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Serine proteases of *Leishmania amazonensis* as immunomodulatory and disease-aggravating components of the crude LaAg vaccine

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ABSTRACT

We previously demonstrated that intradermal and intramuscular vaccination with *Leishmania amazonensis* promastigote antigens (LaAg) increases the susceptibility of BALB/c mice to cutaneous leishmaniasis. In this study, we investigated the role played by serine and cysteine proteases as disease-promoting components of LaAg. Mice were immunized by the intramuscular route with LaAg that was pre-treated with a pool of serine or cysteine protease inhibitors (SPi and CPi, respectively) prior to infection with *L. amazonensis*. Neutralization of either enzyme type reversed the disease-promoting effect of LaAg, as seen by the slower lesion development. However, the parasite burden was only effectively controlled in mice receiving SPi-treated LaAg. Protection was associated with diminished production of TGF-β and particularly IL-10 in response to parasite antigens by the lesion-draining lymph node cells of vaccinated mice relative to control. In vitro, soluble proteases isolated from LaAg (LaSP-Sol) directly activated IL-4, IL-10 and TGF-β production by immune cells. Like native LaAg, vaccination with LaSP-Sol primed mice to respond to parasite challenge with a strong Jones-Mote cutaneous hypersensitivity reaction, and increased susceptibility to infection. Furthermore, neutralization of serine but not cysteine proteases blocked the capacity of LaAg to sensitize mice for Jones-Mote reaction. Together, these results indicate that soluble serine proteases are key components of LaAg responsible for its disease-promoting immunity.

1. Introduction

Leishmaniasis is a complex of diseases caused by different species of the protozoan parasite Leishmania that affects over 12 millions of people worldwide [1]. No vaccine is yet approved for clinical use, despite the fact that protective immunity can be achieved by injecting infective parasites into the skin of healthy individuals, a practice known as leishmanization [2]. Protection seems to require living parasites, as all first-generation vaccines evaluated to date in phase 3 clinical trials failed to protect vaccinated individuals against leishmaniasis [3]. One of the vaccines comprised of killed promastigotes of *Leishmania amazonensis* (Leishvacin®, herein named LaAg) failed to induce protection in a study in Colombia [4] regardless of its immunogenicity to healthy volunteers [5]. Moreover, subcutaneous injections with LaAg alone can significantly increase the susceptibility of Rhesus monkeys to experimental infection with *L. amazonensis,* despite the enhanced IFN-γ production and increased delayed-type cutaneous hypersensitivity [6]. Likewise, intramuscular LaAg was found to increase the susceptibility of BALB/c mice to cutaneous leishmaniasis, in a manner associated with upregulated TGF-β overcoming the increased IFN-γ [7].

The nature of the disease-promoting component(s) accounting for the LaAg failure in humans is unknown. Detrimental effects of vaccination have been ascribed to Leishmanial antigens, including the highly conserved leishmanial homologue of receptors for activated C kinase (LACK) protein responsible for the rapid Th2 responses in *L. major* infection of BALB/c mice [8], the *L. major* lipophosphoglycan (LPG) that promotes disease enhancement when administered by the s.c. route to BALB/c mice [9] and the IL-10 inducing LPG-associated kinetoplastid membrane protein-11 (KMP-11) [10]. However, parasite proteases seem to be critical in LaAg, as demonstrated by the reversion of its in vitro T-suppressive activity by pretreatment with a cocktail of protease inhibitors [11]. Other studies demonstrated that cysteine and metalloproteases are important virulence factors playing a major role in host cell invasion and pathogenicity [12,13]. In addition, cysteine protease B of *L. mexicana* has been shown to inhibit host Th1 responses and protective immunity [14]. Serine proteases have also been demonstrated to occur in *Leishmania* [15–18], and they seem important for effective immunity, as immunization of hamsters with killed *L. braziliensis* amastigotes induced protective immunity only when

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the immunizing parasites were pre-treated with the serine protease inhibitor TLCK [19].

In this work, we investigated the role played by serine as compared with cysteine proteases on the LaAg failure as a vaccine. This study may contribute to a better understanding of the detrimental effect of leishmanial proteases for the host and help improve the efficacy of Leishvacin®, one of the most tested antileishmanial vaccines in man.

2. Materials and methods

2.1. Animals

BALB/c mice were originally obtained from Jackson Laboratory (Bar Harbor, Maine). Animals were bred and maintained at our own facilities using sterilized bedding, filtered water and pelleted food. Female mice were used at 6–8 weeks of age in all experiments. Experimental protocols were previously approved by the Animal Use Committee of the Institute of Biophysics/Federal University of Rio de Janeiro (Brazil).

2.2. Parasites

For antigen preparation, *L. amazonensis* (IFLA/BR/67/PH8) routinely isolated from mouse lesions were transformed and maintained as promastigotes at 26 °C in M199 medium containing 10% heat-inactivated fetal bovine serum (HIFCS, GIBCO Laboratories, Grand Island, NY). For infection, *L. amazonensis* (MHOM/BR/75/Josefa strain) promastigotes rendered fluorescent by transfection with green fluorescent protein (GFP) [20] were used. These were periodically cultured in 1 mg/mL of geneticin for selection of highly fluorescent clones.

2.3. LaAg preparation and treatment with protease inhibitors

L. amazonensis promastigotes antigens (LaAg) were prepared as previously described [7,20]. Briefly, stationary-growth phase promastigotes were washed three times in phosphate buffered saline (PBS) and subjected to three cycles of freezing and thawing. LaAg was lyophilized, stored at −20 °C and reconstituted with PBS immediately prior to use. Serine protease-depleted LaAg (LaAg + SPi) was prepared by treating 1 mg of LaAg with a pool of irreversible serine proteases inhibitors comprised of 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM N-tosyl-lysine chloromethyl ketone (TLCK) and 100 μM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in total of 1 mL of PBS for 4 h on ice. Cysteine protease-depleted LaAg (LaAg + CPi) was prepared by treating LaAg with a pool of cysteine proteases inhibitors containing 100 μM E-64 (irreversible inhibitor) and 100 μM p-hydroxy-mercury-benzoate (PHMB, reversible inhibitor) for the same period of time. LaAg + SPi and LaAg + CPi were dialyzed for 8 h against PBS using a 3 kDa-pore membrane (Sigma–Aldrich) to remove free protease inhibitors. Untreated LaAg was dialyzed in the same way. Protein contents were determined by the Lowry method using bovine serum albumin (BSA) as a standard.

2.4. Preparation of soluble serine proteases (LaSP-Sol)

Soluble serine proteases were purified from LaAg as described by Silva-Lopez and De-Simone [17]. LaAg were prepared in Tris–HCl buffer, pH 7.5, containing 5 mM $CaCl_2$. LaAg was centrifuged (100,000 × *g* for 30 min at 4 °C) and the clear supernatant was loaded onto a pre-equilibrated (10 mM Tris–HCl buffer, pH 7.5, containing 5 mM $CaCl_2$) aprotinin-agarose affinity column (2.5 mL; Sigma–Aldrich). After washing with 20 bed volumes, the material was eluted with 10 mM Tris–HCl buffer, pH 7.5, containing 1.5 M NaCl. 1 mL fractions were collected on ice and the effluents absorption at 280 nm was monitored to detect protein peak. Protein fractions were then pooled (LaSP-Sol), dialyzed against phosphate buffered saline (PBS), and the protein concentration was quantified by the Lowry method.

2.5. Mouse vaccination, infection and Jones-Mote hypersensitivity

For vaccination, BALB/c mice received two intramuscular injections in the thighs with 25 μg of antigens (protein content) in 0.1 mL of PBS with a 7-day interval. When necessary, LaSP-Sol was co-administrated with 100 μg of saponin (Riedel Del Haen's). For infection, 7 days after the second vaccine dose, animals were subcutaneously injected with 2×10^6 stationary-phase *L. amazonensis* promastigotes in the left hind footpad. Lesion sizes were measured with a dial caliper every 3–7 days and expressed as the difference between the thicknesses of infected and the contralateral non-infected footpads. For parasite load determination, the fluorescence intensity of the tissue lysates was determined as previously described [20]. Briefly, at the end of the experiments each infected foot was skinned and individually grinded in 2 mL of PBS. Tissue debris was removed by gravity sedimentation for 10 min, and the fluorescence intensity in twofold dilutions of the supernatants was read in triplicate in a black microtiter plate with a plate reader fluorimeter (Bio-Tek) at 435 nm excitation and 538 nm emission.

2.6. Cytokine production

Single-cell suspensions were isolated from the draining popliteal lymph nodes at the indicated days after infection. Cells were adjusted to 4×10^6 per mL in Dulbecco's minimal essential medium containing 10% HIFCS, 20 mM HEPES, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin sulfate (Sigma–Aldrich) and 50 μg/mL of LaAg or LaSP-Sol, and 1 mL sample plated in 24-well tissue culture plates. Cells were cultured for 48 h and the levels of IL-4, TGF-β, IFN-γ, IL-10 and TNF-α in the supernatants were measured by ELISA following the manufacturer's instructions (R&D Systems, Minneapolis, USA). For TGF-β, the supernatants were heated to 80 °C for 5 min prior to the assay, as previously described [7].

2.7. Statistical analysis

All experiments were reproduced at least twice with very similar pattern of results, and a representative experiment is shown. The statistical differences between the groups were determined by the Student's *t*-test using the GraphPad InStat software, and were considered significant when $p < 0.05$.

3. Results

3.1. Treatment of LaAg with serine protease inhibitors reverses its disease-promoting effect and selectively prevents IL-10 production during infection

To investigate the role played by serine proteases in the disease-aggravating effect of LaAg, BALB/c mice were vaccinated with LaAg treated with a pool of serine protease inhibitors (LaAg + SPi). For comparison, a pool of cysteine protease inhibitors was also used (LaAg + CPi). The results in Fig. 1 show that untreated LaAg expectedly accelerated lesion development and increased parasite growth whereas treatment of LaAg with protease inhibitors reversed its susceptibility enhancing effect. Vaccination with LaAg + SPi induced protection observed by the significantly smaller lesion sizes and parasite burden compared to PBS while vaccination with LaAg + CPi just controlled the lesion growth (Fig. 1). Inhibition of serine proteases was more efficient than cysteine proteases, as

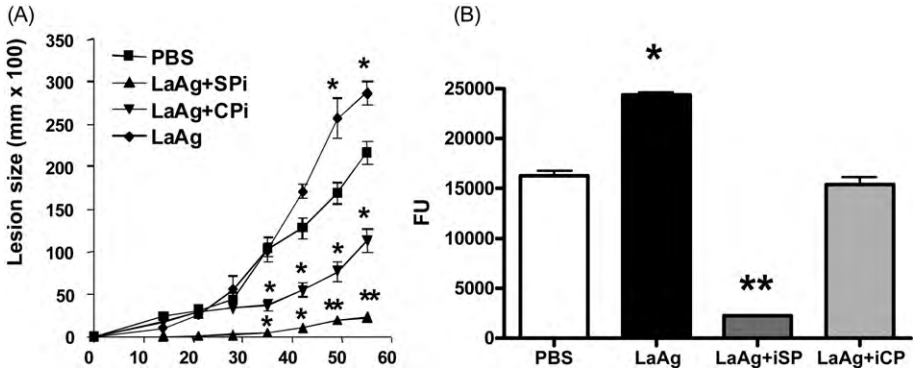

Fig. 1. Effect of protease inhibition in the LaAg efficacy against *L. amazonensis* infection. BALB/c mice were i.m. vaccinated on days −14 and −7 with 25 μg of untreated LaAg; with LaAg that was pre-treated with the serine protease inhibitors PMSF, TLCK and TPCK (LaAg + SPi); or with the cysteine protease inhibitors E-64 and PHMB (LaAg + CPi). Controls received PBS alone. On day 0, animals were infected with 2×10^6 *L. amazonensis*-GFP promastigotes. (A) The increase in lesion sizes was expressed at the indicated days as the thickness increase of the infected footpads. (B) The parasite loads in the infected feet were measured on day 55 and expressed as fluorescence units (FU). Means ± SD ($n = 5$). $^*p \leq 0.05$ in relation to PBS, and $^{**}p \leq 0.01$ in relation to LaAg + CPi.

both the lesion growth (Fig. 1A) and the parasite loads (Fig. 1B) were significantly lower in animals vaccinated with LaAg + SPi compared to LaAg + CPi. This reversing effect was unlikely due to free inhibitors, as these were extensively removed by dialysis. Fig. 2 shows that both vaccination with LaAg + SPi and LaAg + CPi led to a decreased production of IFN-γ, TNF-α and TGF-β cytokines by lesion-draining cells, as compared with non-vaccinated controls. There is a significant reduction of TNF production in LaAg + SPi in relation to LaAg + CPi. However, since only IL-10 was downmodulated in LaAg + SPi but not in LaAg + CPi-vaccinated animals, it appears that the differential production of IL-10 contributed to the protective efficacy of LaAg devoid of serine protease activity.

3.2. Soluble serine proteases of LaAg activate Th2-type cytokines in vitro

Since inactivation of serine proteases reversed the deleterious effect of i.m. LaAg immunization, a soluble serine protease-fraction was isolated from LaAg (LaSP-Sol) and tested for their capacity to activate cytokine production by immune cells in vitro. Fig. 3 shows that LaSP-Sol promoted an enhanced production of IL-4, IL-10 and TGF-β in relation to non-stimulated cells. Although not statistically significant at $p < 0.05$, IFN-γ production was slightly decreased. Together, these findings indicate that soluble serine proteases of LaAg alone can activate Th2-type and/or regulatory T cells.

3.3. Pre-immunization with soluble serine proteases of LaAg sensitizes mice for enhanced susceptibility to infection

To further validate the detrimental role of serine proteases in the LaAg vaccine, BALB/c mice were immunized by i.m. route with LaSP-Sol prior to infection with *L. amazonensis*. Fig. 4 shows that like LaAg, vaccination with LaSP-Sol rendered mice more susceptible to infection, as demonstrated by a faster lesion growth (Fig. 4A) and increased parasite burden (Fig. 4B) in comparison to non-vaccinated animals. Even when associated with saponin, a known Th1-stimulating vaccine adjuvant [21], the disease-promoting effect of LaSP-Sol was further potentiated rather than reversed (Fig. 4C and D). Although the contribution of lipophilic membrane-bound serine proteases cannot be precluded, these results show that hydrophilic serine proteases are strongly immunogenic and important disease-promoting components of LaAg.

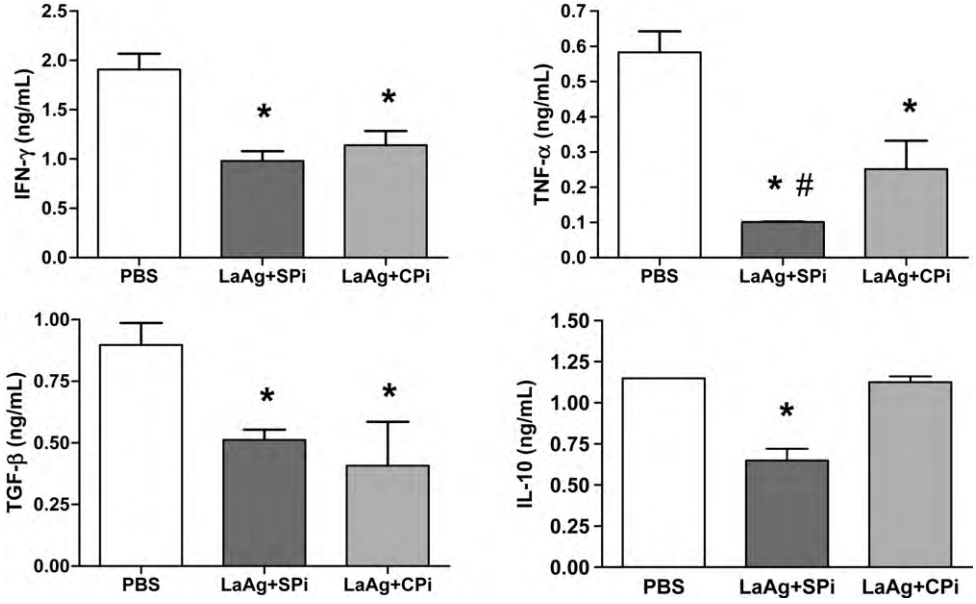

Fig. 2. Cytokine production by mice vaccinated with serine and cysteine protease-depleted LaAg. BALB/c mice were vaccinated and infected as described for Fig. 1. At day 55 of infection, the levels of IFN-γ, TNF-α, TGF-β and IL-10 were measured in the supernatants of LaAg-stimulated draining lymph node cells. Means ± SD ($n = 5$) $^*p \leq 0.05$ in relation to PBS controls, $\#p \leq 0.05$ in relation to LaAg + CPi.

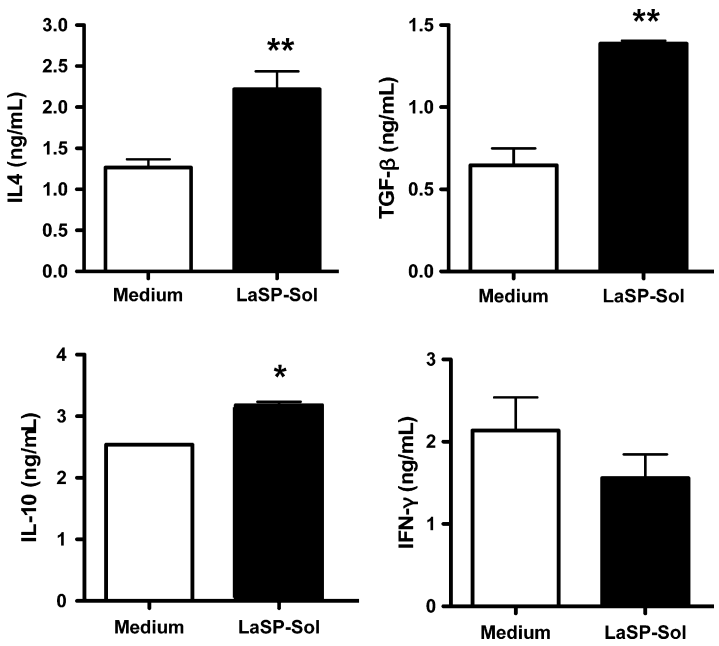

Fig. 3. In vitro cytokine production by immune cells stimulated with LaSP-Sol. Lesion-draining lymph node cells isolated from 7-day infected mice were re-stimulated in vitro with 50 μg/mL of soluble serine proteases of LaAg (LaSP-Sol). After 48 h, the levels of IL-4, TGF-β, IL-10 and IFN-γ were measured in the cell supernatants. Means ± SD ($n = 5$). $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ in relation to unstimulated controls (medium).

3.4. Serine proteases of LaAg drive the disease-associated Jones-Mote reaction

While the classical delayed-type hypersensitivity (DTH) response is characterized by an intense mononuclear cell infiltration within 24–72 h, the Jones-Mote reaction is characterized by a marked basophil infiltration reaching a peak at 18–24 h, and fading or absent at 48 h [22]. DTH has been correlated with protection against *L. major* infection in BALB/c mice recovered from active infection, whereas the Jones-Mote reaction has been associated with the inability to fight infection following immunization by the s.c. or i.d. route with formaldehyde killed *L. major* pro-

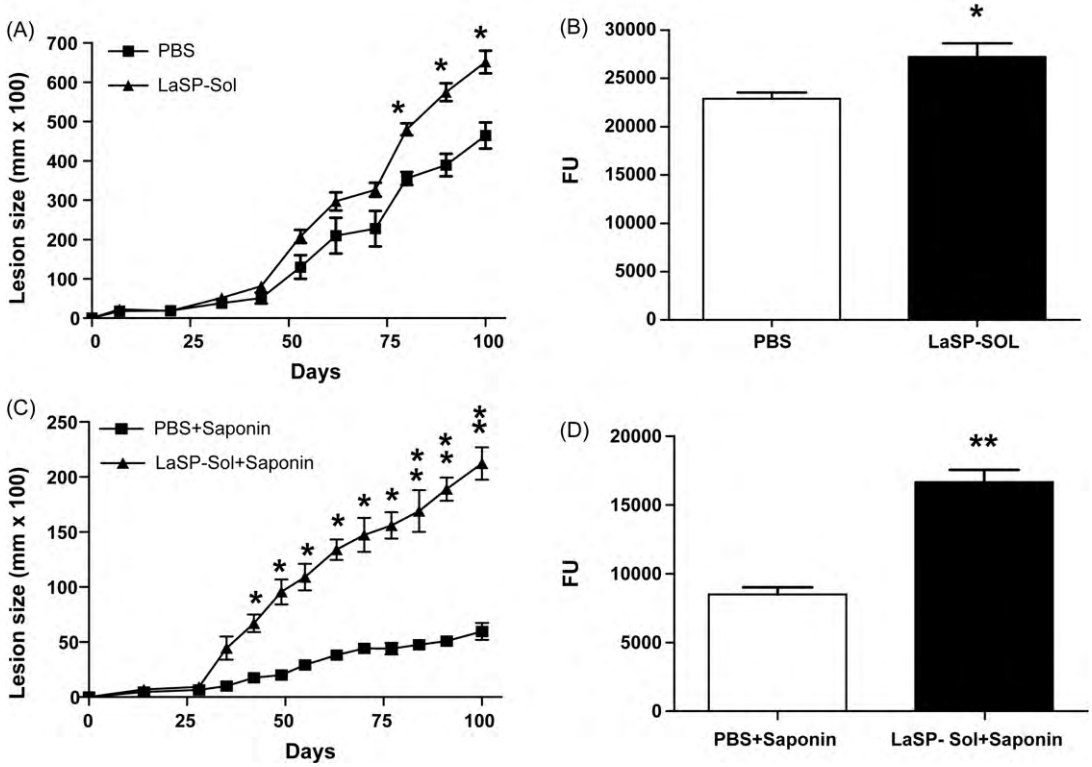

Fig. 4. Pre-vaccination with LaSP-Sol leads to increased susceptibility to infection. BALB/c mice were i.m. vaccinated with 25 μg of LaSP-Sol in the absence (top panels) or in the presence (bottom panels) of 100 μg of saponin on days −14 and −7 and infected on day 0 as described for Fig. 1. (A and C) The lesion sizes are expressed as the thickness increase of the infected footpads. (B and D) The parasite loads in the 100-day-infected feet are expressed as fluorescence units (FU). Means ± SD ($n = 5$). $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ in relation to PBS controls.

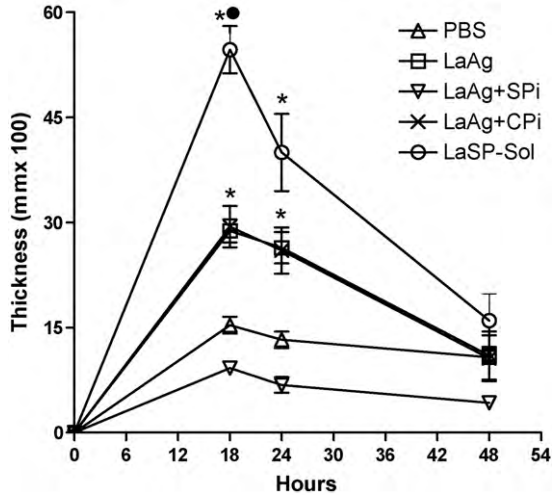

Fig. 5. Effect of LaAg and its proteases in the Jones-Mote-type cutaneous hypersensitivity response. BALB/c mice were vaccinated with LaAg, LaAg + SPi, LaAg + CPi or LaSP-Sol on days −14 and −7 of infection, as described for Figs. 1 and 4. Controls received PBS alone. Animals were then challenged with 2×10^6 *L. amazonensis*-GFP in the footpad and the kinetics of the hypersensitivity response was scored thereafter as the difference between infected and non-infected footpad thicknesses. Means ± SD ($n = 5$) $^*p \leq 0.01$ in relation to PBS controls and $\bullet p \leq 0.01$ in relation to LaAg.

mastigotes [23]. To verify whether LaAg also primes mice to a Jones-Mote-type reaction, mice were immunized twice with LaAg and the footpad thickness was measured at different times after the onset of infection. Fig. 5 shows that LaAg predisposed animals to a Jones-Mote-type reaction, as it faded after 18 h. Neutralization of serine proteases but not cysteine proteases with a cocktail of selective inhibitors abolished the capacity of LaAg to induce Jones-Mote reaction. LaSP-Sol induced a much more pronounced Jones-Mote response than LaAg. Together, these findings further support the notion that soluble serine proteases are important components of LaAg responsible for its contra protective immunity.

4. Discussion

In this study, the use of specific serine protease inhibitors and the isolation of an active fraction enriched in serine proteases pointed out the detrimental role of these enzymes in the disease-promoting effect of LaAg vaccine. Although the use of specific inhibitors showed that cysteine proteases also seem to be involved, compatible with the capacity of cathepsin L-like cysteine protease B of *L. mexicana* to inhibit host Th1 responses and protective immunity [14], serine proteases seem to be more critical, as only inhibition of the latter led vaccinated animals to effectively control the parasite burden (Fig. 1B).

In murine *L. major* infection, protection is clearly associated with the production of macrophage activating IFN-γ [24]. However, in *L. amazonensis* infection IFN-γ seems to be required only in the later stages of infection as demonstrated by the normal capacity of IFN-γ-deficient C57Bl/6 mice to control parasite growth in early but not late infection [25]. Previous studies with LaAg in monkeys and mice showed that the disease-aggravating effect promoted by s.c. or i.m. vaccination was accompanied by an increased capacity of antigen-stimulated cells to produce increased rather than decreased amounts of IFN-γ [6,7]. A later study showing that s.c. vaccination with LaAg led to a protective effect in IL-12p40-deficient C57Bl/6 mice unable to produce normal levels of IFN-γ [26] demonstrated that, indeed, IFN-γ does impair LaAg efficacy.

In the present study, we observed that despite their differential effectiveness in parasite growth control (Fig. 1) animals vaccinated with LaAg devoid of either protease activities responded in vitro in a similar manner to antigen recall. The decreased capacity to produce both the pro-inflammatory IFN-γ and TNF-α and the anti-inflammatory TGF-β (Fig. 2) indicate that those cytokines are not the most relevant for an optimal effect. Although our understanding of the importance of TNF-α in *L. amazonensis* infection is limited, TNF-α could contribute to pathogenesis as observed for *L. chagasi* [27,28] and *L. braziliensis* [29]. The reduction of TNF-α could contribute to the control of lesion growth, and probably the reduction of TNF-α of LaAg + SPi in relation to LaAg + CPi could help in the disease control. Rather, the macrophage deactivating IL-10 was decreased only in LaAg lacking serine but not cysteine proteases (Fig. 2), indicating that IL-10 was critical. This result is in agreement with the loss of immunity in mice that resolved their primary virulent *L. major* infection by inoculation of killed parasite through induction of IL-10 demonstrating that IL-10 is critical for *L. major* and *L. amazonensis* infection [30]. However, the role of other cytokines not studied here such as IL-12 and IL-17 should also be considered, especially in the early stages of infection.

Thus, although not phenotypically identified as such, it appears that serine and to a lesser extent cysteine proteases of LaAg activate functional IL-10- and TGF-β-producing regulatory T cells (T regs). Previous studies have described the beneficial effect of the transient expansion of CD4+ CD25+ T regs cells concomitant with the decreased activation of IFN-γ-producing effector T cells in *L. amazonensis* infection [31]. Allergen peptidases have been associated with the degradation of the CD25 marker of T regs [32], suggesting that inhibition of serine proteases in LaAg could have prevented the degradation of CD25 during vaccination contributing to the observed protection.

We also found that LaAg and LaSP-Sol induced Jones-Mote reaction, whereas inhibition of serine proteases in LaAg reversed this effect (Fig. 5). These results indicate that serine proteases are involved in the sensitization of mice to this early cutaneous hypersensitivity response that is associated with a basophil infiltration [22]. Recently, a number of studies have demonstrated that basophils can function as effective MHC class II-dependent antigen-presenting cells overriding dendritic cells for the in vivo induction of Th2-type responses caused by helminth infection or protease allergens [33–36]. Thus, it is feasible to assume that serine proteases of LaAg could have activated basophils and triggered the resulting Th2 response observed in *L. amazonensis* infection of vaccinated animals.

Despite the indication that the active serine protease(s) is soluble, given that the LaSP-Sol fraction mimicked the parental LaAg immunomodulatory and disease-promoting effects (Figs. 3 and 4), its identity remains unknown. Recently, several serine proteases were identified in the *L. major* genome, including a subtilisin-like protein, prolyl oligopeptidases such as oligopeptidase B, a type I signal peptidase, a 26S regulatory proteasome subunit, a nucleoporin homolog, and various orthologs of the rhomboid-like intramembrane serine peptidase family [37]. The active LaAg serine protease(s) is likely distinct from the 56 kDa serine protease present in the *L. pifanoi* amastigote antigen P-8, or the type I signal peptidase of *L. major,* as these were described as protective, rather than contraprotective vaccine components [38,39]. Contraprotective serine proteases were also suggested to occur in *L. braziliensis*, as only vaccination with TLCK-killed but not untreated *L. braziliensis* amastigotes induced protection against homologue infection in hamsters [19], supporting the notion that they may not be restricted to *L. amazonensis*. We recently cloned two serine peptidases of *L. amazonensis*, the soluble cytosolic oligopeptidase B (OPB) also present in *Trypanosoma cruzi* [40,41] and the oligopeptidase B2 (OPB2) [42] whose localization is as yet unknown. Studies evaluating the immunogenicity of OPB and OPB2 are under way and may help clarify their participation in the LaAg contraprotective effect.

Altogether, the work presented here demonstrates that soluble serine proteases of *L. amazonensis* are important components of LaAg/Leishvacin®, and show that their inhibition can rescue vaccine efficacy encouraging further work on this first-generation vaccine.

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