

Serine Proteases and Vaccines against *Leishmaniasis*: A Dual Role

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Abstract

Serine proteases are involved in several biochemical processes that are essential for the biology of pathogens, including *Leishmania sp.* Considering their importance, an interest in serine proteases for vaccine development against *leishmaniasis* has been raised. As targets, these enzymes have demonstrated a dual role in a vaccine against *leishmaniasis*, both protective and a counter-protective, depending on the conditions that they are evaluated. In this work, serine proteases or inhibitors of them that have been used as components of vaccines to *Leishmania sp.* are presented, aiming to disseminate the knowledge gained about these proteases and their potential in potential vaccine against *leishmaniasis*.

Keywords: Serine proteases; Vaccine; intramuscular immunization; Intranasal immunization; Counter-protective; Protection; TGF-beta

Abbreviations

LaAg: *L. amazonensis* promastigotes antigens; LaE: *L. amazonensis* extract from amastigotes; SP: Serine Proteases; LaSP-Sol: Soluble Serine proteases from *L. amazonensis* promastigotes; LaSP-Ex: Extracellular serine proteases from *L. amazonensis* promastigotes supernatant of culture; pSP: Secreted serine protease from *L. donovani*; Lmjsp: Signal Peptidase I from *L. major*.

Introduction

Leishmaniasis is a protozoonose caused by the etiologic agent *Leishmania sp.* It presents two clinical forms, cutaneous, which varies from a painless, localized and self-healing skin lesion to necrotizing forms in mucosal tissue, and a visceral form that can compromise the function of the liver and spleen with a high incidence of lethality [1]. The disease is found mainly in tropical countries of Asia, Africa, South America and Mediterranean Europe. Current estimates suggest that nearly 11 million people are infected around the world [1]. The World Health Organization (WHO) due to its epidemiology and socio-economic impact classifies it as a neglected tropical disease.

The clinical treatments available for leishmaniasis are based on antimicrobial drugs and amphotericin B, which have serious collateral effects that include cardiotoxicity and nephrotoxicity, respectively. In addition, their high cost and the lack of patients adhering to the long

course of treatment required has made them less than ideal options for controlling leishmaniasis [2]. Unfortunately, there is no vaccine approved for human use against leishmaniasis despite the observation of protective immunity acquired following natural infections, suggesting that the production of an effective vaccine is feasible [3]. Leishvacin[®], which is composed of whole-killed promastigotes of *L. amazonensis*, was extensively studied for use as a vaccine in humans. Immunization with Leishvacin[®] does lead to conversion in the Montenegro skin test and induces IFN- γ responses in human volunteers [4]. However, the vaccination failed in a controlled phase III clinical trial in Colombia [5]. Three veterinary vaccines have been approved and are available against canine visceral leishmaniasis, one in Europe and two in Brazil [6]. These vaccines have inspired the development of other forms for a human vaccine with one currently in phase of clinical trials, known as Leish-111F, which consists of a multi-subunit recombinant protein that has MPL-SE as its adjuvant [7,8].

In the search for new targets for the development of anti-leishmanial drugs and vaccines, knowledge of the parasite biology and the mechanisms involved with the parasite-host interactions are extremely important. Within this framework, leishmania proteases have been described as having a crucial role in parasite survival and infectivity since their activities are necessary for processing exogenous proteins as nutrients [9] and in the invasion of host cells and tissues [10].

Among the defined proteases, serine proteases represent an important group of enzymes with numerous biological functions [11]. From the sequencing of the *L. major* genome, a number of distinct serine proteases have been identified: (i) subtilisin-like, an S9 family

member that also includes prolyloligopeptidase (POP), peptidyl-1 dipeptidase IV and oligopeptidase B (OPB), (ii) a type I signal peptidase (SPase I), (iii) a lysosomal serine carboxypeptidase, (iv) a 26S regulatory proteasome subunit, (v) a nucleoporin homolog and (vi) an orthologous of the rhomboid-like intramembranous serine peptidase family [12]. From the *L. amazonensis* genome, two others of interest are; (vii) Oligopeptidase B [13] and (viii) Oligopeptidase B2 [14].

Among these, subtilisin-like, Oligopeptidase B and SPase I have been studied for their contribution in parasite biology or for host-parasite interactions. Deletion of subtilisin in *L. donovani* reduced parasite differentiation *in vitro* along with virulence in hamster and murine infection models [15]. Oligopeptidase B deficient *L. major* parasites were less capable of infecting and surviving in macrophages *in vitro*, but showed a similar virulence as controls *in vivo* [16]. Oligopeptidase B, along with a new member called have been identified in *L. amazonensis* [13,14], however, their biological roles are unknown. Attempts to create null mutants of SPase I have been unsuccessful, suggesting that this enzyme is crucial to survival. Heterozygote mutants in *L. major* have shown a decreased capacity to infect *in vitro* and the lesions developed in an *in vivo* model with BALB/c infected mice were less severe than controls [17].

Source	Specie	References
<i>Leishmania amazonensis</i>		
Serine oligopeptidase	101 kDa	[18]
SP purified from aqueous extract	68 kDa	[19,20]
SP from a detergentsoluble extract	60 kDa and 45 kDa	[21]
SP from cell free supernatant	110 kDa	[22,23]
<i>Leishmania braziliensis</i>		
SP from a aqueous extract	60 kDa and 45 kDa	[24]
SP from a detergentsoluble extract	130 kDa, 83 kDa, 74 kDa and 30 kDa	[24]
SP from cell free supernatant	62 kDa, 59 kDa, 57 kDa, 49 kDa and 35 kDa	[24]
<i>Leishmania chagasi</i>		
SP from a aqueous extract (LCSII)	105 kDa, 66 kDa, and 60 kDa	[25]
SP from a detergentsoluble extract (LCSI)	60 kDa and 58 kDa	[25]
SP from cell free supernatant (LCSIII)	76 kDa and 68 kDa	[25]
<i>Leishmania donovani</i>		
Intracellular SP	58 kDa	[26]
SP from cell free supernatant	115 kDa	[27,28]

Table 1: Identified serine proteases from *Leishmania spp.*

In addition to the proteases identified by sequence analysis, several proteases have been isolated from promastigotes extracts using chromatography or affinity- chromatography based on binding to aprotinin (Table 1). To date, their identities remain to be determined, which is a priority for complementing the genome-based data to improve our understanding of the complete serine protease profile functioning within the genus *Leishmania*.

The Counter-Protective Effect of Serine Protease

Several studies have demonstrated a protective, anti-leishmanial role for serine protease inhibitors during treatments for infections. These studies demonstrated that serine protease activity could be directly related to host susceptibility to infection. The underlying mechanisms for how serine protease activity enhances susceptibility to infection are unknown. Below, we described serine proteases or serine protease activity related to susceptibility of infection.

TLCK treated *L. braziliensis* and *L. donovani*

N-p-tosyl-L-lysine-chloromethyl ketone (TLCK) is a potent serine protease inhibitor. TLCK treatment of *L. braziliensis* and *L. donovani* parasites was used in a vaccine model for protection against homologous infection events. Initially, hamsters were immunized with *L. braziliensis* (LB) that were exposed to TLCK in culture then challenged with an infection with *L. braziliensis* amastigotes. A gradual proliferation of lymphocytes B and T from lymph nodes that was stimulated by mitogens and a higher response to concanavalin A were observed. In addition, no parasites were located in the lymph nodes after 6 weeks of infection with nodules that were 4 times smaller than those in control hamsters. For *L. donovani*, the pre-immunization of hamsters with treated parasites led to survival for over a year after challenge whereas of the survival period of non-immunized mice was 5 months [29]. This was the first study to address the importance of serine proteases for vaccines against leishmaniasis.

Whole *L. amazonensis* antigens from promastigotes (LaAg) and from amastigotes (LaE)

A role for serine proteases to modulate the immune response of hosts was suggested by observations following the use of whole parasite antigens as an inoculum. When LaAg, which is composed of antigens from whole *L. amazonensis* promastigotes, was associated to *Corynebacterium parvum* and used for immunization, induced protection against *L. amazonensis* infection in C57Bl10 mice [30]. However, LaAg administered to Rhesus monkeys via a subcutaneous route showed an increase in experimental infections with follow up challenges with *L. amazonensis* despite the induction of a higher IFN-gamma release following immunization [31]. In a BALB/c mice cutaneous model, LaAg administered via intramuscular injections also increased the animal susceptibility to infection together with a positive regulation of TGF-beta overcoming the IFN-gamma [32].

To isolate the role of serine proteases in the observed aggravation of infection caused by LaAg, antigens were pre-treated with protease inhibitors (LaAg+SPi) prior to their use as a vaccine in BALB/c mice [33]. In comparison to control mice, vaccinated mice developed lesions more slowly and displayed a lower parasite load suggesting an improved protection that was related to a decrease in IFN-gamma, TNF- alpha, IL-10 and TGF-beta during infection.

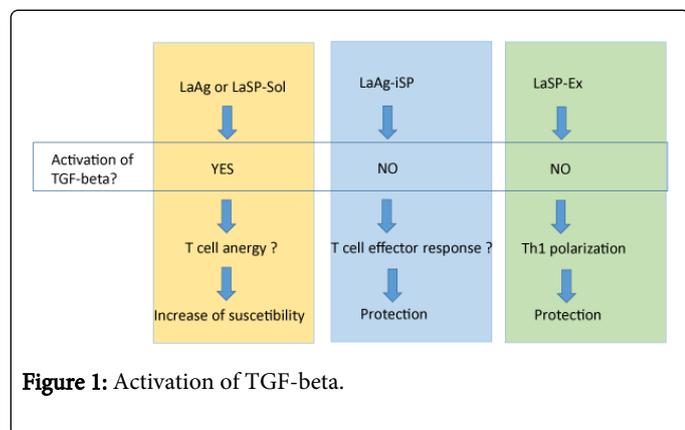
A similar effect was observed in the pre-vaccination treatment when using whole *L. amazonensis* antigen from amastigotes named *L. amazonensis* amastigote extract (LaE) followed by a challenge with *L. braziliensis* [34]. The increase of susceptibility of infection in immunized mice was dependent on the maintenance of serine protease activity. These results suggest a conserved serine protease present in promastigotes and amastigotes that are related to immunomodulation that can enhance the disease progression for *Leishmania* infection.

Soluble serine proteases from *Leishmania amazonensis* (LaSP-Sol)

The first soluble serine protease fraction (LaSP-Sol) was purified by Silva- Lopez et al. [19]. The major protease band displayed a molecular weight of 68 KDa with a number of other minor components. This fraction from LaSP-Sol of *L. amazonensis* was demonstrated to be from membranes of intracellular compartments similar to endocytic/exocytic elements [20].

Following vaccination with LaSP-Sol and a subsequent challenge with parasites, the *in vitro* proliferation of lymphocytes isolated from lymph nodes produced IL-4, IL-10 and TGF-beta in comparison to non-vaccinated mice (seven days post infection). This suggested that LaSP-Sol induced an immune response with a Th2 profile and/or activation of regulatory T cells. However, a comparison between effects using LaSP-Sol to LaAg in BALB/c mice immunized intramuscularly showed a similar profile in lesions and parasite burden compared to non-vaccinated mice [34].

Through these results, it is possible to hypothesize that a possible mechanism for an increase in the susceptibility to a *Leishmania* infection is related to serine protease activity. In support of this hypothesis, it was observed that the application of LaAg or LaSP-Sol increased TGF-beta *in vitro* [32,33]. Proteases can convert latent TGF-beta in active TGF-beta [35]. It has been shown that cysteine peptidase from *L. chagasi* also can enzymatically convert TGF-beta to active form [36,37]; yet, the pre-treatment of LaAg with cysteine peptidase inhibitor was not as effective as the pre-treatment with serine protease inhibitor to overcome the counter-protective effect of LaAg [34]. Since TGF-beta can be related to T cell activity [38-40], it could explain the failure of this vaccination approach within our model system, which is in contrast the success observed in another model, the *Shistosoma manasoni* vaccination [31]. This hypothesis is summarized in Figure 1.



Vaccines Using Serine Proteases as Antigens

Some serine proteases have been evaluated as possible antigens for the development of a vaccine against *leishmaniasis* that are discussed below.

Antigen P8

The first serine proteases evaluated as antigen for vaccine was the P8 antigen from *L. pifanoi*. The association of P8 antigen with *C. parvum* was observed to fully protect BALB/c mice from infection by *L. pifanoi* and partially against *L. amazonensis*. In CBA/J mice, full protection was seen against infections with *L. amazonensis* [42]. This protection was most likely mediated by a Th1 immune response according to the higher levels of gamma interferon produced [42]. The characterization of P8 antigen showed a serine protease immunodominant component with 56 kDa [43].

Signal Peptidase I (Lmjsp)

Using the type I signal peptidase (Lmjsp) cloned from *L. major*, a recombinant protein was created to probe for specific antibodies in the sera of patients that had an acute disease or had recovered from cutaneous and visceral *leishmaniasis*. Independent of their disease state, all patient sera was reactive to Lmjsp [44]. Considering its immunoreactivity, Lmjsp was tested as an antigen within three types of vaccines against *L. major*; DNA/DNA, Protein/Protein and DNA/Protein. All forms of vaccines induced a Th1 profile. When protection against infections by *Leishmania* was evaluated, the DNA/DNA approach was observed to be more effective than the other two approaches with a reduction of 81% in the parasite load [45].

Extracellular Serine proteases (LaSP-Ex) from *L. amazonensis*

An extracellular serine protease fraction (LaSP-Ex) was purified from parasite culture supernatants for the first time by Silva-Lopez et al [22]. A major serine protease of 56 kDa was detected. Through immunohistochemistry, it was localized in the flagellar pocket, cytoplasmic vesicles of promastigotes and other structures that appeared similar to the megasomes of amastigotes. A complete purification and characterization of this protein determined that the enzyme is displayed as a 110 kDa complex in non-reducing condition [23]. It should be noted that other proteins are present in the LaSP-EX preparation after aprotinin-affinity chromatography purification.

The secretion of the 56 kDa suggested that it could be recognized by the host immune system during an infection and a potential vaccine candidate [22,23]. Using immune cells from mice infected for 7-days, stimulation with LaSP-Ex did not increase TGF-β production and inhibited spontaneous IL-10 production [46]. Vaccination with LaSP-Ex via an intranasal delivery was able to control lesion growth and parasite load in BALB/c mice infected with *L. amazonensis*. In addition, isolated spleen cells from mice vaccinated with LaSP-Ex (7 days) and stimulated with LaAg produced IFN-γ, lower levels of IL-4 and IL-10 in comparison to controls and beta TGF was not modulated that suggested a systemic Th1 response profile. In the footpad, there was a reduction in the normally higher levels of gamma IFN and IL-12 with a decrease in TGF-β, suggesting that the mechanism of LaSP-Ex protection is induction of Th1 profile and inhibition of TGF-beta *in vivo* [46].

Secreted serine protease (pSP) from *L. donovani*

A similar serine protease was found in the supernatant culture of *L. donovani* with the same molecular weight of 115 KDa [27] and with the same localization, mainly in flagellar pockets by immunohistochemistry [28]. The expression of the protease was correlated to a high virulence for the parasite and to the metacyclic stage of *L. donovani* promastigotes [28]. When pSP was evaluated as a vaccine against *L. donovani* infection, protection was only obtained when it was associated with IL-12 as an adjuvant, which led to an increase in IFN- γ and TNF- α with a decrease in IL-4 and IL-10 after vaccination [47].

Conclusion and Perspectives

In this review, different formulations were considered for their capacity to induce protection against the wide range of different *Leishmania* strains responsible for infections in endemic regions. The treatment with serine protease inhibitor appears to be a possible approach to improve the efficacy of the most studied vaccine, LaAg, which is similar to Leishvaccine[®]. The addition of specific antigens to vaccine preparations could lead to the development of more effective protection. From the analysis of P8 antigen, LaSP-Ex, pSP and Lmjsp, each demonstrated a capacity to induce protection. Further investigations are needed to determination which serine protease class is represented and their specific biological function that are essential to understand their role in the life cycle of parasite and during infection. Furthermore, other proteins are present in the LaSP-Ex, sSP and Lmjsp that could contribute to their effects and require determination through genomic and proteomic studies. These characterizations can allow the development of the next generation of vaccines that include recombinants proteins and a combination of different serine proteases. Ultimately, these evaluations could allow for the development of a strong vaccine candidate that can induce protection against multiple strains of *Leishmania* parasites.

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