

# Sustained release of recombinant surface antigen 2 (rSAG2) from poly(lactide-co-glycolide) microparticles extends protective cell-mediated immunity against *Toxoplasma gondii* in mice

SHU-CHUN CHUANG<sup>1</sup> and CHUNG-DA YANG<sup>2\*</sup>

<sup>1</sup>Department of Physiology, College of Medicine, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan

<sup>2</sup>Graduate Institute of Animal Vaccine Technology, National Pingtung University of Science and Technology, No. 1, Shuefu Road, Neipu, Pingtung 912, Taiwan

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## SUMMARY

Current development efforts of subunit vaccines against *Toxoplasma gondii*, the aetiological agent of toxoplasmosis, have been focused mainly on tachyzoite surface antigens (SAGs) such as SAG2, due to their attachment roles in the process of host-cell invasion. In the present study, we aimed to produce poly(lactide-co-glycolide) (PLG) microparticles (MPs) containing recombinant SAG2 (rSAG2) to induce improved immunity against *T. gondii*. The resulting PLG-encapsulated rSAG2 (PLG-rSAG2) MPs, 2.14–3.63 µm in diameter, showed 74–80% entrapment efficiency and gradually released antigenic rSAG2 protein (88.3% of the total protein load) for a long 33-day period. Peritoneal immunization with PLG-rSAG2 MPs in BALB/c mice resulted in not only sustained (10 weeks) lymphocyte proliferation and IFN-γ production but also an improved protective capacity (87%) against a lethal subcutaneous challenge of 1 × 10<sup>4</sup> live tachyzoites of *T. gondii* (RH strain). In conclusion, the sustained release of rSAG2 protein from PLG-rSAG2 MPs extends Th1 cell-mediated immunity (lymphocyte proliferation and IFN-γ production) and induces improved protection against *T. gondii* tachyzoite infection in mice.

Key words: *Toxoplasma gondii* (*T. gondii*), recombinant SAG2 (rSAG2), cell-mediated immunity, poly(lactide-co-glycolide) (PLG), PLG-encapsulated rSAG2 microparticles (PLG-rSAG2 MPs).

## INTRODUCTION

*Toxoplasma gondii*, the aetiological agent of toxoplasmosis, is an obligate intracellular protozoan parasite that infects most warm-blooded animals, including humans (Dubey and Jones, 2008; Dubey *et al.* 2012). Toxoplasmosis imposes adverse economic impact due to the induction of severe abortion and neonatal loss of domestic animals (Dubey and Jones, 2008; Dubey *et al.* 2012). During pregnancy, infection may give rise to serious foetal congenital mental retardation, blindness and hydrocephaly (McLeod *et al.* 1985; Fatoohi *et al.* 2002). Toxoplasmosis is also a major opportunistic infection in immunocompromised individuals, often resulting in lethal toxoplasmic encephalitis (Contini, 2008). Although vaccines against *T. gondii* have been investigated for a long time, only one attenuated vaccine has been licensed for use in sheep (Innes *et al.* 2009; Jongert *et al.* 2009). Therefore, the lack of effective

vaccines has become a major burden in controlling toxoplasmosis.

Significant information obtained recently indicates that future investigations on *Toxoplasma* vaccine development have to include potent adjuvants capable of enhancing protective immunity against *T. gondii* in animals (Jongert *et al.* 2009). Adjuvants can enhance the vaccine immunogenicity for induction of the 'appropriate' immune response (Singh and O'Hagan, 2003; Garlapati *et al.* 2009; Heegaard *et al.* 2011), particularly cell-mediated immunity that is urgently required for eliminating tachyzoite-infected cells as well as inhibiting tachyzoite dissemination and cyst formation (Innes *et al.* 2009; Jongert *et al.* 2009, 2010). In addition, IFN-γ, a key cytokine that can favourably strengthen Th1 cell-mediated immunity, has been considered as a decisive mediator of resistance to *T. gondii* (Jongert *et al.* 2010). Thus, efficacious adjuvants that are promised to induce IFN-γ-associated Th1 cell-mediated immunity seem more likely to be approved for use in *Toxoplasma* vaccine development.

In recent years, microparticles (MPs) made from biodegradable poly(lactide-co-glycolide) (PLG) polymer have been developed as potent adjuvants or

\* Corresponding author: Graduate Institute of Animal Vaccine Technology, National Pingtung University of Science and Technology, No. 1, Shuefu Road, Neipu, Pingtung 912, Taiwan. E-mail: cdyang@mail.npust.edu.tw

delivery systems to encapsulate antigens for preparation of controlled-release MP vaccines (Sinha and Trehan, 2003; Heegaard *et al.* 2011; Jain *et al.* 2011). The PLG encapsulation not only allows the sustained release of antigens for a long period (Lim *et al.* 2009; Jain *et al.* 2011) but also promotes the antigen uptake via antigen-presenting cells (APCs) (Newman *et al.* 2002), thereby favourably eliciting strong cell-mediated immunity accompanied by production of Th1 cytokines, such as IFN- $\gamma$  (Sinha and Trehan, 2003; Heegaard *et al.* 2011). Therefore, the MP vaccines made from the PLG polymer can achieve the need for induction of functional IFN- $\gamma$ -associated Th1 cell-mediated immunity against *T. gondii*.

Recent development efforts of subunit vaccines against *T. gondii* have been focused mainly on the tachyzoite surface antigens (SAGs) (Jongert *et al.* 2009; Machado *et al.* 2010; Meng *et al.* 2012; Chuang *et al.* 2013a,b) such as SAG2, due to their attachment roles in the process of host-cell invasion (Grimwood and Smith, 1996). The immune responses elicited by vaccination with SAG2 have previously been demonstrated to protect mice against tachyzoite infection (Yang *et al.* 2003; Jongert *et al.* 2009; Meng *et al.* 2012; Chuang *et al.* 2013a,b) and cyst formation (Mishima *et al.* 2001; Machado *et al.* 2010). In addition, the SAG2 sequence was cloned in our previous work to produce a recombinant SAG2 (rSAG2) protein (Yang *et al.* 2004). However, the further animal study in mice demonstrated that the rSAG2 protein emulsified with an oil adjuvant, Vet L-10, induced only partial protection (53%) against a lethal subcutaneous challenge of *T. gondii* tachyzoites (Yang *et al.* 2004). As long as alternative effective adjuvants such as the PLG polymer are used to make the rSAG2 protein more immunogenic, more protective immunity against *T. gondii* may be achieved in animals.

Although use of PLG as an adjuvant to extend SAG1- and SAG1/2-induced immunity has been described in our previous studies (Chuang *et al.* 2013a,b), both improved immunity and advanced protection against *T. gondii* induced by the more potential antigen, SAG2, encapsulated in PLG MPs are certainly worthy of being studied. In the present study, the *Escherichia coli*-based recombinant SAG2 (rSAG2) protein was prepared to be encapsulated with the PLG polymer for production of PLG-encapsulated rSAG2 (PLG-rSAG2) MPs. The ability of PLG-rSAG2 MPs to induce SAG2-specific protective immunity was then evaluated in BALB/c mice, and compared with rSAG2 emulsified with a Vet L-10 oil adjuvant (rSAG2 (Vet L-10)).

## MATERIALS AND METHODS

### Parasites

The RH strain of *T. gondii* tachyzoites used in the present study were kindly given by Dr David Chao

(Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung, Taiwan) and maintained in Vero cells or BALB/c mice (Chuang *et al.* 2013a). Only two or three passages of tachyzoites in the ascites of BALB/c mice were carried out to recover their virulence before the tachyzoite challenge in mice. The tachyzoites harvested from the mouse peritoneal fluid were used for subcutaneous tachyzoite challenge in immunized mice. On the other hand, for all experiments in our laboratory except the challenge study, the tachyzoites were maintained in tissue culture by passage in Vero cells according to the guidelines of the 'OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013, Chapter 2.9.10. Toxoplasmosis'.

### Animals

BALB/c mice (6–8 weeks of age) purchased from the National Laboratory Animal Center, National Science Council, Taiwan, were used to maintain and passage *T. gondii* tachyzoites as well as to perform the immunization experiments. All mice were housed in high containment facilities and managed in compliance with the Animal Welfare Act. All administrations were reviewed and approved by the Institutional Animal Care and Use Committee, National Pingtung University of Science and Technology.

### Monoclonal antibody (mAb)

Hybridoma cells producing mouse mAb TG-2 (isotype G, subclass 1,  $\kappa$  light chain), which is specific to the tachyzoite SAG2 protein, were prepared by fusion, selection and cloning as described previously (Yang *et al.* 2003; Chuang *et al.* 2013b).

### Antigens

The tachyzoites were harvested, purified and sonicated to prepare the tachyzoite sonicated antigen (TsoAg) as described previously (Chuang *et al.* 2013a,b). The rSAG2 protein used in the present study was produced as the following procedure. The full-length SAG2 sequence was amplified with SAG2 specific primers (the forward primer: 5'-CGCCCCGGGATGTCATTTTCGAAAACCAC-3' and the reverse primer: 5'-ATACCCGGGTCACACAAACGTAATCAGCA-3' contained the underlined *Sma*I sequences) by PCR (Chuang *et al.* 2013a) and then cloned into the plasmid pGEX-6P-1 (GE Healthcare) to be expressed as a glutathione s-transferase (GST) fusion protein, GST-SAG2, in BL21 (DE3) *E. coli* (Yeastern Biotech). Protein expression and purification were then carried out as described previously (Yang *et al.* 2004). The GST-SAG2 protein was expressed by induction with 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) for 4 h at 37 °C. Induced bacteria were obtained by

centrifugation at 3000 *g* for 10 min and resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) supplemented with 1% Triton X-100, 100 µg mL<sup>-1</sup> lysozyme, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM dithiothreitol (DTT). After incubation at 4 °C for 10 min, cells were lysed by repeated freeze/thawing followed by ultrasonication. After centrifugation, supernatants were collected, diluted with an equal volume of PBS containing 1% Triton X-100, filtered through 0.22 µm disk filters (Millipore) and then applied into the GSTrap FF columns (GE Healthcare). The charged columns were washed with 5 mL of PBS and then digested with PreScission protease (160 units) (GE Healthcare) to remove GST protein tag at 4 °C for 16 h. Finally, recombinant SAG2 (rSAG2) was eluted from columns by PreScission protease buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0). The lipopolysaccharide (LPS) in the rSAG2 samples was removed by the Detoxi-Gel Endotoxin Removing column (Thermo Scientific). The LPS level was confirmed by the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) to be below 0.1 EU mL<sup>-1</sup>. The resulting rSAG2 (22 kDa) protein was prepared and its antigenicity was analysed by Western blot analysis using the mouse mAb TG-2, which is specific to SAG2 of *T. gondii* tachyzoites.

#### Preparation of PLG-encapsulated rSAG2 MPs

In this study, PLG MPs containing the rSAG2 protein were fabricated by the water/oil/water double emulsion solvent evaporation technique as described previously (Jeffery *et al.* 1993; Ghaderi and Carlfors, 1997; Chuang *et al.* 2013a,b), with minor modifications. Briefly, 20 mL of a 10% solution of 50:50 PLG (Sigma) in dichloromethane (Sigma) was mixed with 2 mL of a rSAG2 solution (5 mg mL<sup>-1</sup>) by using a PRO200 homogenizer (PRO Scientific) equipped with 10 mm × 150 mm generator at 10000 rpm for 10 min to produce a water/oil emulsion. The resulting emulsion was further homogenized with 20 mL of a 2% polyvinyl alcohol (Sigma) solution at 10000 rpm for 10 min to generate a stable water/oil/water emulsion. The water/oil/water emulsion was then stirred for 18 h at room temperature (RT) and pressurized to promote solvent evaporation and PLG-rSAG2 MP formation in a laboratory fume hood. The MPs were collected by centrifugation at 4000 *g* for 30 min, washed three times with distilled water to remove non-entrapped rSAG2 and then lyophilized by a FD-5030 freeze dryer (Panchum) for storage at -20 °C. The particle morphology was inspected using a S3000N scanning electron microscope (Hitachi) and the particle size was determined by N5 submicron particle size analyser (Beckman Coulter) as before (Chuang *et al.* 2013a,b).

#### Protein entrapment

The entrapment of rSAG2 protein in PLG MPs was then measured with the following procedure. A total of 5 mg of PLG-rSAG2 MPs was first dissolved in 500 µL of 0.1 M NaOH with 2.5% SDS to extract the encapsulated rSAG2 as described previously (Jeffery *et al.* 1993; Chuang *et al.* 2013a,b). After centrifugation at 4000 *g* for 10 min, the content of rSAG2 in the supernatant was assessed with the BCA protein assay (Pierce) and compared with BSA standards and adjusted against empty PLG MPs. Based on this result, the ratio (w/w) of rSAG2 entrapped per dry weight of MPs was determined and the entrapment efficiency (%) was expressed as a ratio of the actual rSAG2 entrapment to the theoretical rSAG2 entrapment (Jeffery *et al.* 1993; Chuang *et al.* 2013a,b). All measurements were performed in triplicate on samples from different batches.

#### In vitro release study

A total of 5 mg of PLG-rSAG2 MPs was suspended in 1 mL of PBS (pH 7.4) with 0.02% sodium azide and shaken at 37 °C in 1.5 mL microfuge tubes. Every 3 days, 1 mL of supernatant was sampled and the amount of rSAG2 in the supernatant was measured using the BCA protein assay (Pierce), compared with BSA standards and adjusted against empty PLG MPs as described previously (Chuang *et al.* 2013a,b). In addition, Western blot analysis using the mouse mAb TG-2, which is specific to SAG2 of *T. gondii* tachyzoites, was performed to determine if released rSAG2 samples on days 3, 9, 15, 21, 27 and 33 could be recognized by mAb TG-2 (Chuang *et al.* 2013a,b). The Amicon Ultra-0.5 Centrifugal Filter Device (10 kDa limit) was used to concentrate these release samples. The same volume (10 µL) of each concentrated sample was then separated by 12% SDS-PAGE and analysed with mouse mAb TG-2.

#### Immunization

Five groups of 35 BALB/c mice each were intraperitoneally injected twice at a 14-day interval with PBS, blank PLG, 10 µg of soluble rSAG2 alone, 10 µg of rSAG2 emulsified with Vet L-10 (Invitrogen) oil adjuvant (rSAG2 (Vet L-10)) as described previously (Yang *et al.* 2004) or PLG-rSAG2 MPs containing 10 µg of rSAG2. During the immunization schedule, the specific anti-*Toxoplasma* immune responses were analysed by the following immunoassays.

#### Serum assay

Two weeks after boosting (week 4), Western blot analysis was performed to study the antigenic specificity of the immunized mouse sera. TsoAg (15 µg well<sup>-1</sup>)

was separated by 12% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking, strips of the membrane were cut and probed with sera from mice immunized with PLG-rSAG2 MPs, rSAG2 (Vet L-10), soluble rSAG2 alone, PLG or PBS for 1 h at 37 °C. The mouse mAb TG-2 was also conducted. The subsequent colour development was processed as described previously (Chuang *et al.* 2013a,b).

#### Lymphocyte proliferation assay

Following immunization, three mice per group were euthanized every 2 weeks to isolate spleen lymphocytes under sterile conditions. Then,  $1 \times 10^5$  cells in 200  $\mu\text{L}$  of RPMI-1640 culture medium (CM) were cultured in each well of 96-well culture plates and stimulated with  $5 \mu\text{g mL}^{-1}$  of rSAG2 as the procedure described previously (Chuang *et al.* 2013a,b). In addition, CM-treated cultures without rSAG2 stimulation were conducted to use as controls. The rSAG2-induced lymphocyte proliferation was then monitored by using the BrdU (5-bromo-2'-deoxyuridine) Colorimetric Cell Proliferation ELISA Kit (Roche) according to the manufacturer's instructions. Finally, the stimulation index (SI =  $\text{OD}_{450}$  values from rSAG2-treated cultures/ $\text{OD}_{450}$  values from CM-treated control cultures) of each group was calculated as described previously (Chuang *et al.* 2013a,b).

#### IFN- $\gamma$ assay

Every 2 weeks, spleen lymphocytes derived from three mice of each group were collected and stimulated with  $5 \mu\text{g mL}^{-1}$  of rSAG2 for 96 h as the procedure for the proliferation assay. Cell-free supernatants were harvested to analyse their IFN- $\gamma$  concentrations by the sandwich ELISA using the OptEIA Mouse IFN- $\gamma$  Set (BD Biosciences) as described previously (Chuang *et al.* 2013a,b). The IFN- $\gamma$  concentrations were determined by comparison to a standard curve created with known amounts of standard recombinant mouse IFN- $\gamma$ .

#### Tachyzoite challenge

Eight weeks after boosting (the 10th week), five groups of 15 mice each were challenged with a subcutaneous injection of  $1 \times 10^4$  live tachyzoites of *T. gondii* (RH strain). The mice were monitored daily for definite distressful signs of acute toxoplasmosis for additional 28 days. Signs of acute toxoplasmosis included ruffled fur, trembling, huddling and loss of activity. The end point occurred when mice showed severest signs of distress for 3 successive days. At the end point, mice with severest signs should be killed by a humane method. The

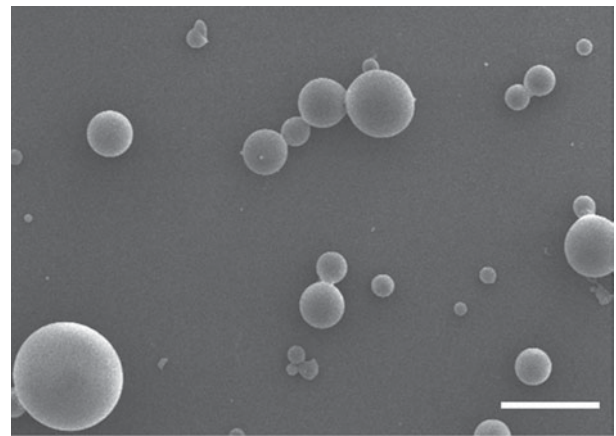


Fig. 1. Scanning electron micrograph of PLG-rSAG2 MPs. PLG-rSAG2 MPs showed spherical particles with a smooth surface (bar represents 2  $\mu\text{m}$ ).

survival rate (number of surviving mice after challenge/number of tested mice in each group) in each group was calculated as described previously (Chuang *et al.* 2013a,b).

#### Statistical analysis

The data in the present study were statistically analysed as described previously (Chuang *et al.* 2013a,b). Particle size and entrapment efficiency of PLG-rSAG2 MPs from different batches were statistically compared using one-way ANOVA. Proliferation SI values and IFN- $\gamma$  production of different immunization groups were statistically compared by using the nested design and the means at each time point in each group were tested by least significant difference (LSD) multiple comparison. The survival rates of different groups were analysed by the chi-square test. A *P* value of less than 0.05 was considered to be significant.

## RESULTS

#### Antigenicity of *E. coli*-based rSAG2

The purified rSAG2 protein prepared for the subsequent PLG encapsulation was analysed by Western blot analysis using the mouse mAb TG-2, which is specific to SAG2 of *T. gondii* tachyzoites. The result demonstrated that the rSAG2 protein (22 kDa) showed the native SAG2 antigenicity recognized by the mouse mAb TG-2 (data not shown).

#### Characteristics of PLG-rSAG2 MPs

The characteristics of PLG-rSAG2 MPs were analysed after PLG encapsulation. The MPs were inspected by scanning electron microscopy and their morphology showed spherical particles with a smooth surface (Fig. 1). In addition, the mean diameter of different batches of PLG-rSAG2 MPs

Table 1. Particle size and entrapment efficiency of PLG-rSAG2 MPs

Batch	Particle size ( $\mu\text{m}$ ) <sup>a</sup>	Entrapment efficiency (%) <sup>b</sup>
SAG2-PV -1	3.02 $\pm$ 1.42 <sup>c</sup>	76 $\pm$ 23 <sup>d</sup>
SAG2-PV -2	3.63 $\pm$ 1.84 <sup>c</sup>	80 $\pm$ 25 <sup>d</sup>
SAG2-PV -3	2.14 $\pm$ 1.25 <sup>c</sup>	74 $\pm$ 18 <sup>d</sup>

<sup>a</sup> The particle size in diameter was measured and expressed as the mean  $\pm$  s.d.

<sup>b</sup> The entrapment efficiency was expressed as a ratio of the actual rSAG2 entrapment to the theoretical rSAG2 entrapment as described in the Materials and Methods.

<sup>c,d</sup> A significant difference ( $P < 0.05$ ) exists between SAG2 particle vaccine (SAG2-PV) batches with different superscript letters.

ranged from 2.14 to 3.63  $\mu\text{m}$  (Table 1). These MPs displayed their entrapment efficiency for the rSAG2 protein ranging from 74 to 80%, without significant differences ( $P > 0.05$ , ANOVA) among different batches (Table 1). In the release study, the cumulative release of rSAG2 protein from PLG MPs gradually increased over the course of a 33-day period with three distinct phases (Fig. 2A). An initial burst released approximately 27.8% of the total protein load in the first 3 days. Afterwards, there was a very slow release (37.7% of the total protein load) for 24 days followed by a rapid release (22.8% of the total protein load) during the last 6 days. Altogether, 88.3% of the total protein load was released from the MPs during the 33-day study (Fig. 2A). Moreover, mouse mAb TG-2, which specifically reacted with the rSAG2 protein (Fig. 2B, lane 1), recognized the released rSAG2 proteins (22 kDa) collected on days 3, 9, 15, 21, 27 and 33 (Fig. 2B, lanes 2–7).

#### SAG2-specific serum response of immunized mice

Two weeks after boosting (week 4), mouse sera were collected to study their antigenic specificity by Western blot analysis (Fig. 3). Results showed that mice immunized with PLG-rSAG2 MPs or oil formulation rSAG2 (Vet L-10) produced serum IgG antibodies in response to the native SAG2 protein in TsoAg (Fig. 3, lanes 1 and 2), which was also recognized by the mAb TG-2 (Fig. 3, lane 6). However, sera from mice immunized with soluble rSAG2 alone, PLG or PBS did not recognize any proteins in TsoAg (Fig. 3, lanes 3–5). Therefore, SAG2-specific serum response could be elicited in mice by immunization with rSAG2 formulated with adjuvants, but not in its soluble form.

#### Prolonged SAG2-specific lymphocyte proliferation induced by PLG-rSAG2 MPs

Spleen lymphocytes stimulated with the purified rSAG2 protein were prepared from mice of different

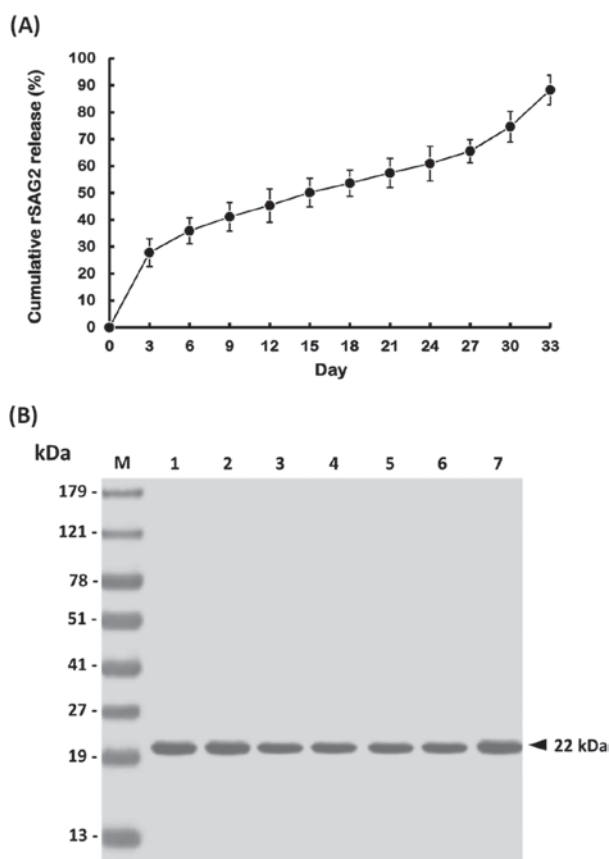


Fig. 2. *In vitro* release of rSAG2 protein from PLG MPs. (A) PLG-rSAG2 MPs were suspended in PBS as described in Materials and Methods. The amount of rSAG2 in the supernatant sampled every 3 days was measured using the BCA protein assay. The release studies were carried out in triplicate, with each point representing the mean  $\pm$  s.d. (B) Western blot analysis of the antigenicity of released rSAG2 protein from PLG MPs. The soluble rSAG2 (lane 1) and released rSAG2 samples on days 3 (lane 2), 9 (lane 3), 15 (lane 4), 21 (lane 5), 27 (lane 6) and 33 (lane 7) were electrophoretically separated on a 12% SDS-PAGE, transferred to a PVDF membrane, and then analysed with mouse mAb TG-2. Standard protein markers (lane M) are shown at the left.

groups every 2 weeks and their SAG2-specific proliferation responses were studied and expressed as SI values (Fig. 4). Before immunization (week 0), rSAG2-stimulated lymphocytes from different groups of mice were found to show similar low proliferative SI values. Two weeks after immunization, PLG-rSAG2 MPs elicited significantly higher SI values ( $P < 0.05$ , nested design) than rSAG2 (Vet L-10) (Fig. 4). The SI values in the PLG-rSAG2 MP group increased from week 0 to week 6 ( $P < 0.05$ , LSD multiple comparison). However, in the same group, there were no significant differences in the SI values from week 6 to week 10 ( $P > 0.05$ , LSD multiple comparison). Therefore, the SI values induced by PLG-rSAG2 MPs reached a maximal level in the 6th week and the enhanced SI values could be maintained until the 10th week (Fig. 4). Although high SI values

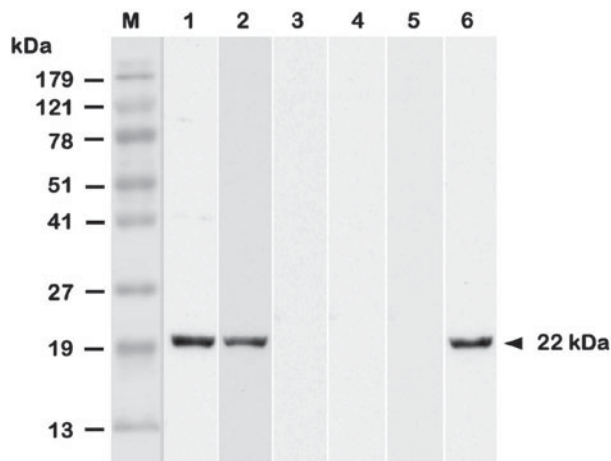


Fig. 3. The antigenic specificity of immunized mouse sera. Two weeks after boosting (week 4), immunized mouse sera were collected and their antigenic specificity analysed. TsoAg was probed with sera from mice immunized with PLG-rSAG2 MPs (lane 1), rSAG2 (Vet L-10) (lane 2), soluble rSAG2 alone (lane 3), PLG (lane 4) or PBS (lane 5). The mouse mAb TG-2 (lane 6) was conducted as a positive control for the native SAG2 in TsoAg. Standard protein markers (lane M) are shown at the left.

were also enhanced by rSAG2 (Vet L-10) from week 0 to week 6 ( $P < 0.05$ , LSD multiple comparison), they gradually decreased starting from week 6 to week 10 ( $P < 0.05$ , LSD multiple comparison) (Fig. 4). Administration with soluble rSAG2 alone, PLG or PBS induced a low proliferation response in mice (Fig. 4). Thus, the PLG encapsulation was better than the oil emulsification in eliciting and maintaining strong SAG2-specific lymphocyte proliferation in mice, indicating the importance of use of the PLG adjuvant.

#### *Sustained SAG2-specific IFN- $\gamma$ production elicited by PLG-rSAG2 MPs*

To evaluate whether immunization with PLG-rSAG2 MPs could induce Th1 cell-mediated immunity, the ability of spleen lymphocytes to produce IFN- $\gamma$ , a Th1 cytokine that plays an important role in cell-mediated immunity, was determined every 2 weeks by sandwich ELISA (Fig. 5). Upon rSAG2 stimulation, lymphocytes from mice immunized with either PLG-rSAG2 MPs or rSAG2 (Vet L-10) produced large amounts of IFN- $\gamma$  following intraperitoneal immunization (Fig. 5). More importantly, 2 weeks after immunization, PLG-rSAG2 MPs elicited significantly higher levels of IFN- $\gamma$  ( $P < 0.05$ , nested design) than rSAG2 (Vet L-10). In the PLG-rSAG2 MP group, the IFN- $\gamma$  levels raised from week 0 to week 6 ( $P < 0.05$ , LSD multiple comparison). However, in the same group, there were no significant differences in the IFN- $\gamma$  levels from week 6 to week 10 ( $P > 0.05$ , LSD multiple comparison). Therefore, the

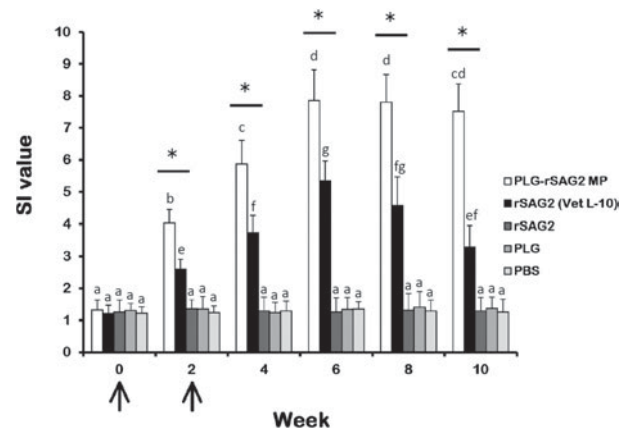


Fig. 4. The proliferation responses of mouse spleen lymphocytes. Groups of mice were intraperitoneally immunized twice ( $\uparrow$ ) with PLG-rSAG2 MPs ( $\square$ ), rSAG2 (Vet L-10) ( $\blacksquare$ ), soluble rSAG2 alone ( $\blacksquare$ ), PLG ( $\square$ ) or PBS ( $\square$ ). After immunization, rSAG2-stimulated spleen lymphocytes were prepared from three mice per group every 2 weeks and their subsequent proliferation responses were analysed and expressed as stimulation index (SI) values. Results were presented as the mean of SI values  $\pm$  s.d. All groups were analysed by the nested design and an asterisk (\*) indicated  $P < 0.05$  when comparing the PLG-rSAG2 group to the rSAG2 (Vet L-10) group. Different letters meant a significant difference at each time point in a group ( $P < 0.05$ , LSD multiple comparison).

enhanced IFN- $\gamma$  levels induced by PLG-rSAG2 MPs in the first 6 weeks were maintained until the 10th week (Fig. 5). However, immunization with rSAG2 (Vet L-10) resulted in an initial IFN- $\gamma$  induction in the first 6 weeks ( $P < 0.05$ , LSD multiple comparison) followed by an IFN- $\gamma$  reduction from week 6 to week 10 ( $P < 0.05$ , LSD multiple comparison) (Fig. 5). Lymphocytes isolated from mice immunized with soluble rSAG2 alone, PLG or PBS produced low amounts of IFN- $\gamma$  (Fig. 5). Therefore, immunization with PLG-rSAG2 MPs in mice could effectively elicit and maintain high levels of SAG2-specific IFN- $\gamma$ .

#### *Strong protection against T. gondii tachyzoites induced by PLG-rSAG2 MPs*

After *T. gondii* tachyzoite challenge, all mice administered with soluble rSAG2 alone, PLG or PBS died within 1 week and displayed no protection (Fig. 6). Three out of 15 mice immunized with rSAG2 (Vet L-10) survived during the challenge study and showed a low protection rate of 20% (Fig. 6). However, in the group of mice immunized with PLG-rSAG2 MPs, only 2 mice died on the 19th day and the 22nd day after challenge. In other words, intraperitoneal immunization with PLG-rSAG2 MPs in mice resulted in the highest survival rate (87%), which was significantly higher ( $P < 0.05$ , chi-square test) than that of the rSAG2 (Vet L-10)

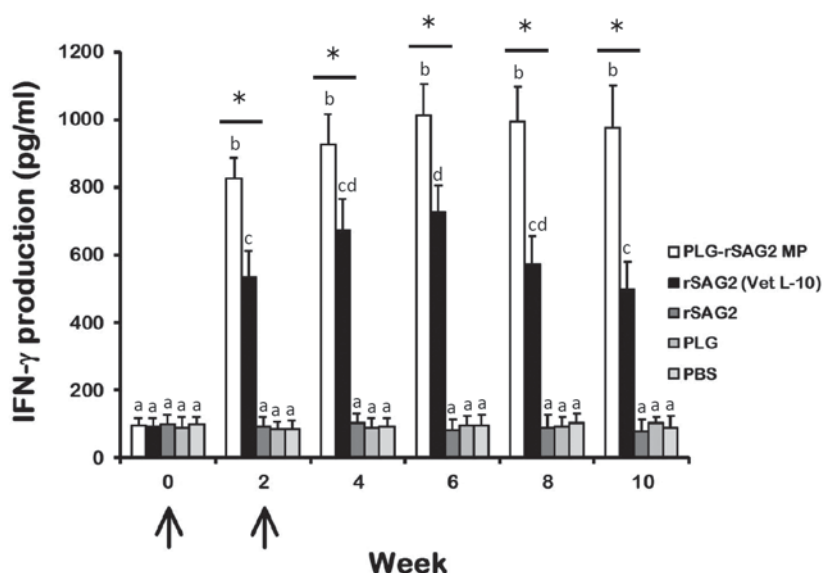


Fig. 5. IFN- $\gamma$  production in lymphocyte cultures of immunized mice. Groups of mice were intraperitoneally immunized twice ( $\uparrow$ ) with PLG-rSAG2 MPs ( $\square$ ), rSAG2 (Vet L-10) ( $\blacksquare$ ), soluble rSAG2 alone ( $\blacksquare$ ), PLG ( $\square$ ) or PBS ( $\square$ ). The production of IFN- $\gamma$  in the culture supernatants of rSAG2-stimulated lymphocytes, collected every two weeks, was calculated and expressed as mean  $\pm$  s.d. All groups were analysed by the nested design and an asterisk (\*) indicated  $P < 0.05$  when comparing the PLG-rSAG2 group to the rSAG2 (Vet L-10) group. Different letters meant significant difference at each time point in a group ( $P < 0.05$ , LSD multiple comparison).

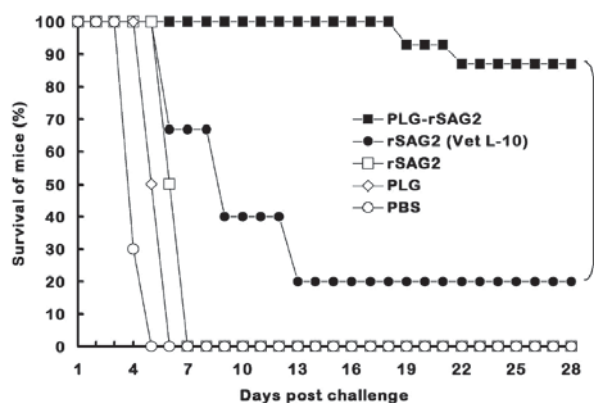


Fig. 6. Survival of immunized mice after a lethal tachyzoite challenge. Groups of mice were intraperitoneally immunized twice with PLG-rSAG2 MPs ( $\blacksquare$ ), rSAG2 (Vet L-10) ( $\bullet$ ), soluble rSAG2 alone ( $\square$ ), PLG ( $\diamond$ ) or PBS ( $\circ$ ). Eight weeks after boosting (the 10th week), five groups of 15 mice each were subcutaneously infected with  $1 \times 10^4$  live tachyzoites of *T. gondii* (RH strain). Animals were observed daily for an additional month (28 days) and the final survival rates were counted. All groups were analysed by the chi-square test and an asterisk (\*) indicated  $P < 0.05$  when comparing the PLG-rSAG2 group with the rSAG2 (Vet L-10) group.

group (Fig. 6). Therefore, the immunity induced by PLG-rSAG2 MPs conferred a substantial resistance to the experimental challenge of *T. gondii* tachyzoites.

#### DISCUSSION

Most modern vaccines based on subunits of pathogens, such as purified proteins, are often poorly

immunogenic and therefore require potent adjuvants to enhance specific immune responses and protection against the given pathogens in vaccinated animals (Singh and O'Hagan, 2003; Garlapati *et al.* 2009; Heegaard *et al.* 2011). In order to prepare efficacious subunit vaccines against toxoplasmosis, more efforts have been put into the adjuvant design and application for induction of anti-*Toxoplasma* protective immune responses, particularly sustained T cell-dependent immunity (Innes *et al.* 2009; Jongert *et al.* 2009, 2010). However, the biodegradable PLG polymer is so far seldom used as an adjuvant for *Toxoplasma* vaccine development. To the best of our knowledge, only three investigations, including our two recent studies, have shown PLG MP-induced immunity and protection against toxoplasmosis in animals (Stanley *et al.* 2004; Chuang *et al.* 2013a,b). However, Stanley and co-authors have found that a PLG MP vaccine containing a tachyzoite extract plus a mucosal adjuvant, cholera toxin, failed to provide protection in sheep (Stanley *et al.* 2004). In our two previous studies, although the adjuvant effects of the PLG encapsulation have been exercised to maintain anti-SAG1 or anti-SAG1/2 immunity (Chuang *et al.* 2013a,b), more effort is needed to improve the potency of a *Toxoplasma* MP vaccine in animals by encapsulating other *Toxoplasma* antigens in PLG MPs. In the present study, we have demonstrated that the rSAG2 protein encapsulated in PLG MPs can induce sustained lymphocyte proliferation and IFN- $\gamma$  production. PLG-rSAG2 MPs also improved anti-*Toxoplasma* protection (87%), which is higher, though not statistically significant, than

either 80% of PLG-rSAG1 MPs (Chuang *et al.* 2013b) or 83% of PLG-rSAG1/2 MPs (Chuang *et al.* 2013a).

In the present study, PLG-rSAG2 MPs suspended in PBS were able to show a triphasic rSAG2 release manner consisted of an initial burst release, a very slow release and a final rapid release (Fig. 2A and B). Similarly, both PLG-rSAG1 MPs (Chuang *et al.* 2013b) and PLG-rSAG1/2 MPs (Chuang *et al.* 2013a), other two PLG MP vaccines prepared in our previous studies, also sustained their critical release of entrapped recombinant proteins in a triphasic controlled-release manner, which has also been addressed by others (Sturesson and Carlfors, 2000; Kavanagh *et al.* 2003; Uchida *et al.* 2006). This reproducible triphasic release manner in PBS indicates the significance of our encapsulation conditions for long, stable *in vitro* release of recombinant proteins from PLG MPs. In the present study, the release assay was done *in vitro* in PBS and therefore may not completely reflect *in vivo* release in mice. However, both sustained lymphocyte proliferation (Fig. 4) and prolonged IFN- $\gamma$  production (Fig. 5) detected in mice immunized with PLG-rSAG2 MPs, rather than rSAG2(Vet L-10), provided notable evidence that the sustained protein release from PLG MPs can repeatedly stimulate the immune effector cells to maintain specific immunity for a long period (Lim *et al.* 2009; Jain *et al.* 2011).

According to previous studies, an indicative hallmark of an efficacious vaccine against *T. gondii* is the ability to induce strong Th1 cell-mediated immunity in vaccinated animals (Innes *et al.* 2009; Jongert *et al.* 2009, 2010). In the present study, after peritoneal immunization in mice, we focused much attention on lymphocyte proliferation and IFN- $\gamma$  production, two activities that positively correlate with Th1 cell-mediated immunity against *T. gondii* (Yang *et al.* 2003, 2004; Chuang *et al.* 2013a, b). Results revealed that vaccination with PLG-rSAG2 MPs resulted in induction of both sustained lymphocyte proliferation and prolonged IFN- $\gamma$  production. These data obviously indicate that PLG-rSAG2 MPs really elicit prolonged Th1 cell-mediated immunity, the expected response that we aimed to induce in mice in the present study. We also found that the two activities displayed similar 10-week profiles. Based on previous studies, IFN- $\gamma$  has been demonstrated to be a critical mediator that has to be secreted for as long as possible in order to maintain anti-*Toxoplasma* immunity (Subauste and Remington, 1993; Casciotti *et al.* 2002). Thus, the sustained IFN- $\gamma$  production detected in mice immunized with PLG-rSAG2 MPs (Fig. 5) could support the prolonged SAG2-specific proliferation response (Fig. 4). However, 6 weeks after immunization, the oil formulation rSAG2 (Vet L-10) could not induce a sufficient amount of IFN- $\gamma$  (Fig. 5) to keep an immunological memory response (Fig. 4).

Based on previous studies, PLG MPs appear to favourably facilitate a size-dependent interaction with APCs, such as macrophages and dendritic cells (Men *et al.* 1999; Newman *et al.* 2002). Generally, the particles, like PLG-rSAG2 MPs (2.14–3.63  $\mu\text{m}$  as indicated by Table 1), smaller than 10  $\mu\text{m}$  in diameter are directly taken by APCs (Newman *et al.* 2002). In our previous studies, the direct size-dependent interaction has also been displayed by PLG-rSAG1 MPs (Chuang *et al.* 2013b) and PLG-rSAG1/2 MPs (Chuang *et al.* 2013a). In further comparison, the mean diameter of PLG-rSAG2 MPs prepared in the present study is smaller than that (4.25–6.58  $\mu\text{m}$ ) of PLG-rSAG1 MPs (Chuang *et al.* 2013b).

More importantly, the immunity induced by PLG-rSAG2 MPs protected 87% of mice against a lethal subcutaneous challenge of  $1 \times 10^4$  *T. gondii* tachyzoites (RH strain), an amount 10 times higher than what we had used in the previous study (Yang *et al.* 2004), and enabled mice to survive for a long period of 28 days after challenge (Fig. 6). Such a high survival rate caused by PLG-rSAG2 MPs has increased by 67% compared with the oil formulation, rSAG2 (Vet L-10). However, different tachyzoite doses used for the challenge experiments in mice immunized with rSAG2 (Vet L-10) led to different survival rates in our present (20%) and previous (53%) studies. In further comparison with our previous studies, PLG-rSAG2 MPs elicited a higher protective rate (87%) in mice than either PLG-rSAG1 MPs (80%) (Chuang *et al.* 2013b) or PLG-rSAG1/2 MPs (83%) (Chuang *et al.* 2013a). Numerous investigations have shown that the *T. gondii* genotypes (Types 1, 2 and 3) (Jongert *et al.* 2009; Rajendran *et al.* 2012) and host susceptibility (Li *et al.* 2012) are critical factors on the disease progression and severity of toxoplasmosis. Therefore, the protective capacity of a *Toxoplasma* vaccine should be evaluated with different parasite strains and different host models. Since the natural *Toxoplasma* infection is initiated by ingesting oocysts released in cat faeces or consuming meat from infected animals containing long-lived tissue cysts (Dubey and Jones, 2008; Dubey *et al.* 2012), further studies are needed to confirm whether administration with PLG-rSAG2 MPs protects different animal models against an oral challenge of *T. gondii* cysts (Types 2 and 3).

## CONCLUSIONS

The controlled-release PLG-rSAG2 MPs can stably release rSAG2 protein in PBS for an extended period. Peritoneal immunization with PLG-rSAG2 MPs in mice not only maintains strong SAG2-specific Th1 cell-mediated immunity (proliferation response and IFN- $\gamma$  production) for 10 weeks but also induces improved protection against *T. gondii* tachyzoite infection. These findings therefore indicate that the adjuvant effects of the PLG encapsulation can



strongly enhance rSAG2 immunogenicity. In addition, the ability of the *Toxoplasma SAG2* MP vaccine to control the stable rate of antigen release would be advantageous for its application in the development of durable vaccines for future use in humans and animals.

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