Total Leishmania antigens with Poly(I:C) induce Th1 protective response

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Summary
Our proposal was to develop a vaccine based on total Leishmania antigens (TLA) adjuvanted with polyinosinic-polycytidylic acid [Poly(I:C)] able to induce a Th1 response which can provide protection against Leishmania infection. Mice were vaccinated with two doses of TLA-Poly(I:C) administered by subcutaneous route at 3-week interval. Humoral and cellular immune responses induced by the immunization were measured. The protective efficacy of the vaccine was evaluated by challenging mice with infective promastigotes of Leishmania (Leishmania) amazonensis into the footpad. Mice vaccinated with TLA-Poly(I:C) showed a high anti-Leishmania IgG titre, as well as increased IgG1 and IgG2a subclass titres compared with mice vaccinated with the TLA alone. The high IgG2a indicated a Th1 bias response induced by the TLA-Poly(I:C) immunization. Accordingly, the cellular immune response elicited by the formulation was characterized by an increased production of IFN-γ and no significant production of IL-4. The TLA-Poly(I:C) immunization elicited good protection, which was associated with decreased footpad swelling, a lower parasite load and a reduced histopathological alteration in the footpad. Our findings demonstrate a promising vaccine against cutaneous leishmaniasis that is relatively economic and easy to develop and which should be taken into account for preventing leishmaniasis in developing countries.

KEYWORDS
adjuvant, leishmaniasis, parasite, vaccination

1 | INTRODUCTION
Leishmaniasis is one of the most important neglected diseases which mainly affect the poorest populations with limited access to health care, located mainly in developing countries.1 Unfortunately, there is no available vaccine to prevent this disease in humans.2

First-generation vaccines are proposed as an economically affordable strategy to prevent leishmaniasis. In such formulations, the
antigens are obtained as a parasite lysate. A clinical trial conducted by Convit et al.\(^2\) showed that a vaccine based on total antigens derived from promastigotes of *Leishmania (Viannia) braziliensis* killed by pasteurization and formulated with Bacillus Calmette-Guérin (BCG) as an adjuvant was safe and effective for treating leishmaniasis. In 2013, Mayrink et al.\(^4\) evaluated the efficacy and safety of a first-generation vaccine based on total antigens of *L. (L.) amazonensis* without adjuvant. That work demonstrated a significant reduction in the incidence of the disease in vaccinated people, as compared to the placebo group. The study also showed that a vaccine formulation based on a single-species antigen of the genus *Leishmania* (single-species vaccine) can reduce the incidence of cutaneous leishmaniasis in the endemic area.

Protection studies in *Leishmania*-murine models and the analysis of the immune profile of self-healed individuals clearly indicate the need to induce a Th1 type response in order to obtain protection.\(^5,6\) New adjuvants have been developed, offering multiple ways to modulate the immune response according to specific requirements.\(^7\)

Natural and synthetic double-stranded RNA (dsRNA), such as the synthetic Poly(I:C) adjuvant, act as toll-like receptor 3 agonist and induce the production of type I interferons (IFN) and other cytokines.\(^8,9\) Poly(I:C) and other synthetic derivatives have been evaluated in animal and human clinical trials, demonstrating good efficacy and safety profiles.\(^10,12\)

In this study, we investigated the potential adjuvant property of Poly(I:C) in a first-generation vaccine formulated with *L. (L.) amazonensis* antigenic extract. We evaluated the ability of this formulation to mediate a Th1 immune response and to provide protective immunity against *L. (L.) amazonensis* infection in BALB/c mice. To the best of our knowledge, we demonstrate for the first time the use of Poly(I:C) as an effective adjuvant in a first-generation *Leishmania* vaccine.

## METHODS

### 2.1 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. All procedures were approved by the institutional animal care unit of Universidad Nacional de Cuyo (Protocol no. 80/2016).

### 2.2 Parasites and antigenic *Leishmania* extract preparation

Promastigotes of *L. (L.) amazonensis* (MHOM/VE/84/MEL) were grown and their infectivity was maintained by serial passage through mice, as described previously.\(^13\) For the preparation of *Leishmania* extract, total *Leishmania* antigens (TLA) were obtained from promastigotes of *L. (L.) amazonensis* in late logarithmic phase. After that, promastigotes were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS) and then disrupted by six to eight cycles of freezing (−80°C) and thawing (56°C).\(^13\)

### 2.3 Formulation and immunization schedule

Mice received TLA alone (100 μg/mouse) (TLA) or formulated with high molecular weight Poly(I:C) (50 μg/mouse) (InvivoGen, San Diego, CA, USA) [TLA-Poly(I:C)]. Negative control groups received PBS and Poly(I:C) alone by subcutaneous route. For booster vaccination, mice received the same vaccine formulation three weeks after priming.

### 2.4 Parasite challenge

Immunized mice were challenged 15 days after the boost. The challenge was performed with \(1 \times 10^5\) of *L. (L.) amazonensis* promastigotes, which were injected into the right footpad (RFP). The infection development, represented as footpad swelling, was followed by measurement using a digital caliper during 11 weeks. The lesion size was calculated by subtracting the thickness value of the contralateral uninfected foot from that of the footpad thickness of the infected foot.\(^13\)

### 2.5 Measurement of humoral immune response

Humoral immune responses induced by vaccinations were evaluated in serum samples 15 days after the boost. Antigen-specific total IgG, IgG1 and IgG2a subtypes were evaluated by enzyme-linked immunosorbent assay (ELISA), as described previously.\(^13\) IgG titres are expressed as the reciprocal of highest serum dilution which yielded ELISA OD values two times the blank.

### 2.6 Preparation of splenocytes and measurement of cellular immune responses

Spleens were recollected aseptically from mice of each group 15 days after the boost. Splenocytes were resuspended in supplemented RPMI 1640 medium (Invitrogen). The cells were cultured in 96-well plates at a density of \(2 \times 10^5\) cells/well. The cells were stimulated in vitro with TLA (1 μg/well), and supernatants were collected after 72 hour of incubation at 37°C with 5% CO\(_2\). Measurements of IFN-γ and IL-4 concentrations in the supernatants were carried out using OptEIA Kits (BD Pharmingen).

### 2.7 Histopathological analysis

The fragments of footpad lesion were fixed in Bouin’s solution 4% and embedded in paraffin. Sections of 5–6 μm were stained with haematoxylin and eosin (H&E) for histopathological analysis. Images were taken with a Nikon Eclipse E200 Microscope (Nikon Corp., Japan) fitted with a Micrometric SE Premium (Nikon Corp., Japan) digital still camera. 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 was evaluated by the degree of inflammation as described by Côrtes DF.\textsuperscript{14} The total score was defined as the sum of all scores.

The parasite load in footpad lesions was performed as described by Rocha-Vieira et al.,\textsuperscript{15} counting the number of parasites per field in 10 noncontiguous fields (magnification 630×).

2.8 | Statistical analysis

Differences between groups were tested for significance by one- and two-way ANOVA followed by Tukey’s post-test using GraphPad Prism v.5.01 Software. \( P \)-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Humoral immune response elicited after immunization

BALB/c mice were injected twice by subcutaneous route with the different vaccines in a period of three weeks. Fifteen days after the boost, the total anti-
Leishmania IgG titre obtained by TLA-Poly(I:C) was greater than that obtained by immunization with TLA (Figure 1A). The IgG1 and IgG2a titres in mice immunized with TLA-Poly(I:C) reached values significantly higher than those obtained in the group of mice immunized with TLA \( (P < .001) \). The inclusion of Poly(I:C) to ATL resulted in an increase of 6-fold, 7-fold and 7.8-fold in IgG1 and IgG2a titres respectively, compared with TLA (Figure 1B).

In accordance with these data, the IgG2a/IgG1 ratio of the TLA-Poly(I:C) group was of 1, 42, polarizing the immune response towards a Th1 profile.

3.2 | Cellular immune response elicited after immunization

To study the T-helper cell cytokine profiles induced after vaccination, the main cytokines of Th1 (IFN-\( \gamma \)) and Th2 (IL-4) were determined in supernatants of spleen cells culture.

IFN-\( \gamma \) was detected in significantly higher levels in antigen-stimulated spleen cells of mice vaccinated with TLA-Poly(I:C), as compared to the levels of mice vaccinated with TLA without adjuvant (Figure 1C). There was no significant difference in the production of IL-4 among all analysed groups (Figure 1D). The high level of IFN-\( \gamma \) production by spleen cells in response to stimulation with \( L. (L.)\) amazonensis antigen indicates that a strong Th1 immune response was generated in mice immunized subcutaneously with TLA-Poly(I:C).

**FIGURE 1** Humoral immune responses: Anti-
Leishmania IgG and IgG subtypes antibodies response in mice vaccinated with two doses (at 3 weeks interval) of PBS, TLA, TLA-Poly(I:C) and Poly(I:C) alone. A, IgG total titre; B, IgG1 and IgG2a subclass titres. Cellular Immune responses: Cytokine levels of C, IFN-\( \gamma \) and D, IL-4 were determined in splenocytes culture in vitro, stimulated with TLA (1 \( \mu \)g/well) or without stimulation. Protection assay: E, Footpad swelling caused by challenge with infective promastigotes of \( L. (L.)\) amazonensis in BALB/c mice vaccinated with PBS, TLA, Poly(I:C) and TLA-Poly(I:C). \( P \)-values:

\*\( P < .05 \); **\( P < .01 \); ***\( P < .001 \).
TABLE 1  Histopathological score indexes and parasite load of the footpad infection site of the different vaccinated groups after eleven weeks post-challenge with L. (L.) amazonensis (MHOM/VE/84/MEL)

| Group          | Histopathological score index | Parasite load (no. parasites/field) |
|----------------|-------------------------------|------------------------------------|
| PBS            | 26.66 ± 3.055                 | 494.1 ± 175.7                      |
| TLA            | 27.66 ± 2.517                 | 608.4 ± 102.9                      |
| TLA-Poly (I:C) | 18.33 ± 4.163**               | 298.6 ± 85.71***                   |
| Poly (I:C)     | 22.66 ± 3.215                 | 546.7 ± 137                        |

Results represent mean values plus SD in each vaccination group. P-values: **P < .01; ***P < .001.

3.3 | Protection assay of mice immunized with TLA-Poly(I:C) against challenge with L. (L.) amazonensis

The mice vaccinated with TLA-Poly(I:C) showed good protection levels against cutaneous leishmaniasis, with a significant reduction in the size of the footpad lesion. On the other hand, immunization with TLA in the absence of adjuvant provided no protection against infection with L. (L.) amazonensis, resulting in swelling levels similar to those of nonvaccinated mice injected with PBS or Poly(I:C). The differences in the footpad thicknesses between the group receiving TLA alone and the group receiving TLA plus Poly(I:C) became statistically significant (P < .05) by week 8 after challenge (Figure 1E).

3.4 | Histopathology analysis of the tissue damage induced after challenge with L. (L.) amazonensis

The analysis of the degree of inflammation of the footpad lesion indicated a significantly lower histological score index in the TLA-Poly(I:C) group compared to the TLA group. Accordingly, the parasite load in the footpad lesions of the TLA-Poly(I:C) group was approximately 3-fold to 5-fold lower (P < .001) than the observed in mice of the TLA and Poly(I:C) groups (Table 1). These results clearly demonstrated that vaccination with the TLA-Poly(I:C) formulation conferred a significant level of protection.

4 | DISCUSSION

Despite extensive efforts, there is currently no effective vaccine for human leishmaniasis. Although, the fact that many candidate antigens have been pinpointed in attempts to develop vaccines against Leishmania, only a first-generation vaccine formulated with BCG as adjuvant succeeded to reach phase III clinical trials. Previous studies reported that an effective vaccine against leishmaniasis requires a multivalent cocktail of various antigens composed of a spectrum of protective epitopes which cover a broad range of MHC types in a population. This fact is consistent with the leishmanization results, which indicate that crude Leishmania antigens such as TLA are appropriate candidates for vaccine development. Mayrink et al. conducted a study in Brazil which showed a significant reduction in the incidence of the Leishmania infection in humans after immunization with a first-generation vaccine produced with TLA of L. (L.) amazonensis (IFLA/BR/67/PH8).

In the present study, we have evaluated the effectiveness of a first-generation vaccine containing whole L. (L.) amazonensis antigenic extract formulated with Poly(I:C), which has the ability to induce an immune response with a strong profile Th1, promoting antigen presentation, induction of IFN type I and potent T-cell immune responses.

First-generation vaccines are still a very attractive option for leishmaniasis control, especially in developing countries. The current approach in the development of whole parasite malaria vaccines is probably the best example of the possibilities of first-generation vaccines. This approach has generated vaccines capable of inducing high-grade protection by parenteral administration.

Our results demonstrate for the first time that the subcutaneous administration of TLA formulated with Poly(I:C) promotes a protective immune response against cutaneous leishmaniasis. This immune response was characterized by a high IgG titre (Figure 1A), as well as high IgG1 and IgG2a subtype titres (Figure 1B). In this work, the immunization with TLA-Poly(I:C) resulted in high titre of IgG2a, which is correlated with a dominate Th1 profile. Accordingly, with this observation, the TLA-Poly(I:C) formulation triggered a cellular immune response characterized by a high production of IFN-γ (5.829 pg/mL) and very low levels of IL-4 (20 pg/mL) (Figure 1C and D).

Results demonstrated that immunization with TLA-Poly(I:C) induced a strong specific Th1-type response, which conferred protection against infection with L. (L.) amazonensis in mice (Figure 1E, and Table 1).

In conclusion, Poly(I:C) is a promising candidate for the development of a new Leishmania vaccine due to its safety and ability to induce a protective Th1 immune response. It would be important to conduct further research on the potential use of Poly(I:C) as an adjuvant and the mechanisms involved for Leishmania vaccines.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.
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