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ORIGINAL ARTICLE

Immunization with antigenic extracts of *Leishmania* associated with Montanide ISA 763 adjuvant induces partial protection in BALB/c mice against *Leishmania (Leishmania) amazonensis* infection

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KEYWORDS
BALB/c;
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Background/Purpose: A proper adjuvant has a relevant role in vaccine formulations to generate an effective immune response. In this study, total *Leishmania* antigen (TLA) formulated with Montanide ISA 763 or R848 as adjuvants were evaluated as a first generation *Leishmania* vaccine in a murine model.
Methods: Immunization protocols were tested in BALB/c mice with a subcutaneous prime/boost regimen with an interval of 3 weeks. Mice immunized with unadjuvanted TLA and phosphate-buffered saline (PBS) served as control groups. On Day 21 and Day 36 of the protocol, we evaluated the humoral immune response induced by each formulation. Fifteen days after the boost, the immunized mice were challenged with 1×10^5 promastigotes of *Leishmania (Leishmania) amazonensis* in the right footpad (RFP). The progress of the infection was followed for 10 weeks; at the end of this period, histopathological studies were performed in the RFP.

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Results: Vaccines formulated with Montanide ISA 763 generated an increase in the production of immunoglobulin G (IgG; $p < 0.05$) compared with the control group. There were no statistically significant differences in IgG1 production between the study groups. However, immunization with TLA-Montanide ISA 763 resulted in an increase in IgG2a compared to the unadjuvanted control ($p < 0.001$). Also noteworthy was the fact that a significant reduction in swelling and histopathological damage of the RFP was recorded with the Montanide ISA 763 formulation.
Conclusion: We conclude that the immunization of BALB/c mice with a vaccine formulated with TLA and Montanide ISA 763 generated a protective immune response against *L. (L.) amazonensis*, characterized by an intense production of IgG2a.

Introduction

Leishmaniasis has a huge economic and public health impact on endemic areas.[1] Treatment of patients infected with *Leishmania* is based solely on the use of chemotherapy, which is costly and has dangerous side effects.[2] Currently, there are no preventive or therapeutic vaccines for any form of human leishmaniasis. Because of this urgent need, much effort is being devoted to the development of an effective vaccine.

The first vaccines tried against *Leishmania* used the whole killed parasite as antigen—this strategy was implemented from the 1940s to the present with variable results.[3] Currently, new vaccines using recombinant DNA technology are being developed, such as vaccines based on recombinant viral vectors expressing parasite proteins[4] or recombinant proteins produced in bacteria.[5] All of these strategies involve sophisticated and expensive production techniques that are difficult to implement in developing countries.

Given the ease of production and some encouraging results, first generation vaccines should still be considered a viable option.

In recent years, valuable basic information has been collected in this field that could certainly help to improve the design of such a strategy. A great deal of knowledge has been already accumulated on the type of immunity that should be induced in the vaccinated organism in order to obtain a protective response. Protection studies in murine models and analysis of the immune profile of self-healed individuals clearly indicate the need to induce in the vaccinated organism a T helper 1 (Th1) type response in order to obtain protection.[6] Also very important is the fact that new adjuvants have been developed that offer multiple ways to modulate the immune response according to specific requirements.[7,8]

Many adjuvants have been tested in either first or second generation experimental vaccines against leishmaniasis and many of these have been shown to induce a Th1 immune response.[9–13]

One adjuvant that has proven very effective is Montanide ISA 720, which is a mix of squalene and mannide oleate emulsifier, to be used in combination with antigen in a 70% V/V emulsion. Montanide ISA 720 has been used in experimental vaccines alone or in combination with other adjuvants with encouraging results.[14–17]

However, although much less toxic than mineral oil adjuvants, the reactogenicity of Montanide ISA 720 may be a cause for concern and it is currently under investigation. Undesirable reactogenicity in the form of sterile abscesses associated with the use of Montanide ISA 720 as adjuvant has been reported.[18–21] Also of concern is the fact that squalene induces chronic inflammatory arthritis in susceptible animal models.[22]

In this study, we tested the efficiency of the adjuvant Montanide ISA 763 in a first generation vaccine compared with the Toll-like receptor (TLR) 7/8 ligand R848, which has been shown to induce an enhanced Th1 response and protective immunity in mice following intradermal/subcutaneous vaccination.[10] Montanide ISA 763 is a biodegradable non-mineral oil adjuvant characterized by its low reactogenicity; it is indicated for bird and fish vaccines (www.seppic.com), but it has never been tried in *Leishmania* vaccines.

Methods

Animals

Inbred female BALB/c mice (8–9 weeks old) were used in this study. Three independent experiments were carried out with five mice per group. Mice were kept in standard conditions with barriers, a controlled light cycle, and controlled temperature. Food and water were provide *ad libitum*. All animals were cared for in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institutes of Health (NIH). All procedures were approved by the Institutional Animal Care and Use Committee of the School of Medical Science, Universidad Nacional de Cuyo (protocol approval no. 18/2013).

Parasites

The strain of *L. (L.) amazonensis* (MHOM/VE/84/MEL) used in this study was kindly provided by Dr Miguel Angel Basombrio, from the Experimental Pathology Institute, National University of Salta, Argentina. Promastigotes of *L.*

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(L.) amazonensis parasites were grown at 22°C in Novy-MacNeal-Nicolle medium (NNN) medium (Invitrogen) with Roswell Park Memorial Institute (RMPI) 1640 (Life Technologies, San Diego, CA, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Life Technologies, San Diego, CA, USA) and subcultured in the same medium after 48—72 hours. Infectivity was maintained by serial passage through mice.

Preparation of *Leishmania* antigens

Total *Leishmania* antigen (TLA) was prepared from late log-phase promastigotes of *L. (L.) amazonensis* after a few passages in liquid culture. Promastigotes were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS) and disrupted by six to eight cycles of freezing (−80°C) and thawing (56°C). A similar procedure was used to obtain soluble *Leishmania* antigen (SLA); after six to eight cycles of freezing and thawing, the suspension was centrifuged at 8000*g* for 20 minutes at 4°C and supernatant containing SLA was collected. Protein contents of the TLA and SLA were estimated by the bicinchoninic acid assay. The antigens were kept frozen at −70°C until use.

Adjuvants

Montanide ISA 763 (Seppic, Puteaux, France) and R848 (InvivoGen, San Diego, CA, USA) were used as adjuvants in this study.

Immunization

TLA (100 μg/mouse) was administered alone or formulated with Montanide ISA 763 or R848. Montanide ISA 763 was used as an oil-in-water emulsion (adjuvant/antigen ratio of 7:3) as per the manufacturer's instructions and administrated subcutaneously in a volume of 200 μL in the interscapular area of each mouse. R848 was prepared as a sterile stock solution (20 mg/mL) in PBS, and the dose used was 20 μg/mouse; this vaccine was formulated in a volume of 50 μL of PBS and administered subcutaneously in the interscapular area of each mouse. For booster vaccinations, mice received the same vaccine formulation at the same site 3 weeks after priming.

Infectious challenge

Mice were challenged with 1×10^5 stationary phase *L. (L.) amazonensis* promastigotes 15 days after the boost. Parasites were injected into the right footpad (RFP) in a volume of 50 μL. Lesion development was followed by weekly measurement of footpad swelling using a digital caliper (ED-10P; Schwyz).

Humoral response

The humoral immune responses induced by the experimental vaccine formulations were evaluated by measuring total specific IgG and IgG1 and IgG2a subtypes by enzyme-linked immunosorbent assay (ELISA) in serum samples collected on Days 0, 21, and 36 of the immunization protocol. Briefly, 96-well microtitration plates (MaxiSorp; Nalge-Nunc International, Pittsburgh, USA) were coated with 100 μL of SLA (3 μg/well) in PBS overnight at 4°C. Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS at room temperature for 1 hour. After washing with PBS containing 0.05% Tween-20 (Sigma Aldrich, St Louis, USA), the plates were incubated at 37°C for 1 hour with 1:100 dilutions of mice sera. The plates were then washed and incubated with horseradish peroxidase-conjugated goat antimouse IgG (Thermo Scientific, Pittsburgh, USA) diluted 1:10,000 for determination of total IgG, or with biotinylated antimouse IgG1 or IgG2a (BD Pharmingen, New Jersey, USA) diluted 1:1000 in blocking buffer. Finally, a color reaction was developed by the addition of 100 μL/well of substrate solution 3,3′,5,5′-tetramethylbenzidine supersensitive (Sigma Aldrich, St Louis, USA) for 30 minutes. Absorbance was determined at 450 nm using ELISA plate reader (Multiskan EX; Thermo Scientific, Pittsburgh, USA).

Histopathological analysis

Fragments of footpad lesion were fixed in Bouin's 4% solution and embedded in paraffin. Sections of 5—6 μm were stained with hematoxylin and eosin (H&E) for histopathological analysis. Images were taken with a Nikon Eclipse E200 Microscope (Nikon Corporation, Japan) fitted with a digital still camera Micrometric SE Premium (Nikon Corporation). Histological damage was calculated from observation of 10 different fields (40× magnification) of H&E-stained sections from each animal. The histopathological score grading system used to evaluate the degree of inflammation was as follows: 0, none; 1, slight infiltration of inflammatory cells; 2, moderate infiltration; 3, severe infiltration.[23] The total score was defined as the sum of all the scores. Each slide was scored by two independent observers and the average score was used.

Statistical analysis

Differences between groups were tested for significance by one- and two-way analysis of variance (ANOVA) followed by Tukey's post-test using GraphPad Prism version 5.01 for Windows (GraphPad Software, California, USA). A p value <0.05 was considered statistically significant. Data shown represent the mean values ± standard error of the mean (SEM) of three independent experiments.

Results

Specific antibodies generated after immunization

BALB/c mice were injected twice by the subcutaneous route at 3-week intervals as indicated above with 100 μg of TLA formulated with Montanide ISA 763 or R848. The levels of anti-*Leishmania*-specific total IgG as determined by ELISA are shown as optical density (OD) values of a 1:100 serum dilution.

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At 21 days and 36 days after the prime immunization, the three experimental vaccine formulations induced seroconversion in all immunized mice. However, the OD values of total IgG induced by TLA formulated with Montanide ISA 763 were greater than those obtained by immunization with TLA or TLA-R848, and showed a marked booster effect on day 36 (Fig. 1).

Regarding the analysis of IgG subtypes induced by the different formulations, there was no significant difference in the production of the IgG1 subtype between all analyzed groups (Fig. 2A).

However, as can be seen in Fig. 2B, the analysis of the IgG2a subtype shows that the group of mice immunized with TLA-Montanide reached values significantly higher than those obtained in the groups of mice immunized with TLA or TLA-R848 ($p < 0.05$).

In accordance with these data, the IgG2a/IgG1 ratio (Fig. 2C) is higher in the TLA-Montanide group (1.14) than in the TLA control (0.15) or TLA-R848 group (0.25).

The difference in magnitude of the total IgG OD values compared with the anti-TLA values of the subtypes IgG1 and IgG2a is likely due to use of different antimouse peroxidase conjugated.

Protection assay of mice immunized with TLA-Montanide or TLA-R848 against challenge with *L. (L.) amazonensis*

The next step in this study was to evaluate if the immune response induced in these groups of vaccinated mice was able to protect the mice against a challenge of live parasites. Fifteen days after the booster, experimental groups were challenged with 1×10^5 *L. (L.) amazonensis* promastigotes and the level of infection was monitored for 10 weeks by measuring footpad swelling. As shown in Fig. 3, immunization with TLA in the absence of an adjuvant provided no protection against infection, resulting in swelling levels similar to those nonvaccinated mice injected with PBS. The mice in the group vaccinated with TLA-R848 showed no significantly greater protection than that obtained in mice vaccinated with TLA without adjuvant. However, the mice in the group vaccinated with TLA-Montanide showed a significant reduction in the size of the lesion in the footpad. The differences in footpad thicknesses between the group receiving TLA alone and the group receiving TLA plus Montanide ISA 763 became statistically significant ($p < 0.05$) by week 8 after the challenge.

Thus we can conclude that TLA formulated with Montanide ISA 763 provided a partial but significant level of protection.

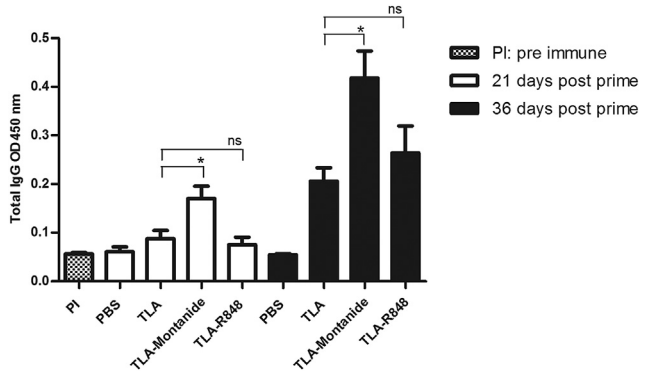

Figure 1. Antileishmania IgG antibody responses to *Leishmania (Leishmania) amazonensis* antigen in vaccinated animals. Animals were vaccinated at two different time points with PBS (control nonvaccinated), TLA alone, or TLA in conjunction with Montanide ISA 763 (TLA-Montanide) or R848 (TLA-R848). Antibody levels were determined on Day 21 and Day 36 postpriming. Data shown indicate mean IgG OD values plus SEM in each vaccination group. * Significant differences in comparison with control group, $p < 0.05$. IgG = immunoglobulin G; ns = not significant; OD = optical density; PBS = phosphate buffered saline; SEM = standard error of the mean; TLA = total *Leishmania* antigen.

Histopathology analysis of the tissue damage induced after challenge of the vaccinated mice with *L. (L.) amazonensis*

Ten weeks postchallenge a histopathological analysis of the footpad lesions was performed in order to identify microscopic features of the lesions in all groups. Microscopic analysis of PBS, TLA, and TLA-R848 groups (Figs. 4A–C) showed an intense inflammatory response characterized by severe local inflammation with intense tissue destruction of the dermis and epidermis. In these groups, extensive collections of vacuolated and heavily parasitized macrophages were observed. Amastigote forms were seen attached to the wall of the parasitophorous vacuoles (Fig. 4F); the number of parasitized macrophages was increased and extensive areas of necrosis were observed. In these areas of necrosis, free amastigote parasites were seen among inflammatory cells. Occasionally, areas of necrosis were infiltrated by granulocytes, giving rise to micro-abscesses (Fig. 4G).

In the TLA-Montanide-vaccinated mice, the presence of a less infected and less vacuolated inflammatory cell population was observed (Fig. 4D). No epidermis destruction was observed.

Histological changes in the lesions were semi-quantitatively graded based on the criteria described by Côrtes et al.[23] A significant decrease in the histological score index resulted from the analysis of the degree of inflammation in the footpad lesion of the TLA-Montanide group compared to the control (Fig. 5). These results further confirm the significant level of protection offered by the vaccination of mice with TLA-Montanide.

Discussion

For many years, many laboratories around the world have worked on the development of an effective vaccine against leishmaniasis. Early attempts (first generation vaccines) used vaccines formulated with whole parasite antigens as a source of antigen. Comprehensive analysis of the clinical trials conducted in humans using this strategy showed that encouraging as well as inconsistent results were obtained.[24] Infecting patients with attenuated strains of *Leishmania* was another strategy, and this gave positive results in some cases.[25]

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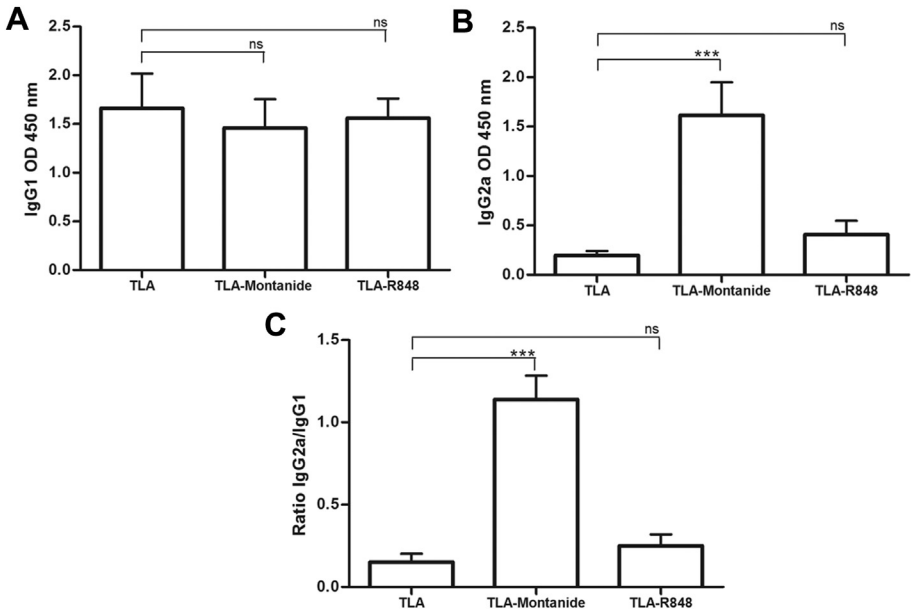

Figure 2. Antileishmania IgG subtype antibody responses to *Leishmania (Leishmania) amazonensis* antigen in vaccinated animals. Antibody levels were determined 36 days after the prime immunization. (A) IgG1 subtype; (B) IgG2a subtype; (C) IgG2a/IgG1 ratio. Data shown indicate mean IgG subtype OD values and ratio plus SEM in each vaccination group. *** Significant differences in comparison with control group, $p < 0.001$. IgG = immunoglobulin G; ns = not significant; OD = optical density; SEM = standard error of the mean.

With the advent of recombinant DNA technology, many new strategies for the development of a vaccine are available. Under this technology, vaccines based on purified recombinant proteins of the parasite, recombinant viral vectors, and genetic vaccines have been developed.[4,5,26] However, these technologies involve production methods that are highly sophisticated and are expensive to implement in many endemics areas, implying that the provision of vaccines for these areas would rely on an external source.

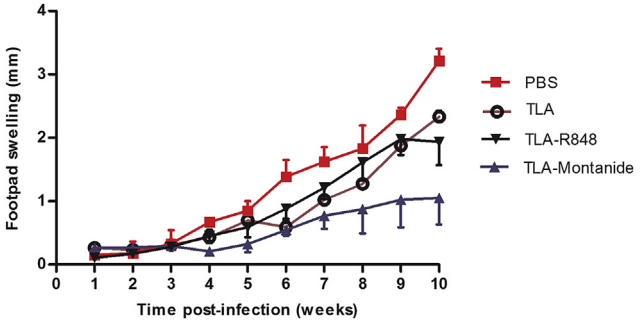

Figure 3. Footpad swelling caused by challenge with infective promastigotes of *Leishmania (Leishmania) amazonensis* in BALB/c mice vaccinated with PBS (control nonvaccinated), TLA alone, or TLA in conjunction with Montanide ISA 763 (TLA-Montanide) or R848 (TLA-R848). Fifteen days after the boost, mice were challenged in the footpad with 1×10^5 promastigotes of *L. (L.) amazonensis* (MHOM/VE/84/MEL). Following challenge, the mice were monitored every week for 10 weeks by measurement of lesion swelling using a digital caliper. Footpad swelling is given as means plus SEM. PBS = phosphate buffered saline; SEM = standard error of the mean; TLA = total *Leishmania* antigen.

In an early development, the group of Mayrink prepared a vaccine against cutaneous leishmaniasis utilizing whole parasite antigens obtained from stocks derived from patients.[27] Later, the same group developed a vaccine based on *L. (L.) amazonensis* that was effective in preventing the disease, and when administered in combination with antimonium salts, demonstrated immunotherapeutic activity.[28] In a recent contribution, the same group[29] showed in a clustered, randomized clinical trial a significant reduction in the number of cases of American cutaneous leishmaniasis in a group vaccinated with a nonadjuvanted first generation vaccine obtained from *L. (L.) amazonensis* compared with the placebo group.

A vaccine developed by Convit's group[30] in Venezuela using whole antigenic extracts combined with Bacillus Calmette–Guérin (BCG) as adjuvant administered to patients with American cutaneous leishmaniasis had an efficacy of 91.2–98.7% clinical remission. The use of BCG as adjuvant is based on the known fact that when it is coadministered with antigen, it induces a strong immune response with a Th1 profile.

Analysis of those human trials that have yielded positive results indicates that there is still the possibility of developing a vaccine formulated with parasite extracts. For this goal to be successful it will be essential to formulate the vaccine with the appropriate adjuvant. Analysis of the data obtained in the murine model as well as analysis of the clinical course of infected patients clearly indicates the importance of the development of Th1 immunity to protect organisms from infection.[6]

In the present study, we have investigated two first generation vaccine formulations containing whole *L. (L.)*

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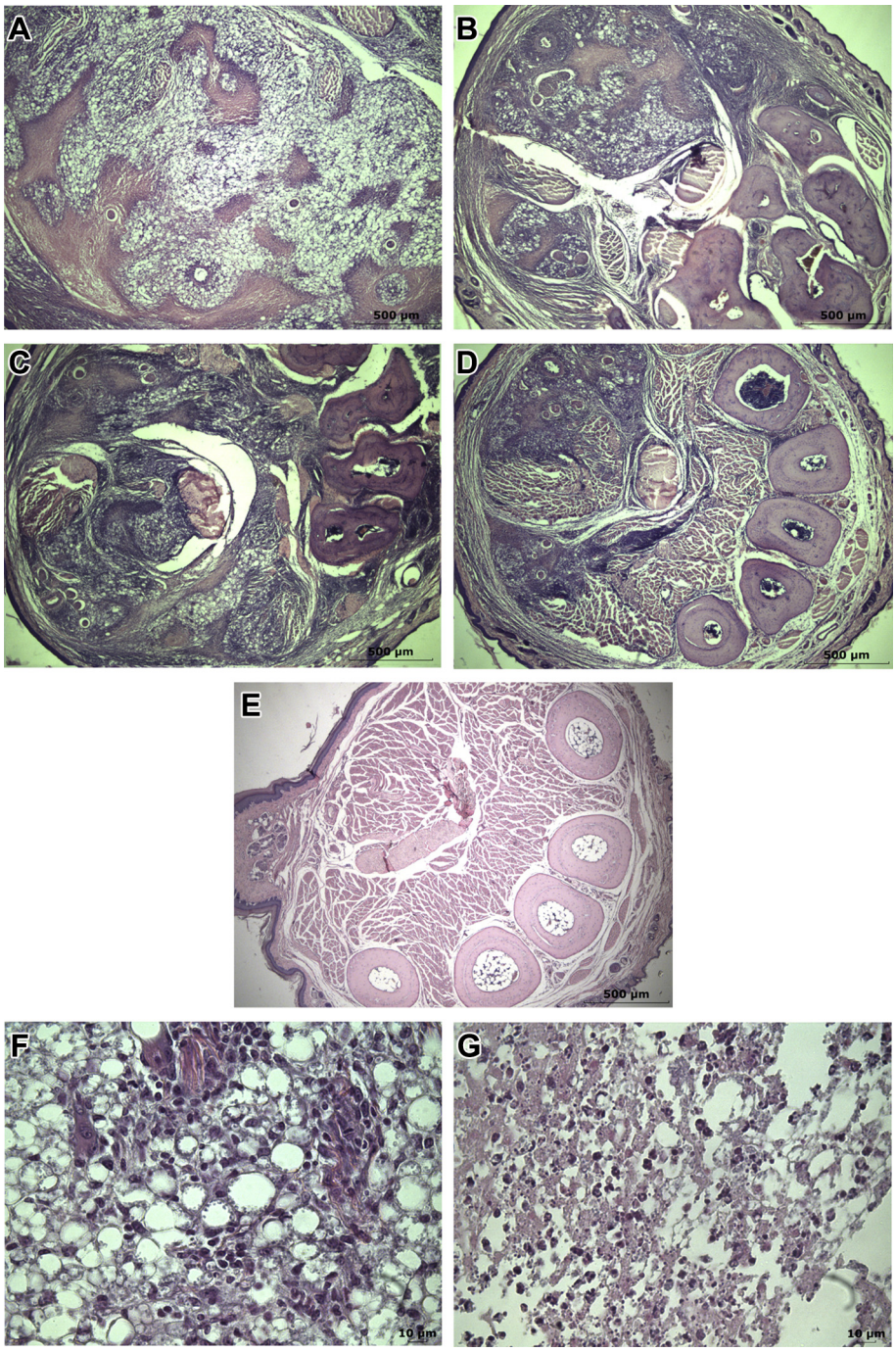

Figure 4. Histological outcome in the footpad infection site of the different vaccinated groups after 10 weeks of the challenge. (A) Mice vaccinated with PBS (control nonvaccinated). (B) Mice vaccinated with TLA. (C) Mice vaccinated with TLA formulated with R848 (TLA-R848). (D) Mice vaccinated with TLA formulated with Montanide ISA 763 (TLA-Montanide). (E) The histological outcome of noninfected mice was used as an indicator of the normal histoarchitecture of the footpad. (F) Amastigote forms attached to the wall of the parasitophorous vacuoles and (G) necrosis areas infiltrated by granulocytes were seen in groups PBS, TLA, and TLA-R848. Figures are representative of three animals analyzed in each vaccinated group. PBS = phosphate buffered saline; TLA = total *Leishmania* antigen.

amazonensis antigenic extract formulated with a TLR7/8 agonist or with Montanide ISA 763 as adjuvants. The efficacy of these vaccines was evaluated by the level of protection generated in susceptible BALB/c mice after challenge with live parasites.

The results of this work demonstrate that the subcutaneous administration of TLA formulated with Montanide ISA 763 generates a protective immune response against infection by *L. (L.) amazonensis* in BALB/c, characterized by a high production of the IgG2a subtype with a low

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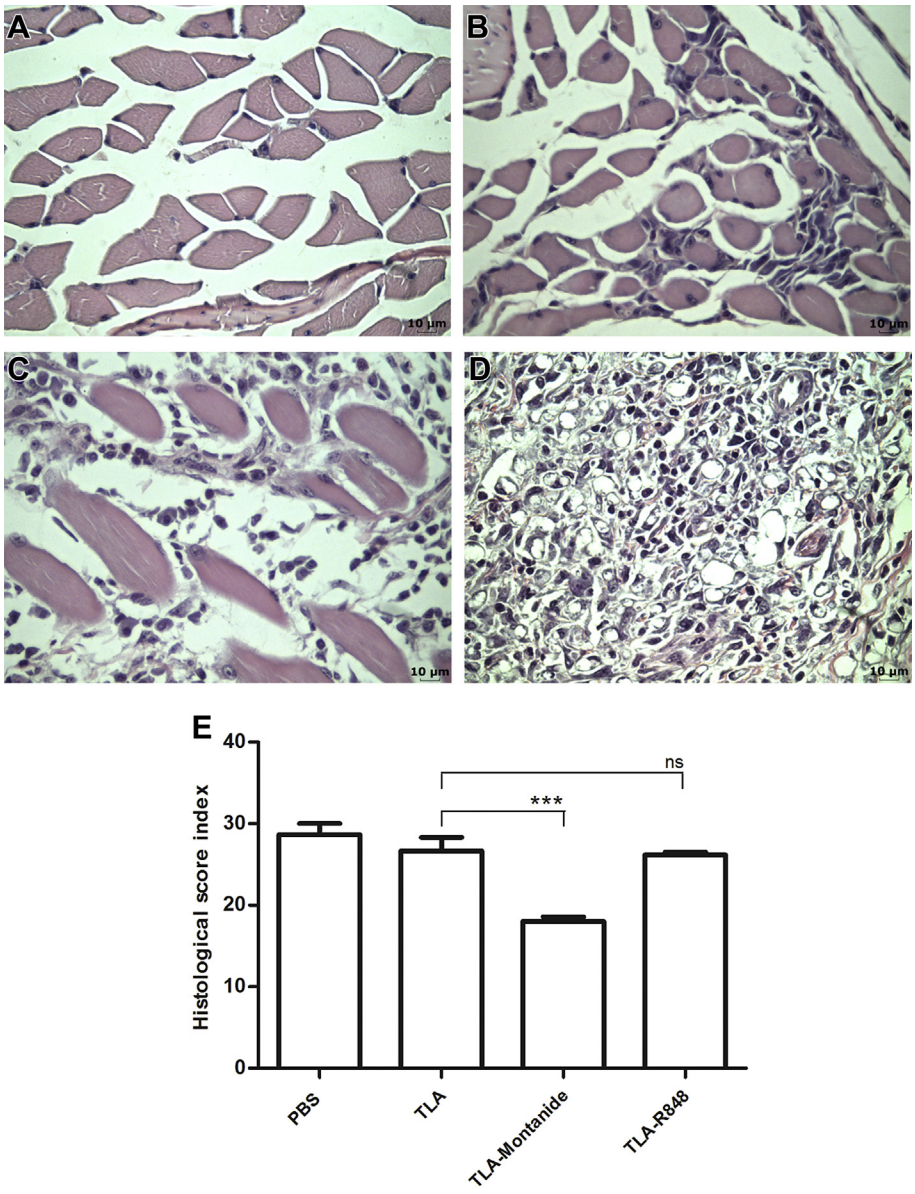

Figure 5. Histopathological indexes of the footpad infection site of the different vaccinated groups after 10 weeks of the challenge. Histopathological damage scores used to evaluate the degree of inflammation were: (A) none; (B) slight infiltration of inflammatory cells; (C) moderate infiltration; (D) severe infiltration. Panel (E) shows the score indexes of the different groups calculated after 10 weeks of the challenge. Data shown indicate mean values plus SEM in each vaccination group. *** Significant differences in comparison with control group, $p < 0.001$. ns = not significant; PBS = phosphate buffered saline; TLA = total *Leishmania* antigen.

production of IgG1 (Figs. 2A and B) and a IgG2a/IgG1 ratio greater than 1.

Because IgG2a and IgG1 have been used as indicators of the induction of Th1 and Th2 responses, respectively, the IgG2a/IgG1 ratio can help to define the T cell phenotype induced by vaccination.[31–33] Thus, IgG2a/IgG1 ratios were used as indicators of Th1- or Th2-based responses induced by immunization.

It is interesting to note that the TLA-Montanide formulation was able to induce a high IgG2a/IgG1 ratio (Fig. 2C) and protection of mice against experimental challenge with *L. (L.) amazonensis*, while the vaccine formulated with R848 induced much higher IgG1 than IgG2a subtype and failed to generate protection in infected mice (Fig. 3).

The vaccine formulated with R848 did not generate protective immunity against the challenge with *L. (L.) amazonensis* and produced low levels of IgG2a (Fig. 2B) and an IgG2a/IgG1 ratio lower than one (Fig. 2C). This result might disagree with the work of Zhang and Matlashewski[10], which demonstrated in a murine model of infection with *L. major* that immunization in the footpad with TLA-R848 protects mice from infectious challenge, while the same formulation administered intramuscularly did not protect against infection.

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However, it should be taken into account that it is well known that the route of immunization has a significant influence on the effectiveness of a vaccine. In the case of the work of Zhang and Matlashewski[10], the delivery of the vaccine was by injection in the footpad, which is one of the most frequently used methods in murine models for evaluation of experimental vaccine formulations against *Leishmania*. This route of vaccine delivery is assumed to be a mixed subcutaneous and intradermal route.[34] However, in our case the vaccines were administered in the mouse interscapular area, which is considered a purely subcutaneous route,[35] while in the work of Zhang and Matlashewski,[10] the TLA-R848 was effective when it was injected in the footpad, which, as mentioned above, is considered a combination of the subcutaneous and intradermal route. We believe that the different routes used for the administration of vaccines could explain the different results obtained in our work compared to those of Zhang and Matlashewsky.[10]

In conclusion, Montanide ISA 763 adjuvant appears to be a promising candidate for the development of a new *Leishmania* vaccine due to its safety and ability to induce a protective immune response, characterized by a high production of the IgG2a subtype with a low production of IgG1, and skewing the immune response toward a Th1 profile when it is given by the subcutaneous route. It would be important to carry out further investigations on the potential use of Montanide ISA 763 as an adjuvant for *Leishmania* vaccines in nonhuman primate models. Such studies would provide a guide for the design of vaccine formulations for clinical trials.

Based on the results shown in this study in terms of the quality of the immune response obtained with a vaccine formulated with Montanide ISA 763 and the fact that the vaccine confers partial protection against a challenge of living parasites, we suggest that Montanide ISA 763 should be considered as a candidate adjuvant for the development of a new vaccine against *Leishmania*.

Conflicts of interest

All authors declare no conflicts of interest.

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