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

69 candidates based on native or recombinant SAG1 proteins have been evaluated in animal
70 models against, acute, chronic and congenital toxoplasmosis, using various adjuvant
71 formulations to overcome the low intrinsic immunogenicity of the protein [8]. Immune
72 responses and significant protections were obtained in most of these studies.
73 With the continuous improvement of knowledge and awareness regarding the immune
74 system, the application of vaccine delivery strategies such as Poly (D, L-lactic-co-glycolic
75 acid) (PLGA) particles could bring hope to improve vaccine efficacy [9]. Indeed, we
76 previously showed that rSAG1 adsorbed on the surface of PLGA nanoparticles or rSAG1
77 encapsulated in PLGA nanoparticles elicited higher systemic IFN-γ and specific anti-*T. gondii*
78 IgG antibodies than rSAG1 alone and conferred significant protection against acute
79 toxoplasmosis [10]. Similar results were also obtained with rSAG1 protein [11] and a
80 multimeric recombinant *T. gondii* vaccine including SAG1 epitopes [12] or a recombinant
81 chimeric protein rSAG1/2 [13] combined with PLGA nano-or microparticles.
82 TLRs agonists belong to a class of adjuvant known as immune stimulating agents. TLRs
83 not only mediate the activation of innate immune cells, but also directly modulate vaccine
84 specific response [14]. MPL, a portion of *Salmonella minnesota* lipopolysaccharide
85 peptidoglycan, as specific agonist of TLR4 has been applied in some vaccination studies
86 against *T. gondii* infection. Golkar et al. [15] demonstrated the protective efficacy of
87 recombinant GRA2 with MPL against chronic infection by *T. gondii* and confirmed the role
88 of MPL as an efficient immunostimulator to induce a protective Th1 immune response. The
89 MPL adjuvant with toxofilin DNA vaccine alsoinduced significantly enhanced humoral and
90 Th1-biased immune responses compared to the non-adjuvant toxofilin DNA vaccine [16].
91 IMQ, a synthetic analog of imidazoquinoline family, as an agonist of TRL7 has been
92 approved by US Food and Drug Administration (FDA) as an immunopotentiator agent for
93 local topical administration [17]. Recently, IMQ has been considered as a candidate for use
94 against *Toxoplasmosis* both therapeutically and prophylactically [18, 19], but there is not any
95 research on vaccine design against *T. gondii*. Although TLR agonists are powerful immune
96 stimulators, induced unwanted cytokine release syndrome leading to potential adverse events
97 and safety concerns is a major factor limiting their usage. To minimize these side effects and
98 to enhance their efficacy, TLR agonists are increasingly combined with delivery systems
99 including PLGA particles [20-22].
100 As PLGA nanoparticles can serve for dual-delivery of antigens and immunomodulators, in
101 present study, mice vaccination was performed with rSAG1-PLGA nanoparticles in
102 combination with IMQ or MPL encapsulated into PLGA nanoparticles in order to assess their

103 impacts on eliciting immune responses and protection against *T. gondii* infection. Since, we
104 previously proved that adsorption is a more suitable approach than encapsulation in antigen
105 loading on PLGA nanoparticles [10], rSAG1-PLGA was prepared by adsorption method. It is
106 worthy to note that during encapsulation process, protein exposure to water-oil interface,
107 harsh mechanical, thermal and chemical stresses could affect protein integrity and
108 consequently its immunogenicity [23]. Protection was evaluated against chronic infection in
109 CBA/J mice susceptible to cyst formation and development of toxoplasmosis encephalitis. In
110 addition, mice were challenged bythe oral route, the major natural route of infection, with
111 tissue cysts.
112
113
114 **2. Materials and Method**
115
116 ***2.1 Materials***
117
118 Poly (D, L-lactide-co-glycolide) polymer (PLGA), Resomer®RG503 (50:50, lactide:
119 glycolide ratio) (viscosity 0.32-0.44 dl/g) was purchased from Boehringer Ingelheim,
120 Germany. PVA [poly vinyl alcohol; molecular weight (MW) 30,000–70,000 Da, 88%
121 hydrolyzed] was obtained from Sigma Chemical Company. The materials applied for SDS
122 PAGE gel electrophoresis and protein molecular weight marker were supplied from Roche
123 Applied Sciences (Mannheim, Germany) and Fermentas, Vilnius, Lithuania, respectively.
124 Dichloromethane (DCM) (analytical grade) was obtained from Merck Ltd. Cell culture
125 reagents including RPMI-1640, Fetal Calf Sera (FCS), HEPES, L-glutamine, sodium
126 pyruvate, penicillin, and streptomycin, were obtained from Gibco (Life Technologies GmbH,
127 Karlsruhe, Germany). Concanavalin A (conA), lyophilized powder of MPL (monophosphoryl
128 from *Salmonella enterica* serotype *minnesota Re 595*) were purchased from Sigma-Aldrich
129 (Darmstadt, Germany). Imiquimod Vacci Grade™ was supplied from InvivoGen (CA, USA).
130 All other chemicals used were of analytical reagent grade. All solutions were prepared by
131 MilliQ™ ultrapure (Milli-QSystem, Millipore, Molsheim, France).
132
133 ***2.2 Purification and characterization of rSAG1 protein***
134
135 rSAG1 protein was cloned, expressed and purified according to our previous studies and its
136 purity, antigenicity, and immunogenicity were confirmed [24]. In order to remove of

137 contaminants including LPS, and *E.coli* DNA, Sartobind® Q strong 0.08 mL (Sartorius) was
138 used. Briefly, rSAG1 buffer was exchanged to binding buffer of Sartobind® Q strong (Tris-
139 HCl 20 mM, NaCl 50 mM, pH 8) by MicroSpin G-25 column. After column equilibration by
140 mentioned buffer, rSAG1 was applied to Sartobind® Q strong. The Sartobind® Q strong
141 operated as negative chromatography, so DNA attached to the column, and rSAG1 passed
142 through Sartobind® Q strong. The concentration of bacterial endotoxin in purified rSAG1
143 was measured by limulus amboebocyte lysate assay using LAL chromogenic endotoxin
144 quantitation kit (Pierce®, Thermo scientific, USA).
145
146 ***2.3 The preparation of different nanoparticles***
147
148 Encapsulation of IMQ and MPLwas carried out completely according to the preparation of
149 blank PLGA mentioned in our previous study [10] using double emulsion solvent evaporation
150 technique. The only distinction was the substitution of IMQ or MPL ligands for PBS. The
151 IMQ and MPL were usedin concentration of 5 mg/mL and 2.5 mg/mL, respectively. rSAG1
152 was adsorbed on blank PLGA as stated by Allahyari et al. [25].
153 Various batches of individual PLGA nanoparticles loaded by IMQ, MPL, and rSAG1 were
154 prepared to provide sufficient quantity of homogenous nanoparticles. rSAG1-PLGA
155 nanoparticles were mixed with either IMQ-PLGA or MPL-PLGA nanoparticles to make
156 different formulations just before each immunization.
157
158 ***2.4 Nanoparticles characterization***
159
160 *2.4.1 Particle size, zeta potential and polydispersity index*
161 The particle size (Z-average mean), particle size distribution (PSD), polydispersity index
162 (PDI) and surface charge of all nanoparticles were determined based on Allahyari et al. [25].
163 Both types of measurements were performed at 25°C using a ZetasizerNano ZS (Malvern
164 Instruments, Worcestershire, UK). All measurements were performed in triplicate.
165
166 *2.4.2 Process yield determination*
167 Process yield for both blank PLGA nanoparticles and PLGA nanoparticles loaded by
168 rSAG1, IMQ, and MPL were assessed in three preparation batches as mentioned in Allahyari
169 et al. [10].
170

171 *2.4.3 Evaluation of encapsulation efficiency*

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

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

192

193 ***2.5 Vaccination studies***

194

195 *2.5.1 Mice immunization and Vaccination schedules*

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

205 group in second experiment: only four mice for cellular analysis. All groups were immunized
206 subcutaneously (s.c.) two times at 3-week intervals with different formulations containing 20
207 µg rSAG1, IMQ (64.5µg), and MPL (34 µg), as represented in Table 1and 2, respectively.
208
209 *2.5.2 T. gondii extract (TE) preparation*
210 Preparation of TE containing both cytoplasmic and membrane antigens was prepared from
211 tachyzoites of the RH strain obtained by serial passaging in human foreskin fibroblast (HFF)
212 cell monolayers, as previously described [27]. Briefly, the obtained tachyzoites were washed
213 in PBS and sonicated for three 10-min periods at 60 W/s. The *Toxoplasma* sonicate was
214 centrifuged at 2,000 g for 30 min. The protein concentration was determined in the
215 supernatant by the Micro BCA protein assay reagent kit using bovine serum albumin (BSA)
216 as the standard (Pierce, Rockford, III.). The TE was stored at -20°C until use.
217
218 *2.5.3 Humoral response*
219 Anti SAG1-specific IgG antibodies were measured by ELISA on sera collected three weeks
220 after second immunization as previously described [27]. Briefly, Flat-bottomed 96-well plates
221 (Nunc) were coated with 10 µg/mL TE. Serial two-fold dilutions of serum were performed
222 (starting at a 1:100 dilution) and added to the wells. Sample of naive mice (untreated) served
223 as negative controls. Bound antibodies were detected with Goat anti-Mouse IgG alkaline
224 phosphatase (1:5,000, Sigma). The optical density of each sample was read at 405 nm. The
225 endpoint antibody titer for each sample is given as the reciprocal of the highest dilution
226 producing an OD that was 2.5-fold greater than that of the serum of naïve mice (serum of
227 naïve mice gave optical density readings of less 0.1).
228 The levels of anti-SAG1 IgG subclasses were measured by ELISA as described above, except
229 that sera were added to the plates at a single dilution (1:100 for sera of mice immunized with
230 rSAG1, rSAG1-PLGA, rSAG1-PLGA and IMQ-PLGA, PLGA, IMQ-PLGA or MPL-PLGA
231 nanoparticles and 1:800 for sera of mice immunized with rSAG1-PLGA and MPL-PLGA
232 nanoparticles). The alkaline phosphatase-conjugated Rat anti-Mouse IgG1 and IgG2a were
233 used at 1:1,000 (BD Pharmingen). IgG subclasses were evaluated using optical density.
234
235 *2.5.4 Western Blotting*
236 Western blottings were performed as previously described with either rSAG1 or *T. gondii*
237 extractas the source of antigenand pooled sera obtained from mice in each group three weeks
238 after second immunizations [27]. rSAG1 (6 µg/1-cm-wide slot) or TE (60 µg/1-cm-wide slot)

239 were electrophoresed in a 12 % SDS–polyacrylamide gel (SDS-PAGE) under non-reducing
240 conditions, transferred to nitrocellulose, and probed with pooled sera from untreated mice (T-
241), *T. gondii* infected mice (inf), mice immunized with soluble rSAG1 in PBS (G1), rSAG1-
242 PLGA (G2), rSAG1-PLGA and IMQ-PLGA (G3), PLGA (G4) or IMQ-PLGA (G5). Sera
243 were diluted at 1:100. A mouse monoclonal antibody (mAb), anti-SAG1 MAb 1E5 (diluted
244 at 1:100) isused as positive control [28]. Anti-SAG1 mAB 1E5 was kindly provided by Jean-
245 François Dubremetz.
246
247 *2.5.5 Cellular response (cytokine assay)*
248 Four mice in each group were sacrificed three weeks after the last immunization. Single
249 spleen cell suspensions were individually obtained by filtration through nylon mesh.
250 Erythrocytes were removed by lysis (hypotonicshock) and the remaining cells were washed
251 and suspended in RPMI 1640 medium supplemented with 5% FBS, 25 mM HEPES, 2 mM L-
252 glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/mL penicillin, and 100
253 μg/mL streptomycin. Cells (5×10^5 cells/well) were stimulated in triplicate with 4 μg/mL
254 endotoxin depleted rSAG1, or with medium alone (negative control). Concanavalin A (5
255 μg/mL) was used as a positive control for proliferation. Supernatants were harvested and
256 assayed for IL-2 after 24 h and for IFN-γ, IL-5, IL-10 after 72 h. The concentrations of
257 cytokines were determined using ELISA kits (eBioscience, San Diego, CA) according to the
258 manufacturer's protocol. Cytokine concentrations were determined by reference to standard
259 curves constructed with known amounts of cytokines provided by the kits.
260
261 *2.5.6 Challenge*
262 Mice (8/group) were orally challenged with 15 cysts of the 76K *T. gondii* strain, three
263 weeks after the last immunization. To evaluate the protection, brain cyst loads were evaluated
264 one month after challenge. Mouse brains were homogenized in 5 mL of RPMI medium, and
265 the number of tissue cysts per brain was determined microscopically by counting 8 samples
266 (10 μl) of each mouse brain by light microscope.
267
268 ***2.6 Statistical analyses***
269
270 Statistical significance was analyzed using GraphPad Prism software. Statistical analysis
271 was done by one-way ANOVA followed by a Tukey's multiple comparison test or using a

272 Kruskall–Wallis test followed by Dunn's multiple comparison test. $p < 0.05$ was considered
273 to be statistically significant.
274
275 **3. Results**
276
277 ***3.1 Production of rSAG1***
278
279 rSAG1 was expressed and purified, its antigenicity and immunogenicity were confirmed as
280 described in our previous study [24]. Endotoxin concentration in purified protein was
281 determined less than 0.05 EU/µg by limulus amboebocyte lysate assay.
282
283 ***3.2 Nanoparticles characterization***
284
285 All different nanoparticles were identified regarding zeta average size, PDI, zeta potential,
286 encapsulation efficiency and process yield. The results were summarized in Table 3.
287 The sizes of IMQ-PLGA and MPL-PLGA were about 451.3 ± 27and 403 ± 25 nm,
288 respectively. There was significant difference ($p < 0.05$) between mean sizes of MPL-PLGA
289 and IMQ-PLGA or rSAG1-PLGA nanoparticles. The difference between mean sizes of IMQ-
290 PLGA and rSAG1-PLGA was not significant. All prepared nanoparticles share a PDI less
291 than 0.2.
292 Surface charges of IMQ-PLGA, MPL-PLGA, nanoparticleswere negative, -4.9 ± 0.26 and -
293 5.6 ± 0.75 mV,respectively. Among all nanoparticles, rSAG1-PLGA showed the lowest
294 negative charge about -2.37 ± 0.3 mV. Zeta potential in rSAG1-PLGA was significantly ($p <$
295 0.001) lower than all other PLGA nanoparticles.
296 All statistical analysis was done by one way ANOVA. The efficacy of IMQ encapsulation
297 into IMQ-PLGA nanoparticles measured by spectrophotometry method was about 70 ± 3.1 %
298 (Table 3), which was calculated through interpolating of absorbance with the concentration.
299 In addition, as shown in Table 3, the encapsulation efficacy of MPL into MPL-PLGA
300 nanoparticles was quantified by RP-HPLC through comparison of AUC of encapsulated MPL
301 and AUC related to defined amount of MPL, nearly 73.1 ± 1.2 (Fig.1).
302 The adsorption efficacy of rSAG1-PLGA nanoparticles was 69.01 ± 1.8 %. Blank PLGA
303 and rSAG1-adsorbed PLGA were prepared as mentioned by Allahyari et al. [25]. The results
304 represent mean ± SD of 5 independent PLGA nanoparticle preparations.
305

306 Fig. 1. **Quantification of encapsulated MPL in MPL-PLGA nanoparticles by RP-HPLC**
307 **(Knauer, AZURA).** The numbers presented on graphs demonstrated area under curve
308 (AUC). Std, standard, is referred to defined amount of MPL. The column specification;
309 TSKgel Octadecyl-4PW column (2.0 mm ID × 15.0 cm length, particle size 7.0 μm) (TOSOH
310 Bioscience).
311
312 ***3.3Vaccination with rSAG1-PLGA in combination with IMQ-PLGA***
313
314 The effect of IMQ adjuvant, when encapsulated into PLGA and co-administrated with
315 rSAG1-PLGA was first investigated. CBA/J mice (12 mice/group) were immunized
316 subcutaneously (s.c.) two times at 3 weeks interval with rSAG1-PLGA nanoparticles or with
317 the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles. Control mice were immunized
318 with rSAG1 in PBS, blank PLGA nanoparticles or IMQ-PLGA nanoparticles (Table 1).The
319 induced humoral and cellular immune responses and the protective efficacy against chronic
320 toxoplasmosis following oral challenge, were evaluated.
321
322 *3.3.1 Humoral immune responses*
323 The humoral response was analyzed three weeks after the second immunization.
324 Immunoblot analysis of mouse sera was first performed against rSAG1 (Fig. 2A). Sera from
325 mice immunized either with rSAG1, rSAG1-PLGA or rSAG1-PLGA and IMQ-PLGA reacted
326 mainly with two protein bands corresponding to rSAG1 monomers at the expected size
327 (around 30 kDa). These sera reacted also with dimers (around 70 kDa) and probably
328 multimers of rSAG1 (> 70 kDa). These bands are also recognized by serum IgG antibodies
329 from *T. gondii* infected mice (inf). Monoclonal antibodie 1E5 reacted only with the two
330 protein bands corresponding to rSAG1 monomers. Monoclonal antibodie 1E5 recognizes a
331 conformational epitope which may not be accessible (or problem of conformation) in the
332 multimeric forms of rSAG1. Importantly, immunoblot analysis of mouse sera performed
333 against *T. gondii* extract, showed that sera from mice immunized either with rSAG1, rSAG1-
334 PLGA or rSAG1-PLGA and IMQ-PLGA reacted strongly with native SAG1 (Fig. 2B). As
335 expected, sera from mice immunized with PLGA (G4) and IMQ-PLGA (G5), and untreated
336 mice (T-) identified no protein in rSAG1 or *T. gondii* extract.
337 ELISA using *T. gondii* extract containing native SAG1 as coating antigen was used to
338 determine endpoint anti-SAG1 IgG antibodies titers (Fig. 2C). In our experimental conditions,
339 anti-SAG1-specific IgG antibodies were detected only in 6/12 mice immunized with rSAG1

340 in PBS (for 6 mice, OD value failed to reach 2.5 times the background value at the initial
341 dilution tested), whereas all mice immunized with rSAG1-PLGA nanoparticles produced
342 detectable anti-SAG1-specific IgG antibodies. These results indicated that a stronger humoral
343 immune response was induced in mice immunized with rSAG1-PLGA nanoparticles
344 compared to mice immunized with rSAG1 alone, however, the difference between the two
345 immunized groups did not reach statistical significance. In mice immunized with rSAG1-
346 PLGA and IMQ-PLGA nanoparticles, higher antibodies titers were found compared to mice
347 immunized either with rSAG1 in PBS or rSAG1-PLGA nanoparticles, however, a significant
348 statistical difference was found only between the rSAG1 and rSAG1-PLGA+IMQ-PLGA
349 groups ($p < 0.01$). Mice vaccinated with PLGA nanoparticles or IMQ-PLGA nanoparticles
350 did not produce any anti-SAG1 antibodies. To find out whether a Th1 and/or a Th2 humoral
351 response was induced by immunization, the IgG subclasses were analyzed (Fig 2D). The
352 levels of IgG1 exceeded those of IgG2a in sera of mice immunized with PLGA nanoparticles
353 with or whithout IMQ (IgG1/IgG2a OD ratio > 2), suggesting mixed Th1/Th2 humoral-type
354 response with a bias towards a Th2 response. In conclusion, vaccination with the mixture of
355 rSAG1-PLGA and IMQ-PLGA enhanced a mixed Th1/Th2 humoral response witha bias
356 towards a Th2 response.
357
358 **Fig. 2**. **Specific antibody response in miceimmunized with rSAG1-PLGA nanoparticles**
359 **or the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles.** Analysis of humoral
360 response was done three weeks after second immunization. For immunoblot analysis, rSAG1
361 (**A**) or *T. gondii* extract (**B**) electrophoresed and transferred to nitrocellulose, were probed
362 with pooled sera from immunized mice, *T. gondii* infected mice (inf) and untreated mice (T-).
363 A mouse mAb (anti-SAG1 MAb 1E5) was used as positive control (mAb). The molecular
364 weights are shown on the left side. Sera and and Mab 1E5 were diluted at 1:100. mAb (anti-
365 SAG1 MAb 1E5), inf (*T. gondii* infected mice), G1 (rSAG1), G2 (rSAG1-PLGA), G3
366 (rSAG1-PLGA + IMQ-PLGA), G4 (PLGA), and G5 (IMQ-PLGA), T- (untreatedmice).
367 **C)** Determination of specific anti-SAG1 antibody titers by ELISA using *T. gondii* extractas
368 coating antigen. Sera from each mouse in each group (n = 12/group) were analyzed
369 individually. The antigen-specific antibody titer (endpoint titer) was given as the reciprocal of
370 the highest dilution making an optical density (OD) that was 2.5-fold greater than that of the
371 serum of non-immunized mice. Dotted line represent the lowest mouse sera dilution tested.
372 Titers below the limit of detection (< 100) were assigned a value of 50 for analysis. Symbols

373 represent individual animals. Results are presented on scatter plots as geometric mean with
374 the 95% confidence interval. Kruskall-Wallis, Dunn's multiple comparisons test. $^{**}p$ 0.01
375 **D)** Determination of the IgG subclasses profiles by ELISA using *T. gondii* extractas coating
376 antigen. Sera were collected from six mice in each group and evaluated individually. Results
377 are expressed as the mean of the optical density (OD) ± SEM. Sera were tested at a single
378 dilution (1 :100).
379
380 *3.3.2 Cellular immune responses*
381 The potency of the various formulations to induce T cell immune responses was
382 investigated by measuring the specific cytokine responses in spleen three weeks after second
383 immunization. Splenocytes from mice immunized with rSAG1-PLGA or the mixture of
384 rSAG1-PLGA and IMQ-PLGA nanoparticles elicited significant ($p < 0.05$) amounts of IFN-γ,
385 IL-2, TNF-α (Th1 cytokines) and IL-5 (Th2 cytokine) in response to rSAG1 stimulation
386 compared to their respective control (PLGA and IMQ-PLGA, respectively). However, any
387 significant difference in cytokine production was shown between the two above-mentioned
388 immunized groups. Furthermore, splenocytes from mice immunized with rSAG1 elicited very
389 low levels of IFN-γ, IL-2, TNF-α and IL-5 as compared to mice immunized with rSAG1-
390 PLGA or the mixture of rSAG1-PLGA and IMQ-PLGA. In addition, the amounts of IFN-γ,
391 TNF-α, IL-5 ($p < 0.05$) in mice immunized with rSAG1-PLGA and the level of IL-2 ($p <$
392 0.01) in mice immunized with the mixture of rSAG1-PLGA and IMQ-PLGA were
393 significantly higher than those in mice immunized by rSAG1. Although the level of IL-5
394 significantly increased in groups immunized with rSAG1-PLGA and the mixture of rSAG1-
395 PLGA and IMQ-PLGA nanoparticles in comparison with control groups, all immunized
396 groups and control groups share a considerably low amount of induced IL-5.
397
398 **Fig. 3. Cellular immune response in mice immunized with rSAG1-PLGA nanoparticles**
399 **or the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles.** Three weeks after the
400 second immunization, splenocytes were collected from four mice in each group, and cultured
401 with 4 μg/mL rSAG1 or with medium alone (no stimulation). Cell-free supernatants were
402 harvested after 24 (IL-2) or 72 h (IFN-γ, IL-5, and TNF-α) for cytokine assays. Results are
403 expressed as the median and interquartile range. Kruskall–Wallis, Dunn's multiple
404 comparisons test. $^{*}p < 0.05$; $^{**}p < 0.01$ significant differences between the immunized
405 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).

440

441 *3.4.1 Humoral immune responses*

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

449

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

466

467

468 *3.4.2 Cellular immune responses*

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

473 More IL-2 and IFN-γ were found in the supernatants of restimulated splenocytes from mice
474 immunized with the mixture of rSAG1-PLGA and MPL-PLGA nanoparticles compared to
475 those from mice immunized with rSAG1-PLGA and IMQ-PLGA. However the differences
476 did not reach statistical significance.
477
478 **Fig. 6. Cellular immune response in mice immunized with the mixture of rSAG1-PLGA**
479 **and MPL-PLGA or with the mixture of rSAG1-PLGA and IMQ-PLGA nanoparticles.**
480 Three weeks after the second immunization, splenocytes were collected from four mice in
481 each group, and cultured with 4 μg/mL rSAG1or medium alone (no stimulation). Cell-free
482 supernatants were harvested after 24 (IL-2) or 72 h (IFN-γ, IL-5, and TNF-α) for cytokine
483 assays. Results were expressed as the median and interquartile range. Mann Whitney test was
484 done. "ns", no significant difference between the two immunized groups.
485
486
487 *3.4.3 Challenge studies*
488 Significant protection was displayed in the group of mice immunized with the mixture of
489 rSAG1-PLGA and MPL-PLGA compared to its control group (MPL-PLGA) ($p < 0.01$),
490 demonstrating 41% brain cyst reduction. The brain cyst number of mice immunized with the
491 mixture of rSAG1-PLGA and MPL-PLGA was similar to that of mice immunized with the
492 mixture of rSAG1-PLGA and IMQ-PLGA (1583 +/- 427 versus 1698 +/- 519). These results
493 suggest that in our experimental conditions, MPL and IMQ have similar protective effects
494 against chronic toxoplasmosis when encapsulated into PLGA nanoparticles and co-
495 administrated with rSAG1-PLGA nanoparticles.
496
497 **Fig. 7. Evaluation of the protection against chronic toxoplasmosis in mice immunized**
498 **with the mixture of rSAG1-PLGA and MPL-PLGA or with the mixture of rSAG1-**
499 **PLGA and IMQ-PLGA nanoparticles.** Mice in all groups were orally challenged with15
500 cysts of the 76 K strain, 3 weeks after second immunization. One month after challenge, all
501 mice were sacrificed and the number of brain cysts in each mouse was calculated
502 individually. Data represent the mean ± SEM of measurements from eight mice. ANOVA,
503 Tukey's multiple comparison test. $^{**}p < 0.01$; "ns", no significant difference.
504
505

506 **4. Discussion**

507

508 The main goal of this study was to investigate the adjuvanticity of IMQ and MPL loaded
509 into PLGA nanoparticles when administrated with rSAG1 antigen adsorbed on PLGA
510 nanoparticles and to compare the protective efficacies against *T. gondii* chronic infection in
511 CBA/J after challenge with *T. gondii* cysts by the oral route.It has been well established that
512 vaccine delivery systems can compensate for poor immunogenicity of subunit vaccines.
513 Hence, both PLGA micro and nanoparticles were applied to serve as delivery system and
514 adjuvant dual role for recombinant SAG1 proteins [11, 29], a multimeric recombinant *T.*
515 *gondii* vaccine including SAG1 epitopes [12] or a recombinant chimericprotein rSAG1/2
516 [13]. To further potentiate protective immunity, particulate delivery of rSAG1 can be co-
517 administrated with an immunostimulant molecule.

518 In our design, IMQ and MPL that target TLR7/8 and TLR4, respectively, were
519 encapsulated in PLGA nanoparticles to be co-administrated with rSAG1 adsorbed on PLGA
520 nanoparticles. Among the properties of PLGA nanoparticles including size, shape, surface
521 charge and hydrophobicity, particle sizes are of high priority and play considerable role in
522 antigen uptake by antigen presenting cells (APCs) [30, 31]. In the present study, all prepared
523 PLGA nanoparticles (rSAG1-PLGA, MPL-PLGA, IMQ-PLGA) shared diameters less than
524 500 nm which are compatible with desired size mentioned by others. It is worthy to note that
525 PLGA nanoparticles < 500 nm more potently elicit CTLs (cytotoxic T lymphocytes) response
526 than larger ones (> 2 μm) [32].

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

537 PLGA nanoparticles, while rSAG1-PLGA showed less negative charge because of rSAG1
538 adsorption on PLGA nanoparticles [25]. It is worthy to note that we previously proved that
539 adsorption is a more suitable approach than encapsulation in antigen loading on PLGA
540 nanoparticles [10]. It should be mentioned that mixing different nanoparticles to prepare two
541 formulations (PLGA-IMQ + rSAG1-PLGA and PLGA-MPL + rSAG1-PLGA) may affect
542 nanoparticles features. Thus, the characterizations of different nanoparticles after mixing and
543 preparing final vaccine formulations are necessary in order to provide better understanding of
544 nanoparticles behavior at least *in vitro*. Unfortunately, we did not perform these
545 characterizations before vaccination studies in this research. It should be emphasized that one
546 of the most critical consequences of stability issues regarding nanopoarticles is aggregation
547 and particle precipitation. As this problem often occurs during time, different PLGA
548 nanoparticles of each vaccine formulation were mixed just before injection. Fortunately, any
549 precipitation or aggregation was not observed after mixing different PLGA nanoparticles of
550 each vaccine formulation. Absolutely, if aggregation was happened, the needle would be
551 clogged and syringe aspiration and injection to mice would be impossible.
552 Another possible outcome of mixed formulations would be its influence on rSAG1 adsorption
553 efficiency. In the light of this fact, the mixing of two nanoparticles (rSAG1-PLGA and IMQ-
554 PLGA or rSAG1-PLGA and MPL-PLGA), and formulations preparation were done exactly
555 before injection to mice in order to eliminate or decrease any adverse effect of nanoparticles
556 mixing on rSAG1 adsorption *in-vitro*. Of course, if rSAG1 adsorption was measured after
557 mixing of different nanoparticles, it could provide satisfying insight in this regard.
558
559 In first experiment, mice vaccinated with the mixture of rSAG1-PLGA and IMQ-PLGA
560 developed the highest amount of SAG1-specific IgG antibodies, with amixed Th1/Th2
561 humoral response biased towards a Th2 response similar to rSAG1-PLGA group (IgG1/IgG2a
562 OD ratio >2). While, SAG1-specific IgG antibodies titers were higher in mice vaccinated with
563 the mixture of rSAG1-PLGA and MPL-PLGA in comparison with those vaccinated with the

564 mixture of rSAG1-PLGAand IMQ-PLGA in second experiment, with a more balanced
565 Th1/Th2-type antibody response (IgG1/IgG2a OD ratio < 2). Therefore, we could deduce that
566 the formulation rSAG1-PLGA, MPL-PLGA resulted in more potent humoral responses than
567 rSAG1-PLGA, IMQ-PLGA against SAG1. These findings clearly confirmed the role of IMQ
568 and MPL in eliciting antibody response against SAG1, although MPL showed higher impact
569 than IMQ. Mice vaccinated with rSAG1-PLGA and those with the mixture of rSAG1-PLGA
570 and IMQ-PLGA did not show any significant differences in eliciting IFN-γ, IL-2 and TNF-α
571 indicating Th1-biased immunity. Moreover, rSAG1-PLGA + MPL-PLGA group elicited
572 higher amounts of mentioned cytokines than rSAG1-PLGA + IMQ-PLGA group, though no
573 significant difference was observed. The less cyst numbers in mice immunized with rSAG1-
574 PLGA, the mixture of rSAG1-PLGA and IMQ-PLGA, and the mixture of rSAG1-PLGA and
575 MPL-PLGA were supported by higher amounts of Th1-associated cytokines and anti-SAG1
576 IgG antibodies in comparison with control groups.
577 The protective efficiency against toxoplasmosis depends on the Th1 immune response [5].
578 Although antibodies action is limited to extracellular parasites, B-cell antibody responses play
579 a role in preventing persistent proliferation of tachyzoites in the brain and lung during the
580 chronic phase of infection [6]. SAG1 contains T epitopes [33, 34] and neutralizing B epitopes
581 [34-36] and both the cellular responses [34, 38, 39] and the humoral responses [37, 40]
582 directed against SAG1 were shown to play a role in the protection observed against acute or
583 chronic *T. gondii* infections. More specifically, in CBA/J or C3H mice (with H-2k haplotype),
584 parenteral immunizations with SAG1 or SAG1 peptides conferred protection against chronic
585 *T. gondii* infection following oral challenge with *T. gondii* cysts, when Th1 immune
586 responses were induced [33, 41]. The brain cyst number in rSAG1-PLGA + MPL-PLGA
587 group was less than those in rSAG1-PLGA + IMQ-PLGA group. Nevertheless, this difference
588 was no significant. The lack of any significant difference in cyst number reduction between
589 rSAG1-PLGA group and rSAG1-PLGA + IMQ-PLGA group is in accordance with their
590 cytokine profiles and IgG1/IgG2a OD ratio.
591 Immune-enhancing effects of MPL have been reported for a number of antigens, including
592 *T. gondii* antigens in mouse model [15, 16]. Adjuvant/delivery vehicles containing MPL have
593 been developed and licensed in FDA-approved vaccines for human use, such as AS04 and
594 AS01 [42]. Compared to Aluminum salts, AS04 (Aluminium salt + MPL) is more efficient in
595 inducing the amplification and differentiation of $CD4^+$ T cells and promotes a Th1-biased
596 response, MPL therefore provides a counterbalance to the Th2-differentiating properties of
597 alum [43]. In our experimental conditions, co-administration of rSAG1-PLGA and MPL-

598 PLGA resulted in a more potent humoral response than rSAG1-PLGA and rSAG1-PLGA
599 plus IMQ-PLGA, with a more polarized Th1 humoral response, confirming the ability of
600 MPL to promote Th1 bias response. Compared to PLGA, IMQ potentiated the humoral
601 response but did not potentiate the Th1 humoral response and did not improve the magnitude
602 of the Th cell response. In present study, the enhanced humoral response obtained by co-
603 administration of IMQ may be due to B cell–intrinsic TLR7 signaling. This signaling has
604 been shown to promote vigorous memory B cell responses following co-immunization of an
605 antigen with IMQ [44]. However, compared to PLGA, IMQ did not potentiate the Th1
606 humoral response and did not improve the magnitude of the Th cell response. This could be
607 due to the modest Th1-polarizing responses from TLR7 pDC signaling [45].
608 Our results are similar to those obtained by Kasturi et al. [46] who compared the humoral
609 responses induced in C57BL/6 mice subcutaneously immunized with hemagglutinin (HA)
610 from avian influenza H5N1 virus encapsulated in PLGA nanoparticles combined or not with
611 either PLGA-MPL or PLGA-IMQ nanoparticles. Similar results were also obtained with IMQ
612 in an anionic liposome formulation, administrated by the intramuscular route to C57BL/6
613 mice with a recombinant *Plasmodium berghei* circumsporozoite protein which was compared
614 to the TLR4 agonist Glucopyranosyl lipid adjuvant (GLA) [47]. Importantly, compared to
615 immunization with a single TLR ligand, either IMQ or MPL in PLGA nanoparticles,
616 immunization with PLGA nanoparticles including both TLR ligands has been shown to
617 induce synergistic responses. It would be of interest to investigate if whether or not, in our
618 experimental conditions, combination of PLGA-MPL and PLGA-IMQ could synergize to
619 enhance the protection. Dendritic cell targeting is also a promising strategy to provide
620 protection against *T. gondii*. SAG1 targeting to DEC205^{+} dendritic cells via an antibody
621 fragment single-chain fragment variable (scFv) by intranasal and subcutaneous administration
622 to CBA/J mice has been shown to improve the protection against chronic *T. gondii* infection
623 [48]. This targeting strategyto dendritic cells, applied to ovalbumin as a model antigen
624 encapsulated in targeted PLGA nanoparticles together with TLR agonists, led to strong
625 enhancement of vaccine potency and induction of T cell responses compared to non-specific
626 delivery of nanoparticles to dendritic cells [49, 50]. Furthermore, as *Toxoplasma* infection is
627 mainly acquired by consumption of oocysts in contaminated water or vegetables or by
628 ingestion of tissue cysts contained in infected meat, a vaccine strategy able to induce both
629 systemic and mucosal immune responses would be of great interest to tackle the parasite at
630 the portal of entry. Nasal administration is a suitable route to induce such immune responses
631 and nanoparticles based on PLGA or PLGA derivatives have great potential for thisstrategy.

632 For example, PLGA nanoparticulate intranasal administration of a combined TLR7/NOD2
633 agonist with HIV p24 antigen was recently shown to induce high-quality humoral and
634 adaptive responses both in systemicand mucosal compartments [51]. Since *T. gondii* is an
635 intracellular parasite with various life cycles and antigenic variations, the development of
636 effective vaccine against *T. gondii* is a challenging endeavour. This study used SAG1, a well
637 known candidate vaccine antigen. SAG1 is a major and stage-specific antigen expressed in
638 *Toxoplasma* tachyzoite and is highly conserved among virulent strains of *T. gondii*.
639 Combining SAG1 with antigens from the different *T. gondii* stages would undeniably
640 improve vaccine efficacy.
641 Nowadays, vaccine strategy is implied as a puzzle that all its parts should be accurately
642 designed and selected in order to achieve immunization goal. These pieces in a vaccine
643 consist of antigen, adjuvant, delivery system, route of immunization and model.
644
645 **Conclusion**
646 Using SAG1 as a potential candidate vaccine and PLGA nanoparticles as delivery system,
647 this study indicated that co-delivery of immunomodulators such as TLR agonists and antigens
648 with PLGA nanoparticles could be appropriate to develop efficacious vaccines against
649 toxoplasmosis.
650
651
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895 **Table1.** First experiment

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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

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929 **Table 2.** Second experiment

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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

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932 * In this group, 4 mice were injected and were used for the analysis of cellular immune
933 response.
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963 **Table 3.** Characterization of IMQ-PLGA, MPL-PLGA, rSAG1-PLGA and blank PLGA
964 nanoparticles. Results represent mean ± SD of five independent PLGA particle preparation
965 batches.
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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

967 PDI; Poly Dispersity Index, A; adsorption, E; encapsulation.
968 * Encapsulation efficiency, ** Adsorption efficiency
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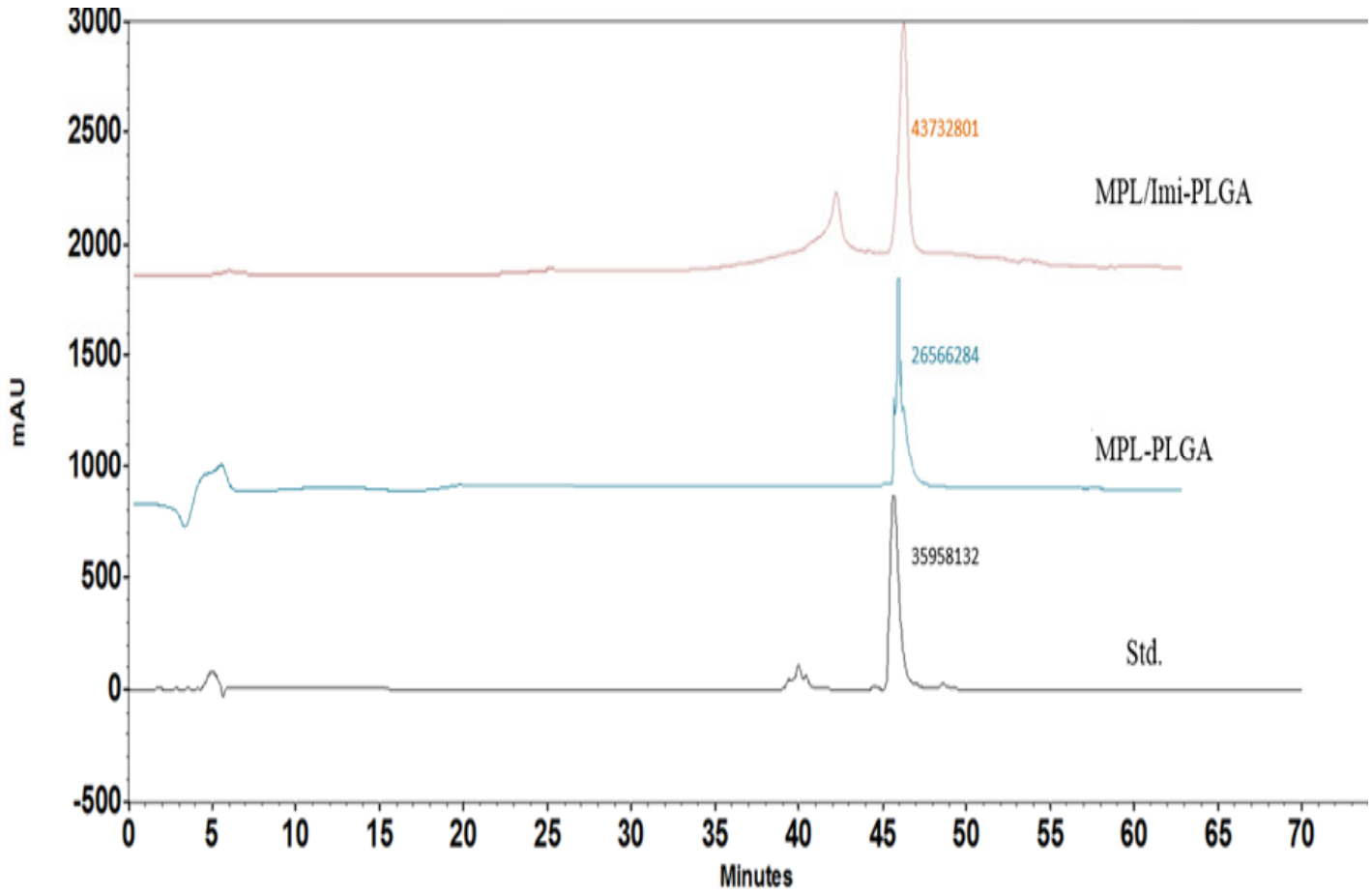
3000
2500
2000
1500
1000
500
0
-500
mAU
43732801
MPL/Imi-PLGA
26566284
MPL-PLGA
35958132
Std.
0
5
10
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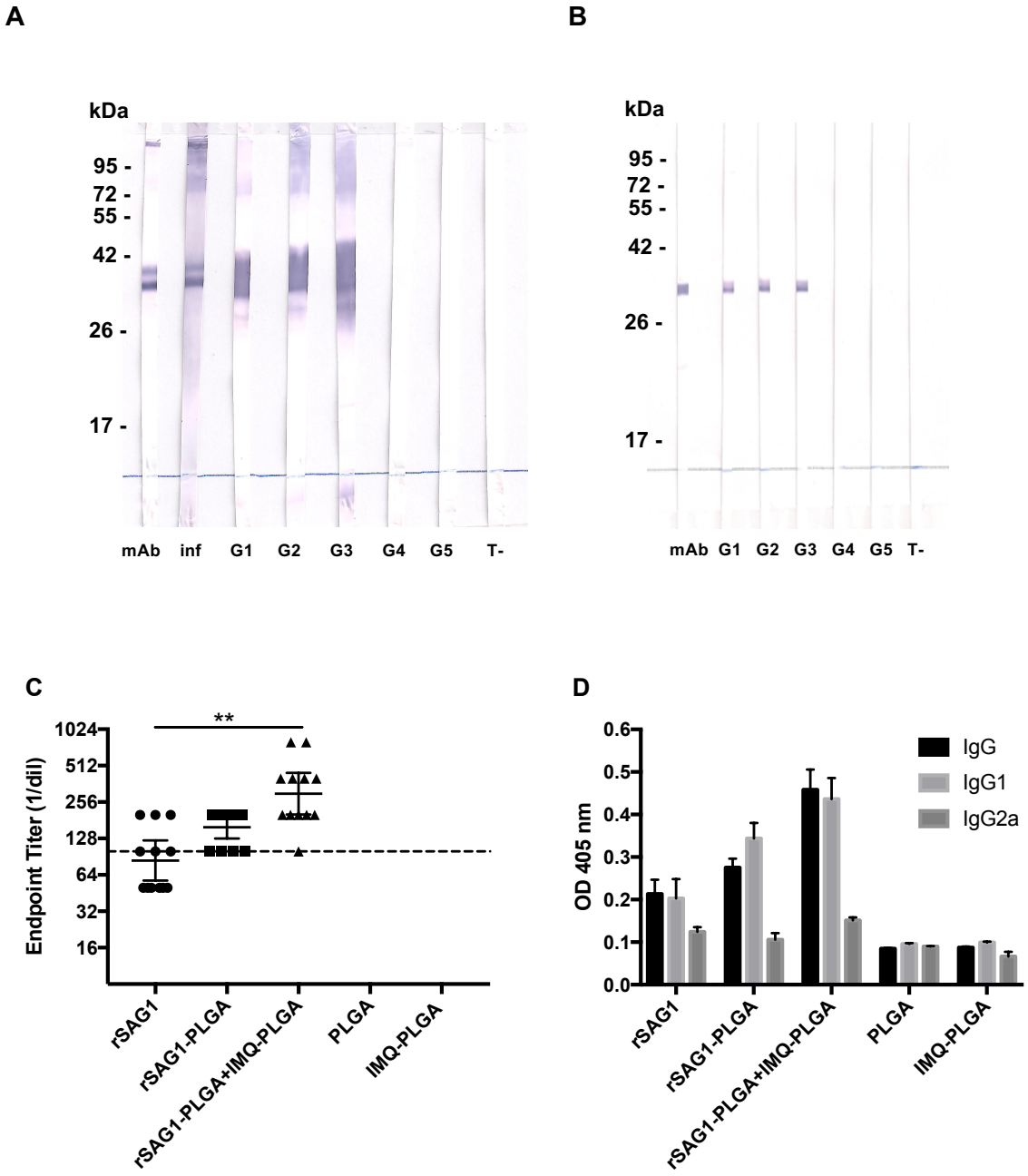
Minutes

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
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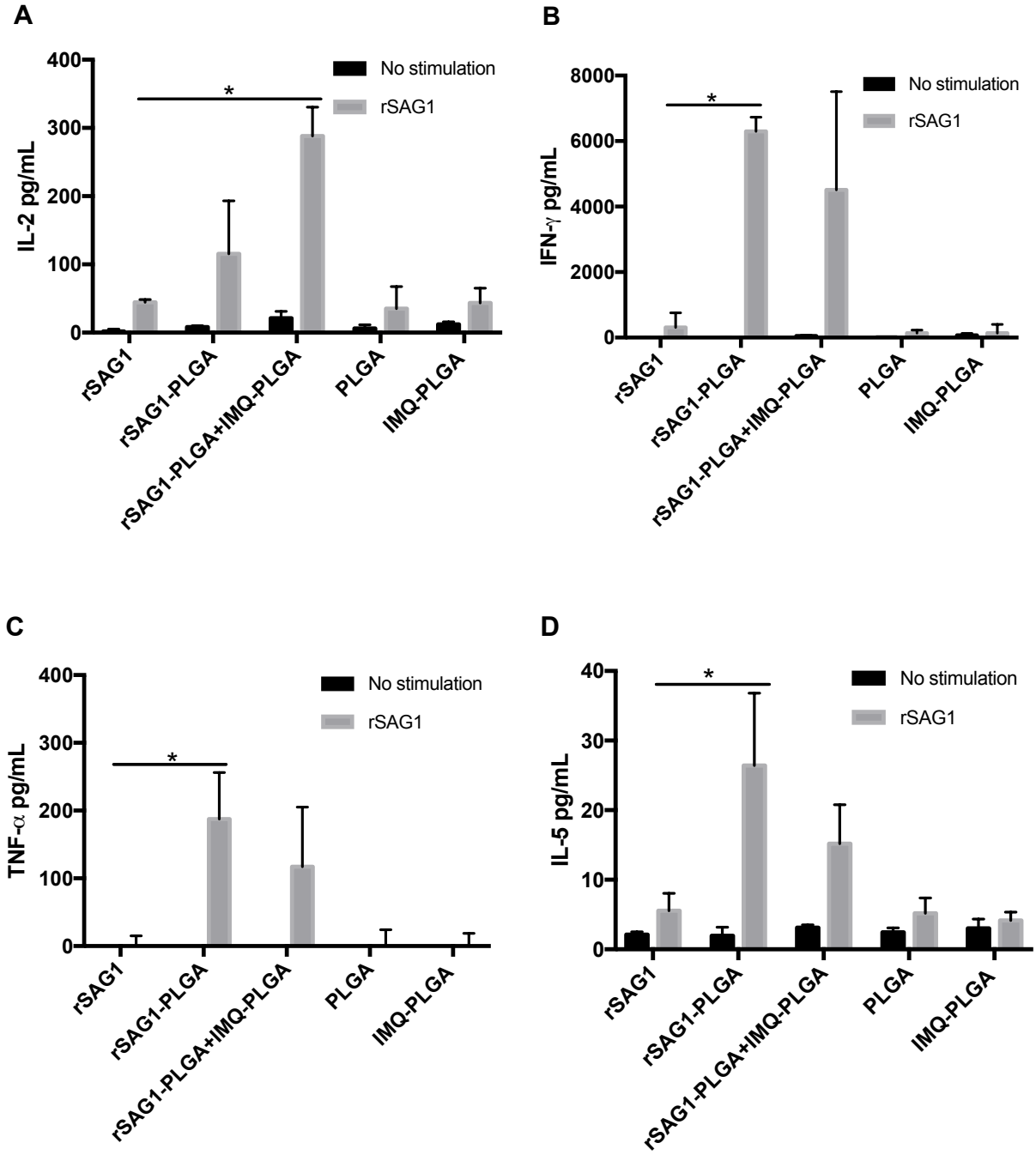
Endpoint Titer (1/dil)
1024
512
256
128
64
32
16
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA
D
OD 405 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
IgG
IgG1
IgG2a
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA

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
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No stimulation
rSAG1
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA

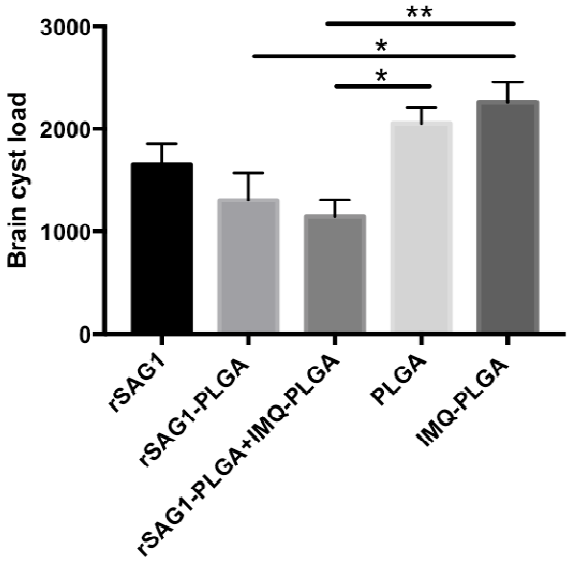
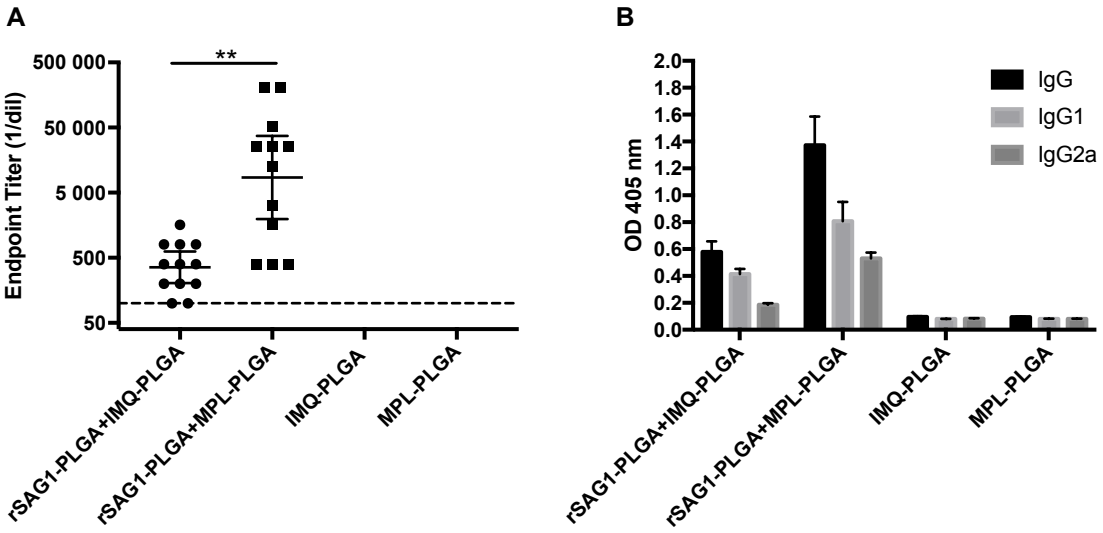

Brain cyst load
3000
2000
1000
0
**
*
*
rSAG1
rSAG1-PLGA
rSAG1-PLGA+IMQ-PLGA
PLGA
IMQ-PLGA

Figure 5


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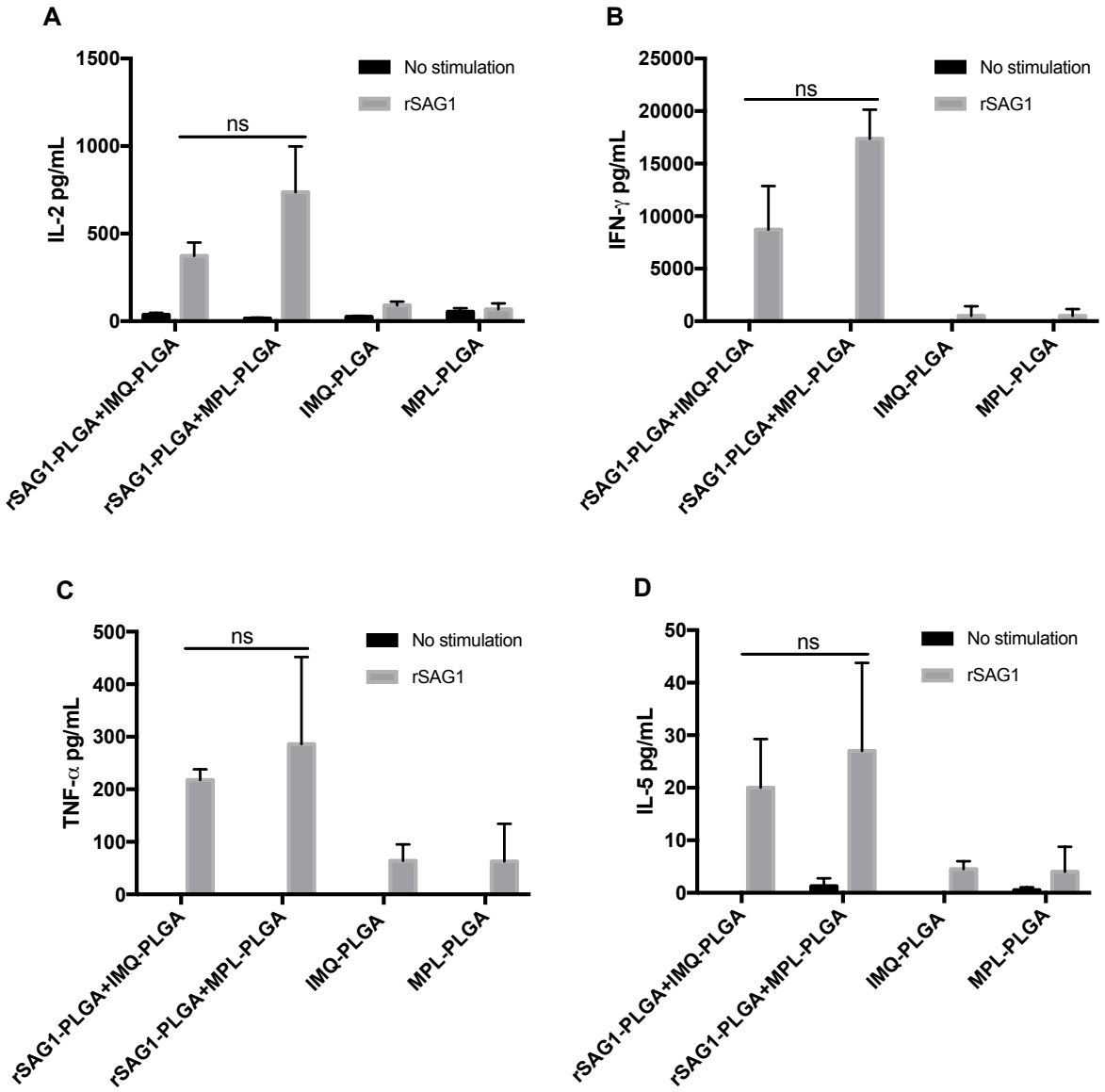
500 000
50 000
5 000
500
50
Endpoint Titer (1/dil)
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA
B
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 405 nm
IgG
IgG1
IgG2a
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA

Figure 6


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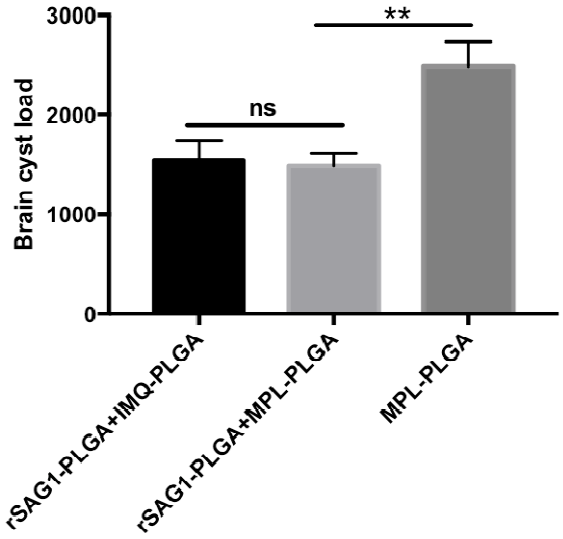
ns
No stimulation
rSAG1
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
IMQ-PLGA
MPL-PLGA


3000
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Brain cyst load
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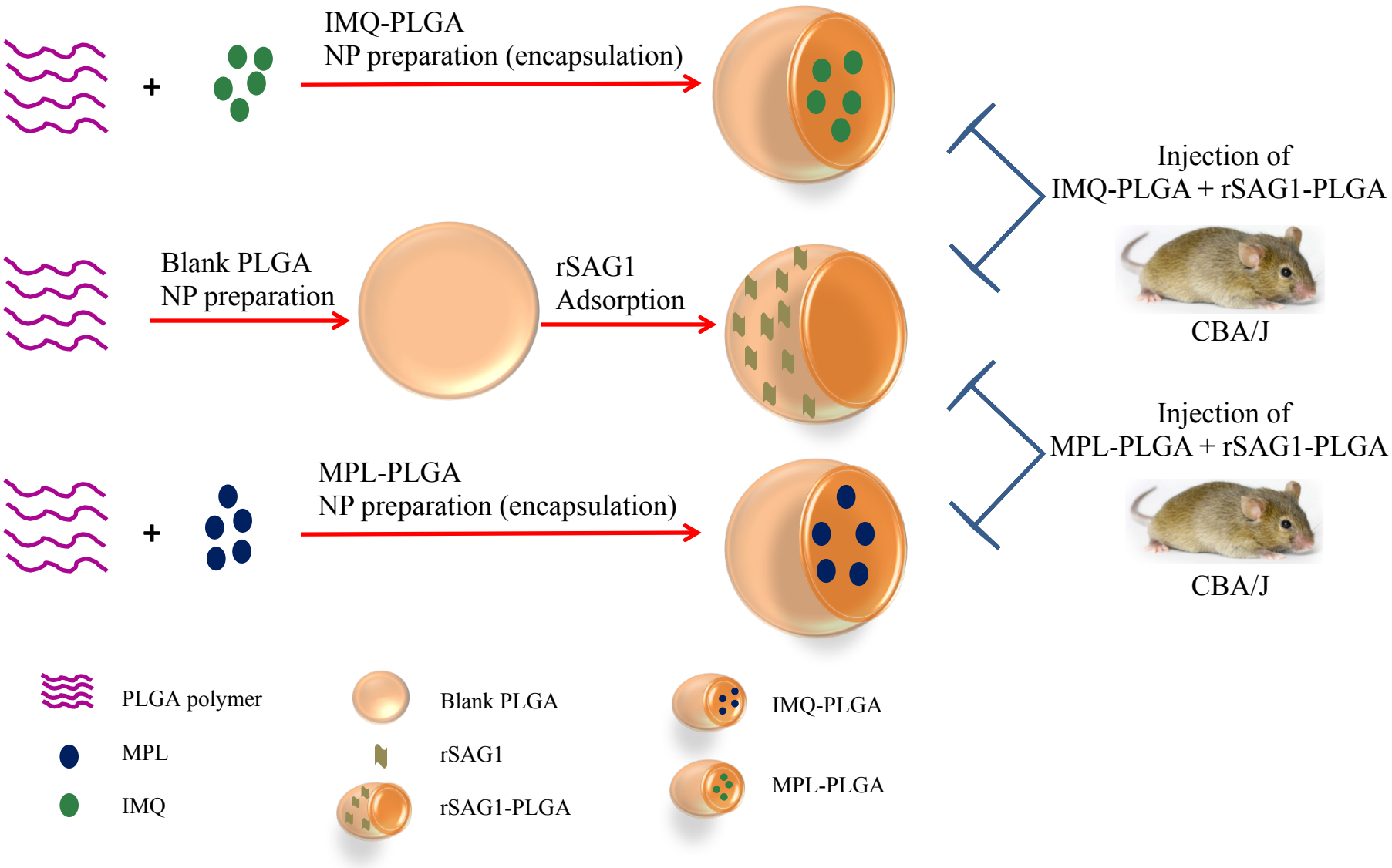
rSAG1-PLGA+IMQ-PLGA
rSAG1-PLGA+MPL-PLGA
MPL-PLGA


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