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Effect of *Leishmania* proteins in mice: Investigation of *Leishmania amazonensis* virulence molecules for therapy and prophylaxis of leishmaniasis

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ABSTRACT. It has been reported that a single injection of *Leishmania amazonensis* amastigote extract (*La*E) in BALB/c mice exacerbates the infection of these animals with *Leishmania braziliensis*. This study aimed to investigate proteins of *La*E that confers susceptibility to *L. braziliensis* infection. Methods BALB/c mice were injected with saline, *La*E, isolate fractions of the *La*E (68, 45, 36, and 28 kDa), or *L. infantum* recombinant protein Lci1 (Hsp70). After a week, the animals were infected with *L. braziliensis*, and the lesion's size was monitored weekly. Six weeks later, the parasite burden in the footpads and the activities of IgG1 and IgG2a anti-*La*E antibodies were determined. Additionally, the 68 and 28 kDa fractions were supplemented with serine and cysteine protease inhibitors and evaluated in the same described experimental model. The intradermal injection of BALB/c mice with 68, 36, and 28 kDa fractions of *La*E, and the recombinant protein Lci1 conferred significant susceptibility to *L. braziliensis* infection, but not the 45 kDa fraction. This potentiating effect was associated with the increase in increment of the lesion size, parasite load, and IgG1 anti-*La*E antibodies. Furthermore, the biological activity of the 68 and 28 kDa fractions was inhibited by the pretreatment of the extract with serine protease inhibitors. Our study found the biological activity of the 68, 36, and 28 kDa fractions, as well as the recombinant protein Lci1, exacerbating the infection in our experimental model.

Keywords: *Leishmania amazonensis*; *Leishmania braziliensis*; Antigen; Infection; Leishmaniasis.

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Introduction

In 2021, Afghanistan, Algeria, Brazil, Colombia, Iran, Iraq, Pakistan, Peru, and the Syrian Arab Republic reported the highest number of cases of Cutaneous Leishmaniasis, which together account for 88% of global reported disease incidence (World Health Organization [WHO], 2023). In Brazil, the main species causing the infection are *Leishmania braziliensis* and *L. amazonensis* (Almeida et al., 2021).

Transformation of *Leishmania* into amastigotes form in the vertebrate host contributes to the establishment of infection since phagocytosis of amastigotes triggers less oxidative metabolism in macrophages than promastigotes (Pearson & Steigbigel, 1981).

In studies performed by our group, it was observed that four intravenous injections within a 2-week interval of *Leishmania amazonensis* amastigote extract *La*E (Silva et al., 2011), or even a single intradermal injection (Araújo et al., 2014), potentiate *L. braziliensis* infection in BALB/c mice, which are normally resistant to infection by this specie of the parasite. The functional characterization of *La*E molecules may clarify the pathogenesis of leishmaniasis and contribute to the development of biotechnological products related to the therapeutic or a vaccine component.

Since the discovery of the *Leishmania* genome, various proteins have already been described. The Heat shock protein (Hsp) family is composed by chaperones directly involved in parasite survival and immunomodulatory mechanisms (Rico et al., 2002; Teixeira et al., 2011). Moreover, the 46 kDa promastigote protein from *L. amazonensis* stimulates the production of IFN-γ by lymphocytes of C57BL/10 mice infected by *L. amazonensis* (Cardoso et al., 2003). Another molecule with immunostimulatory activity is the *Leishmania*-

activated C-kinase antigen (LACK) (Fernández et al., 2018), able to trigger the activation of T helper 2 cells (Th2), stimulating the synthesis of IL-4 and potentiating the infection (Julia et al., 1996; Schilling & Glaichenhaus, 2001). Recently, a novel protein named LAX28 (for *Leishmania* axonemal protein, 28 kDa) was characterized in *L. mexicana*, with a role in motility (Beneke et al., 2020). With the same molecular mass, the cysteine protease B is involved in suppressing the Th1 $TCD4^{+}$ lymphocyte response (Mottram et al., 1996).

In a pilot experiment performed by our group, BALB/c mice (4 per group) received intradermal injections with fractions of molecules with estimated molecular weights of 96, 68, 52, 45, 36, 28, 19, and 15 kDa of *Leishmania amazonensis* amastigote extract (*La*E), and after infected with *L. braziliensis*. At different levels, the fractions increased the size of the lesions. Therefore, the present study aims to investigate *La*E fractions with a biological effect of potentially exacerbating or controlling murine infection by *L. braziliensis*.

Material and methods

Mice and ethical considerations

Specific-pathogen-free, 8 to 12-week-old BALB/c mice were maintained at the animal facilities of the Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Brazil. All procedures involving animals were conducted according to the Brazilian Federal Law on Animal Experimentation (Lei nº 11.794, 2008), and with the Oswaldo Cruz Foundation guidelines for research with animals. This study was approved by the ethics committee for the use of animals in research (CPqGM-FIOCRUZ, CEUA license 010/2012) (Instituto Oswaldo Cruz [IOC], 2012).

Parasites, extract, and fractions

Leishmania amazonensis (MHOM/Br87/Ba125) and *L. braziliensis* (MHOM/Br/3456) strains were used. The parasites were maintained as previously described (Araújo et al., 2014). Briefly, *L. amazonensis* infectivity was maintained by regular inoculations of promastigotes into susceptible BALB/c mice and *L. braziliensis* into golden hamsters. To obtain the *La*E, promastigotes were cultured at 23°C in Schneider's medium (Sigma Chemical Co., St. Louis, MO, USA), pH 7.2, supplemented with 50 μg mL^{-1} of gentamicin and 10% heat-inactivated fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY) for *L. amazonensis* or 20% FBS for *L. braziliensis*. *L. amazonensis* axenic amastigotes extract was obtained by the differentiation of promastigotes in axenic cultures. The amastigotes were washed three times in ice-cold saline, resuspended in saline, and lysed by exposure to ultrasound (10 pulses, 1-minute, 300-W, with 30-second intervals in between, on ice; Sonifier Cell Disruptor; Branson Sonic Power Company, Danbury, CT, USA). The lysates were centrifuged at 16,000 x g for 10 min. at 4°C, and the supernatants were filtered on membranes with 0.22-μm diameter pores (Millipore, São Paulo, Brazil) and immediately aliquoted (Teixeira et al., 2002).

To isolate fractions, the *La*E was subjected to 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970), under non-denaturing conditions, in which the sample was not heated, the buffer did not contain SDS and 2-mercaptoethanol, and the entire methodology was performed at 4°C. Then, the sample was stained with Coomassie blue to visualize the bands. Fragments of the gel with proteins with molecular weights of 68, 45, 36, and 28 kDa, chosen from previous results, were excised from the *La*E gel and subjected to electroelution separately. The material was placed on dialysis membranes and eluted by electrophoresis in running buffer (0.025 M Tris, 0.192 M glycine, 1% SDS) using an Electro-Eluter (model 422; BioRad) at 10 mA for 5 h. After elution, the proteins were dialyzed against 0,15 M phosphate-buffered saline (PBS), 4°C, which were replaced three times over 17 hours and visualized by 12% SDS-PAGE with Coomassie blue staining.

The Lci1 (an Hsp70 heat shock protein polypeptide segment) was obtained as described previously (Teixeira et al., 2011). *L. infantum* amastigote recombinant antigens, encoded by plasmids constructed with *L. infantum* amastigote cDNA, were expressed in *Escherichia coli* and purified by an inclusion body (insoluble aggregate of over-expressed proteins) isolation protocol or by immobilized metal-ion affinity chromatography using nickel-chelating Sepharose Fast Flow columns (GE Healthcare, Uppsala, Sweden). The degree of purity of the recombinant antigens was analyzed by 10% SDS-PAGE, followed by Coomassie blue staining, as described previously (Laemmli, 1970).

The protein content of each antigen preparation was determined by protein reaction with fluorescamine (Lorenzen & Kennedy, 1993), and all the samples were stored at – 70ºC.

Experimental design

Each BALB/c from groups of 8 to 6 mice was injected intradermally with 50 µL of saline (negative control), or 5 µg *La*E (positive control), or the following *La*E fractions 68, 45, 36, and 28 kDa. Furthermore, *La*E, fractions 68 and 28 kDa were supplemented or not with serine (5.4 mM *N*-tosyl-L-lysine chloromethyl ketone hydrochloride and 5.7 mM *N*-*p*-tosyl-L-phenylalanyl chloromethyl ketone) or cysteine (5 mM iodoacetamide) protease inhibitors. The concentration of the administered fractions corresponded to the percentage of each fraction (68 kDa, 4.4%; 45 kDa, 4%; 36 kDa; 11.2%; 28 kDa, 13.7%) present in 5 µg of protein contained in the *La*E. Another group of BALB/c was subjected to intradermal immunization with 5 µg of the Lci1 protein *L. infantum* amastigote recombinant antigen (an Hsp70 heat shock protein polypeptide segment). After one week, 10^7 *L. braziliensis* promastigotes, obtained from stationary-phase cultures, were subcutaneously inoculated into one of the hind footpads of BALB/c mice. The hind pad thicknesses were weekly monitored with a digital caliper until the sixth-week post-infection and the lesion sizes were estimated by subtracting the thickness of the uninfected pad from the thickness of the infected footpad (Silva et al., 2011). Six weeks after infection the animals were euthanized.

Quantification of tissue parasitism

The parasite burden in the footpads was estimated by limited dilution (Lima et al., 1997). Briefly, the infected footpads were macerated in Schneider's medium and centrifuged at 50 *x* g for 10 min., at 4°C. The supernatants were recentrifuged at 1,540 *x* g for 10 min. at 4°C, and the pellets were resuspended in 700 µl Schneider's medium supplemented with 50 µg mL^{-1} gentamicin and 20% FBS. The suspension was serially diluted in 2-fold dilutions and distributed in triplicate in 96-well culture plates. The parasite load of the lesion was determined after 7 days of incubation at 23ºC and determined by the formula:

$$\frac{700\ \mu l\ (total\ lesion\ pellet\ volume)}{200\ \mu l\ (volume\ of\ suspension\ by\ well)}\ x\ \frac{1}{last\ dilution\ in\ which\ parasites\ were\ detected}$$

Antibody activity

For determination of IgG1 and IgG2a anti-*Leishmania* antibodies, 96-well microtiter plate wells were coated by overnight incubation at 4° C with 100 µL of E*La* (containing 10 µg of protein mL^{-1}) diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6. The plates were blocked by incubation for 1 h at room temperature with 150 µl of 0.15 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 (PBS-T) and 10% FBS. Serum samples were diluted 1:1.000 (IgG1) and 1:25 (IgG2a) in PBS-T with 5% FBS and incubated for 1 h at room temperature. Bound antibodies were detected by incubation with biotinylated rat anti-mouse IgG1 or IgG2a (Pharmingen, Minneapolis, MN) for 1 h, followed by incubation with avidin-conjugated peroxidase for 30 min, at room temperature. The reaction was developed in 100 µL of 50 mM citrate-phosphate buffer, pH 5.2, containing 1.1 µM o-phenylenediamine and 0.015 volume of H_2O_2, and stopped by the addition of 25 µL of 4 M H_2SO_4. The absorbance at 490 nm was determined in an enzyme-linked immunosorbent assay microtiter plate reader.

Statistical analysis

The type of data distribution was determined by the Shapiro-Wilk test. The samples with normal distribution were submitted to the one-ANOVA test, followed by the Bonferroni post-test, and non-Gaussian distributions were submitted to the Kruskal-Wallis test, followed by Dunn's post-test. Results were considered significant when the value of P was < 0.05.

Results

Fractions of 68, 45, 36, and 28kDa enhanced *Leishmania braziliensis* infection in BALB/c mice

*La*E proteins with estimated molecular weights of 68, 45, 36, and 28 kDa were purified, under non-denaturing conditions, by electroelution from 12% SDS-PAGE gel (Figure 1).

In the third week after the *L. braziliensis* infection, the BALB/c mice that received *La*E and the fractions of 68 and 28 kDa had significantly ($p < 0.05$) larger footpad lesions than the animals injected with saline, 45 or

36 kDa fractions. In the following week, lesion sizes in the mice in all experimental groups were significantly larger than those in the negative control group. At the fifth week post-infection, there was a reduction in lesion size in the mice injected with the 45 kDa fraction. In the last week, the mice that received the 36 and 45 kDa fractions had no statistically significant increase in lesion size, although they maintained larger lesions than those in the saline group. The groups of animals injected with *La*E, 68 and 28 kDa maintained a significant lesion increase in the control group from the third to the sixth week after infection (Figure 2A).

The result of the parasite loads confirmed the findings of the lesion sizes. The groups of mice injected with *La*E and the 68, 36, and 28 kDa fractions differed statistically from the saline group. In contrast, the group of animals that received the 45 kDa fraction did not differ from the saline group (Figure 2B).

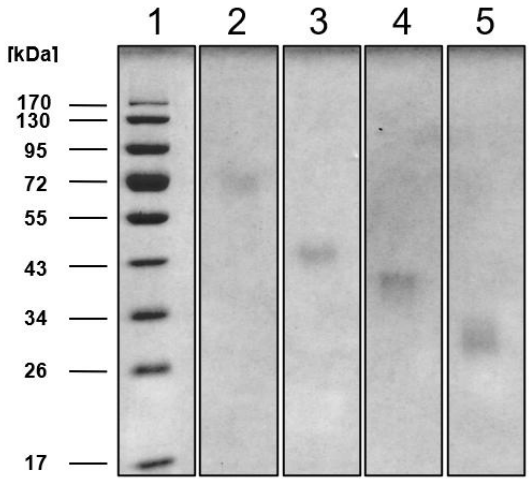

Figure 1. 12% SDS-PAGE stained with Coomassie blue. *Leishmania amazonensis* extract (*La*E) fractions by polyacrylamide gel electrophoresis under non-denaturing conditions followed by electroelution. Column 1, molecular weight markers in kilodaltons (kDa); column 2, 68 kDa; column 3, 45 kDa, column 4, 36 kDa; and column 5, 28 kDa.

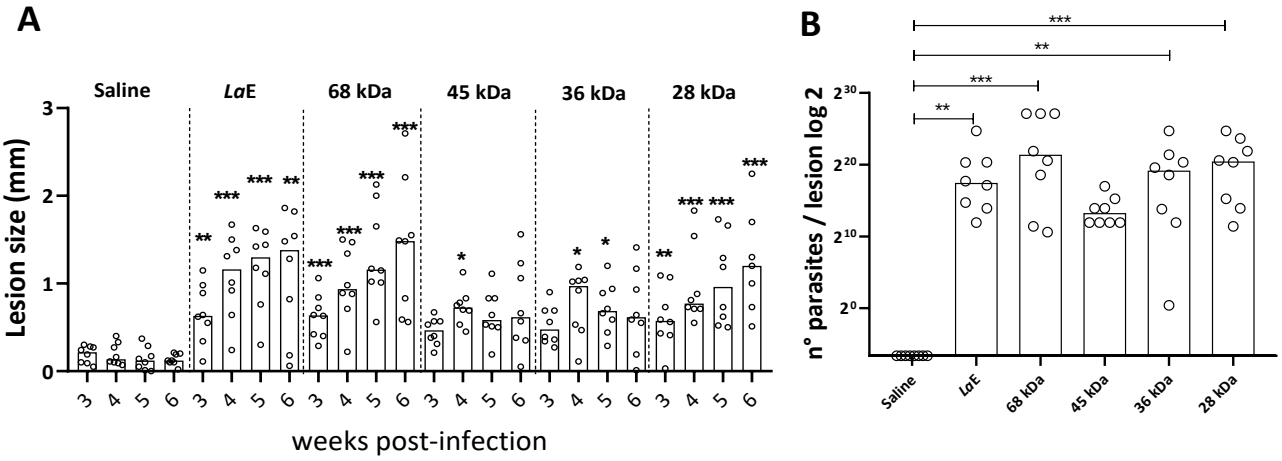

Figure 2. BALB/c mice were intradermally injected with saline, *Leishmania amazonensis* extract (*La*E), or *La*E fractions of 68, 45, 36, or 28 kDa. After one week the animals were infected in the footpad with 10^7 *Leishmania braziliensis* stationary-phase promastigotes. (A) Lesion size from the 3rd to the 6th week after infection of BALB/c mice with *L. braziliensis*. (B) Parasite burden 6 weeks after infection with *L. braziliensis*, in the footpads of the mice showed in panel A. Each circle represents the result obtained from an individual animal. The horizontal lines represent the medians of the results. Statistical significances of differences between groups were evaluated by Bonferroni's multiple comparisons test for lesion size and Dunn´s multiple comparison test for nº parasites $lesion^{-1}$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (relative to the Saline group of the respective weeks).

Fractions of 68, 45, 36, and 28 kDa increased IgG1 antibody serum levels in BALB/c mice infected with *Leishmania braziliensis*

Anti-*La*E IgG1 antibodies levels were significantly higher in the group of mice treated with *La*E and 68, 36, or 28 kDa fractions, but not injected with 45 kDa, than in the group injected with saline at 6 weeks after being infected with *L. braziliensis* (Figure 3A). The levels of anti-*La*E IgG2a antibodies did not differ statistically among the groups of mice (Figure 3B).

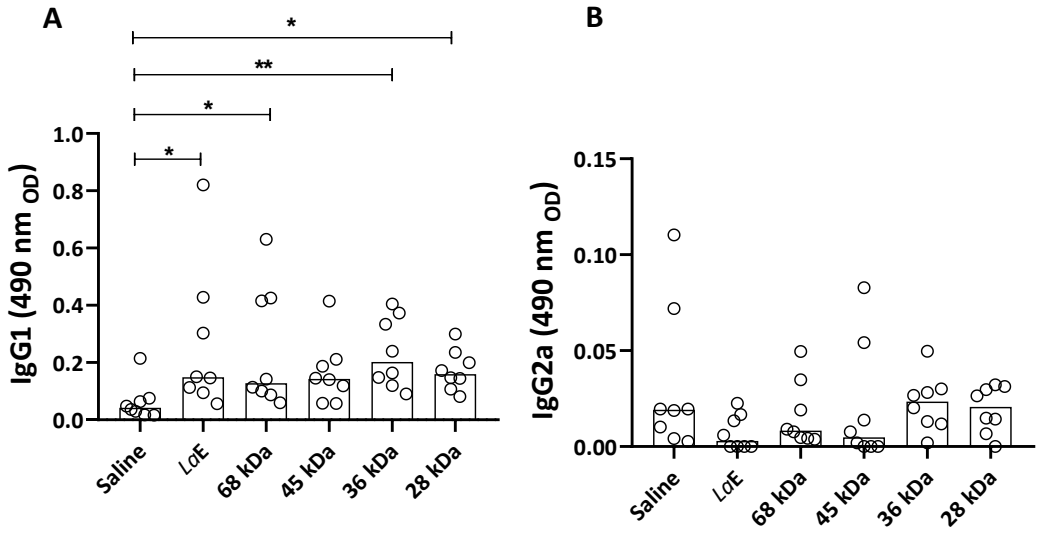

Figure 3. BALB/c mice were intradermally injected with saline, *Leishmania amazonensis* extract (*La*E), or *La*E fractions of 68, 45, 36, and 28 kDa. After one week the animals were infected in the footpad with 10^7 *Leishmania braziliensis* stationary-phase promastigotes. Levels of IgG1 (A) and IgG2a (B) anti-*L. amazonensis* extract was measured in sera, prepared from blood collected 6 weeks after the infection. Each circle represents the result obtained from an individual animal. The horizontal lines represent the median for each group. Statistical significance of differences between groups was evaluated by Dunn´s multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$, relative to the Saline group.

Serine protease inhibitors reduced the experimental leishmaniasis-promoting activity of *La*E

Our research group previously described that serine and cysteine protease inhibitors interfered with the potentiating action of *La*E. In this sense, we chose to test these protease inhibitors on fractions that aggravated the lesion after six weeks of infection.

The addition of serine protease inhibitors to the *La*E decreased the ability of the extract and the 68 and 28 kDa fractions (Figure 4A) promote *L. braziliensis*-induced lesions in BALB/c mice (Figure 4A) and parasite loads (Figure 4B).

The cysteine protease inhibitor supplementation did not statistically interfere with the lesion size and the parasite load of the *La*E group or their fractions of 68 and 28 kDa in the infection of BALB/c mice by *L. braziliensis* (data not shown).

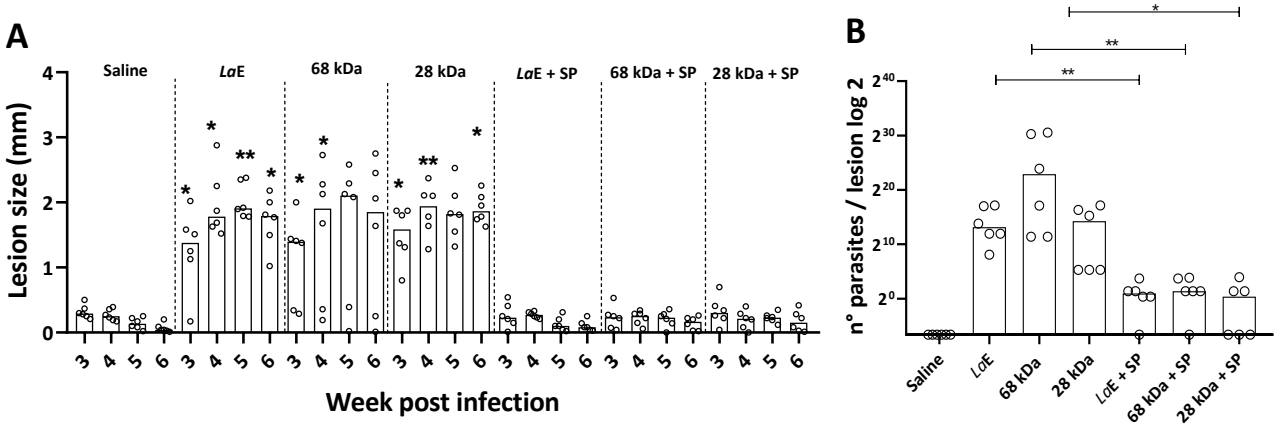

Figure 4. BALB/c mice were intradermally injected with saline, *Leishmania amazonensis* extract (*La*E), or *La*E fractions of 68 or 28 kDa supplemented or not with serine protease inhibitors. After one week the animals were infected in the footpad with 10^7 *Leishmania braziliensis* stationary-phase promastigotes. (A) Lesion sizes and (B) parasite loads were determined 6 weeks after the infection. Each circle represents the result obtained from an individual animal. The horizontal lines represent the median for each group. Statistical significances of differences between groups were evaluated by Dunn's multiple comparisons tests. *, $p < 0.05$ (relative to the respective fraction group without serine protease inhibitor).

Lci1 recombinant enhanced *Leishmania braziliensis* infection in BALB/c mice

The injection of Lci1 recombinant, clearly promoted *L. braziliensis* infection in BALB/c mice, increasing the lesion size (Figure 5A) and the tissue parasitism (Figure 5B).

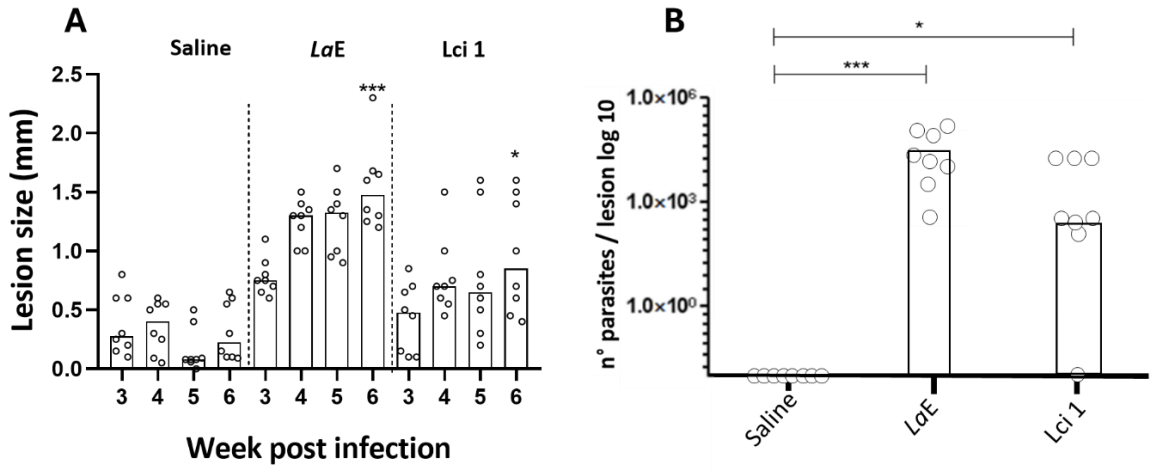

Figure 5. BALB/c mice were intradermally injected with saline, *Leishmania amazonensis* extract (*La*E), and Lci1 protein *Leishmania infantum* amastigote recombinant (Lcir). After one week the animals were infected in the footpad with 10^7 *Leishmania braziliensis* stationary-phase promastigotes. (A) Lesion size from the 3rd to the 6th week after infection of BALB/c mice with *L. braziliensis*. (B) Parasite burden, as assessed by limiting dilution 6 weeks after infection with *L. braziliensis*. Each circle represents the result obtained from an individual animal. The horizontal lines represent the median for each group. Statistical significance of differences between groups was evaluated by Dunn´s multiple comparison test. *, $p < 0.05$; ***, $p < 0.001$ (relative to the Saline group of the respective weeks).

Discussion

Several *Leishmania* molecules expressed by this protozoan are virulence factors, which contribute to pathogenesis and modulate the host´s immune response (Aoki et al., 2019). To investigate the infection-enhancing activity role of these antigens, we previously observed that BALB/c mice injected intravenously or intradermally with *La*E become susceptible to *L. braziliensis* infection (Araújo et al., 2014; Silva et al., 2011). Therefore, for refining this investigation, the present study analyzed the action of selected *La*E fractions in the disease exacerbation murine model.

In the present work, the axenic amastigote native *La*E proteins with apparent molecular masses of 68, 45, 36, and 28 kDa were purified. For the same purpose, (Garg et al., 2006) separated immunostimulatory molecules from *L. donovani* with molecular weights between 97.4 and 68 kDa. Subsequently, these proteins were sub-fractionated and, in combination with BCG, protected hamsters against *L. donovani* challenge (Garg et al., 2006). Analysis of these molecules revealed that elongation factor-2, p45, Hsp70/83, aldolase, enolase, triosephosphate isomerase, disulfide isomerase, and calreticulin were the major immunostimulatory molecules (Kumari et al., 2008).

A similar methodology to the present work was carried out by Bhowmick and Ali (2009) to identify molecules that induce a Th1 immune response in *L. donovani* amastigotes extract. Furthermore, immunogenic proteins, 42, 46, 63, 66, 73, 87, 97, and 160 kDa, from the nonliving promastigote polyvalent *Leishmania* vaccine against American tegumentary leishmaniasis (Leishvacin®) showed protection levels between 28.57 and 57.14% in *L. amazonensis* infected C57BL/10 mice (Cardoso et al., 2003). However, only our study investigated molecules capable of exacerbating *Leishmania* infection.

BALB/c mice that received the 45 and 36 kDa fractions showed no significant enhancement in lesion size six weeks post-infection. However, the 36 kDa fraction significantly increased the parasite load, so this fraction had a higher infection enhancing activity than the 45 kDa.

On the other side, 68 and 28 kDa fractions increased lesion size and parasite burden, proving that these molecules aggravate the disease. The elevation of the IgG1 subclass by these fractions also evidences the immunomodulatory activity of these molecules (Chu et al., 2010; Rostamian et al., 2017). Our previous results had already shown that *La*E injection promotes an intense cutaneous disease associated with higher levels of IgG1 anti-*Leishmania* antibodies and IL-4, in contrast to lower levels of IFN-γ, after *L. braziliensis* infection (Silva et al., 2011). In addition to the current findings, the results indicate that the injection of the 68 and 28 kDa fractions triggered a Th2 immune response, which could be responsible for the progression of the infection.

In the literature, there are many *L. amazonensis* proteins described and some have molecular masses like the purified molecules. Silva-Lopez and Giovanni-De-Simone (2004) described a promastigote monomeric serine protease of *L. amazonensis* involved in the parasite-host interaction with a molecular mass of 68 kDa. A year later, the same research group described that this 68 kDa enzyme is in membranes of intracellular

compartments and plasma membranes and seems to play a role in intracellular traffic (Morgado-Díaz et al., 2005). Indeed, soluble serine proteases are key components of *L. amazonensis* promastigote antigens responsible for disease-promoting immunity (Matos-Guedes et al., 2010). It was also reported by Nandan et al. (1994) proteins isolated from *L. donovani* with 68 and 69 kDa which are members of the 70 kDa heat shock protein family (Hsp70).

The role of Hsp70 in leishmanial infection is still controversial. In the murine model of the present study, Lci1 aggravated the disease and increased IgG1 antibody activity. Similarly, recombinant Hsp70 and Hsp83 induced proliferation in purified BALB/c spleen B-cell populations (Rico et al., 2002). In apparent contradiction to these findings, Lci1 induced a Th1-type immune response in dogs experimentally infected with *L. infantum* (Teixeira et al., 2011). Corroborating this finding, BALB/c mice immunized with Hsp70 showed an increased Th1 response, in addition to reduced parasite load and IL-4 and IL-10 productions (Domínguez-Bernal et al., 2012; Kaur et al., 2011).

Another molecule of similar molecular weight to that described in our work, the 46 kDa promastigote from *L. amazonensis*, when combined with live *Corynebacterium parvum*, could stimulate the production of IFN-γ by lymphocytes of C57BL/10 mice infected by *L. amazonensis* (Cardoso et al., 2003). Another possibility would be PP2C, with approximately 45 kDa, which seems to potentiate the microbicidal activities of macrophages by increasing nitric oxide, TNF-α, and IL-6 *in vitro* (Pranai et al., 2018). Compared to our results, the group of BALB/c immunized with the 45 kDa fraction did not play a role as a promoter of infection, however, showed higher values than the saline group regarding footpad size and parasite load. Thus, it should be possible that these are the same molecules.

LACK could be responsible for the infection-enhancing activity of the 36 kDa fraction since the immune response against this protein has been shown to favor infection of BALB/c mice by *L. donovani* (Julia et al., 1996). In the same direction, LACK can bind to the Vα8 and Vβ4 receptors present on $CD4^+$ T cells, stimulating IL-4 production in *L. major* infection (Schilling & Glaichenhaus, 2001). In addition, LACK drives the induction of higher levels of IL-10 e IFN-γ in patients (Azeredo-Coutinho et al., 2008; Maasho et al., 2000) and the expansion of IL-4 secreting T cells in mice (Julia et al., 1996). For this reason, LACK vaccination trials used approaches to redirect early Th2-type immune response to protect Th1 (Fernández et al., 2018).

The 28 kDa protein detected in *La*E has the same apparent mass as the cysteine protease B, found abundantly in the amastigotes of the *L. mexicana* complex, which is involved in suppressing the Th1 $TCD4^+$ lymphocyte response (Mottram et al., 1996). Strong recognition of a 28 kDa protein in the serum of patients with visceral leishmaniasis by IgG2 has already been demonstrated (Ghosh et al., 1995) and associated with the most severe form of American Tegumentary Leishmaniasis (Magalhães et al., 2021).

One more prominent finding of this work was that the 68 and 28 kDa *La*E fractions supplemented with serine and cysteine protease inhibitors, respectively, inhibited or had no effect on the infection-enhancing activity in BALB/c infected with *L. braziliensis*. Similar results were observed in mice injected intravenously with *La*E in combination with serine protease inhibitors (Silva et al., 2011). On the other hand, supplementing *L. amazonensis* promastigotes antigen with cysteine or serine inhibitors resulted in infection control in *L. amazonensis*-infected BALB/c mice, accompanied by decreased production of TGF-β, IL-10, and TNF-α (Morgado-Díaz et al., 2005). The additive action of the cysteine protease inhibitor can be easily explained by the difference in protein compositions and concentrations of amastigotes and promastigotes extracts. We expected to find inhibition only in the 68 kDa fraction, since its molecular weight is compatible with a serine protease described in the literature (Silva-Lopez & Giovanni-De-Simone, 2004).

Despite the intravenous injection of protease inhibitors alone did not affect the infection of BALB/c mice by *L. braziliensis* or by *L. amazonensis* (Silva et al., 2011), it is important to consider that serine protease inhibitors could interfere with the infection-enhancing activity of the fractions in a manner independent of the presence of serine in the *La*E. The inhibitors may be still present in effective concentrations even a week after their injection and after undergoing dilution in aqueous compartments *in vivo* and could therefore act on the serine proteases of parasites that were injected a week later. *In vivo,* concentrations of the inhibitors would be between 45 and 50 μM. Recently, BALB/c mice infected with *L. amazonensis* or *L. infantum* and treated with TPCK showed a reduction in the parasite loads (Machado et al., 2022).

An alternative is the action of inhibitors by promoting a Th1-type immune response (Matos-Guedes et al., 2010), consequently inhibiting a Th2 response. In agreement with this hypothesis, it has been reported in the literature that serine protease inhibitors stimulate the Th1 response in experimental models of respiratory allergy and neoplasia (Ishizaki et al., 2008; Kim et al., 2004).

Molecules described in the literature as protective against *Leishmania* infection may not be the potentiating proteins in our murine model. For instance, β tubulin from *L. donovani*, incorporated into a liposome, induced a reduction in parasite load in BALB c^{-1} mice (Bhowmick & Ali, 2009). Similarly, elongation factor-2 (LeLF-2), stimulated PBMC from patients cured of visceral leishmaniasis and individuals exposed to *Leishmania* infection to produce the cytokines IL-12, IFN-γ, and TNF-α (Kushawaha et al., 2011). Protein disulfide isomerase (PDI) was related to Th1 response in individuals residing in an anthroponotic visceral leishmaniasis endemic area and individuals cured of this disease (Kushawaha et al., 2012). Vaccination with a liposomal EF1- α (leishmanial elongation factor-1α, 36 kDa protein) directs a Th1 protect response in BALB/c mice (Sabur et al., 2018). In this order, mice immunized with a mixed 31, 36, and 51 kDa formulation showed significantly higher INF-γ and IgG2a compared to infected controls in an experimental model of visceral leishmaniasis (Kaur et al., 2011). Therefore, these molecules are unlikely to promote infection. However, to determine the potentiating activity of these proteins in our murine model, the polypeptides would have to be tested individually, purified from *La*E, or obtained by recombinant DNA technique.

Another hypothesis to explain the promotion of infection would be that it results from the protocol used, rather than from an intrinsic property of the proteins. This hypothesis would imply that any *Leishmania* protein, when injected intradermally at a low dose, would potentiate the infection. Although some authors have described that subcutaneous, but not intravenous (Bhowmick & Ali, 2009) and epidermal (Weiss et al., 2007) injection of *Leishmania* extract potentiates infection, a similar effect of intradermal injection of antigens has not been reported.

Conversely, it has been described that dendritic cells from the dermis of leishmaniosis infection-resistant mice inoculated with *L. major* migrate to the lymph nodes carrying parasite antigens and induce T lymphocyte proliferation (Mayerova et al., 2004), initiating a protective immune response. Another finding that can be used as evidence is that the 45 kDa fraction did not significantly potentiate the infection, although the mice in this group showed higher values for lesion size and parasite load when compared to the negative control. Thus, the introduction of antigens into the dermal compartment alone should not promote infection, and this targeting would depend on the nature of the antigen.

Conclusion

Our findings confirm the biological capability of the protein fractions to potentiate *L. braziliensis* infection in our murine model. This effect may be attributed to one or more proteins. However, it is necessary to determine the composition of these fractions to characterize the molecules in the future. We highlight that LACK and the serine protease with a molecular weight of 68 kDa may be present in the fractions. We also emphasize that HsP70 may be involved in infection potentiation, corroborating the observed findings of Lci1. This work brings prospects for identifying molecules aiming to determine therapeutic targets and antigenic formulations in vaccines against leishmaniasis.

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