Co-delivery of PLGA nanoparticles loaded with rSAG1 antigen and TLR ligands: An efficient vaccine against chronic toxoplasmosis

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Abstract

Although vaccination is a promising approach for the control of toxoplasmosis, there is currently no commercially available human vaccine. Adjuvants such as delivery vehicles and immunomodulators are critical components of vaccine formulations. In this study, Poly (D, L-lactide-co-glycolide) (PLGA) nanoparticles were applied to serve as delivery system for both surface antigen-1 (SAG1), a candidate vaccine against toxoplasmosis and two TLR ligands, monophosphoryl lipid A (MPL) and imiquimod (IMQ), respectively.

Compared to rSAG1 alone, CBA/J mice immunized with rSAG1-PLGA produced higher anti-SAG1 IgG antibodies titers. This response was increased by the co-administration of

IMQ-PLGA ($p < 0.01$). Compared to IMQ-PLGA co-administration, MPL-PLGA co-administration further increased the humoral response ($p < 0.01$) and potentiated the Th1 humoral response. Compared to rSAG1 alone, rSAG1-PLGA, or rSAG1-PLGA mixed with IMQ-PLGA or MPL-PLGA similarly enhanced the cellular response characterized by the production of IFN-γ, IL-2, TNF-α and low levels of IL-5, indicating a Th1-biased immunity. The induced immune responses, led to significant brain cyst reductions ($p < 0.01$) after oral challenge with *T. gondii* cysts in mice immunized with either rSAG1-PLGA, rSAG1-PLGA + IMQ-PLGA, rSAG1-PLGA + MPL-PLGA formulations.
Taken together the results indicated that PLGA nanoparticles could serve as a platform for dual-delivery of antigens and immunomodulators to provide efficacious vaccines against toxoplasmosis.

Keywords
Toxoplasma gondii, rSAG1, Poly (D, L-lactide-co-glycolide) PLGA, Monophosphoryl lipid A, Imiquimod.

1. Introduction

Toxoplasma gondii is an obligatory intracellular protozoan parasite with a considerably worldwide distribution which estimates to infect more than one-third of human population. Although immunocompetent individuals are rarely affected by toxoplasmosis, it still remains as major health concern and makes serious consequences in immunocompromised patients caused by AIDS or chemotherapy and in developing fetus due to mother's primary infection [1]. Undoubtedly, vaccination is known as the most promising approach against toxoplasmosis. Firstly, primary infection confers protective immunity against re-infection confirming the potential of vaccination [2]. On the other hand, available therapeutics do not eradicate tissue cysts and share severe side effects [3]. Protective immune response to *T. gondii* and other intracellular parasites is complicated and involves the collaboration of innate immunity, humoral and cellular acquired immunity, directed against the multi stages of the parasite [4]. Appropriate adaptive immune response depends mainly on the ability of $CD4^+$ and $CD8^+$ T lymphocytes to produce IFN-γ [5]. Along with immune T cells, antibodies contribute also to protection [6]. Therefore, if vaccination enhances potent Th1 T cell responses, it can successfully provide protective immunity to *Toxoplasma* infection.

Among numerous *T. gondii* antigens, surface antigen 1 (SAG1) has attracted many attentions in vaccine development due to remarkable characteristics [7]. Subunit vaccine

candidates based on native or recombinant SAG1 proteins have been evaluated in animal models against, acute, chronic and congenital toxoplasmosis, using various adjuvant formulations to overcome the low intrinsic immunogenicity of the protein [8]. Immune responses and significant protections were obtained in most of these studies.

With the continuous improvement of knowledge and awareness regarding the immune system, the application of vaccine delivery strategies such as Poly (D, L-lactic-co-glycolic acid) (PLGA) particles could bring hope to improve vaccine efficacy [9]. Indeed, we previously showed that rSAG1 adsorbed on the surface of PLGA nanoparticles or rSAG1 encapsulated in PLGA nanoparticles elicited higher systemic IFN-γ and specific anti-*T. gondii* IgG antibodies than rSAG1 alone and conferred significant protection against acute toxoplasmosis [10]. Similar results were also obtained with rSAG1 protein [11] and a multimeric recombinant *T. gondii* vaccine including SAG1 epitopes [12] or a recombinant chimeric protein rSAG1/2 [13] combined with PLGA nano-or microparticles.

TLRs agonists belong to a class of adjuvant known as immune stimulating agents. TLRs not only mediate the activation of innate immune cells, but also directly modulate vaccine specific response [14]. MPL, a portion of *Salmonella minnesota* lipopolysaccharide peptidoglycan, as specific agonist of TLR4 has been applied in some vaccination studies against *T. gondii* infection. Golkar et al. [15] demonstrated the protective efficacy of recombinant GRA2 with MPL against chronic infection by *T. gondii* and confirmed the role of MPL as an efficient immunostimulator to induce a protective Th1 immune response. The MPL adjuvant with toxofilin DNA vaccine alsoinduced significantly enhanced humoral and Th1-biased immune responses compared to the non-adjuvant toxofilin DNA vaccine [16]. IMQ, a synthetic analog of imidazoquinoline family, as an agonist of TRL7 has been approved by US Food and Drug Administration (FDA) as an immunopotentiator agent for local topical administration [17]. Recently, IMQ has been considered as a candidate for use against *Toxoplasmosis* both therapeutically and prophylactically [18, 19], but there is not any research on vaccine design against *T. gondii*. Although TLR agonists are powerful immune stimulators, induced unwanted cytokine release syndrome leading to potential adverse events and safety concerns is a major factor limiting their usage. To minimize these side effects and to enhance their efficacy, TLR agonists are increasingly combined with delivery systems including PLGA particles [20-22].

As PLGA nanoparticles can serve for dual-delivery of antigens and immunomodulators, in present study, mice vaccination was performed with rSAG1-PLGA nanoparticles in combination with IMQ or MPL encapsulated into PLGA nanoparticles in order to assess their

impacts on eliciting immune responses and protection against *T. gondii* infection. Since, we previously proved that adsorption is a more suitable approach than encapsulation in antigen loading on PLGA nanoparticles [10], rSAG1-PLGA was prepared by adsorption method. It is worthy to note that during encapsulation process, protein exposure to water-oil interface, harsh mechanical, thermal and chemical stresses could affect protein integrity and consequently its immunogenicity [23]. Protection was evaluated against chronic infection in CBA/J mice susceptible to cyst formation and development of toxoplasmosis encephalitis. In addition, mice were challenged bythe oral route, the major natural route of infection, with tissue cysts.

2. Materials and Method

2.1 Materials

Poly (D, L-lactide-co-glycolide) polymer (PLGA), Resomer®RG503 (50:50, lactide: glycolide ratio) (viscosity 0.32-0.44 dl/g) was purchased from Boehringer Ingelheim, Germany. PVA [poly vinyl alcohol; molecular weight (MW) 30,000–70,000 Da, 88% hydrolyzed] was obtained from Sigma Chemical Company. The materials applied for SDS PAGE gel electrophoresis and protein molecular weight marker were supplied from Roche Applied Sciences (Mannheim, Germany) and Fermentas, Vilnius, Lithuania, respectively. Dichloromethane (DCM) (analytical grade) was obtained from Merck Ltd. Cell culture reagents including RPMI-1640, Fetal Calf Sera (FCS), HEPES, L-glutamine, sodium pyruvate, penicillin, and streptomycin, were obtained from Gibco (Life Technologies GmbH, Karlsruhe, Germany). Concanavalin A (conA), lyophilized powder of MPL (monophosphoryl from *Salmonella enterica* serotype *minnesota Re 595*) were purchased from Sigma-Aldrich (Darmstadt, Germany). Imiquimod Vacci Grade™ was supplied from InvivoGen (CA, USA). All other chemicals used were of analytical reagent grade. All solutions were prepared by MilliQ™ ultrapure (Milli-QSystem, Millipore, Molsheim, France).

2.2 Purification and characterization of rSAG1 protein

rSAG1 protein was cloned, expressed and purified according to our previous studies and its purity, antigenicity, and immunogenicity were confirmed [24]. In order to remove of

contaminants including LPS, and *E.coli* DNA, Sartobind® Q strong 0.08 mL (Sartorius) was used. Briefly, rSAG1 buffer was exchanged to binding buffer of Sartobind® Q strong (Tris-HCl 20 mM, NaCl 50 mM, pH 8) by MicroSpin G-25 column. After column equilibration by mentioned buffer, rSAG1 was applied to Sartobind® Q strong. The Sartobind® Q strong operated as negative chromatography, so DNA attached to the column, and rSAG1 passed through Sartobind® Q strong. The concentration of bacterial endotoxin in purified rSAG1 was measured by limulus amboebocyte lysate assay using LAL chromogenic endotoxin quantitation kit (Pierce®, Thermo scientific, USA).

2.3 The preparation of different nanoparticles

Encapsulation of IMQ and MPLwas carried out completely according to the preparation of blank PLGA mentioned in our previous study [10] using double emulsion solvent evaporation technique. The only distinction was the substitution of IMQ or MPL ligands for PBS. The IMQ and MPL were usedin concentration of 5 mg/mL and 2.5 mg/mL, respectively. rSAG1 was adsorbed on blank PLGA as stated by Allahyari et al. [25].

Various batches of individual PLGA nanoparticles loaded by IMQ, MPL, and rSAG1 were prepared to provide sufficient quantity of homogenous nanoparticles. rSAG1-PLGA nanoparticles were mixed with either IMQ-PLGA or MPL-PLGA nanoparticles to make different formulations just before each immunization.

2.4 Nanoparticles characterization

2.4.1 Particle size, zeta potential and polydispersity index

The particle size (Z-average mean), particle size distribution (PSD), polydispersity index (PDI) and surface charge of all nanoparticles were determined based on Allahyari et al. [25]. Both types of measurements were performed at 25°C using a ZetasizerNano ZS (Malvern Instruments, Worcestershire, UK). All measurements were performed in triplicate.

2.4.2 Process yield determination

Process yield for both blank PLGA nanoparticles and PLGA nanoparticles loaded by rSAG1, IMQ, and MPL were assessed in three preparation batches as mentioned in Allahyari et al. [10].

2.4.3 Evaluation of encapsulation efficiency

Encapsulation efficiency of IMQ, and MPL into PLGA nanoparticles was determined by direct methods. Therefore, after hydrolysis of individual encapsulated PLGA nanoparticles, the quantity of encapsulated IMQ and MPL was calculated in definite amount of PLGA nanoparticles. The quantification of IMQ was done using spectrophotometry. Standard calibration curve of IMQ was drawn by ascertaining adsorption of definite concentration of IMQ at 320 nm. Afterwards, IMQ encapsulated in PLGA nanoparticles was quantified against standard curve. The amount of encapsulated MPL into PLGA nanoparticles was calculated by reverse phase (RP) HPLC (Kenuver, AZURA) through the area under curve (AUC), in comparison with defined amount of MPL. Encapsulated MPL was extracted by methanol, choloroform solvents (2:1 v/v). After centrifugation (at 20000 rpm, 20 mins), 50 µl of supernatant was loaded on TSKgel Octadecyl-4PW column (2.0 mm ID × 15.0 cm length, particle size 7.0 µm) (TOSOH Bioscience). The elution was carried out with a linear gradient at a flow rate of 0.5 mL/min (Sykam delivery system S2100 and at 210 nmusing UV detector (Sykam UV/Vis detector S3210) according to Kazzaz et al. [26]. Evaluation of all encapsulation efficiencies was evaluated by direct method according to following equation in triplicate:

Encapsulation efficiencies % = "Amount of TLR ligand encapsulated into PLGA nanoparticles"/"Total amount of TLR ligand used for encapsulation" × 100. Blank PLGA nanoparticles were used as negative control.The amount of rSAG1 adsorbed on PLGA nanoparticles was calculated as described by Allahyari et al. [25].

2.5 Vaccination studies

2.5.1 Mice immunization and Vaccination schedules

The 6-week-old female CBA/J (H-2k) mice (Janvier, Le Genest St. Isle, France) resistant to acute toxoplasmosis infection and susceptible to cyst formation in chronic infection were applied in present study. Mice were kept under pathogen-free conditions in the animal house of University of Tours. Experiments were performed in accordance with the guideline for animal experimentation (EU Directive 2010/63/EU) and the protocol was approved by the local ethics committee (number 00807-02, Comité d'Ethique en Expérimentation Animale Val de Loire). *In-vivo* studies were divided in two experiments. Vaccination groups in first experiment and second experiment were shown in table 1 and table 2, respectively. CBA/J (H-2k) mice were randomly divided into groups of 12 mice, except for IMQ-PLGA control

group in second experiment: only four mice for cellular analysis. All groups were immunized subcutaneously (s.c.) two times at 3-week intervals with different formulations containing 20 µg rSAG1, IMQ (64.5µg), and MPL (34 µg), as represented in Table 1and 2, respectively.

2.5.2 T. gondii extract (TE) preparation

Preparation of TE containing both cytoplasmic and membrane antigens was prepared from tachyzoites of the RH strain obtained by serial passaging in human foreskin fibroblast (HFF) cell monolayers, as previously described [27]. Briefly, the obtained tachyzoites were washed in PBS and sonicated for three 10-min periods at 60 W/s. The *Toxoplasma* sonicate was centrifuged at 2,000 g for 30 min. The protein concentration was determined in the supernatant by the Micro BCA protein assay reagent kit using bovine serum albumin (BSA) as the standard (Pierce, Rockford, III.). The TE was stored at -20°C until use.

2.5.3 Humoral response

Anti SAG1-specific IgG antibodies were measured by ELISA on sera collected three weeks after second immunization as previously described [27]. Briefly, Flat-bottomed 96-well plates (Nunc) were coated with 10 µg/mL TE. Serial two-fold dilutions of serum were performed (starting at a 1:100 dilution) and added to the wells. Sample of naive mice (untreated) served as negative controls. Bound antibodies were detected with Goat anti-Mouse IgG alkaline phosphatase (1:5,000, Sigma). The optical density of each sample was read at 405 nm. The endpoint antibody titer for each sample is given as the reciprocal of the highest dilution producing an OD that was 2.5-fold greater than that of the serum of naïve mice (serum of naïve mice gave optical density readings of less 0.1).

The levels of anti-SAG1 IgG subclasses were measured by ELISA as described above, except that sera were added to the plates at a single dilution (1:100 for sera of mice immunized with rSAG1, rSAG1-PLGA, rSAG1-PLGA and IMQ-PLGA, PLGA, IMQ-PLGA or MPL-PLGA nanoparticles and 1:800 for sera of mice immunized with rSAG1-PLGA and MPL-PLGA nanoparticles). The alkaline phosphatase-conjugated Rat anti-Mouse IgG1 and IgG2a were used at 1:1,000 (BD Pharmingen). IgG subclasses were evaluated using optical density.

2.5.4 Western Blotting

Western blottings were performed as previously described with either rSAG1 or *T. gondii* extractas the source of antigenand pooled sera obtained from mice in each group three weeks after second immunizations [27]. rSAG1 (6 µg/1-cm-wide slot) or TE (60 µg/1-cm-wide slot)

were electrophoresed in a 12 % SDS–polyacrylamide gel (SDS-PAGE) under non-reducing conditions, transferred to nitrocellulose, and probed with pooled sera from untreated mice (T-), *T. gondii* infected mice (inf), mice immunized with soluble rSAG1 in PBS (G1), rSAG1-PLGA (G2), rSAG1-PLGA and IMQ-PLGA (G3), PLGA (G4) or IMQ-PLGA (G5). Sera were diluted at 1:100. A mouse monoclonal antibody (mAb), anti-SAG1 MAb 1E5 (diluted at 1:100) isused as positive control [28]. Anti-SAG1 mAB 1E5 was kindly provided by Jean-François Dubremetz.

2.5.5 Cellular response (cytokine assay)

Four mice in each group were sacrificed three weeks after the last immunization. Single spleen cell suspensions were individually obtained by filtration through nylon mesh. Erythrocytes were removed by lysis (hypotonicshock) and the remaining cells were washed and suspended in RPMI 1640 medium supplemented with 5% FBS, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells (5×10^5 cells/well) were stimulated in triplicate with 4 µg/mL endotoxin depleted rSAG1, or with medium alone (negative control). Concanavalin A (5 µg/mL) was used as a positive control for proliferation. Supernatants were harvested and assayed for IL-2 after 24 h and for IFN-γ, IL-5, IL-10 after 72 h. The concentrations of cytokines were determined using ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's protocol. Cytokine concentrations were determined by reference to standard curves constructed with known amounts of cytokines provided by the kits.

2.5.6 Challenge

Mice (8/group) were orally challenged with 15 cysts of the 76K *T. gondii* strain, three weeks after the last immunization. To evaluate the protection, brain cyst loads were evaluated one month after challenge. Mouse brains were homogenized in 5 mL of RPMI medium, and the number of tissue cysts per brain was determined microscopically by counting 8 samples (10 µl) of each mouse brain by light microscope.

2.6 Statistical analyses

Statistical significance was analyzed using GraphPad Prism software. Statistical analysis was done by one-way ANOVA followed by a Tukey's multiple comparison test or using a

Kruskall–Wallis test followed by Dunn's multiple comparison test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Production of rSAG1

rSAG1 was expressed and purified, its antigenicity and immunogenicity were confirmed as described in our previous study [24]. Endotoxin concentration in purified protein was determined less than 0.05 EU/μg by limulus amboebocyte lysate assay.

3.2 Nanoparticles characterization

All different nanoparticles were identified regarding zeta average size, PDI, zeta potential, encapsulation efficiency and process yield. The results were summarized in Table 3.

The sizes of IMQ-PLGA and MPL-PLGA were about 451.3 ± 27and 403 ± 25 nm, respectively. There was significant difference ($p < 0.05$) between mean sizes of MPL-PLGA and IMQ-PLGA or rSAG1-PLGA nanoparticles. The difference between mean sizes of IMQ-PLGA and rSAG1-PLGA was not significant. All prepared nanoparticles share a PDI less than 0.2.

Surface charges of IMQ-PLGA, MPL-PLGA, nanoparticleswere negative, -4.9 ± 0.26 and -5.6 ± 0.75 mV,respectively. Among all nanoparticles, rSAG1-PLGA showed the lowest negative charge about -2.37 ± 0.3 mV. Zeta potential in rSAG1-PLGA was significantly ($p < 0.001$) lower than all other PLGA nanoparticles.

All statistical analysis was done by one way ANOVA. The efficacy of IMQ encapsulation into IMQ-PLGA nanoparticles measured by spectrophotometry method was about 70 ± 3.1 % (Table 3), which was calculated through interpolating of absorbance with the concentration. In addition, as shown in Table 3, the encapsulation efficacy of MPL into MPL-PLGA nanoparticles was quantified by RP-HPLC through comparison of AUC of encapsulated MPL and AUC related to defined amount of MPL, nearly 73.1 ± 1.2 (Fig.1).

The adsorption efficacy of rSAG1-PLGA nanoparticles was 69.01 ± 1.8 %. Blank PLGA and rSAG1-adsorbed PLGA were prepared as mentioned by Allahyari et al. [25]. The results represent mean ± SD of 5 independent PLGA nanoparticle preparations.

Fig. 1. **Quantification of encapsulated MPL in MPL-PLGA nanoparticles by RP-HPLC (Knauer, AZURA).** The numbers presented on graphs demonstrated area under curve (AUC). Std, standard, is referred to defined amount of MPL. The column specification; TSKgel Octadecyl-4PW column (2.0 mm ID × 15.0 cm length, particle size 7.0 μm) (TOSOH Bioscience).

3.3Vaccination with rSAG1-PLGA in combination with IMQ-PLGA

The effect of IMQ adjuvant, when encapsulated into PLGA and co-administrated with rSAG1-PLGA was first investigated. CBA/J mice (12 mice/group) were immunized subcutaneously (s.c.) two times at 3 weeks interval with rSAG1-PLGA nanoparticles or with the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles. Control mice were immunized with rSAG1 in PBS, blank PLGA nanoparticles or IMQ-PLGA nanoparticles (Table 1).The induced humoral and cellular immune responses and the protective efficacy against chronic toxoplasmosis following oral challenge, were evaluated.

3.3.1 Humoral immune responses

The humoral response was analyzed three weeks after the second immunization. Immunoblot analysis of mouse sera was first performed against rSAG1 (Fig. 2A). Sera from mice immunized either with rSAG1, rSAG1-PLGA or rSAG1-PLGA and IMQ-PLGA reacted mainly with two protein bands corresponding to rSAG1 monomers at the expected size (around 30 kDa). These sera reacted also with dimers (around 70 kDa) and probably multimers of rSAG1 (> 70 kDa). These bands are also recognized by serum IgG antibodies from *T. gondii* infected mice (inf). Monoclonal antibodie 1E5 reacted only with the two protein bands corresponding to rSAG1 monomers. Monoclonal antibodie 1E5 recognizes a conformational epitope which may not be accessible (or problem of conformation) in the multimeric forms of rSAG1. Importantly, immunoblot analysis of mouse sera performed against *T. gondii* extract, showed that sera from mice immunized either with rSAG1, rSAG1-PLGA or rSAG1-PLGA and IMQ-PLGA reacted strongly with native SAG1 (Fig. 2B). As expected, sera from mice immunized with PLGA (G4) and IMQ-PLGA (G5), and untreated mice (T-) identified no protein in rSAG1 or *T. gondii* extract.

ELISA using *T. gondii* extract containing native SAG1 as coating antigen was used to determine endpoint anti-SAG1 IgG antibodies titers (Fig. 2C). In our experimental conditions, anti-SAG1-specific IgG antibodies were detected only in 6/12 mice immunized with rSAG1

in PBS (for 6 mice, OD value failed to reach 2.5 times the background value at the initial dilution tested), whereas all mice immunized with rSAG1-PLGA nanoparticles produced detectable anti-SAG1-specific IgG antibodies. These results indicated that a stronger humoral immune response was induced in mice immunized with rSAG1-PLGA nanoparticles compared to mice immunized with rSAG1 alone, however, the difference between the two immunized groups did not reach statistical significance. In mice immunized with rSAG1-PLGA and IMQ-PLGA nanoparticles, higher antibodies titers were found compared to mice immunized either with rSAG1 in PBS or rSAG1-PLGA nanoparticles, however, a significant statistical difference was found only between the rSAG1 and rSAG1-PLGA+IMQ-PLGA groups ($p < 0.01$). Mice vaccinated with PLGA nanoparticles or IMQ-PLGA nanoparticles did not produce any anti-SAG1 antibodies. To find out whether a Th1 and/or a Th2 humoral response was induced by immunization, the IgG subclasses were analyzed (Fig 2D). The levels of IgG1 exceeded those of IgG2a in sera of mice immunized with PLGA nanoparticles with or whithout IMQ (IgG1/IgG2a OD ratio > 2), suggesting mixed Th1/Th2 humoral-type response with a bias towards a Th2 response. In conclusion, vaccination with the mixture of rSAG1-PLGA and IMQ-PLGA enhanced a mixed Th1/Th2 humoral response witha bias towards a Th2 response.

Fig. 2. **Specific antibody response in miceimmunized with rSAG1-PLGA nanoparticles or the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles.** Analysis of humoral response was done three weeks after second immunization. For immunoblot analysis, rSAG1 (**A**) or *T. gondii* extract (**B**) electrophoresed and transferred to nitrocellulose, were probed with pooled sera from immunized mice, *T. gondii* infected mice (inf) and untreated mice (T-). A mouse mAb (anti-SAG1 MAb 1E5) was used as positive control (mAb). The molecular weights are shown on the left side. Sera and and Mab 1E5 were diluted at 1:100. mAb (anti-SAG1 MAb 1E5), inf (*T. gondii* infected mice), G1 (rSAG1), G2 (rSAG1-PLGA), G3 (rSAG1-PLGA + IMQ-PLGA), G4 (PLGA), and G5 (IMQ-PLGA), T- (untreatedmice).
C) Determination of specific anti-SAG1 antibody titers by ELISA using *T. gondii* extractas coating antigen. Sera from each mouse in each group (n = 12/group) were analyzed individually. The antigen-specific antibody titer (endpoint titer) was given as the reciprocal of the highest dilution making an optical density (OD) that was 2.5-fold greater than that of the serum of non-immunized mice. Dotted line represent the lowest mouse sera dilution tested. Titers below the limit of detection (< 100) were assigned a value of 50 for analysis. Symbols

represent individual animals. Results are presented on scatter plots as geometric mean with the 95% confidence interval. Kruskall-Wallis, Dunn's multiple comparisons test. $^{**}p$ 0.01 **D)** Determination of the IgG subclasses profiles by ELISA using *T. gondii* extractas coating antigen. Sera were collected from six mice in each group and evaluated individually. Results are expressed as the mean of the optical density (OD) ± SEM. Sera were tested at a single dilution (1 :100).

3.3.2 Cellular immune responses

The potency of the various formulations to induce T cell immune responses was investigated by measuring the specific cytokine responses in spleen three weeks after second immunization. Splenocytes from mice immunized with rSAG1-PLGA or the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles elicited significant ($p < 0.05$) amounts of IFN-γ, IL-2, TNF-α (Th1 cytokines) and IL-5 (Th2 cytokine) in response to rSAG1 stimulation compared to their respective control (PLGA and IMQ-PLGA, respectively). However, any significant difference in cytokine production was shown between the two above-mentioned immunized groups. Furthermore, splenocytes from mice immunized with rSAG1 elicited very low levels of IFN-γ, IL-2, TNF-α and IL-5 as compared to mice immunized with rSAG1-PLGA or the mixture of rSAG1-PLGA and IMQ-PLGA. In addition, the amounts of IFN-γ, TNF-α, IL-5 ($p < 0.05$) in mice immunized with rSAG1-PLGA and the level of IL-2 ($p < 0.01$) in mice immunized with the mixture of rSAG1-PLGA and IMQ-PLGA were significantly higher than those in mice immunized by rSAG1. Although the level of IL-5 significantly increased in groups immunized with rSAG1-PLGA and the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles in comparison with control groups, all immunized groups and control groups share a considerably low amount of induced IL-5.

Fig. 3. Cellular immune response in mice immunized with rSAG1-PLGA nanoparticles or the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles. Three weeks after the second immunization, splenocytes were collected from four mice in each group, and cultured with 4 μg/mL rSAG1 or with medium alone (no stimulation). Cell-free supernatants were harvested after 24 (IL-2) or 72 h (IFN-γ, IL-5, and TNF-α) for cytokine assays. Results are expressed as the median and interquartile range. Kruskall–Wallis, Dunn's multiple comparisons test. $^{*}p < 0.05$; $^{**}p < 0.01$ significant differences between the immunized groups.

406

407 *3.3.3 Challenge studies*

408 In order to evaluate the protective effect of these vaccine formulations against chronic
409 toxoplasmosis, mice were orally challenged with15 cysts of the 76K strain 3 weeks after the
410 second immunization and sacrificed one month after challenge.

411 The numbers of brain cyst after challenge were significantly decreased in mice immunized
412 with the mixture of rSAG1-PLGA and IMQ-PLGA compared to both control groups ($p < 0.05$
413 compared to PLGA control group; $p < 0.01$ compared to IMQ-PLGA control group,
414 respectively). In addition, the reduction in brain cyst load was attained in rSAG1-PLGA
415 immunized mice compared to IMQ-PLGA control group ($p < 0.05$), but not compared to
416 PLGA control group. Mice immunized with the mixture of rSAG1-PLGA and IMQ-PLGA
417 and mice immunized with rSAG1-PLGA shared the least brain cyst loads about 51% and 42%
418 brain cyst reduction, respectively, versus control IMQ-PLGA group ($p < 0.01$). However, the
419 difference between these two groups did not meet any statistical significance. Moreover, mice
420 immunized with rSAG1 alone did not show any significant reduction in brain cyst compared
421 to control groups.

422

423 **Fig. 4. Evaluation of the protection against chronic toxoplasmosis in mice immunized**
424 **with rSAG1-PLGA nanoparticles or the mixture of rSAG1-PLGA and IMQ-PLGA**
425 **nanoparticles**. Mice in all groups were orally challenged with 15 cysts of the 76 K strain 3
426 weeks after the second immunization. One month after challenge, all mice were sacrificed (n
427 = 8/group) and the number of brain cysts in each mouse was calculated individually. Data
428 represent the mean ± SEM of measurements from eight mice. ANOVA, Tukey's multiple
429 comparison test. *$p < 0.05$ and **$p < 0.01$.

430

431 ***3.4 Vaccination with rSAG1-PLGA in combination with IMQ-PLGA or MPL-PLGA***

432

433 After the investigation of the effect of IMQ, effect of MPL adjuvant, when encapsulated
434 into PLGA and co-administrated with rSAG1-PLGA, was evaluated. CBA/J mice were
435 immunized subcutaneously (s.c.) two times at 3 week intervals with the mixture of rSAG1-
436 PLGA and MPL-PLGA nanoparticles (12 mice) or with the mixture of rSAG1-PLGA and
437 IMQ-PLGA nanoparticles (12 mice). Control mice were immunized with MPL-PLGA (12
438 mice) or IMQ-PLGA nanoparticles (4 mice) for the analysis of the cellular immune response
439 (Table2).

3.4.1 Humoral immune responses

Higher endpoint anti- SAG1 IgG titers were found in mice immunized with rSAG1-PLGA and MPL-PLGA nanoparticles (Fig 5A), compared to mice immunized with rSAG1-PLGA and IMQ-PLGA nanoparticles ($p < 0.01$). In both groups, the levels of IgG1 exceeded those of IgG2a (Fig 5B) with a IgG1/IgG2a OD ratio slightly different (rSAG1-PLGA and IMQ-PLGA nanoparticles, ratio > 2; rSAG1-PLGA and MPL-PLGA nanoparticles, ratio < 2). Compared to IMQ, MPL potentiated the humoral response and induced a more balanced Th1/Th2 humoral response (IgG1/IgG2a OD around 1.5).

Fig. 5. Specific antibody response in mice immunized with the mixture of rSAG1-PLGA and MPL-PLGA or with the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles. Analysis of humoral response was done three weeks after the second immunization. **A**). Determination of specific anti-SAG1 antibody titers by ELISA using *T. gondii* as coating antigen. Sera from each mouse in each group were analyzed individually (n = 12/group, except for IMQ-PLGA nanoparticles group, n = 4). The antigen-specific antibody titer (endpoint titer) was given as the reciprocal of the highest dilution making an optical density (OD) that was 2.5-fold greater than that of the serum of non-immunized mice. Dotted line represent the lowest mouse sera dilution tested. Symbols represent individual animals. Results are presented on scatter plots as geometric mean with the 95% confidence interval. Kruskall-Wallis, Dunn's multiple comparisons test. $^{**}p < 0.01$. **B)** Determination of the IgG subclasses profiles by ELISA using *T. gondii* extract as coating antigen. Sera (n = 6/group, except for IMQ-PLGA nanoparticles group n = 4) were evaluated individually.Results are expressed as the mean of the optical density (OD) ± SEM. Sera were tested at a single dilution (rSAG1-PLGA + IMQ-PLGA group and control groups, dilution 1:100 ; rSAG1-PLGA + IMQ-PLGA group, dilution 1:800).

3.4.2 Cellular immune responses

Splenocytes from mice immunized with the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles or rSAG1-PLGA and MPL-PLGA nanoparticles elicited significant ($p < 0.05$) levels of IFN-γ, IL-2, TNF-α (Th1 cytokines) and IL-5 (Th2 cytokine) in response to rSAG1 stimulation compared to their respective control (IMQ-PLGA and MPL-PLGA, respectively).

More IL-2 and IFN-γ were found in the supernatants of restimulated splenocytes from mice immunized with the mixture of rSAG1-PLGA and MPL-PLGA nanoparticles compared to those from mice immunized with rSAG1-PLGA and IMQ-PLGA. However the differences did not reach statistical significance.

Fig. 6. Cellular immune response in mice immunized with the mixture of rSAG1-PLGA and MPL-PLGA or with the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles. Three weeks after the second immunization, splenocytes were collected from four mice in each group, and cultured with 4 μg/mL rSAG1or medium alone (no stimulation). Cell-free supernatants were harvested after 24 (IL-2) or 72 h (IFN-γ, IL-5, and TNF-α) for cytokine assays. Results were expressed as the median and interquartile range. Mann Whitney test was done. "ns", no significant difference between the two immunized groups.

3.4.3 Challenge studies

Significant protection was displayed in the group of mice immunized with the mixture of rSAG1-PLGA and MPL-PLGA compared to its control group (MPL-PLGA) ($p < 0.01$), demonstrating 41% brain cyst reduction. The brain cyst number of mice immunized with the mixture of rSAG1-PLGA and MPL-PLGA was similar to that of mice immunized with the mixture of rSAG1-PLGA and IMQ-PLGA (1583 +/- 427 versus 1698 +/- 519). These results suggest that in our experimental conditions, MPL and IMQ have similar protective effects against chronic toxoplasmosis when encapsulated into PLGA nanoparticles and co-administrated with rSAG1-PLGA nanoparticles.

Fig. 7. Evaluation of the protection against chronic toxoplasmosis in mice immunized with the mixture of rSAG1-PLGA and MPL-PLGA or with the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles. Mice in all groups were orally challenged with15 cysts of the 76 K strain, 3 weeks after second immunization. One month after challenge, all mice were sacrificed and the number of brain cysts in each mouse was calculated individually. Data represent the mean ± SEM of measurements from eight mice. ANOVA, Tukey's multiple comparison test. $^{**}p < 0.01$; "ns", no significant difference.

4. Discussion

The main goal of this study was to investigate the adjuvanticity of IMQ and MPL loaded into PLGA nanoparticles when administrated with rSAG1 antigen adsorbed on PLGA nanoparticles and to compare the protective efficacies against *T. gondii* chronic infection in CBA/J after challenge with *T. gondii* cysts by the oral route.It has been well established that vaccine delivery systems can compensate for poor immunogenicity of subunit vaccines. Hence, both PLGA micro and nanoparticles were applied to serve as delivery system and adjuvant dual role for recombinant SAG1 proteins [11, 29], a multimeric recombinant *T. gondii* vaccine including SAG1 epitopes [12] or a recombinant chimericprotein rSAG1/2 [13]. To further potentiate protective immunity, particulate delivery of rSAG1 can be co-administrated with an immunostimulant molecule.

In our design, IMQ and MPL that target TLR7/8 and TLR4, respectively, were encapsulated in PLGA nanoparticles to be co-administrated with rSAG1 adsorbed on PLGA nanoparticles. Among the properties of PLGA nanoparticles including size, shape, surface charge and hydrophobicity, particle sizes are of high priority and play considerable role in antigen uptake by antigen presenting cells (APCs) [30, 31]. In the present study, all prepared PLGA nanoparticles (rSAG1-PLGA, MPL-PLGA, IMQ-PLGA) shared diameters less than 500 nm which are compatible with desired size mentioned by others. It is worthy to note that PLGA nanoparticles < 500 nm more potently elicit CTLs (cytotoxic T lymphocytes) response than larger ones (> 2 μm) [32].

One of the most critical parameters affecting the size of PLGA-based nanoparticles is hydrophobicity orwater solubility of their cargo [25]. MPL was solved in organic solvents (oil phase, first emulsion) like PLGA, while IMQ and rSAG1 were solved in aqueous solutions. Hence, the disruption of MPL into PLGA oil phase resulted in smaller nanoparticles than those of IMQ and rSAG1. The above-mentioned reason well justifies why the size of MPL-PLGA was different from the size of IMQ-PLGA and rSAG1-PLGA nanoparticles. All prepared nanoparticles shared a PDI less than 0.2 indicating more or less homogeneity in the size of individual nanoparticles. The surface charge similarities among IMQ-PLGA and MPL-PLGA and blank PLGA nanoparticles were attributed to the method of preparation (encapsulation) and confirmed that IMQ and PLGA completely have been encapsulated into

PLGA nanoparticles, while rSAG1-PLGA showed less negative charge because of rSAG1 adsorption on PLGA nanoparticles [25]. It is worthy to note that we previously proved that adsorption is a more suitable approach than encapsulation in antigen loading on PLGA nanoparticles [10]. It should be mentioned that mixing different nanoparticles to prepare two formulations (PLGA-IMQ + rSAG1-PLGA and PLGA-MPL + rSAG1-PLGA) may affect nanoparticles features. Thus, the characterizations of different nanoparticles after mixing and preparing final vaccine formulations are necessary in order to provide better understanding of nanoparticles behavior at least *in vitro*. Unfortunately, we did not perform these characterizations before vaccination studies in this research. It should be emphasized that one of the most critical consequences of stability issues regarding nanopoarticles is aggregation and particle precipitation. As this problem often occurs during time, different PLGA nanoparticles of each vaccine formulation were mixed just before injection. Fortunately, any precipitation or aggregation was not observed after mixing different PLGA nanoparticles of each vaccine formulation. Absolutely, if aggregation was happened, the needle would be clogged and syringe aspiration and injection to mice would be impossible.

Another possible outcome of mixed formulations would be its influence on rSAG1 adsorption efficiency. In the light of this fact, the mixing of two nanoparticles (rSAG1-PLGA and IMQ-PLGA or rSAG1-PLGA and MPL-PLGA), and formulations preparation were done exactly before injection to mice in order to eliminate or decrease any adverse effect of nanoparticles mixing on rSAG1 adsorption *in-vitro*. Of course, if rSAG1 adsorption was measured after mixing of different nanoparticles, it could provide satisfying insight in this regard.

In first experiment, mice vaccinated with the mixture of rSAG1-PLGA and IMQ-PLGA developed the highest amount of SAG1-specific IgG antibodies, with amixed Th1/Th2 humoral response biased towards a Th2 response similar to rSAG1-PLGA group (IgG1/IgG2a OD ratio >2). While, SAG1-specific IgG antibodies titers were higher in mice vaccinated with the mixture of rSAG1-PLGA and MPL-PLGA in comparison with those vaccinated with the

mixture of rSAG1-PLGAand IMQ-PLGA in second experiment, with a more balanced Th1/Th2-type antibody response (IgG1/IgG2a OD ratio < 2). Therefore, we could deduce that the formulation rSAG1-PLGA, MPL-PLGA resulted in more potent humoral responses than rSAG1-PLGA, IMQ-PLGA against SAG1. These findings clearly confirmed the role of IMQ and MPL in eliciting antibody response against SAG1, although MPL showed higher impact than IMQ. Mice vaccinated with rSAG1-PLGA and those with the mixture of rSAG1-PLGA and IMQ-PLGA did not show any significant differences in eliciting IFN-γ, IL-2 and TNF-α indicating Th1-biased immunity. Moreover, rSAG1-PLGA + MPL-PLGA group elicited higher amounts of mentioned cytokines than rSAG1-PLGA + IMQ-PLGA group, though no significant difference was observed. The less cyst numbers in mice immunized with rSAG1-PLGA, the mixture of rSAG1-PLGA and IMQ-PLGA, and the mixture of rSAG1-PLGA and MPL-PLGA were supported by higher amounts of Th1-associated cytokines and anti-SAG1 IgG antibodies in comparison with control groups.

The protective efficiency against toxoplasmosis depends on the Th1 immune response [5]. Although antibodies action is limited to extracellular parasites, B-cell antibody responses play a role in preventing persistent proliferation of tachyzoites in the brain and lung during the chronic phase of infection [6]. SAG1 contains T epitopes [33, 34] and neutralizing B epitopes [34-36] and both the cellular responses [34, 38, 39] and the humoral responses [37, 40] directed against SAG1 were shown to play a role in the protection observed against acute or chronic *T. gondii* infections. More specifically, in CBA/J or C3H mice (with H-2k haplotype), parenteral immunizations with SAG1 or SAG1 peptides conferred protection against chronic *T. gondii* infection following oral challenge with *T. gondii* cysts, when Th1 immune responses were induced [33, 41]. The brain cyst number in rSAG1-PLGA + MPL-PLGA group was less than those in rSAG1-PLGA + IMQ-PLGA group. Nevertheless, this difference was no significant. The lack of any significant difference in cyst number reduction between rSAG1-PLGA group and rSAG1-PLGA + IMQ-PLGA group is in accordance with their cytokine profiles and IgG1/IgG2a OD ratio.

Immune-enhancing effects of MPL have been reported for a number of antigens, including *T. gondii* antigens in mouse model [15, 16]. Adjuvant/delivery vehicles containing MPL have been developed and licensed in FDA-approved vaccines for human use, such as AS04 and AS01 [42]. Compared to Aluminum salts, AS04 (Aluminium salt + MPL) is more efficient in inducing the amplification and differentiation of $CD4^+$ T cells and promotes a Th1-biased response, MPL therefore provides a counterbalance to the Th2-differentiating properties of alum [43]. In our experimental conditions, co-administration of rSAG1-PLGA and MPL-

PLGA resulted in a more potent humoral response than rSAG1-PLGA and rSAG1-PLGA plus IMQ-PLGA, with a more polarized Th1 humoral response, confirming the ability of MPL to promote Th1 bias response. Compared to PLGA, IMQ potentiated the humoral response but did not potentiate the Th1 humoral response and did not improve the magnitude of the Th cell response. In present study, the enhanced humoral response obtained by co-administration of IMQ may be due to B cell–intrinsic TLR7 signaling. This signaling has been shown to promote vigorous memory B cell responses following co-immunization of an antigen with IMQ [44]. However, compared to PLGA, IMQ did not potentiate the Th1 humoral response and did not improve the magnitude of the Th cell response. This could be due to the modest Th1-polarizing responses from TLR7 pDC signaling [45].

Our results are similar to those obtained by Kasturi et al. [46] who compared the humoral responses induced in C57BL/6 mice subcutaneously immunized with hemagglutinin (HA) from avian influenza H5N1 virus encapsulated in PLGA nanoparticles combined or not with either PLGA-MPL or PLGA-IMQ nanoparticles. Similar results were also obtained with IMQ in an anionic liposome formulation, administrated by the intramuscular route to C57BL/6 mice with a recombinant *Plasmodium berghei* circumsporozoite protein which was compared to the TLR4 agonist Glucopyranosyl lipid adjuvant (GLA) [47]. Importantly, compared to immunization with a single TLR ligand, either IMQ or MPL in PLGA nanoparticles, immunization with PLGA nanoparticles including both TLR ligands has been shown to induce synergistic responses. It would be of interest to investigate if whether or not, in our experimental conditions, combination of PLGA-MPL and PLGA-IMQ could synergize to enhance the protection. Dendritic cell targeting is also a promising strategy to provide protection against *T. gondii*. SAG1 targeting to $DEC205^{+}$ dendritic cells via an antibody fragment single-chain fragment variable (scFv) by intranasal and subcutaneous administration to CBA/J mice has been shown to improve the protection against chronic *T. gondii* infection [48]. This targeting strategyto dendritic cells, applied to ovalbumin as a model antigen encapsulated in targeted PLGA nanoparticles together with TLR agonists, led to strong enhancement of vaccine potency and induction of T cell responses compared to non-specific delivery of nanoparticles to dendritic cells [49, 50]. Furthermore, as *Toxoplasma* infection is mainly acquired by consumption of oocysts in contaminated water or vegetables or by ingestion of tissue cysts contained in infected meat, a vaccine strategy able to induce both systemic and mucosal immune responses would be of great interest to tackle the parasite at the portal of entry. Nasal administration is a suitable route to induce such immune responses and nanoparticles based on PLGA or PLGA derivatives have great potential for thisstrategy.

For example, PLGA nanoparticulate intranasal administration of a combined TLR7/NOD2 agonist with HIV p24 antigen was recently shown to induce high-quality humoral and adaptive responses both in systemicand mucosal compartments [51]. Since *T. gondii* is an intracellular parasite with various life cycles and antigenic variations, the development of effective vaccine against *T. gondii* is a challenging endeavour. This study used SAG1, a well known candidate vaccine antigen. SAG1 is a major and stage-specific antigen expressed in *Toxoplasma* tachyzoite and is highly conserved among virulent strains of *T. gondii*. Combining SAG1 with antigens from the different *T. gondii* stages would undeniably improve vaccine efficacy.

Nowadays, vaccine strategy is implied as a puzzle that all its parts should be accurately designed and selected in order to achieve immunization goal. These pieces in a vaccine consist of antigen, adjuvant, delivery system, route of immunization and model.

Conclusion

Using SAG1 as a potential candidate vaccine and PLGA nanoparticles as delivery system, this study indicated that co-delivery of immunomodulators such as TLR agonists and antigens with PLGA nanoparticles could be appropriate to develop efficacious vaccines against toxoplasmosis.

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Table1. First experiment

Groups	Immunization with	Amounts injected to each mouse			Control/Vaccine group	Mice No.	Injection volume
		rSAG1	IMQ	PLGA			
1	rSAG1	20 µg	-	-	Vaccine group	12	100 µl
2	rSAG1-PLGA	20 µg	-	4 mg	Vaccine group	12	100 µl
3	rSAG1-PLGA + IMQ-PLGA	20 µg	64.5 µg	4 mg	Vaccine group	12	100 µl
4	PLGA	-	-	4 mg	Control group	12	100 µl
5	IMQ-PLGA	-	64.5 µg	4 mg	Control group	12	100 µl

Table 2. Second experiment

Groups	Immunization with	Amounts injected to each mouse				Control/Vaccine group	Mice No.	Injection volume
		rSAG1	IMQ	MPL	PLGA			
1	rSAG1-PLGA + IMQ-PLGA	20 µg	64.5 µg	-	4 mg	Vaccine group	12	100 µl
2	rSAG1-PLGA + MPL-PLGA	20 µg	-	34 µg	4 mg	Vaccine group	12	100 µl
3	IMQ-PLGA	-	64.5 µg	-	4 mg	Control group	4*	100 µl
4	MPL-PLGA	-		34 µg	4 mg	Control group	12	100 µl

* In this group, 4 mice were injected and were used for the analysis of cellular immune response.

Table 3. Characterization of IMQ-PLGA, MPL-PLGA, rSAG1-PLGA and blank PLGA nanoparticles. Results represent mean ± SD of five independent PLGA particle preparation batches.

Formulation	Size (nm)	PDI	Zeta potential (mV)	A/E efficiency (%)	Yield (%)
IMQ-PLGA	451.3 ± 27	0.15 ± 0.02	-5.2 ± 0.26	70 ± 3.1*	87.3 ± 2.8
MPL-PLGA	403 ± 25	0.18 ± 0.04	-5.6± 0.75	73.1± 1.2*	88.4± 2
rSAG1-PLGA	447 ± 9	0.11 ± 0.05	-2.37 ± 0.3	69.01 ± 1.8**	88.4 ± 5.1
Blank PLGA	412 ± 13	0.10 ± 0.02	-6.15 ± 0.7	-	90.3 ± 2.7

PDI; Poly Dispersity Index, A; adsorption, E; encapsulation.
* Encapsulation efficiency, ** Adsorption efficiency

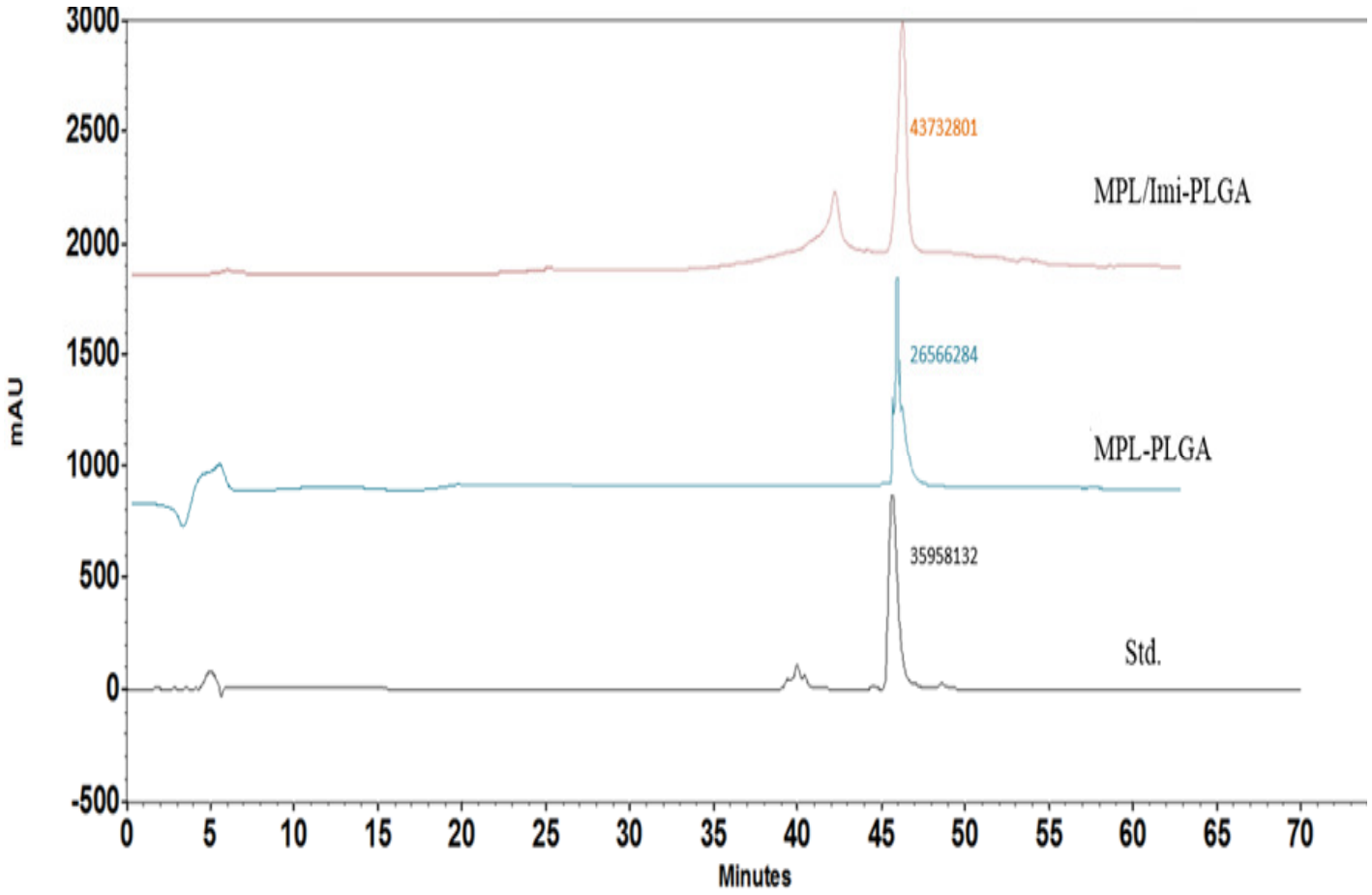

3000
2500
2000
1500
1000
500
0
-500
mAU
43732801
MPL/Imi-PLGA
26566284
MPL-PLGA
35958132
Std.
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Minutes

Figure 2

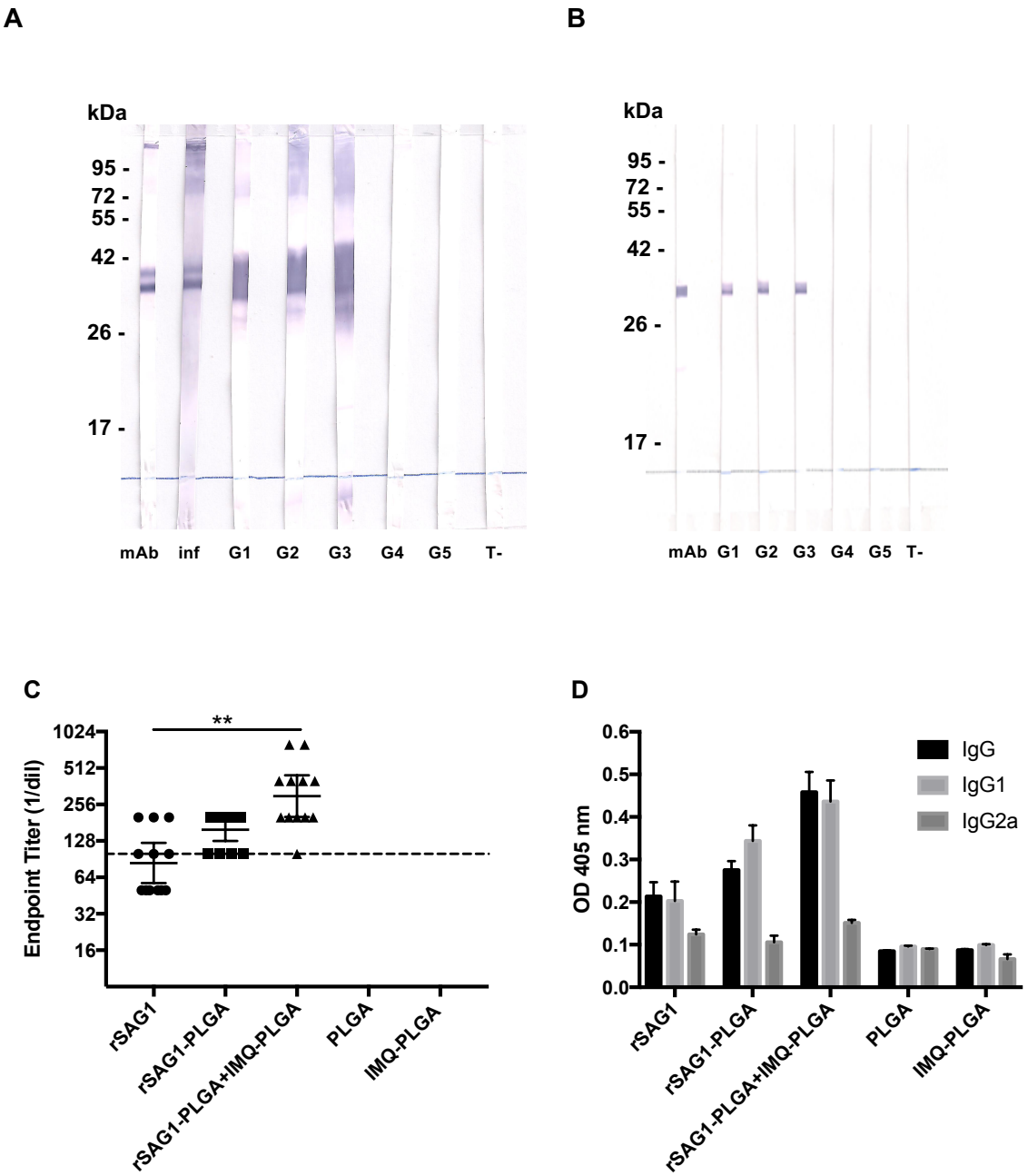
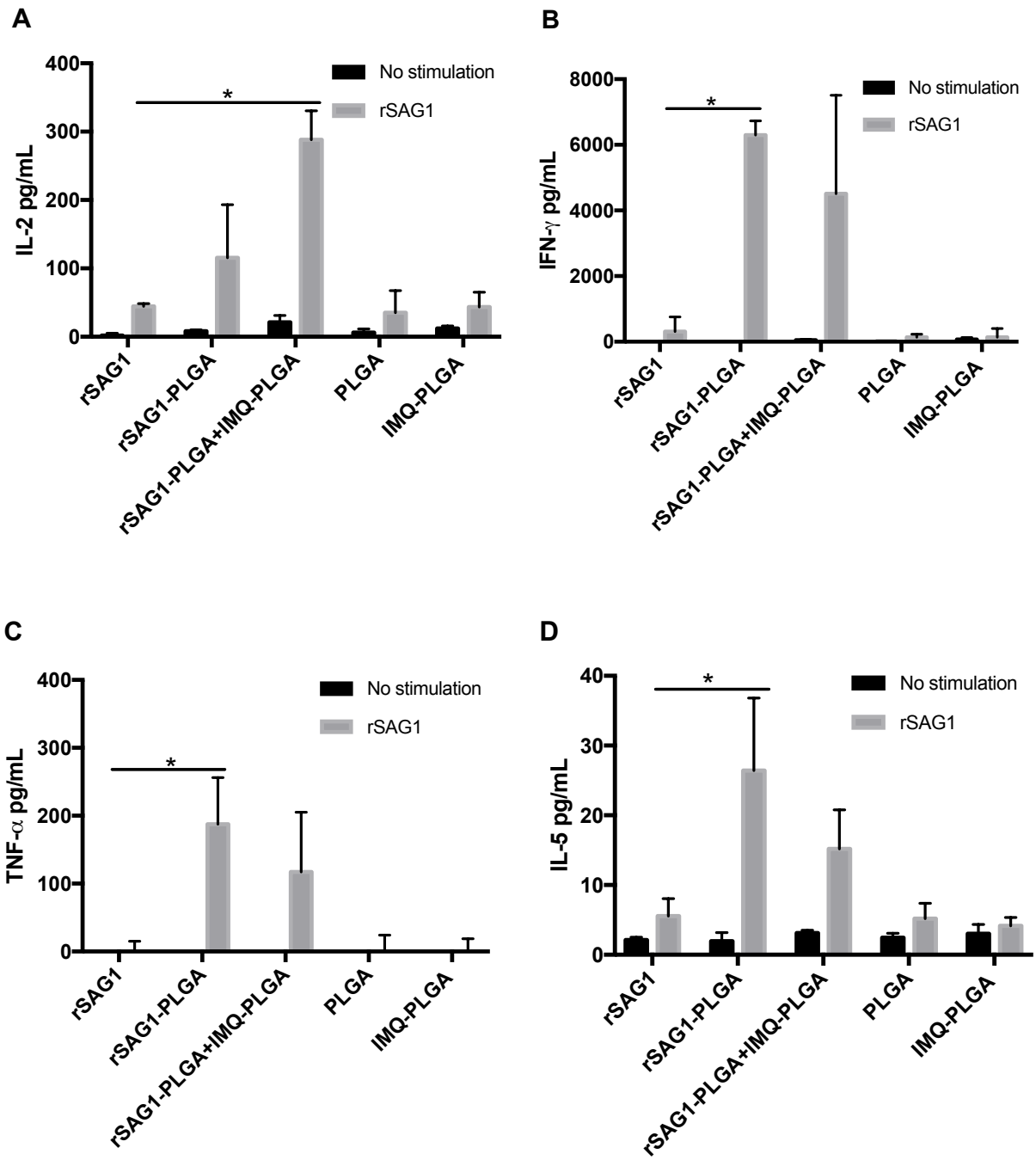

A
kDa
95 -
72 -
55 -
42 -
26 -
17 -
mAb inf G1 G2 G3 G4 G5 T-
B
kDa
95 -
72 -
55 -
42 -
26 -
17 -
mAb G1 G2 G3 G4 G5 T-
C
Endpoint Titer (1/dil)
1024
512
256
128
64
32
16
**
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA
D
OD 405 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
IgG
IgG1
IgG2a
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA

Figure 3


A
IL-2 pg/mL
400
300
200
100
0
No stimulation
rSAG1
*
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA
B
IFN-γ pg/mL
8000
6000
4000
2000
0
No stimulation
rSAG1
*
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA
C
TNF-α pg/mL
400
300
200
100
0
No stimulation
rSAG1
*
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA
D
IL-5 pg/mL
40
30
20
10
0
No stimulation
rSAG1
*
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA

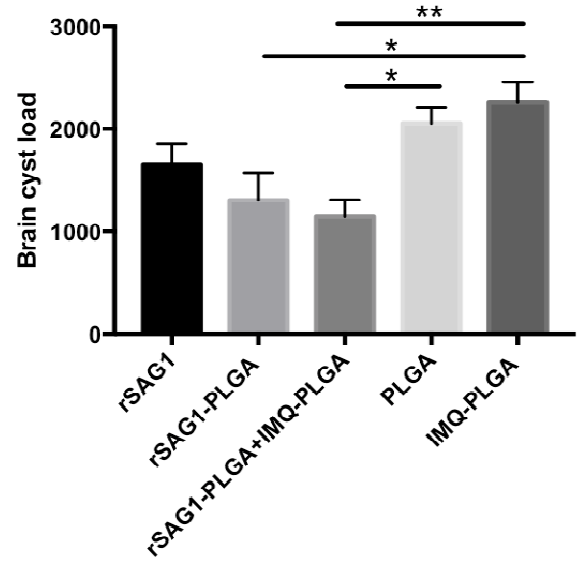

Brain cyst load
3000
2000
1000
0
**
*
*
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA

Figure 5

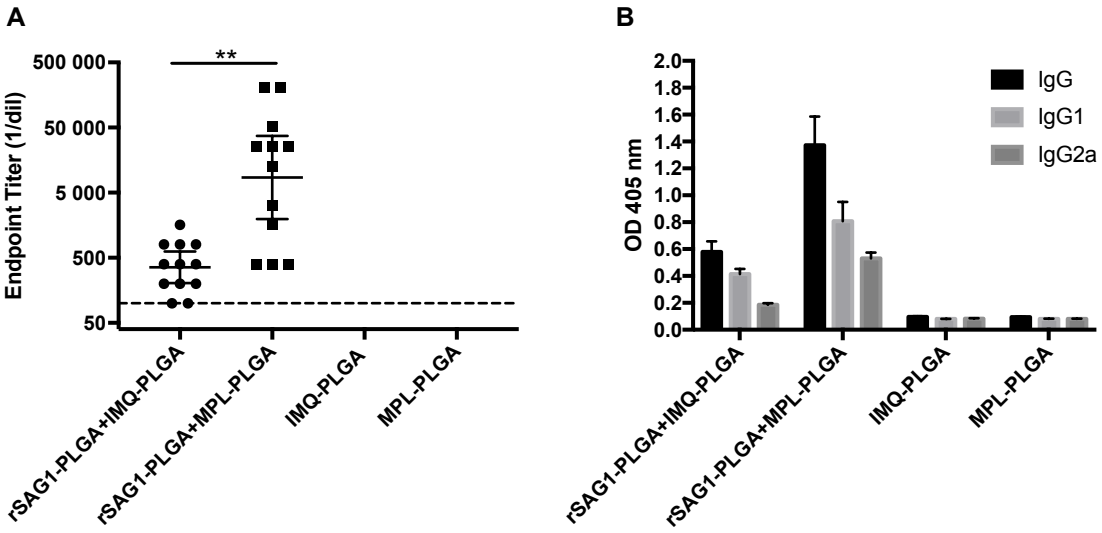
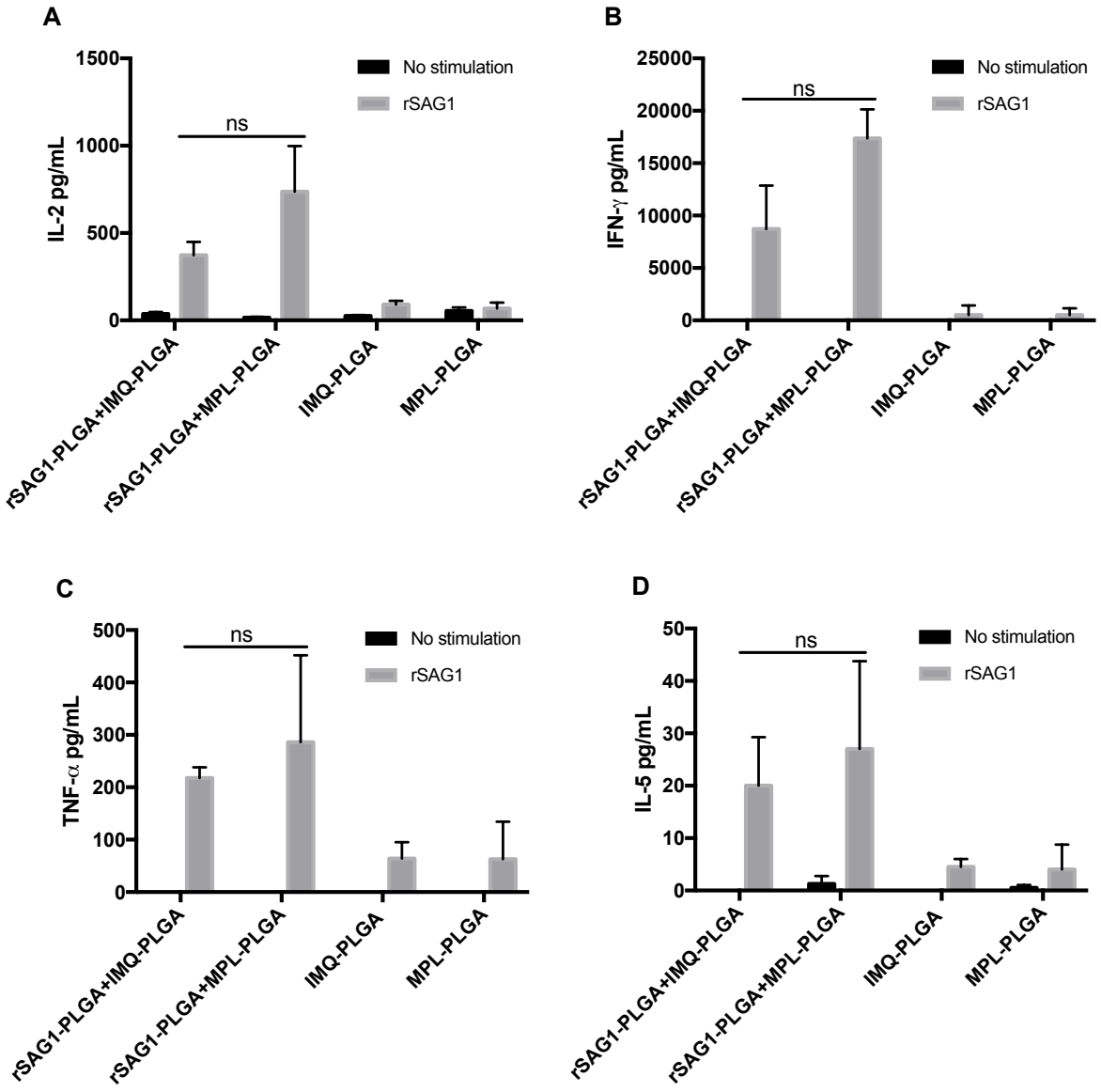
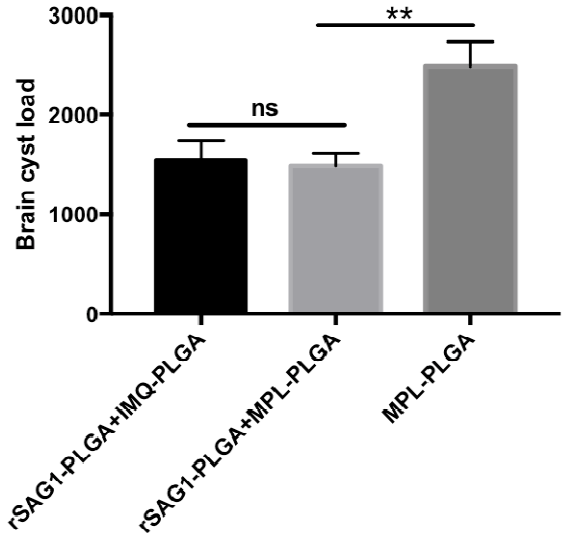
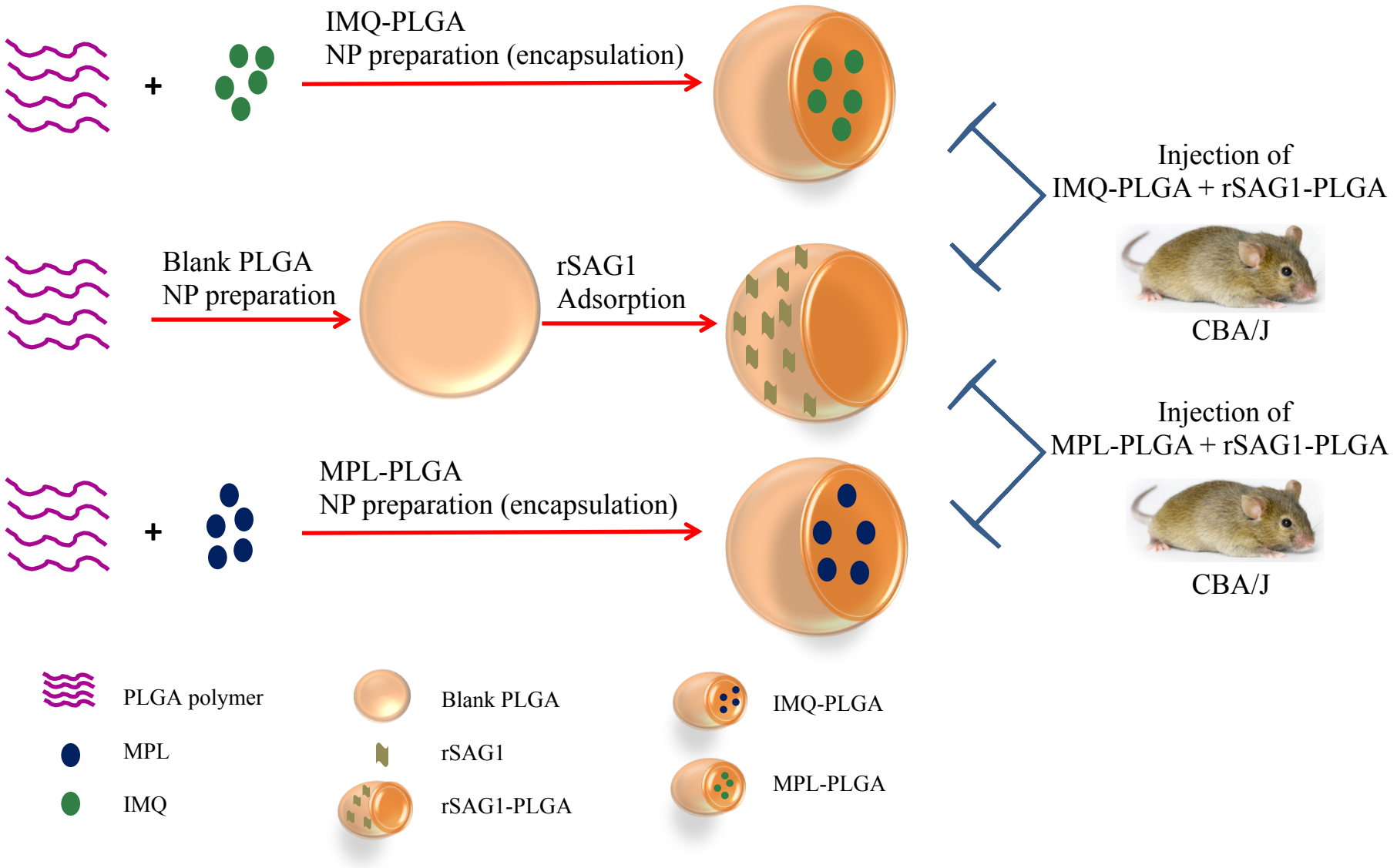

A
Endpoint Titer (1/dil)
500 000
50 000
5 000
500
50
**
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA
B
OD 405 nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
IgG
IgG1
IgG2a
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA

Figure 6


A
1500
1000
500
0
IL-2 pg/mL
No stimulation
rSAG1
ns
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA
B
25000
20000
15000
10000
5000
0
IFN-γ pg/mL
No stimulation
rSAG1
ns
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA
C
500
400
300
200
100
0
TNF-α pg/mL
No stimulation
rSAG1
ns
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA
D
50
40
30
20
10
0
IL-5 pg/mL
No stimulation
rSAG1
ns
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA


3000
2000
1000
0
Brain cyst load
**
ns
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
MPL-PLGA


IMQ-PLGA
NP preparation (encapsulation)
Blank PLGA
NP preparation
rSAG1
Adsorption
MPL-PLGA
NP preparation (encapsulation)
Injection of
IMQ-PLGA + rSAG1-PLGA
CBA/J
Injection of
MPL-PLGA + rSAG1-PLGA
CBA/J
PLGA polymer
MPL
IMQ
Blank PLGA
rSAG1
rSAG1-PLGA
IMQ-PLGA
MPL-PLGA