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Research Paper

Development and assessment of a new cage-like particle adjuvant

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Abstract

Objectives To obtain and assess stable cage-like particles with low surface charge density, which can be prepared using a standardized, economic and scalable method.

Methods To form these nanoparticles, the lipid composition and proportion as well the method were modified in relation to cage-like particles previously described elsewhere. Bovine albumin was used to compare ISPA performance with that of other adjuvants in mice and to assess stability. Adjuvant efficacy was analysed using a mouse model of *Trypanosoma cruzi* infection, which shows protection against an intracellular infection that needs a strong cellular response.

Key findings The new particles were better in terms of level, kinetics and profile of humoral responses than Freund Adjuvant, aluminium hydroxide and Montanide TM ISA 206; they also tended to improve ISCOMATRIX™ performance. Particle size and adjuvant performance were conserved during the 6-month period assessed after preparation. In the model of *Trypanosoma cruzi* infection, mice immunized with ISPA and trans-sialidase developed high protection.

Conclusions The obtained nanoparticles were stable and outperformed the other assessed adjuvants in joining together the capacity of most adjuvants to enhance the immune response against specific antigen, to reduce the number of doses, to homogenize the response between individuals and to reach a balanced TH1/TH2 response.

Introduction

Adjuvants are used for a number of purposes, namely to enhance the immune response against vaccine antigens, to increase the number of individuals that respond, to reach homogenous responses in vaccinated individuals, to decrease the amount of antigen used, to reduce the number of vaccine doses and to direct a beneficial profile of the response.[1] This goal is achieved using molecules that stimulate Pattern Recognition Receptors (PRRs), using strategies that improve the antigen uptake and presentation by dendritic cells, by promoting antigen transport to draining lymph nodes or through depot formation.[2] In 1984, Morein and coworkers developed an adjuvant based on a lipid cage-like particle composed of Quillaja Saponaria, phospholipids (PL), cholesterol (CHOL) and hydrophobic antigens associated in a single nanostructure.[3] Cage-like particles composed of saponin, CHOL and PL are among the most promising new-generation adjuvants.[4] Moreover, their safety and tolerability in humans have been confirmed in a recent meta-analysis.[5]These particles trigger a balanced TH1 and TH2 response and stimulate a cytotoxic cellular response that is hardly obtained with other adjuvants.[4,6]

After their initial description, cage-like particles were found to be also functional when they are simply mixed with both hydrophilic and hydrophobic antigen before the inoculation.[4,7] This observation contributed to the expansion of the number and type of antigen formulations with these particles. However, a theoretical drawback for this

approach is that the glucuronic acid groups of the Quil-A glycosides confer high density of negative charge on particles, limiting the number of antigens that can be associated with the particles.[8] This topic may be important, as the delivery action of this particulate adjuvant would act in a synergic way with immunostimulant effect, allowing the transport and delivery of specific antigens to be more efficiently taken up by the antigen-presenting cells (APC).[6,9–11] Indeed, in general terms, the importance of the association between antigen and adjuvant has been widely shown to enhance the action of different kinds of antigens and adjuvants.[12] Accordingly, some authors have proposed increasing the surface charge of cage-like particles to improve antigen adsorption.[13,14] To modify the charge of these particles, positive phospholipids or cationic cholesterol derivative has been used. Positive charged or low surface charged density particles have been obtained using these approaches. Only one of these particles has been assessed in immunological studies and it has been shown that it could reproduce but not improve the performance of classical cage-like particles.[14] However, as suggested in that report, more extensive assessments including different antigens are necessary to confirm the potential benefit of using positive cage-like particles. Furthermore, these previous reports did not take into account the cost of reagents used for the particle preparation, the scalable feasibility of methods or the stability of the particles.

Several approaches to obtain anionic classical cage-like particles were described in the early 2000s, in a simple way and without using detergents, which are expensive and hard to clear.[15] [16,17] Although these reports made important contributions, many important aspects in vaccine production were not considered, such as cost of reagents and processes, physical stability and immunogenicity regarding storage time. Thus, the aim of this study was to obtain stable low surface charge density lipid cage-like particles that could be prepared using a standardized, economic and scalable method. Here, we developed a method to obtain cage-like particles of similar structure to classical ones but displaying low charge on the surface to promote the binding antigens that are mainly of negative charge. The developed method also takes into account the scalability of preparation steps by avoiding lipid film or dialysis, and the cost of reagents by avoiding expensive detergents. In addition, tocopherol, which was not previously used in these particles, was introduced as antioxidant and immunostimulant. The obtained particles were characterized in terms of physicochemical features and their immunostimulant properties were compared with those of a commercial empty cage-like particle (ISCOMATRIX™, Isconova) and three conventional adjuvants, using bovine albumin as model antigen. The level, kinetics, response homogeneity among individuals and profile of the humoral immune responses were evaluated. In addition, stability was assessed over time. Finally, the efficacy of the adjuvant was assessed in a formulation used to protect against an intracellular infection. In a previous work, we have described that trans-sialidase formulated with the commercial classical cage-like particle ISCOMATRIX™ (IMX) had a comparable protection to that obtained with viral or naked DNA delivery systems.[18] In this study, the same model was used to assess the new adjuvant performance.

Methods and Materials

Materials

Quil-A as lyophilized powder was acquired from Brenntag Biosector (Frederikssund. Denmark), egg-derived phosphatidylcholine/dipalmitoyl phosphatidylcholine (DPPC) was purchased from Lipoid GmbH, Cholesterol (CHOL) was purchased from Fluka, stearylamine (STEA) was purchased from Sigma-Aldrich and Tocopherol (TOCOP) was purchased from Sigma-Aldrich (Missouri, USA). The buffer used was sodium acetate (Merck, Darmstadt, Germany) 50 mM pH 5.5 or PBS (PO_4Na_2 (Merck, Darmstadt, Germany), $PO_4H_2Na_1$ (Merck, Darmstadt, Germany) 0.16 mM pH 7.4. acetate 50 mM pH 5,5 with a composition of 50 mM sodium acetate and the volume of acetic acid needed to reach the pH. Ethanol was purchased from Merck, Darmstadt, Germany.

For immunization, commercial adjuvants used were ISCOMATRIX™ (Isconova, Sweden), Freund Adjuvant (FA) (Sigma-Aldrich, USA), aluminium hydroxide (AH) (Alhydrogel™, USA) and Montanide™ ISA 206 VG w/o/w emulsion (ISA 206) (Seppic, France). Bovine serum albumin (BSA) (Sigma-Aldrich, USA) was employed as antigen in immunization assays. Mutated Trans-sialidase mTS was kindly provided by Dr. Nico Callewaert (Ghent University, Belgium).

Preparation of cage-like particles

To prepare 20 ml, the method was as follows: (i) 61 mg of DPPC and 29 mg of CHOL were dissolved in 2.3 ml of ethanol; (ii) 30 mg of STEA was dissolved in 1 ml of ethanol and 144 µl of this stock solution was added to DPPC and CHOL stock solution; (iii) then, 80 µl of 1% ethanolic stock solution of TOCOP was added as antioxidant; and (iv) the resulting solution was injected in of acetate buffer pH 5.5 at 65 °C. The final proportions of components in the formed liposomes were as follows DPPC: 0320% (4,35 mM), COL: 0.143% (3.70 mM), STEA: 0.0216% (0.8 mM) and TOCOP: 0.00074% (0.017 mM). The suspension was extruded with a 50-nm membrane pore. Then,

Quil-A saponin solution in acetate buffer was added to liposomes (6.5 mg/300ul per 1 ml of liposomes). The suspension was extruded five times with a 50-nm membrane pore. The final solution was sterilized with a 200-nm filter. These particles were named immunostimulant particles (ISPA).

Size and zeta potential

Average particle diameters were measured via dynamic light scattering (DLS). A photometer (Brookhaven Instruments Inc.) was employed, fitted with a vertically polarized He-Ne laser at 632.8 nm, and a digital correlator (Model BI-2000 AT). Measurements were taken at 30 °C in an aqueous medium and at a detection angle of 90°. *Z*-average diameters were calculated through the quadratic cumulants method. In addition, particle size distribution and its number average were estimated using a self-designed software, which requires numerically solving an inverse problem through a standard regularization technique. Zeta potential was measured with a Zetasizer Nano (Malvern Instruments). Structure of particles was analysed using electron microscopy (JEM 1200 EX Jeol microscope); for this purpose, a droplet of the suspension was placed on a grid coated with a carbon-reinforced formvar film. After 30 s, the excess fluid was removed by absorbing with filter paper and the grids stained with 2% (w/v) phosphotungstic acid pH 5.2 (with KOH)..

Mice

BALB/c female mice (6–8 weeks old) used in all experimental procedures were obtained from the Centro de Medicina Comparada, ICIVET-CONICET-UNL, Argentina. All protocols for animal studies were approved by the Animal Care & Use Committee (IACUC) according to Institutional guidelines (Acta n° 06/11-24/5/2011).

Immunization schedules

Six groups of BALB/c mice (n = 5/group) were used for the immunization assay. Mice were immunized by subcutaneous injection (sc) on days 0, 14 and 28 with 10 μg of BSA in 100 μl of PBS buffer alone (BSA group) or same quantities of BSA formulated in 100 ul of acetate buffer pH 5,5 with 3 μl of ISPA (BSA-ISPA) or BSA formulated in 100 μl of PBS buffer pH 7,4 with 10 μg of ISCOMATRIX™ (BSA-IMX), or 15% of aluminium hydroxide (BSA-AH), or 50% of Freund Adjuvant (BSA-FA) complete in the first dose and incomplete the second and third ones, or 50% of ISA 206 (BSA-I206) prepared as indicated by the manufacturer. Blood was collected before each immunization and 14 days after the last dose application to analyse specific antibodies. Three independent experiments were performed.

Specific IgG 1 and IgG2a antibody determination

Microtitre plates (Greiner Bio One) were coated with BSA (0.05 μg/well) in carbonate–bicarbonate buffer (0.05 M; pH 9.6), and blocked with PBS/milk (5%). Serum samples from immunized mice, diluted 1 : 1000 in PBS/milk (1%), were incubated by duplicate in coated wells. and then peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (1 : 10.000 dilution) was added as appropriate (Southern Biotechnology). Fifty microlitres of ready-to-use trimethylbenzidine (TMB, Invitrogen, USA) was added to wells and after 10 min of incubation, colorimetric reaction was stopped with 2 N sulfuric acid, and plates were read at 450 nm in an ELISA reader (Bio-Tek Instruments).

Delayed-type hypersensitivity (DTH) test

DTH test was performed by intradermal (id) challenge with 5 μg of BSA in 20 μl of PBS in the left footpads 14 days after completion of the immunization schedule with different adjuvants. The thickness of hind footpads was measured before and 48 h after the antigen injection with a Vernier calliper (Stronger). Results were expressed as the difference in thickness of footpads after and before the inoculation (ΔA).

Immune response evaluation using a murine model of *T. cruzi* infection

Two groups of BALB/c mice (n = 5/group) were used for the antibody assay and challenge. Mice were immunized by sc injection on days 0, 14 and 28 with 10 μg of mTS formulated with 3 μl of ISPA (mTS-ISPA) or with 10 μg of IMX (mTS-IMX). Blood was collected on days 7, 21 and 35 postimmunization to analyse specific antibodies.

IgG1 and IgG2a subclasses were determined by ELISA as previously described, but using mTS as coating antigen. DTH test was performed at day 14 after the last immunization, by administering 5 μg of mTS in 20 μl of PBS in the footpad of vaccinated mice and measuring the ΔA as described above.

In a separate experiment, groups of mice were immunized with mTS-ISPA or mTS-IMX according to the immunization schedule formerly described, but a control group was included, which was inoculated only with PBS. Two weeks after the last immunization, animals were challenged intraperitoneally (ip) with 1000 bloodstream trypomastigotes of *T. cruzi* Tulahuen strain. Survival was recorded daily during 100 days.

For IFN-γ detection upon *ex-vivo* stimulation, spleen cell culture was performed in complete RPMI 1640 (Gibco) at 1.106 cell/ml in a 48-well plate and stimulated with mTS (10 μg/ml) during 24 h. Subsequently, cells were incubated with 1 μg/ml of 12-myristate 13-acetate (PMA, Sigma-Aldrich), 0.5 μg/ml of ionomycin (Sigma-Aldrich) and brefeldin A (GolgiPlug, BD Biosciences) at 37 °C and 5% CO_2. After 4 h, cells were washed twice with PBS, incubated with anti-FcγIII/II receptor antibody for 30 min and stained with anti-CD8-APC-Cy7 and anti-CD4 FITC during 30 min. Then, cells were washed and re-suspended in fixation/permeabilization solution (eBiosciences) during 1 h, according to the manufacturer's instructions, and subsequently stained with PE-Cy7-conjugated anti-IFN-γ Ab (eBiosciences) in permeabilization buffer. Cell acquisition was performed on FACS ARIA II flow cytometer (Becton Dickinson, San Jose, CA, USA) operating FACSDiVa software (BD Biosciences). Living cells were gated on the basis of forward and side cell scatter. Data were analysed using DiVA software (BD) Biosciences, CA).

Statistical analysis

Statistical analyses were performed using GraphPad Instat 4.0 software (GraphPad, California, USA). Differences between groups were analysed by applying the nonparametric Kruskal–Wallis test, followed by Mann–Whitney *U*-test for comparisons between two groups. Mantel–Cox long-rank test was used to evaluate survival curves. Significance is indicated with (*) when $P < 0.05$ and with (**) when $P < 0.01$ compared between the indicated groups.

Results

A simple procedure was developed to prepare ISPA cage-like particles

A new method was developed by modifying the liposomal precursor method.[15] First, liposomes were obtained by the proposed method of ethanolic injection [19] adding an extruding step. Liposomes with *Z*-average diameters of 80.6 ± 1 and with positive surface charges were obtained (zeta potential = 40 mV and number average diameter = 50.1 nm). Then, the stock solution of Quil-A was added, and stable particles of *Z*-average diameter equal to 290 nm were obtained and further extruded to acquire typically *Z*-average diameters of 73.0 ± 1.5 nm with a number average diameter of 42.1 nm. The zeta potential was −1.96 mV. The expected cage-like structure of ISPA particles was confirmed by electron microscopy (Figure 1). The number average diameter observed in the micrography was lower than 50 nm. No other colloidal particles, such as liposomes, micelle ring-like or worm-like micelles, were observed.

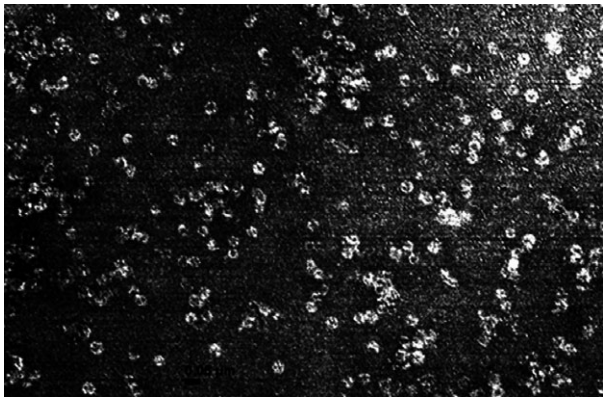

Figure 1 Transmission electron microscopy (TEM) of the obtained particles.

Immunostimulant behaviour of ISPA adjuvant is highly efficient compared to other adjuvants

To test the performance of ISPA as adjuvant, we carried out a mice immunization experiment using BSA as antigen and different adjuvants: IMX, AH, FA and ISA206. After finishing the vaccination schedule of three doses for each formulation, specific responses of IgG1 and IgG2a subclasses were determined and compared. IgG1 subclass was high for every formulation, except for AH and I206 (Figure 2a). For IgG2a, ISPA and IMX groups developed a stronger response than the other adjuvants ($P < 0.001$).

The analysis of the kinetics of the response during the vaccination schedule (Figures 2a and 2b) showed that specific IgG1 and IgG2a responses for ISPA and IMX developed earlier than for the other adjuvants (from the first dose, $P < 0.05$). In addition, both for IgG1 and IgG2a, the levels achieved with the second and third doses of ISPA were similar ($P > 0.05$), indicating that two doses may be enough to obtain an adequate humoral immune response with ISPA adjuvant. In addition, global IgG1 and IgG2a responses over time produced with the different adjuvants were compared through AUC (area under the curve) analysis (Table 1). Concerning IgG1, ISPA and IMX gave similar and significantly higher AUC values with respect to the other groups ($P < 0.01$). Regarding IgG2a subclass, only BSA-ISPA and BSA-IMX were able to elicit high levels and significant increases of this antibody subclass as compared with the other groups ($P < 0.01$) (Figure 2b). Although no statistical differences were detected between ISPA and IMX subclasses, a tendency to produce a higher antibody response was clearly observed in the former, which was more marked for IgG2a subclass. The analysis of the

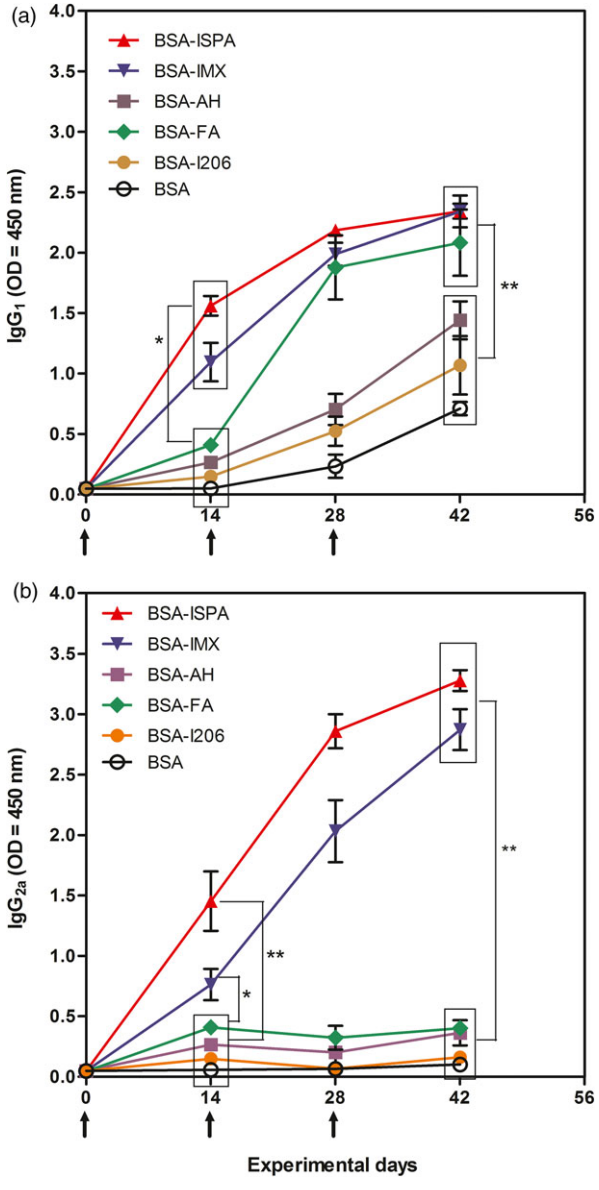

Figure 2 Kinetic curves of antibody production. Groups of BALB/c mice (n = 5) were immunized with three doses every 14 days (indicated with arrows in the abscissas axis) consisting of 10 μg BSA formulated with different adjuvants: BSA-ISPA, BSA-IMX, BSA-AH, BSA-FA or BSA-I206. The control group was inoculated with BSA alone. IgG[1] (a) and IgG2a (b) subclasses were determined in mouse serum before the application of each dose and 2 weeks after the last immunization. Mean O.Ds ± SEM are shown for each time. **The difference between groups in different times is indicated as *(P < 0,05) or ** (P < 0,01).

variation coefficient for the global response of each group of mice also showed that the homogeneity of the response was better for ISPA than for most adjuvants when considering both IgG1 and gG2a (Table 1 and 2). The immune response profile was estimated by analysing the IgG2a/IgG1 ratio. For ISPA and IMX groups, this ratio was >1, corresponding to a TH1 immune response profile. In contrast, the ratio was <1 for the other groups, suggesting a TH2 profile (Figure 2b). In agreement with these results, DTH assay (Figure 3) indicated that ISPA and IMX adjuvants elicited a similar inflammatory cellular response.

Adjuvant stability

To assess the stability of ISPA adjuvant, the size of particles and immunogenicity over time were determined at the initial time, and 2 and 6 months after a lot preparation. The sizes obtained by DLS measurement were 73.0 nm, 73.4 nm and 73.4 nm, respectively. Figure 4 and table 3 shows the IgG1 and IgG2 humoral responses obtained with the same lot of ISPA at the three times (0, 2 and 6 months). At the end of the immunization schedule, no significant difference was observed in the IgG1 levels induced by ISPA at different storage times. Nevertheless, the comparison of AUCs showed that curves obtained using ISPA stored during 2 or 6 months were slightly but significantly smaller than the one obtained immediately after manufacture (P < 0.05). For IgG2a, ISPA stored for 2 and 6 months showed significant decreases in the levels of these antibodies after 3 immunizations (P < 0.01) and also when comparing AUC curves corresponding to the whole response (P < 0.01). Interestingly, there was neither difference in final IgG2a levels nor in AUC curves between the ISPA stored for 2 and 6 months, indicating that there was no activity loss during this period. Notably, after the storage period, the immunogenicity of the ISPA adjuvant remained similar to that of IMX and still elicited higher specific IgG1 and IgG2a responses than the other groups of adjuvants.

The immune response elicited by ISPA adjuvant is highly protective in the framework of Trypanosoma cruzi infection used as intracellular infection model

As a strong cellular response is required to control intracellular pathogens, the most successful vaccine approaches evaluated to control *T. cruzi* infection have been based on gene immunization or recombinant viruses. However, in a previous work we described that the formulation of the *T. cruzi* antigen mTS with IMX adjuvant accomplished a protection as effective as genic or viral delivery system approaches.[18] Using mTS-IMX formulation, we achieved 100% survival after *T. cruzi* challenge eliciting a Th1 immune response profile that included CD8 + -activated T lymphocytes. Here we compared the performance of ISPA adjuvant with commercial IMX in this infection model. Mice were immunized with *T. cruzi* mTS formulated with ISPA or IMX. After a *T. cruzi* challenge, 100% of mice

Table 1 Areas under the curve (AUC) for IgG_1 production. AUCs were determined for the kinetics of IgG_1 production obtained for each adjuvant. The mean AUCs ± SEM and coefficient of variation (CV) are shown

	AUC of IgG_1					
	BSA-ISPA	BSA-IMX	BSA-AH	BSA-FA	BSA-I206	BSA
Mean±SEM	70.2 ± 2.2**	59.9 ± 3.7**	24,0 ± 2.9	47.0 ± 2.7	17.3 ± 2.6	2.8 ± 1.6
CV (%)	7.1	14	26.9	12.8	33.0	38.5

**Statistical differences compared with conventional adjuvants ($P < 0.01$).

Table 2 Areas under the curve (AUC) for IgG_{2a} production. AUCs were determined for the kinetics of IgG_{2a} production obtained for each adjuvant. The mean AUCs ± SEM and coefficient of variation (CV) are shown

	AUC of IgG_{2a}					
	BSA-ISPA	BSA-IMX	BSA-AH	BSA-FA	BSA-I206	BSA
Mean±SEM	83.7 ± 5.8**	59.6 ± 4.9**	9.5 ± 2.1	13,4 ± 1.8	nd	nd
CV (%)	15.5	18.3	49.1	29.7	–	–

**Statistical differences compared with conventional adjuvants ($P < 0.01$). nd: AUC was not determined because specific antibodies were not developed.

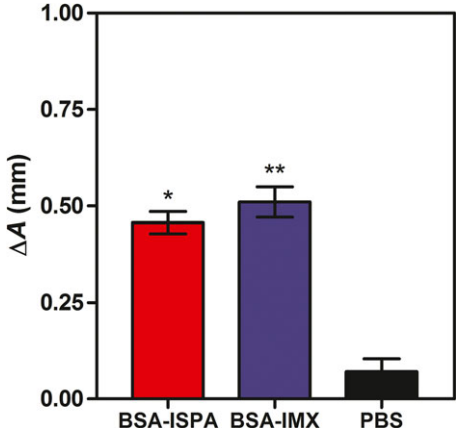

Figure 3 Delayed-type hypersensitivity reaction. Two weeks after completion of immunization schedule, mice were inoculated in the footpad with 5 μg of BSA. Footpad thickness was measured before and 48 h after inoculation and results are expressed as difference between both measures (ΔA). Mean ΔA ± SEM are shown. The difference of BSA-ISPA and BSA-IMX groups with PBS group is indicated as *($P < 0,05$) or ** ($P < 0,01$).

survived in both groups immunized with mTS-IMX or mTS-ISPA, whereas 40% of survival was recorded in control groups at day 30 after challenge (Figure 5a). Fifteen days after finishing the immunization schedule, immune parameters were assessed and compared. Anti-mTS IgG1 and IgG2a levels were similar in the groups immunized with mTS-ISPA and IMX mTS (Figure 5b). Furthermore, both adjuvants yielded an IgG2a/IgG1 ratio greater than 1, which is consistent with a TH1 response profile. This ratio was significantly higher in the ISPA group ($P < 0.05$). DTH assay was performed to determine specific cellular response. When the specific antigen mTS was inoculated in the footpads of mice, variations in footpad thickness were similar in both groups immunized with mTS-IMX and mTS-ISPA, being significantly increased with respect to the control group (Figure 5c).

Activation of CD4 + or CD8 + lymphocytes was analysed in mice vaccinated with the ISPA-TS formulation by determining INF-y in these cells. CD8 + splenocytes from ISPA-TS-vaccinated mice showed a higher percentage of IFN-y expression than mice vaccinated with mTS alone or PBS (Figure 6). Concerning CD4 $^{+}$ lymphocytes, an increased production of INF-y was also observed in vaccinated mice as compared with control groups, although the difference was not significant.

Discussion

Here, we describe a standardized, detergent-free and economic method for preparing cage-like particles with low surface charge density. The proposed methodology is scalable and allowed us to obtain stable particles. Previous works described that the proportion of cage-like particles in relation to other formed structures depends on the relationship among PL, CHOL and Quil-A, along with the method of preparation [17,20,21] Accordingly, to select the preparation method and conditions, it is crucial to establish the proportions necessary to obtain mainly cage-like particles. In our method, the ratio used allowed us to obtain cage-like particles without observing other structures. Our technique combines the advantages of the liposomal precursor method with the scalable alcoholic injection technique,[19] allowing homogeneous and stable particle dispersion with low density of surface charges. Tocopherol

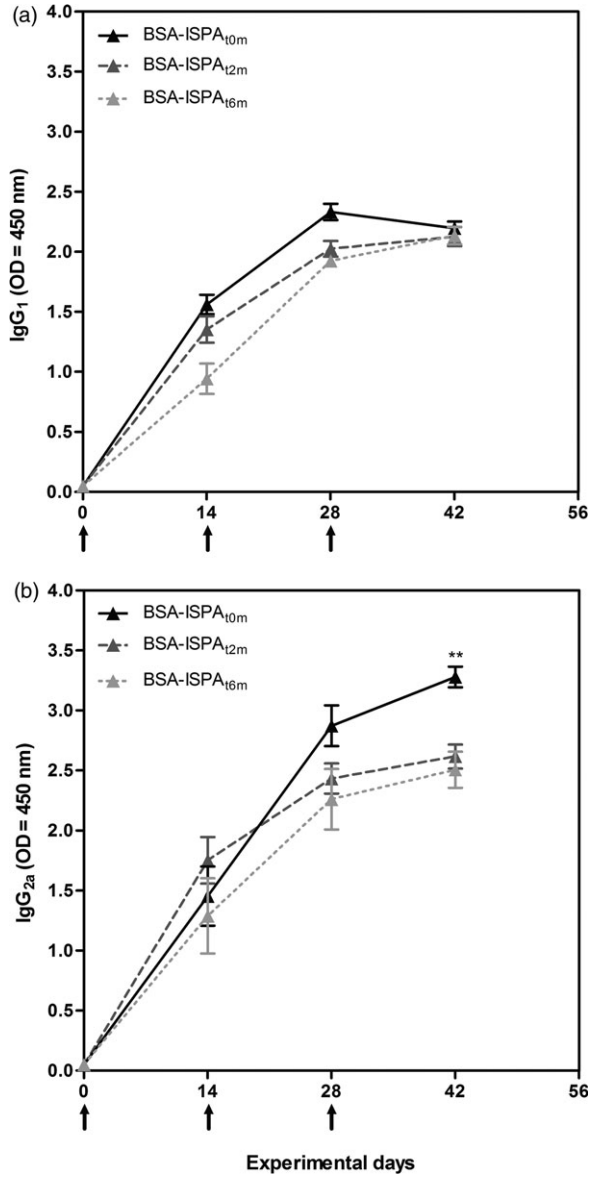

Figure 4 Stability of ISPA adjuvant over time. The same lot of ISPA was evaluated in mice immediately after preparation (BSA-ISPA$_{t0\ m}$), 2 months (BSA-ISPA$_{t2\ m}$) and 6 months (BSA-ISPA$_{t6\ m}$). Each experiment consisted of inoculation of three doses of BSA-ISPA every 14 days (indicated with arrows in abscissas axis). IgG$_1$ (a) and IgG$_{2A}$ (b) subclasses were monitored during the experimental days for obtaining the respective kinetic curves. Mean O.Ds ± SEM for each time are shown. **Significant differences compared with other groups ($P < 0.01$).

has not been previously used in the preparation of any cage-like particle. We added this lipid component to the particles, taking into account that this molecule is able to induce significant antibody titters.[22] As an additional benefit, this molecule is an antioxidant that improves conservation. Indeed, commercial adjuvants AS03 from Glaxo, an oil-in-water emulsion composed by squalene, has incorporated alpha tocopherol in the formulation as immunostimulant component.[23] In our preparation, this component did not alter the structure of cage-like particles, as demonstrated by electron micrography.

In previous reports, different strategies were proposed to increase the charge of cage-like particles. The cationic phospholipids N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate(DOTAP) and 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC cholesterol) have been incorporated to obtain partially or completely positive particles, respectively.[13,14] Only the latter strategy was immunologically assessed. In that study, although the adsorbed antigen in the particle increased, the immune response was not enhanced. Furthermore, the stability of the obtained particles was not studied. Here we propose a new method to increase the charge using STEA instead of DOTAP or DC-Chol, which are more expensive lipids.

In relation to the pH of the solution, pH = 5.5 was chosen taking into account that is a typical isoelectrical point for many proteins, and thus, antigen–particle interaction is favoured in that pH environment. As buffer, acetate was selected because it is an authorized solvent [24] and has been useful to obtain optimal and stable particles in our preliminary studies (data not shown).To assess the biological activity of the obtained adjuvant, BSA was chosen as antigen. Particularly for BSA, a pH = 4.5–5.5 has been reported to be very favourable for electrostatic interaction.[25] Acid treatment of BSA has been previously found to expose its hydrophobic regions, improving the interaction between the protein and ISCOM cage-like particles and, therefore, the immunological performance of the formulation.[26] However, this approach may alter the antigen. Here, we describe a specific high immunostimulant performance of BSA when this non-modified antigen is formulated with low density charged cage-like particles.

The suspension obtained with the proposed method was translucent, with a *Z*-average diameter of 73 nm and a number average diameter of 42.1 nm. The latter parameter was in agreement with transmission electronic micrography observations. The larger *Z*-average diameter is probably due to the fact that the sample is not strictly monodisperse and to changes in the particle–solvent interactions.[27] Notably, no mixed particles, such as worm-like micelles, ring-like micelles or cholesterol crystals, which are frequently obtained by other methods,[28] were observed in ISPA suspension.

Particle adjuvanticity was compared with other three widely used commercial adjuvants as well as with the classical IMX cage-like particles. The magnitude of the response of different antibody subclasses after different numbers of doses was determined. It is notable that the

Table 3 Areas under the curve (AUC) for IgG_1 and IgG_{2a} production. AUCs were determined for the kinetic curves of IgG_1 and IgG_{2a} production obtained for BSA-ISPA with different preparation times. The mean AUCs ± SEM and coefficient of variation (CV) are shown

	AUC of IgG1			AUC of IgG_2		
	BSA-ISPA$t_{0\ m}$	BSA-ISPA$t_{2\ m}$	BSA-ISPA$t_{6\ m}$	BSA-ISPA$t_{0\ m}$	BSA-ISPA$t_{2\ m}$	BSA-ISPA$t_{6\ m}$
Mean±SEM	70.2 ± 2.2*	62.5 ± 2.3	61.4 ± 1.9	70.6 ± 5.5**	65.9 ± 4.0	59.5 ± 3.8
CV (%)	7.1	8.6	8.0	15.6	11.7	15.0

Asterisks indicate statistical differences compared with BSA-ISPA$_{t6\ m}$. (*$P < 0.05$, **$P < 0.01$).

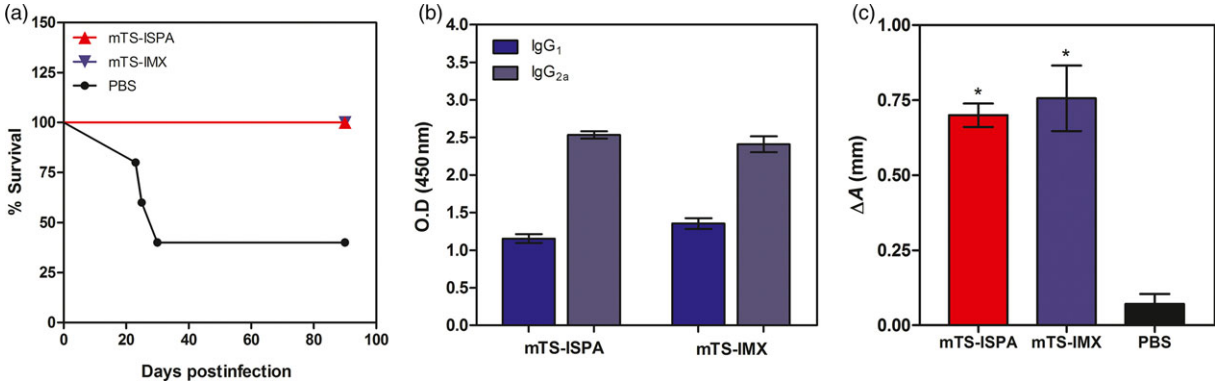

Figure 5 Evaluation of the cellular response evoked with ISPA in the framework of *T. cruzi* infection. Groups of mice were immunized with three doses s.c of 10 μg of mTS formulated with ISPA (mTS-ISPA) or IMX (mTS-IMX) and further challenged through i.p inoculation of 1000 trypomastigotes (a) IgG_1 and IgG_{2a} subclasses determined 7 days after last dose. (b) Delayed-type hypersensitivity reaction was performed 14 days after completion the immunization schedule. Footpad thickness was measured before and 48 h after inoculation of 5 μg of mTS and results are expressed as difference between both measures (ΔA). (c) Survival rates after mice challenge. Mean ± SEM are shown. *Significant differences with respect to the control group ($P < 0.05$).

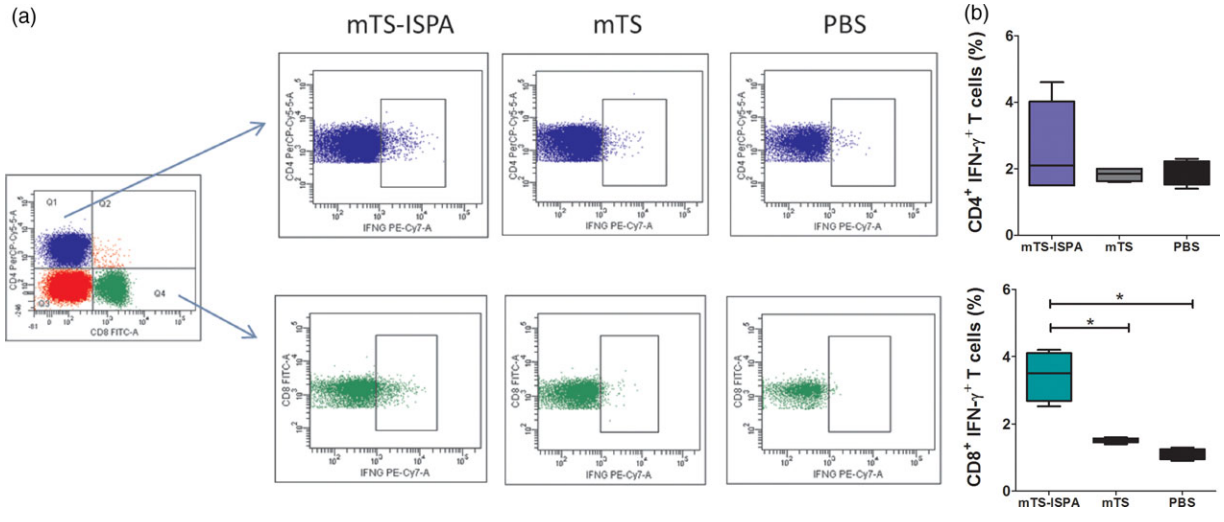

Figure 6 Specific activation of CD4 + or CD8 + lymphocytes of mTS-ISPA-immunized mice. Splenocytes were obtained 15 days after immunization and restimulated during 24 h *ex vivo* with TS to evaluated IFN-γ secretion by mTS-primed lymphocytes. After that, to improve IFN-γ detection in T lymphocytes, splenocytes were briefly cultured with a mix of PMA/brefeldin A/Ionomycin and afterwards were stained for CD4, CD8 and IFN-γ. (a) Representative dot plots illustrate IFN-γ intracellular staining profiles among CD4 + and CD8 + populations (square regions indicate CD4 + INFγ + or CD8 + INFγ + cells). (b) Frequency of IFN-γ production among splenic CD4 + (upper panel) and CD8 + (bottom panel) T cells restimulated with TS (n = 3–5/group). *$P < 0.05$.

obtained IgG1 levels were similar for most adjuvants. Conversely, IgG2a levels were much higher for IMX and ISPA. This result is consistent with the observation of a balanced immune response that is not obtained with other adjuvants, except for IMX. As expected, AH adjuvant directed mainly a TH2 antibody profile.[29] This

adjuvant has been used in veterinary and human vaccines for more than 70 years, being the most used adjuvant in human vaccines. AH has been safe and effective in vaccines designed to elicit mainly neutralizing antibodies. However, it does not trigger antibodies or cellular response with a TH1 profile needed to control intracellular pathogens.[29] Freund Adjuvant is considered one of the most powerful adjuvants to reach humoral and cellular responses, but its use is not allowed for human and even veterinary vaccines.[30] Notably, the response reached with FA was lower than that obtained with ISPA and IMX mainly for IgG2a response. I206 is a commercial emulsion-based adjuvant indicated by the manufacturer to formulate veterinary vaccines for pigs, sheep or cows. Only low levels of specific IgG1 antibodies were reached with this adjuvant. As expected, the use of this adjuvant improved the response in relation to non-adjuvanted formulation. These results indicate the usefulness of ISPA adjuvant to elicit both high IgG1 and IgG2a humoral response, which may be critical to reach immune protection against infections, when solely IgG1 neutralizing antibodies are inefficient. The comparison of these adjuvants in terms of the speed of the initial response, the number of doses required to reach high antibody levels and the homogeneity of the response obtained in different mice revealed an outstanding performance of ISPA adjuvant. Notably, a low dispersion of the response was obtained only with the ISPA adjuvant, a behaviour that is required to obtain uniform protection within a vaccinated population. The magnitude of the response obtained after each dose was also compared among the different adjuvants. Our formulation reached a similar response between the second and third doses, whereas the others increased their response significantly between the second and third doses. This indicates that the ISPA adjuvant has the potential to be effective when applied in a vaccination scheme of only two doses. However, this analysis should be performed for each particular antigen used for vaccine development formulation. Although the levels of IgG2a antibodies reached higher values with ISPA than with IMX, the comparison of the response after the first, second and third doses or the analysis of the whole response using AUC curve during the complete scheme of immunization showed that the difference was not significant. Taken together, these results show that ISPA particles comprise the particular behaviours required for an adjuvant, as they enhance the immune response against specific antigens, reduce the number of doses, homogenize the response among individuals and reach a balanced TH1/TH2 response. None of the other compared adjuvants has all these benefits.

In our experiment, although a remarkable humoral response was triggered, most parameters of this response were only slightly improved when the charge of the particle was changed. Whether this slight improvement in the response is due to the low charges or tocopherol composition remains to be elucidated. This analysis should involve other antigens to confirm this behaviour. Among the parameters that may account for ISPA efficiency, the influence of the low charge of ISPA remains to be fully determined. In a previous work, a net positive cage-like particle was developed using cationic cholesterol and the adjuvant performance was compared with classical ISCOM using ovoalbumin (OVA) as antigen.[14] These authors described that the cationic particles adsorbed more amounts of OVA and that vaccinated mice triggered more INF-γ from specific antigen restimulated $CD8^+$ cells than mice vaccinated with OVA but not with classical ISCOM. In addition, the levels of antibodies elicited by vaccination were lower with positive antibodies than with classical ISCOM. These results, along with those obtained in the present study, may indicate that protein adsorption to particles as well as charge of the particles may be important but not critical for the outstanding performance of cage-like particles. Indeed, the beneficial effect of using positively charged particles in adjuvants has been extensively evaluated using liposomes for this purpose and it has been widely described that positively charged liposomes are better adjuvants than negatively charged liposomes, as the negative nature of cell membranes allows particles to be highly taken up by cells.[31,32] As the liposomes and cage-like particles are likely to share their action mechanisms, future analysis with cage-like particles should be performed to clarify this topic.

Vaccine stability is a main concern for vaccine producers and regulatory agencies due to the natural decay in the potency during storage. Vaccine manufacturers have to define a shelf life during which a vaccine, if stored correctly, is expected to comply with the specification.[33] ISPA particles were stored at 4–8 °C, which is the storage condition that we propose to keep the suspension before use. We determined the size stability of the particles and the preservation of the specific antibody triggered. Interestingly, concerning IgG1 antibodies, the same response was reproduced over the storage period. For IgG2a levels, a slight but significant decrease was determined after the second month of the preparation, with ISPA stored for 2 or 6 months having the same response. When considering the AUC curves at time 0 and 6 months, a 15.72% decrease in the response of global antibodies was obtained. Notably, when compared with the levels of antibodies obtained with the other adjuvants, 6 months after ISPA preparation, IgG2a remained as high as the obtained for IMX and higher than levels obtained with the other adjuvants. Thus, the best immunological performance elicited by stored ISPA was conserved during the storage period, at least up to the sixth month of storage. The evaluation of a longer storage time is in

progress to determine the shelf life of the particles. The analysis performed allows us to foresee the utilization of the adjuvant to formulate vaccines.

Finally, ISPA adjuvancy was tested for the ability to protect against an intracellular pathogen using a mice model of *T cruzi* infection. In a recent work, we have described the efficacy of IMX™ to develop a vaccine against *T. cruzi*.[18] The present results indicate that ISPA is equally effective, as it generates a complete protective immune response against a challenge with *T. cruzi* parasites. We considered that an infection challenge is required to assess the adjuvant behaviour, as immunological parameters are only a partial aspect of the complex network developed during an effective immune response. Then parasitemia and survival experiments provide complete information about adjuvant efficacy, integrating all immunological mechanisms, including specific antibodies or cell response, which are necessary but not enough to fight against *T. cruzi* infection. Accordingly, both antibody response and cellular immune response contribute important, but not complete, information about possible mechanistic effects of the immune response triggered by the vaccine formulation. In this study, immunological parameters that have previously shown to correlate with *T. cruzi* protection were determined and compared between IMX and ISPA. The results show similar or even improved (although not significantly) values for ISPA. Particularly, mTS-ISPA immunization triggered a strong IFN-γ secretion by mTS-specific CD8 + T lymphocytes. This result is in agreement with the protection reached with mTS-ISPA formulation, considering that a cytotoxic cellular response is essential to protect against *T. cruzi*.[34] In particular, the activation of CD8 + cells with exogenous antigens needs the occurrence of cross-presentation after the antigen is taken up. It has been previously described that this mechanism is improved by ISCOM particles.[6,9–11] The complex of cage-like particles and antigen improves its endocytosis by the recognition of saponin glucidic residues by the receptor of dentritic cells DEC205 (CD205), by the binding to cholesterol membrane structure and by actin-dependent endocytosis. Once internalized, these complexes induce the maturation of APC and promote the translocation of the antigen to the cytosol, possibly via a pore formation to access the MHCI pathway.[6] As ISPA has a similar composition to IMX, it probably elicits the same antigen processing by the MHCII presentation pathway. This result together with the high specific antibody levels of TH1 profile correlates with the high protection that was obtained against the intracellular infection.

Conclusions

ISPA has many desirable behaviours for an adjuvant: (i) it elicits homogenous response in individuals of a population, (ii) it requires a lower number of doses, (iii) it promotes a balanced cellular/humoral response, and (iv) it increases the speed and magnitude of the primary response. In addition, stability of the formulation allows for long-term storage that is critical for vaccine production. The simple steps used for the adjuvant preparation, ethanolic injection, struding and direct mixing of components, are indicative of a highly scalable processes with low-cost components that reduce the cost of the final product. Overall, the results reported here indicate that ISPA is a very suitable adjuvant for vaccine development.

Declaration

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