and Immune Responses Elicited by rSAG1-PLGA Nanoparticles in C57BL/6 Against *Toxoplasma gondii*

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ABSTRACT

Introduction: This study aimed to evaluate rSAG1-PLGA efficacy as a particulate vaccine in conferring protection against *Toxoplasma gondii* infection in C57BL/6 mice. In light of our previous studies, we studied mice genotype role in eliciting immune responses by rSAG1-PLGA nanoparticles in this study. **Methods:** Poly (DL-lactide-co-glycolide) (PLGA) nanoparticles loaded by rSAG1 as a subunit vaccine were prepared, and C57BL/6 mice were subcutaneously immunized twice at a 3-week interval by rSAG1-PLGA, soluble rSAG1, blank PLGA, and one group kept unvaccinated. The characteristics of PLGA nanoparticles, the amounts of produced IFN-γ, IL-10, specific anti-*Toxoplasma*IgGs, and the conferred protection against infection by *T. gondii* RH tachyzoite were assessed. **Results:** rSAG1-PLGA nanoparticles shared a z-average of about 450nm with negative Zeta potential. Compared with the negative control group, the mice vaccinated with rSAG1-PLGA nanoparticles produced significantly higher amounts of IFN-γ, specific anti-*T. gondii* IgG antibodies and higher titer of IgG2a, which resulted in longer survival times. **Conclusion:** The efficiency of rSAG1-PLGA nanoparticles in inducing humoral and cellular responses and consequently partial protection against acute toxoplasmosis in C57BL/6 was confirmed.

INTRODUCTION

new generation of vaccines, including preventive and therapeutic ones, takes advantage of vaccine delivery vehicles to overcome the poor immunogenicity of recombinant protein-based vaccines. *Toxoplasma gondii* is well known as an intracellular parasite with a multi-stage life cycle; tachyzoites and bradyzoites are cases in point. *T. gondii*, as an intracellular parasite with a wide distribution globally, is considered the most successful parasite [1, 2]. Toxoplasmosis rarely develops any severe symptoms in immunocompetent individuals. In contrast, it causes toxoplasmic encephalitis in immune-compromised patients [3, 4] and severe consequences in congenitally-infected newborns [5]. In order to reduce the global burden of *T. gondii* and control toxoplasmosis, vaccine development is of high importance [6].

Despite designing and evaluating various experimental formulations against *T. gondii* infection, no approved human vaccine is available yet. The development of a potent vaccine against *T. gondii* seems to be challenging [7]. The major *T. gondii* tachyzoite surface antigen, SAG1, is one of the most immunogenic antigens and has been thoroughly applied in various experiments as one of the encouraging immunogens [7-11].

In order to overcome the poor antigenicity of rSAG1 [12, 13], we applied it as a nanoparticulate vaccine delivery system [14-16]. Nowadays, PLGA nanoparticles have achieved success as a suitable delivery system thanks to their impressive features [17-19]. Accordingly, PLGA nanoparticles have been successfully employed as antigen-carrying vehicles in many designed vaccines against infections by intracellular parasites, including hepatitis B [20], leishmaniasis [21], malaria [22], tuberculosis [23], and toxoplasmosis [11, 17, 24].

An efficient strategy for vaccine design and development relies on selecting most immunogenic antigen (s), potent adjuvants, and immunostimulating agents (e.g., TLR ligands), a suitable delivery vehicle such as a PLGA-based particulate delivery system, and finally, an appropriate animal model to assess the efficacy of the desired formulation.

Various mice strains have differences in susceptibility to *T. gondii* infection [25].

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It is worth noting that H2-b background mice such as C57BL/6 are susceptible to toxoplasmosis infection [26], while BALB/c inbred mice (H2-d) and all outbred mice (such as NMRI strain) are regarded resistant to toxoplasmosis [25, 27].

Previously, we confirmed the immunogenicity of purified rSAG1 antigen [28] and showed the efficiency of PLGA nanoparticles as vehicles for carrying rSAG1 and rGRA2 antigens against toxoplasmosis [29]. Then, we proved the advantages of adsorption method to encapsulan method in the loading of rSAG1 antigen into PLGA nanoparticles [30] against *T. gondii* in BALB/c mice. Hence, in this research, we vaccinated C57BL/6 mice with rSAG1-PLGA nanoparticles to examine the formulation efficacy in eliciting protective immune responses against acute toxoplasmosis.

MATERIALS AND METHODS

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) was obtained from Boehringer Ingelheim, Germany. PVA [poly vinyl alcohol; molecular weight (MW) 30,000–70,000 Da, 88% hydrolyzed], all cell culture reagents were purchased from Sigma (Darmstadt, Germany). The SDS PAGE gel electrophoresis and protein molecular weight marker materials were purchased from Roche Applied Sciences and Fermentas. Dichloromethane (DCM) analytical grade was purchased from Merck Ltd. RPMI-1640, and Fetal Calf Sera (FCS) was supplied from Gibco (Life Technologies GmbH). Water for solution preparations was prepared by MilliQ™ ultrapure (Milli-QSystem).

rSAG1 protein. rSAG1 protein (amino acids 49 to 311 of native one) was cloned, produced, and purified and its purity (SDS-PAGE), antigenicity (Western blotting), folding (RP-HPLC), and immunogenicity was confirmed based on our previous studies [28]. Endotoxin concentration in purified rSAG1 was determined less than 0.05 EU/μg by Limulus amebocyte lysate assay.

Nanoparticles preparation and characterizations. The preparation of blank PLGA was performed entirely, as mentioned in our previous study, using the double emulsion solvent evaporation technique [29], and the adsorption of rSAG1 on blank PLGA nanoparticles was done by Allahyari *et al*. (2020) [30]. Several batches of rSAG1 adsorbed-PLGA were produced individually and then blended into each other to supply adequate quantities of a homogenous mixture of nanoparticles. PLGA nanoparticles were identified based on particle size (Z-average mean), particle size distribution (PSD), and polydispersity index (PDI), and surface charge by zeta-potential (laser-Doppler electrophoresis) by a Zetasizer Nano ZS (Malvern Instruments) at 25°C as described in our previous study. Process yield for both blank PLGA and rSAG1-PLGA nanoparticles was determined, and protein adsorption efficiency was calculated by the direct method, as described by others [30]. The efficiency of rSAG1 adsorption on blank PLGA nanoparticles was estimated as described previously [29].

Vaccination studies. Mice and Parasite. Female C57BL/6 mice (6–8 weeks old) supplied from Pasteur Institute of Iran were kept in plastic cages located in the controlled animal facility with free access to tap water and standard rodent pellets. The animal ethics committee of Pasteur institute of IRAN (IR.PII.REC.1397.025) confirmed the procedures for animal trial such as maintenance, feeding, and taking blood samples, in agreement with the Guide for the Care and Use of Laboratory Animals by the National Research Council (US) Institute for Laboratory Animal Research (1996).

Tachyzoites of *T. gondii* (RH strain) were extracted from peritoneal fluid of infected Swiss mice by Percoll purification based on Wu *et al*. [31]. Then tachyzoites were washed with phosphate-buffered saline (PBS) and were frozen at -70 °C. Challenge study was performed with freshly-harvested tachyzoites.

Vaccination schedules. C57BL/6 mice were categorized into four groups (12 mice/group) by chance, then were subcutaneously (s.c) vaccinated in the right-hind footpad as follows: blank PLGA as a control group (G1) and rSAG1-PLGA nanoparticles (G2), rSAG1 and (G3), and mice in G4 kept unvaccinated (Table 1). The same regimen was injected in the second immunization three weeks after the first immunization. Three weeks later, blood samples were collected from the mice's eye vein; the sera were separated and stored at −20 °C. Simultaneously, mice were challenged by freshly prepared tachyzoites, and every 12 hours were monitored accordingly to record their survival.

Table 1. Vaccine formulations indifferent groups of C57BL/6.

Groups	Mice	First Immunization	Second Immunization	Amount of rSAG1
1	C57BL/6	Blank PLGA	Blank PLGA	-
2	C57BL/6	rSAG1-PLGA	rSAG1-PLGA	rSAG1 (20 μg)
3	C57BL/6	rSAG1	rSAG1	rSAG1 (20 μg)
4	C57BL/6	Unvaccinated	Unvaccinated	-

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Preparation of soluble *T. gondii* antigen (STAg). Freshly prepared RH tachyzoites were sonicated in PBS by an ultrasonic probe sonicator (400S Sonotrode, Hielscher, 0.5-second Cycle) and centrifuged (at 2000×*g* for 30 min) [29]. After evaluating total protein concentration through Bio-Rad DC protein assay, aliquots were kept at -70°C.

Cytokine assays. Three weeks after the booster and before the challenge infection, the spleen of four mice in each group was aseptically removed, and single-cell suspensions of mice spleen were individually prepared as previously described [30]. Spleen cells were cultured in RPMI-1640 supplemented with fetal calf serum (FCS) 5%, L-glutamine1%, HEPES 1%, beta-mercaptoethanol 50 μM, Penicillin-Streptomycin (10000U/ml), and in microtiter plates at 3×10^5 cells per well. Spleen cells were stimulated with 2 μg/ml of rSAG1 or 20 μg/ml of STAg. The stimulation of positive control cells was performed by Concanavalin A (ConA) 5 μg/ml. In contrast, culture media was substituted for ConAin negative controls. The concentrations of IFN-γ (Interferon-gamma) and IL-10 (Interleukin 10) in cell-free supernatants were investigated after 96 and 72h, respectively, by relevant ELISA kits (eBioscience). All samples were analyzed in triplicate. The limit of detection of IFN-γ and IL-10 were 15 pg/ml and 30 pg/ml, respectively.

Evaluation of specific anti-rSAG1 IgGs. ELISA method was applied to develop specific anti-rSAG1 IgGsin serum samples of each mouse in different groups three weeks after the second immunization. Hence, the titers of total IgG, IgG1, and IgG2a antibodies were determined in sera collected from four mice in each group, as described by Allahyari *et al*. (2016) [29]. Briefly, 96-well tissue culture plates (Maxisorp) were coated with rSAG1(1 μg/ml) in 50 mM sodium carbonate, after plate incubation at 37 °C, serum samples (in 1/100 dilution) were added to wells. Following different washing steps, anti-mouse HRP (Horseradish peroxidase) conjugated IgG (BD Pharmingen) (1/20,000, 100μl) was applied to detect bounded IgG. After the addition of streptavidin-peroxidase (0.75 μg/ml) at 37 °C, O-phenylenediamine (OPD) revealed the enzymatic activity for 15 min. Sulfuric acid (1M) was used to stop the reaction, then the absorbance was read at 492 nm. The mean OD related to the samples of unvaccinated mice plus three standard deviations (SD) was defined as the cutoff.

Challenge infection. The protection induced by different vaccine formulations against acute toxoplasmosis was evaluated by intraperitoneally (i.p.) infection of eight mice in each group with 1×10^3 tachyzoites of virulent RH *T. gondii*, three weeks after booster. Mice were checked every 12 h, their survival was documented, and the results were examined by the Kaplen-Miere method.

Statistical analyses. Statistics analyses were carried out using GraphPad Prism 5.0 software (Inc2007, San Diego, USA). The means of variables were assessed among all groups by one-way ANOVA (Tukey multiple comparison tests). Survival studies were performed by the Kaplan-Miere approach, and survival curves were compared by log-rank test (Mantel-Cox). When $P< 0.05$, the differences were regarded as statistically significant.

RESULTS

Features of PLGA nanoparticles. Physico-chemical properties of PLGA nanoparticles, including size, PDI, zeta potential, adsorption efficiency, and yield, were evaluated, as shown in Table 2. The average sizes of blank PLGA and rSAG1-PLGA nanoparticles were about 441 ± 15 nm and 452 ± 13nm, respectively, without any significant difference. The size distributions of rSAG1-PLGA and blank PLGA were lower than 0.12. Both nanoparticles shared a negative charge; blank PLGA with a zeta potential of -6.71 ± 0.43 mV, while rSAG1-PLGA had a lower negative charge of nearly -2.3 ± 0.81 mV. The zeta potentials were significantly different.

Blank PLGA nanoparticles were prepared by the W/O/W solvent evaporation method [29]. Ten batches of blank PLGA and 5 batches of rSAG1-PLGA were prepared. The adsorption efficiency of rSAG1 on the surface of PLGA nanoparticles was 64.91 ± 2.7%.

Table 2. Characterization of rSAG1-PLGA and blank PLGA nanoparticles. Results represented as mean ± SD of different batches of PLGA particles.

Formulation	Size (nm)	PDI	Zeta potential (mV)	Adsorption efficiency (%)	Yield (%)
Blank PLGA	441 ± 15	0.11 ± 0.03	-6.71 ± 0.43	-	89.6 ± 4.2
rSAG1-PLGA	452 ± 13	0.12 ± 0.03	-2.3 ± 0.81	64.91 ± 2.7	87.5 ± 3.2

Evaluation of cytokine production. Cellular immunity induced by different formulations was individually evaluated through the secretion of IFN-γ and IL-10 in each mouse three weeks after the second immunization. C57BL/6 mice immunized with rSAG1-PLGA (G2) produced significantly higher amounts of IFN-γ (307.4 ± 40 pg/ml) compared to mice in G1 (24.25 ± 3 pg/ml) and mice in G3 (26.8 ± 2 pg/ml) in response to rSAG1 ($P<0.001$) as shown in Fig.1A. Besides, splenocytes from mice immunized with rSAG1-PLGA

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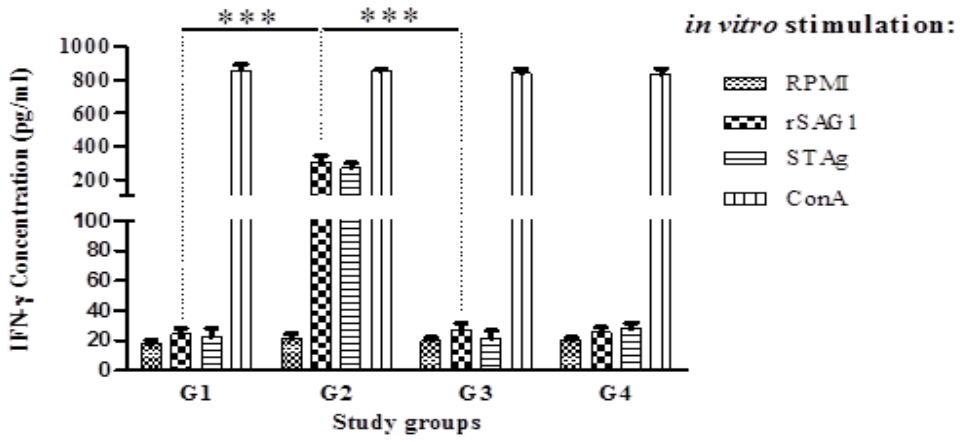
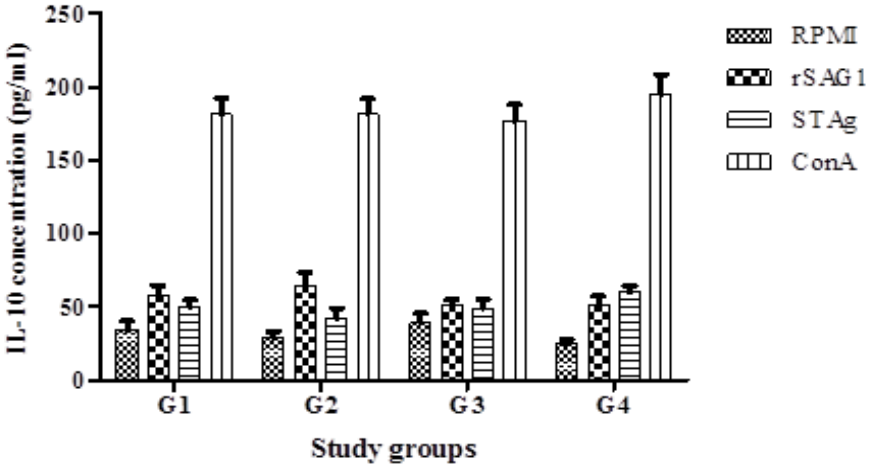
(G2) elicited a significant level of IFN-γ in response to STAg (269.3 ± 33 pg/ml).

However, no statistically significant differences were observed in the amounts of IL-10 among all vaccinated groups (Fig.1B).



1A



1B

Fig. 1. Cytokine assessments. Spleen cells cultured in triplicate for five days in the presence of rSAG1 (2 μg/ml), soluble *Toxoplasma* antigen (STAg, 20 μg/well), Con A (as positive control), and RPMI (as negative control). IFN-γ (A) and IL-10 (B) were measured in the four groups. Each bar represents the mean ± SD of four mice in each group. "***" represents a significant difference among groups with $P<0.001$. G1, Blank PLGA; G2, rSAG1-PLGA; G3, soluble rSAG1, G4, unvaccinated.

Investigation of humoral responses. Specific anti-rSAG1 IgGs, including total IgG, IgG1, and IgG2a titers of four mice in each vaccinated group, were individually assessed three weeks after the second immunization. C57BL/6 mice vaccinated with rSAG1-PLGA (G2) demonstrated significantly higher total IgG titers ($P<0.001$) than those vaccinated with rSAG1 (G3) or blank PLGA (G1) (Fig. 2A). The titers of both IgG1 and IgG2a in C57BL/6 vaccinated with rSAG1-PLGA (G2) were substantially higher compared to mice in G1 (blank PLGA) and G3 (rSAG1) ($P<0.001$), as shown in Fig. 2B. The differences of IgG1 among G1, G2, and G3 groups did not display in Fig 2B.

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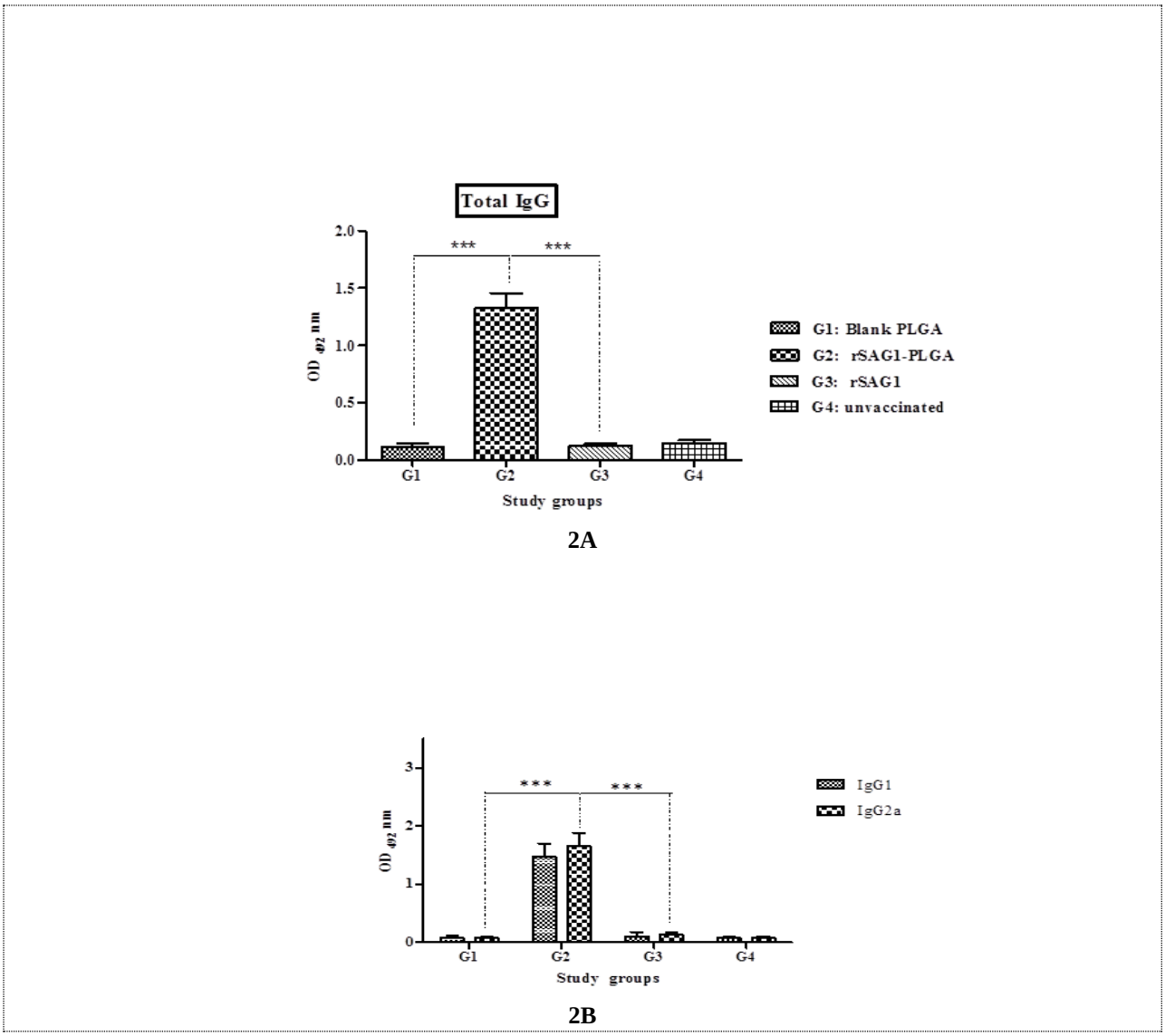

Fig. 2. Evaluation of specific anti-*T.gondii* IgG antibodies. (A) Total specific IgG, (B) IgG1 and IgG2a subclasses. Sera were collected three weeks after the second immunization and analyzed individually. Results were expressed as mean ± SD of four mice in each group. "***" represents a significant difference among groups with $P< 0.001$. G1, Blank PLGA; G2, rSAG1-PLGA; G3, soluble rSAG1; G4, unvaccinated.

Challenge study. C57BL/6 mice vaccinated by rSAG1-PLGA (G2) significantly survived longer than those vaccinated with blank PLGA (G1) as the control group and rSAG1 (G3) ($P<0.0001$) by log-rank; Mantel-cox). On average, C57BL/6 vaccinated with rSAG1-PLGA succumbed the day after the challenge. In contrast, mice in other groups survived no more than 7 days (Fig. 3).

DISCUSSION

In novel vaccine development, there are several issues to keep in mind when designing a vaccine: selecting immunodominant antigen(s), use of efficient adjuvant and delivery vehicles, and application of suitable mice model to evaluate vaccine efficacy reliably. In the present study, we assessed the efficacy of rSAG1-PLGA in C57BL/6 mice in eliciting protection against acute T. gondii infection. We previously demonstrated the dominance of adsorption on the encapsulation method in the preparation ofrSAG1-PLGA nanoparticles [30]. Hence, in the present study, we adsorbed rSAG1 antigen on blank PLGA nanoparticles.

There is no doubt that PLGA nanoparticle characteristics such as size, shape, hydrophobicity, and charge influence its delivery capability [32, 33]. Mean diameters of prepared rSAG1-PLGA nanoparticles and blank PLGA (452 ± 13 and 441 ± 15 nm, respectively) meet a size range less than 500 nm, as described by others [30].

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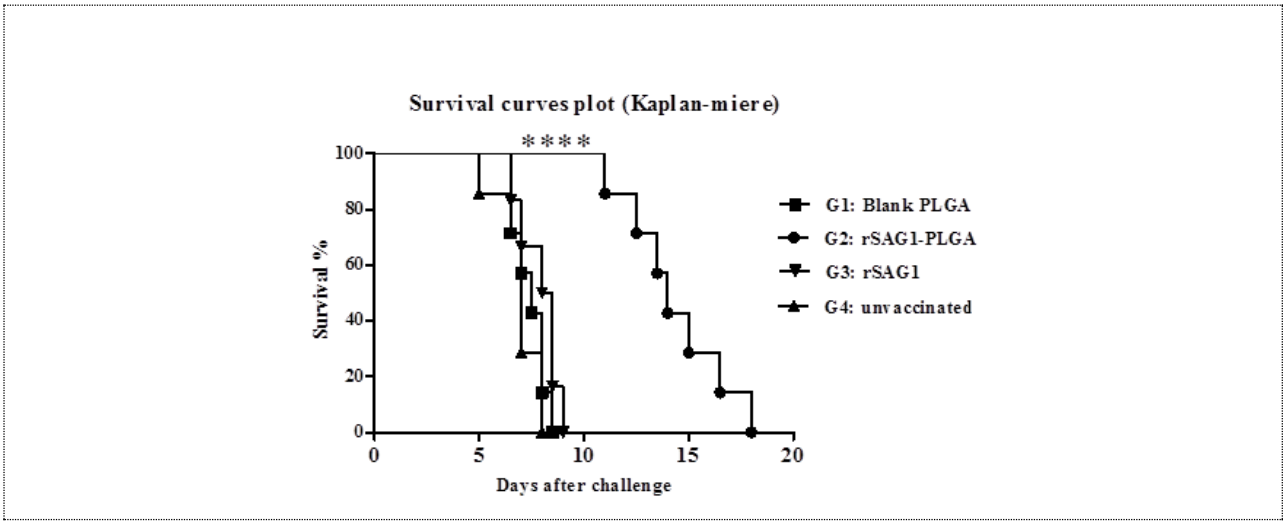

Fig. 3. Kaplan-Meier plot comparing survival time of vaccinated BALB/c mice after challenging with 1×10^3 tachyzoites of highly lethal RH strain of *T.gondii*, three weeks after the second immunization. Each group contained eight mice. "****" represents a significant difference among groups with $P< 0.0001$. *p* values mentioned are based on log-rank (Mantel-Cox) test. G1, Blank PLGA; G2, rSAG1-PLGA; G3; soluble rSAG1; G4, unvaccinated.

It is worth noting that the trafficking of PLGA particles to lymph nodes and their uptake happens in a size-dependent manner. It has been clearly observed that PLGA nanoparticles < 500 nm elicit more efficient CTLs (Cytotoxic T lymphocytes responses compared to particles larger than 2 μm) [17, 34]. The negativity of the surface charge of rSAG1-PLGA was less than that of blank PLGA due to rSAG1 adsorption, as we discussed previously [29]. Based on *in vivo* analyses, it was clarified that rSAG1-PLGA elicited a higher amount of anti-*Toxoplasma* total IgG, IgG1, and IgG2a than mice vaccinated with rSAG1 (G3) or blank PLGA (G1). In addition, the intracellular pathway of PLGA nanoparticles and passive targeting of PLGA nanoparticles towards dendritic cells results in Th1 (T helper1) polarization in PLGA-based vaccine [15]. Furthermore, rSAG1-PLGA vaccination produced higher IFN-γ, as the main cytokine against *T. gondii* infection [35], compared to the mice vaccinated with rSAG1, proving PLGA nanoparticles function as an efficient delivery vehicle in eliciting high humoral and cellular responses. Consequently, partial protection based on longer survival times after challenge was obtained in mice vaccinated with rSAG1-PLGA unlike the control group. It was verified that the survival times among vaccinated groups are almost proportional to the amounts of IFN-γ. Accordingly, rSAG1-PLGA induced high titer of specific humoral and cellular immune responses in C57BL/6, similar to BALB/c mice [36, 37]. In this study, we proved the efficacy of rSAG1-PLGA against acute *Toxoplasma* infection in C57BL/6 as we investigated its role in conferring partial protection in BALB/c [29, 30] and CBA/j (unpublished data).

Among experimental animals, mice are a susceptible group to *T. gondii* infection; however, these animals demonstrate different immunological and pathological aspects. Hence they are used in experimental assays based on the purpose of the study [38]. As a rule of thumb, mice's genotype directly affects infection outcomes [25, 39, 40].

Although some studies have investigated the efficiencies of PLGA-based vaccines using SAG1 antigen against *T. gondii* in BALB/c mice [11, 24, 36, 38], to the best of our knowledge, there is no similar report in C57BL/6 mice. The efficacy of other *T. gondii* antigens without PLGA nanoparticles has been evaluated in C56BL/6 [41-44].

The efficacy of adenovirus expressing rSAG1 protein (AdSAG1) as viral vectors in conferring protection against *T. gondii* in C57BL/6 [45] exhibited reduced mortality and cyst formation following challenges with ME49 strain due to the activation of $CD8^+$Tcell [45]. Administration of AdSAG1 in BALB/c mice revealed similar results (partial protection) [46].

In conclusion, we showed the efficiency of rSAG1-PLGA in conferring partial protection against *T. gondii* acute infection in C57BL/6 associated with high titers of IFN-γ and specific anti-*Toxoplasma* total IgG, as we previously proved the eficacy of rSAG1-PLGA formulation in BALB/c.[11, 30, 36].

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests associated with this manuscript.

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