ARTIGO

DIFFERENTIAL PROTECTION INDUCED BY IMMUNIZATION WITH VARIABLE DOSES OF A *LEISHMANIA* ANTIGENIC EXTRACT AGAINST *LEISHMANIA AMAZONENSIS*

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ABSTRACT

Experimental vaccines have been developed to protect against leishmaniasis using the BALB/c mice model immunized with different immunogens, but an effective vaccine still does not exist. To determine factors inherent to immunogens that might abrogate vaccine-induced efficacy, our research sought to investigate the impact of immunization using variable doses (low, medium and high) of a known soluble Leishmania antigenic extracts (SLA), associated or not with alum, in order to determine the best dose of this vaccine immunogen able to induce the best level of protection in BALB/c mice against L. amazonensis infection. This work shows that the immunization´ model using a high inoculum (100 μg) of SLA results in the best level of protection against challenge. These mice presented significant reductions in the footpad swelling and parasite load; high levels of IFN-γ and IL-12, and low levels of IL-4, IL-10, TGF-β and Leishmania-specific IgG and IgE antibodies. Mice immunized with 50 μg of SLA present intermediate results of protection; on the other hand, mice immunized with 1 μg showed the worst results. Considering all the elements, it could be concluded that the model employing a high dose of SLA in BALB/c mice can bring about the development of a protective immune response in the animals, thus allowing for the protection against the disease. In addition, we understand that the definition of an ideal dose for each vaccine candidate appears to be fundamental to determining the phenotype of resistance and/or susceptibility in murine models to study leishmaniasis.

Keywords: Leishmania amazonensis; vaccine; differential protection; size inoculum; soluble Leishmania antigenic extracts.

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INTRODUCTION

Leishmaniasis is a complex of diseases caused by the intracellular protozoan parasite *Leishmania*, which affects over 12 million people worldwide [1]. Depending on the parasite species and the immunological status of the host, the clinical manifestations of the disease may range from single cutaneous lesions to fatal visceral infection [2].

Among the *Leishmania* species reported as sources of the disease, Leishmania amazonensis has been considered an important etiological agent of leishmaniasis due to presents a wide spectrum of clinical diseases, accounting for cutaneous leishmaniasis until the visceral infection [3–5].

Experimental vaccines employing animal models have been developed to induce protection against leishmaniasis, however, an effective and safe vaccine still does not exist [6–11]. BALB/c mice have represented the most commonly employed murine model in this form of research. In this mouse strain, resistance to L. major has been associated with the development of a parasite-specific T cell-mediated immune response characterized by the production of IFN-γ. Susceptibility is related to a Th2 immune response characterized by the production of IL-4, IL-10, as well as by the presence of high titers of specific-parasite antibodies [7,10]. The characterization of such well-defined roles for Th1/Th2 responses in the L. major model is controversial for infection by *L. amazonensis*. The ability to develop a Th1 predominant response is reported as a resistant phenotype, but the susceptible has been reported be due to a exacerbated Th2 immune response [12], by the absence or poor Th1 immune response [13], or a mixed Th1/Th2 response [14,15].

It has been demonstrated that an important challenge for the development of an effective vaccine against leishmaniasis is to find a well-defined dose and route of administration of immunogens [16,17]. Antigen dose has been shown to influence both the type of immune response and the production of cytokines [18], which may influence the efficacy of a vaccine. Pinto et al. (2003) showed that injecting 10 μg of total Leishmania antigenic extracts (LaAg) by subcutaneous route rendered BALB/c mice and rhesus monkeys susceptible to cutaneous leishmaniasis; however, an opposing protective effect was observed when immunogen was administered by oral route into the animals [19]. In this case, there was a association between protection and high levels of IFN-γ in the draining lymph nodes, and a concomitant suppression of parasite-specific Th2 immune response in the protected animals.

Since the concentration used of an immunogen can too influence the development of a protective or susceptible T cell-mediated response; the present study was carried out to investigate the impact of immunization using variable doses (low, medium and high) of a known soluble *Leishmania* antigenic extracts (SLA), associated or not with alum, in order to determine the best dose of this vaccine immunogen able to induce the best level of protection in BALB/c mice against *L. amazonensis* infection.

2. MATERIALS AND METHODS

2.1. Mice and parasites

Female BALB/c mice (6-weeks old) were purchased from the Biological Sciences Institute from the Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil. Leishmania amazonensis (IFLA/BR/1967/PH-8) parasites were grown at 24ºC in Schneider's medium (Sigma, St. Louis, MO, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Sigma), 20 mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin at pH 7.2. The Animal Use Ethics Committee of the UFMG approved the experimental protocols.

2.2. Antigen preparation

Soluble *Leishmania* amazonensis antigenic extracts (SLA) was prepared from stationary-phase promastigotes after few passages in liquid culture, as described [10]. Briefly, 109 promastigotes were washed 3 times in cold and sterile phosphate-buffered saline (PBS). The pellet was resuspended in sterile PBS added with a protease inhibitor cocktail (Sigma, catalog P8340). After 6 cycles of freezing and thawing followed by ultrasonication (Ultrasonic processor, GEX600), with 5 cycles of 30 sec at 38 MHz, the suspension was centrifuged at 8.000 x g for 30 min at 4oC, and supernatant was collected and stored at -70oC, until used. The proteins concentration was estimated by the Bradford method [20].

2.3. Immunization and challenge infection

BALB/c mice (n=8 per group) were immunized subcutaneously in their left hind footpad with 1, 50 or 100 μg of SLA with or without alum (1.5 μg protein: 1 μg alum; Rehydragel Low Viscosity Gel, Reheis, Inc., Berkeley Heights, USA). Three doses were administered, at 2-weeks interval. Control mice (n=8 per group) received 20 μL of sterile PBS with or without alum. One month after the last immunization, 106 stationary-phase promastigotes of *L. amazonensis* were injected in the mice (n=4 per group) into their right hind footpad.

2.4. Cutaneous lesion development

The course of disease was monitored at weekly intervals by measuring footpad thickness with a metric calliper and expressed as the increase in thickness of the infected hind foot compared to the uninfected left foot. Mice were evaluated for lesion development for 8 weeks, when animals were sacrificed and infected tissue fragments, sera samples and spleens were harvested for parasitological and immunological analysis.

2.5. Parasite quantitation

The infected skin fragments were collected for parasite quantitation, following a technical protocol [21]. Briefly, total infected footpads were collected, weighted and homogenized using a glass tissue grinder in sterile PBS. Tissue debris was removed by centrifugation at 150 × g and cells were concentrated by centrifugation at 2000 × g. Pellets were resuspended in 1 ml of Schneider's insect medium supplemented with 20% FBS. Two hundred and twenty microliters were plated onto 96-well flat-bottom microtiter plates (Nunc, Nunclon®, Roskilde, Denmark) and diluted in log-fold serial dilutions in supplemented Schneider's culture medium with a 10-1 to 10-10 dilution. Each sample was plated at 24oC in triplicate and read 7 to 10 days after the beginning of the cultures. Pipette tips were discarded after each dilution to avoid carrying adhered parasites from one well to another. Results are expressed as the negative log of the titer (i.e., the dilution corresponding to the last positive well) adjusted per microgram of tissue.

2.6. Cytokine production

Splenocytes culture and production of cytokines were evaluated like described [10]. Briefly, single-cell suspensions were collected of mice immunized and/or infected, homogenized and plated in duplicate in 24-well plates (Nunc) at 5 x 106 cells/ml. Cells were incubated in DMEM medium (unstimulated, background control) or stimulated with SLA (50 μg/ml), at 370C in 5% CO2 for 48 h. IFN-γ, IL-4, IL-10 and TGF-β levels were assessed by ELISA using monoclonal antibodies (capture and detection) provided in commercial kits (Pharmingen, San Diego, CA, USA), according to manufacturer's instructions.

2.7. Analysis of the humoral response

Leishmania-specific IgE, IgG, IgG1 and IgG2a antibodies were measured by ELISA. A titration curve was performed to determine the best SLA concentration and antibodies dilution. In brief, 96-well plates (Falcon) were sensitized with SLA (1 μg per well) overnight at 40C. Plated were blocked with sterile PBS/10% bovine albumin at 370C for 2 h, and, lately, sera samples (1:100 dilution) were added and incubation occurs for 1 h at 370C. Peroxidase-labeled antibodies specific to mouse IgE, IgG, IgG1 and IgG2a isotypes (Sigma, St. Louis, MO, USA) were diluted at 1:5000 and added for 2 h at 370C, then incubated with H2O2, o-phenylenediamine and citrate buffer pH 5.0 for reactions´ development. Optical densities were read at 492 nm in a spectrophotometer (BioRad).

2.8. Statistical analysis

Comparisons among the groups were carried out by two-way ANOVA and Bonferroni´s post-test. Differences were considered significant when $P<0.01$. The GraphPad Prism version 5.0 for Windows (GraphPad Instat Software, San Diego, California) was used to perform the analysis.

3. RESULTS

3.1. Evaluation of the cellular and humoral response in the immunized BALB/c mice

Since the activation of a Th1 immune response with sustained IFN-γ production is considered an important requirement for protection against the most of Leishmania species, we analysed the production of IFN-γ, IL-12, IL-4, IL-10, and TGF-β in the spleen cells of immunized BALB/c mice, one

month after the last vaccine dose and before challenge infection.

In the results, we observed that spleen cells cultures from mice immunized with 50 or 100 µg of SLA with (SLA/alum) or without (SLA) alum produced significantly higher levels of IFN-γ and IL-12 in comparison to levels detected in the splenic cultures from the control groups (saline and alum groups), and those immunized with 1 µg of SLA or SLA/alum (Fig. 1). A higher production of IFN-γ and IL-12 was observed in mice immunized with 100 µg, when compared to animals immunized with 50 µg of SLA or SLA/alum. No significant production of IL-4, IL-10, and TGF-β was observed in the cultures of the mice immunized with SLA or SLA/alum (Fig. 1). The IFN-γ and IL-12 production was higher in animals that did not receive alum as adjuvant when compared to those immunized with alum; however, no significant difference between the groups was observed.

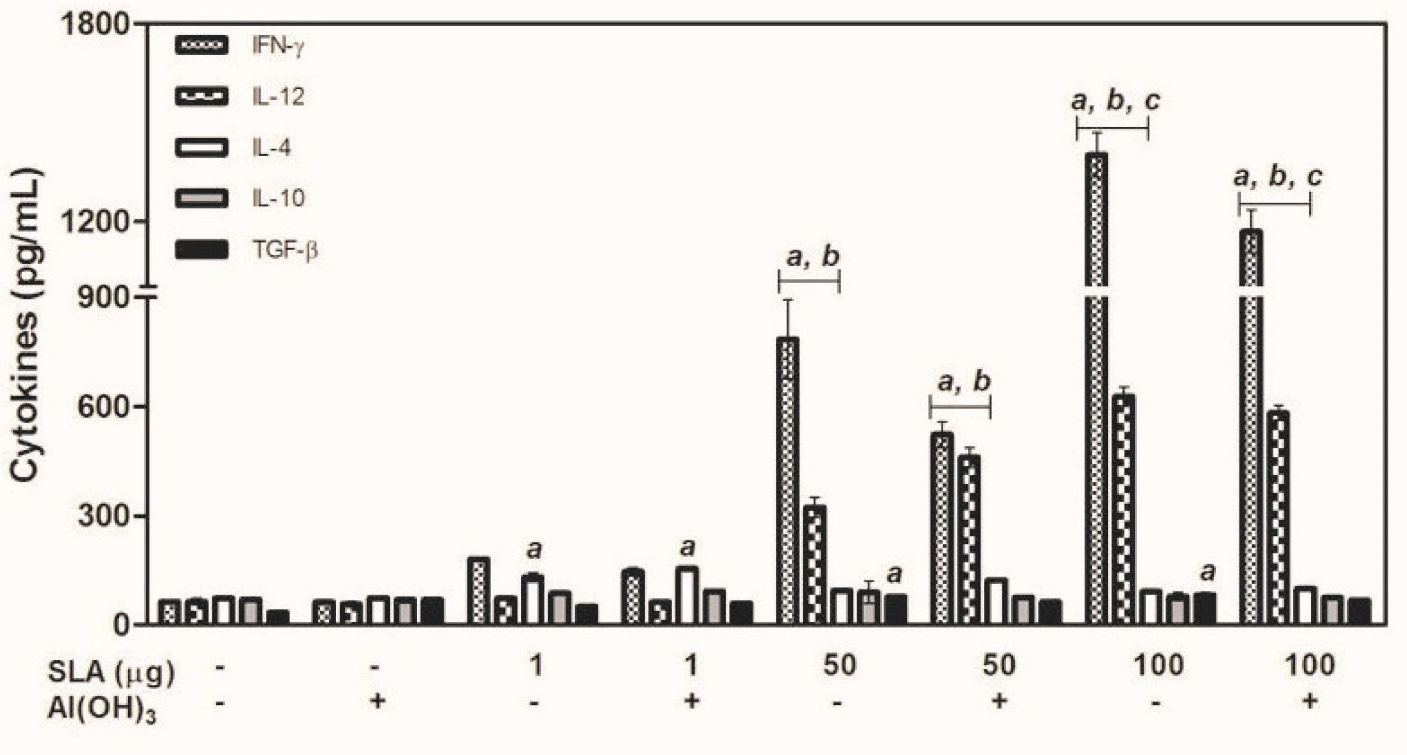
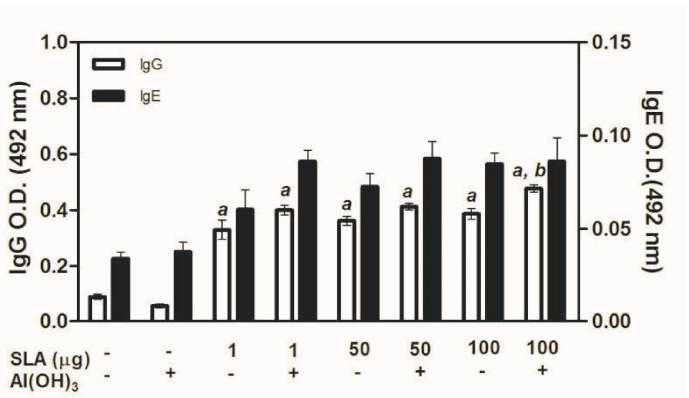


Figure 1. Cellular response elicited in BALB/c mice immunized with variable doses of SLA with or without alum. *Spleen cells obtained from mice one month after the last vaccine dose were cultured in vitro and unstimulated (DMEM medium; background control) or stimulated with SLA (50 µg/ml), at 37oC with 5% CO2 for 48 h. IFN-µ, IL-12, IL-4, IL-10 and TGF-β levels were assessed by capture ELISA in the culture supernatants. Each bar represents the mean ± standard deviation (SD) of data from four individual mice per group. Differences were considered statistically significants between animals immunized with 1, 50 or 100 µg of SLA with (SLA/alum) or without (SLA) alum and control groups (A: P<0.005); between mice immunized with 1, 50 or 100 µg of SLA in comparison to SLA/alum groups (B: P<0.005), and between mice immunized with 50 or 100 µg of SLA in comparison to SLA/alum groups (C: P<0.005). Data shown are representative of three independent experiments with similar results.*

In the evaluation of the humoral response, we observed that the production of antibodies was directly proportional to the size of the received immunogen dose. In this way, mice immunized with 100 µg of SLA or SLA/alum produced higher levels of IgG and IgE antibodies, when compared to animals immunized with 50 or 1 µg of SLA or SLA/alum (Fig. 2A); however significant differences were not observed. The IgG and IgE production was higher in the animals that received alum as adjuvant when compared to the mice that had not been inoculated with this adjuvant. In the evaluation of the levels of IgG1 and IgG2a, it could be observed that mice immunized with 100 and 50 µg of SLA or SLA/alum produced more elevated levels of Leishmania-specific IgG2a antibodies in comparison to IgG1 levels, however, in the group immunized with 1 µg, the levels of IgG1 and IgG2a isotypes were similar (Fig. 2B).



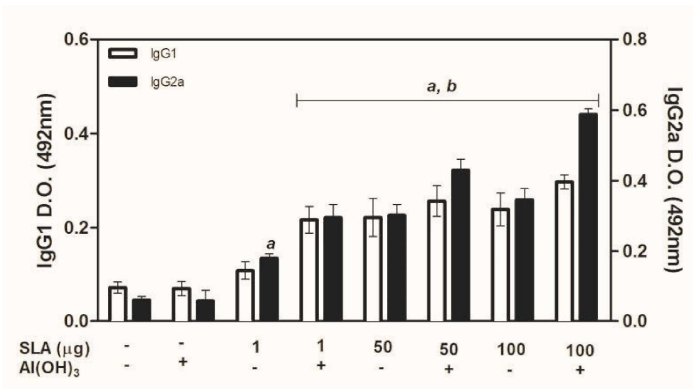


Figure 2. Antibodies´ production in BALB/c mice immunized with variable doses of SLA with or without alum. *Sera samples obtained from mice one month after the last vaccine dose were tested by ELISA to detect the presence of Leishmania-specific IgE, IgG, IgG1 and IgG2a antibodies. Each bar represents the mean ± standard deviation (SD) of data from four individual mice per group. Sera were tested by ELISA to determine the presence of IgE and IgG (Panel A) antibodies, and IgG1 and IgG2a (Panel B) isotypes. Differences were considered statistically significant between mice immunized with 1, 50 or 100 µg of SLA with (SLA/alum) or without (SLA) alum and control groups (A: P<0.005), and between mice immunized with 1, 50 or 100 µg of SLA in comparison to SLA/alum groups (B: P<0.005). Data shown are representative of three independent experiments with similar results.*

Therefore, in the general analysis of the immune response mounted before challenge infection in the animals, it was observed that the immunization using 50 and, mainly, 100 µg of SLA or SLA/alum primed the animals for the development of a Th1 immune response. In contrast, mice immunized with a low dose of immunogen (1 µg), with or without alum, presented a mixed Th1/Th2 response.

3.2. Efficaccy of protection against L. amazonensis in the immunized mice

The protective effect of the immunization using variable doses (low, medium and high) of SLA, associated or not with alum as adjuvant, was evaluated by the measuring of the lesion development (Fig. 3A) and parasite load (Fig. 3B) in the infected footpads. We observed a low reduction in the parasite load in the animals immunized with 1 µg of SLA or SLA/alum; however, the mice immunized with 50 and, mainly with 100 µg, displayed the best results of protection represented by significant reductions in the footpad swellings (Fig. 3A) and in the parasite load in the infected footpads (Fig. 3B). Mice immunized with the association of alum into the immunogens presented the worst results of protection in comparison to animals that not received this adjuvant.

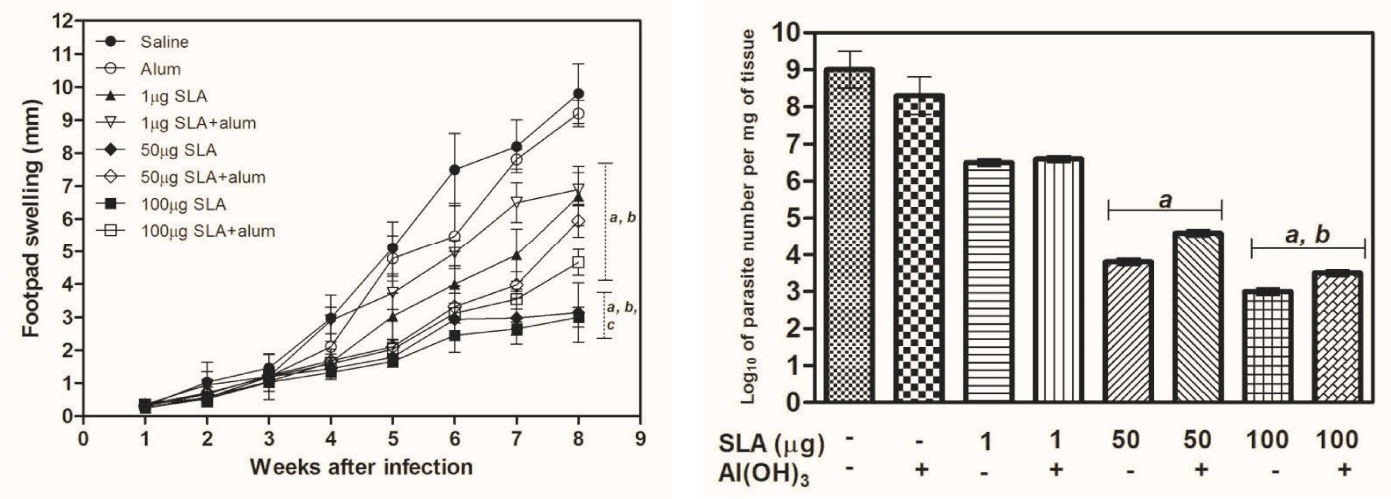


Figure 3. Leishmania amazonensis protection assays. *BALB/c mice (n=8 per group) were immunized with three subcutaneous injections, in fifteen day interval, with 1, 50 or 100 μg of SLA or SLA/alum in their left hind footpad. One month after, mice (n=4 per group) were infected with 106 stationary-phase promastigotes of L. amazonensis into their right hind footpad. Control mice received sterile PBS with or without alum. Panel A shows the lesion development (footpad swelling) in the infected mice, monitored weekly with a calliper. Panel B shows the parasite load detected in the infected footpads, 8 weeks after challenge. Number of viable parasites was determined by a limiting dilution assay as described in the Material and Methods Section. Mean μ standard deviation (SD) of each group is shown. Differences were considered statistically significant between animals immunized with 1, 50 or 100 μg of SLA with (SLA/alum) or without (SLA) alum and control groups (A: P<0.005); between mice immunized with 1, 50 or 100 μg of SLA in comparison to SLA/alum groups (B: P<0.005); and between mice immunized with 50 or 100 μg of SLA in comparison to SLA/alum groups (C: P<0.005). Data shown are representative of three independent experiments with similar results.*

3.3. Immune response in the immunized BALB/c mice and infected with L. amazonensis

About eight weeks after the challenge infection, we detected that the profile of immune response generated in the mice immunized with 50 or 100 μg of SLA or SLA/alum was maintaned, once that spleen cells cultures of these animals produced significantly higher levels of IFN-γ and IL-12 in comparison to animals immunized with 1 μg and control groups (Fig. 4A). As witnessed before the challenge infection, a higher production of IFN-γ and IL-12 was observed in the mice immunized with 100 μg of SLA or SLA/alum, when compared to levels obtained in animals immunized with 50 μg of the immunogenic preparation. The SLA-driven production of IL-4, IL-10, and TGF-β was also analyzed (Fig. 4B). Mice immunized with 50 or 100 μg of SLA or SLA/alum showed a lower production of these cytokines, when compared to the control groups, which presented the higher levels of production of these cytokines, mainly, of IL-10, after *L. amazonensis* challenge infection.

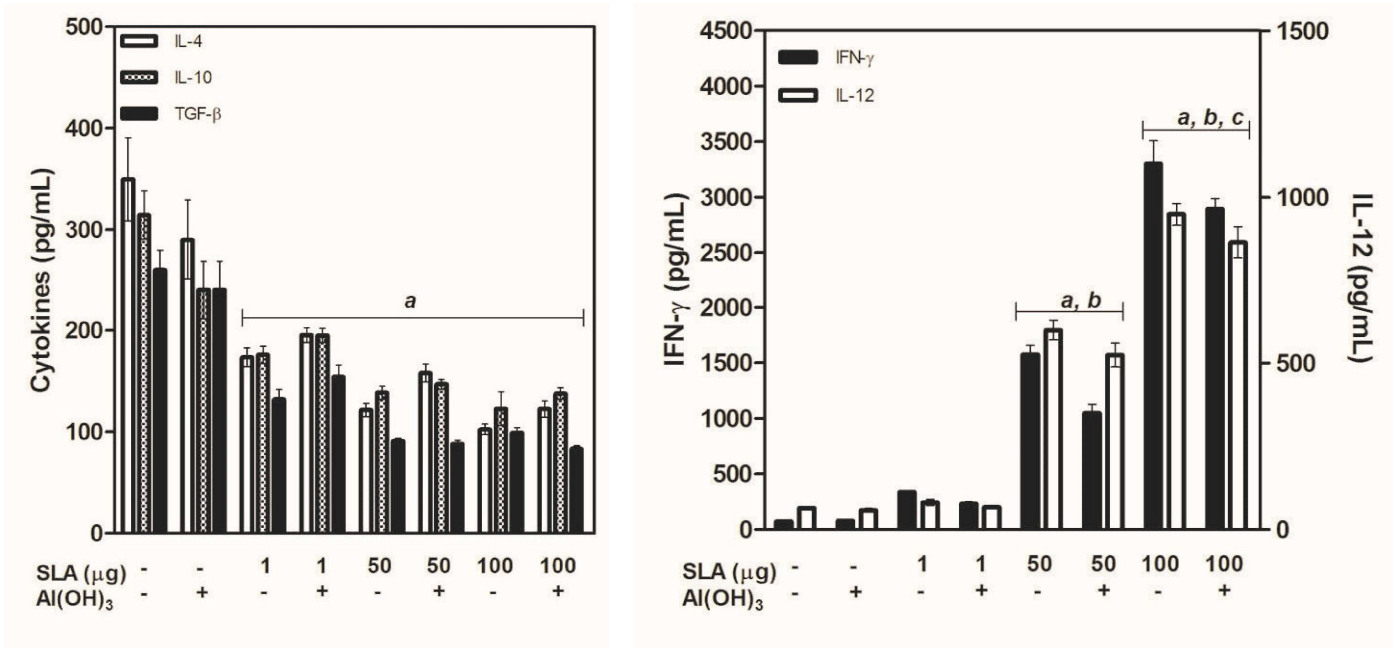


Figure 4. Cytokines´ production by spleen cells of BALB/c mice infected with L. amazonensis. *Spleen cells suspensions were obtained from immunized and infected mice, 8 weeks after L. amazonensis challenge infection. Cells were unstimulated (DMEM medium; background control) or stimulated with SLA (50 μg/ml), at 37°C with 5% CO2 for 48 h. IFN-γ and IL-12 (Panel A); IL-4, IL-10 and TGF-β (Panel B) levels were assessed by capture ELISA in the culture supernatants. Each bar represents the mean ± standard deviation (SD) of data from four individual mice per group. Differences were considered statistically significant between animals immunized with 1, 50 or 100 μg of SLA with (SLA/alum) or without (SLA) alum and control groups (A: P<0.005); between mice immunized with 1, 50 or 100 μg of SLA in comparison to SLA/alum groups (B: P<0.005), and between mice immunized with 50 or 100 μg of SLA in comparison to SLA/alum groups (C=P<0.005). Data shown are representative of three independent experiments with similar results.*

The levels of Leishmania-specific IgG and IgE antibodies after infection in the animals immunized with SLA or SLA/alum were lower in comparison to control groups (Fig. 5A). The association of alum did not induce a higher production of antibodies in the immunized animals, as it was observed before of the challenge infection. In the evaluation of IgG1 and IgG2a isotypes, we observed that mice immunized with 50 and, mainly, with 100 μg of SLA or SLA/alum produced higher levels of IgG2a in comparison to IgG1 levels (Fig. 5B). Animals of control groups presented significantly higher Leishmania-specific IgG1 levels in comparison to IgG2a levels obtained after the challenge infection.

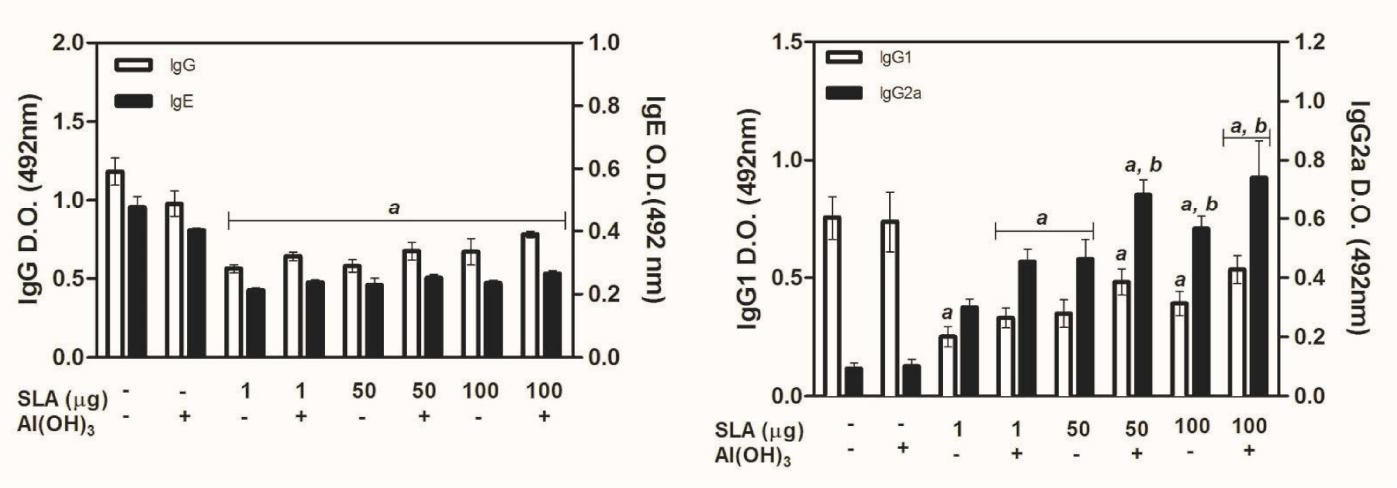


Figure 5. Antibodies´ production after L. amazonensis challenge infection. *Mice were immunized and challenged with L. amazonensis and eight weeks after sera samples were collected. Sera were tested by ELISA to determine the presence of Leishmania-specific IgG and IgE antibodies (Panel A), and IgG1 and IgG2a (Panel B) isotypes. Each bar represents the mean ± standard deviation (SD) of data from four individual mice per group. Differences were considered statistically significant between mice immunized with 1, 50 or 100 μg of SLA with (SLA/alum) or without (SLA) alum and control groups (A: P<0.005), and between mice immunized with 1, 50 or 100 μg of SLA in comparison to SLA/alum groups (B: P<0.005). Data shown are representative of three independent experiments with similar results.*

4. DISCUSSION

Experimental vaccines have been tested to protect against *Leishmania*, however, few have shown to induce an effective protection against disease. Lysates antigenic extracts from parasites are considered a reasonable alternative due to their immunogenicity, relatively simple preparation, and low cost [22,23]. The present study tested the outcome of the immunization using different doses (low, medium and high) of a known vaccine immunogen, namely, soluble Leishmania amazonensis antigenic (SLA) extracts, administered by subcutaneous route with or without alum, in order to evaluate if the employ of different doses can influence to the efficacy of protection induced in a known murine model against *L. amazonensis* infection.

Results suggest that a well-define immunogen dose is critical in determining the direction in which the immune response of the infected mice will take, given that inoculation with low, medium, and high doses leads to the development of variable profiles of immune responses in the animals and, consequently, to the rapid or delayed development of the disease, as reflected by the low parasite load and high levels of Th1 cytokine observed in mice immunized with 100 μg of SLA.

In order to assess the cytokines´ response induced by immunization with variable doses of SLA, we observed that the immunization using 50 and 100 μg of SLA was able to induce a specific Th1 immune response before challenge, characterized by high levels of IFN-γ and IL-12, which was maintaned after infection. IFN-γ and IL-12 are considered critical cytokines to induce protection against several *Leishmania* species [13,24–26]. It has been postulated that high levels of IFN-γ and IL-12 are related to the generation of a protective immunity against *L. amazonensis* in BALB/c mice. Recently, Chávez-Fumagalli et al. (2010) showed that these cytokines have a fundamen-

tal role to induce protection in BALB/c mice vaccinated with *L. infantum* ribosomal proteins against *L. amazonensis* or *L. chagasi* infections [11]. In these cases, immunized and protected animals presented significant reductions in the dermal pathology represented by significant reductions of footpad swellings and parasite load at the site of infection by *L. amazonensis*, and a protective Th1 immune response in all protected animals.

In this study, we observed that immunized and protected mice presented low levels of IL-4, IL-10 and TGF-β before and after challenge infection. It is postulated that combined effects of low levels of IFN-γ, and high levels of IL-4 and IL-10 can promote the rapid recruitment of immature or insufficiently activated macrophages, which favors the replication of amastigotes and the progression of the disease [27,28]. IL-10 produces multiple effects in suppressing microbicidal activity in the macrophages, lowering IFN-γ production and, consequently, preventing parasite clearance in highly susceptible mice [29–33]. Zanin et al. (2007) showed that BALB/c mice immunized with a plasmid which encoded the A2 protein were protected against *L. amazonensis* or *L. donovani* infections, and that protection was related to high levels of IFN-γ and low levels of IL-10 in the both cases [34]. TGF-β is too considered an cytokine related with the susceptibility of BALB/c mice to *L. amazonensis* [35]. This cytokine can, in fact, enhance the progression and/or prevent the cure of leishmaniasis in murine models [36-39].

The evaluation of humoral response in the immunized and protected mice demonstrates that the production of *Leishmania*-specific IgG and IgE antibodies was negatively correlated with the efficacy of protection against *L. amazonensis*. High levels of these antibodies were encountered in the control mice, which presented a rapid evolution of the infection and disease. On the other hand, immunized and protected mice presented the lowest levels of IgG and IgE antibodies and, contrariously to observed to the control mice, the levels of IgG2a supplanted, significantly, the IgG1 levels after challenge infection in the protected animals.

It has been shown that circulating antibodies play a critical role in the pathogenesis of *L. amazonensis* infection in mice [40]. Investigations of the mechanism by which antibodies modify the presentation of antigens to T cells have suggested that internalization via the Fc receptor affects the endocytic transport of the internalized molecules, which leads to either an enhanced or a diminished presentation of epitopes [41,42]. Antibody opsonization of *Leishmania* amastigotes may increase the efficiency of parasite internalization and/or qualitatively modify the host's response to infected macrophages. These facts corroborate with the idea of a direct participation of antibodies in the phenotype of the susceptibility for L. amazonensis [40].

In conclusion, not only the careful choice of route of immunization of a vaccine candidate is important, but also how much of immunogens should be administered to protect against *Leishmania* [43,44]. These are important indicators, considering that the routinely used models of doses for immunization may especially under or upestimate the potential of vaccine candidates by altering the animals' immune response, when exposed to a low or excessively high vaccine inoculum. The present investigation concluded that the model employing a high dose of a known vaccine immunogen in BALB/c mice can bring about the development of a protective immune response in the animals, thus allowing for the protection against the disease. In addition, we understand that the definition of an ideal dose for each vaccine candidate appears to be fundamental to determining the phenotype of resistance and/or susceptibility in murine models to study leishmaniasis.

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