



LALLT (*Loxosceles* Allergen-Like Toxin) from the venom of *Loxosceles intermedia*: Recombinant expression in insect cells and characterization as a molecule with allergenic properties

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ABSTRACT

Bites evoked by Brown spiders (*Loxosceles* genus) are associated with skin injuries (cutaneous rash, itching, swelling, erythema and dermonecrosis) and systemic manifestations. Transcriptome analyses of *Loxosceles* venom glands showed that the venom has a complex composition containing toxins such as phospholipases-D, metalloproteases and hyaluronidases. Here, by screening the RNA from *L. intermedia* venom glands, we cloned a novel allergen toxin, and named LALLT (*Loxosceles* Allergen-Like Toxin). Sequence analysis showed that LALLT is closely related to allergens from other spiders and RNA screening indicated the presence of LALLT orthologues in the venom of other *Loxosceles* spiders. Recombinant LALLT was expressed (~45 kDa) in baculovirus-infected insect cells and purified by affinity chromatography. Antibodies against different *Loxosceles* venoms cross-reacted with LALLT and antibodies against LALLT recognized three *Loxosceles* venoms, revealing epitopes identity. LALLT triggered paw edema in mice and erythema, edema and leukocyte infiltration into the dermis of rabbit skin. Also, LALLT induced vascular permeability in mice, degranulation of rat mesentery mast cells, as well as prompted degranulation and increased calcium influx in RBL-2H3 cells. Data reported suggest for the first time the existence of allergens in *Loxosceles* venoms and make LALLT available for clinical studies about allergenic events arisen by *Loxosceles* envenoming.

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1. Introduction

Accidents caused by bites of Brown spiders, named loxoscelism or necrotic arachnidism, are reported worldwide and constitute a public health problem in several countries. According to SINAN-Brazil (Sistema de Informação de Agravos de Notificação) [1], only in 2019, spiders of the genus *Loxosceles* were responsible for most of the accidents in Brazil, corresponding to 8.391 notified cases. Loxoscelism manifestations include skin lesions characterized by edema, erythema, itching and cutaneous rash surrounding the spider bite, which can evolve to necrotic lesions with gravitational spreading. Into a lesser extent, systemic manifestations can occur including hematological dysfunctions such as intravascular hemolysis, disseminated intravascular coagulation and acute renal failure [2–5].

The venom from *Loxosceles* spiders is a colorless liquid, enriched in proteins and glycoproteins, with a predominance of molecules ranging from 5 to 40 kDa [2–6]. Literature has described in the crude venom three families of highly expressed toxins designated as phospholipases-D (PLDs), metalloproteases (astacin-like proteases) and inhibitor cystine knot peptides (ICKs) [2,4,5,7–12]. Moreover, toxins with low level of expression, such as hyaluronidase, serine proteases, serine protease inhibitors, and a translationally controlled tumor protein (a histamine-releasing factor), also have been described in the venom [9,13–16].

The molecular mechanism by which crude venom induces deleterious activities is under investigation. Despite the complexity of venom composition, just few toxins as PLDs have their participation better understood in the pathology triggered by the accidents [2,4]. There is strong evidence for the participation of PLDs family members, also named dermonecrotic toxin, as playing a role in the skin lesions, which are distinguished by macroscopic dermonecrosis and diffuse inflammatory response at the bite site (histologically described as an

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aseptic coagulative necrosis) [17,18]. Besides that, the PLDs are responsible for systemic events such as intravascular hemolysis, platelet aggregation and renal disturbances [2,4,19–23]. Additionally, studies have shown that other Brown spider venom constituents as hyaluronidases and metalloproteases play a key role in the gravitational spreading of the skin lesion and systemic diffusion of other venom toxins. These molecules act as “spreading factors”, which was experimentally observed and it is based on their hydrolytic activity on hyaluronic acid and other extracellular matrix (ECM) components as fibronectin, fibrinogen and entactin – molecules responsible for tissue integrity and stability. By degrading ECM constituents, venom hyaluronidases and metalloproteases render the extracellular matrix less rigid, increasing the tissue permeability and, consequently, facilitating the spread of other venom components [4,6,8,13,15,24].

Nevertheless, some clinical signs described during the development of loxoscelic lesion as itching, urticaria, swelling and especially cutaneous rash surrounding the bite site are just partially explained by the participation of PLDs family members and spreading factors as hyaluronidases and metalloproteases. Therefore, other families of toxins must be investigated in order to provide a final understanding on the molecular basis of the signs previously mentioned. These clinical signs suggest the involvement of histamine-releasing factor(s) or allergen toxins and justify the use of antihistamines combined with anti-inflammatory drugs for loxoscelism therapy in different parts of the world [2–4,16].

A great difficulty to study *Loxosceles* venom toxins is the low volume of venom produced by spiders and the low protein content obtained by extraction, which prevent the purification of native toxins for biochemical and cell biology studies [2,4,25]. Thus, molecular biology approaches become essential as they allow the obtaining of purified recombinant proteins in sufficient quantities to be tested. Expression of recombinant proteins has been possible mainly due to organisms such as bacteria, yeast and more recently to the baculovirus system, which uses as hosts cell lines from *Spodoptera frugiperda* (Sf9 or Sf21). The prokaryotic system, represented by *Escherichia coli*, although it is the most used, has several disadvantages as: proteins without post-translational modifications and incorrectly folded, accumulation of misfolded proteins in inclusion bodies and presence of bacterial contaminants after purification steps [26,27]. The eukaryotic system using Sf9 cells transfected with recombinant baculovirus is most similar to expression in arachnid cells since these host cells have endoplasmic reticulum and Golgi apparatus. These organelles provide the molecular machinery needed to set post-translational modifications (disulfide bonds and the glycosylation, for example) and, ultimately, enable the correct folding of proteins, which are the most important advantages of the eukaryotic system over the prokaryotic model. For allergens production these features are very important factors, since allergens are molecules that need to have their disulfide bonds formed and their correct folding established, as these will be crucial for the conformational epitopes of B cells [28]. The produced recombinant baculovirus specifically infects the insect cells and have been used to produce proteins of scientific interest as well as for the production of vaccines for both human and veterinary uses [29].

Over the last years, through molecular biology techniques, insights into the toxinology area were enormous and allowed transcriptome analysis, gene cloning and recombinant expression of toxins. Researchers have obtained large amounts of several recombinant molecules from Brown spider venoms such as PLDs isoforms, metalloproteases, ICK peptides, hyaluronidase and TCTP, which deeply improved studies into the molecular action of these toxins [4,5].

Herein, we report the cloning, heterologous expression in insect cells, purification and functional characterization of a novel allergen toxin in the *L. intermedia* venom gland, which was denominated LALLT (*Loxosceles* Allergen-Like Toxin). This study shows for the first time in the literature a broad evaluation of an allergen molecule present in the venom of a *Loxosceles* spider and strengthens the previous clinical

data that suggested allergenic factors are involved on noxious effects after *Loxosceles* spiders envenoming.

2. Material and methods

2.1. Reagents

The venoms of *L. intermedia*, *L. laeta* and *L. gaucho* were kindly provided by CPPI (Production and Research Center of Immunobiological Products, State Department of Health, Piraquara, Paraná, Brazil). The pENTR/D-TOPO vector (pENTR™/D-TOPO™ Cloning Kit), the baculovirus recombination kits (BaculoDirect™ C-Term Transfection Kit), Cellfectin® II Reagent and Clonase® II Enzyme mix were obtained from Invitrogen (Waltham, USA). Both cell culture media for Sf9 cells, Sf-900™ II SFM and Grace's Insect Medium Unsupplemented, were acquired from Invitrogen. Histrap™ Excel 1 mL column was purchased from GE Healthcare Life Sciences (Marlborough, USA). Cromolyn sodium salt (cromolyn) was purchased from Sigma Aldrich (Darmstadt, Germany) and Evans blue from Vetec (Duque de Caxias, Brazil). Coomassie Blue G was purchased from BioRad Laboratories (Hercules, USA).

2.2. LALLT sequence obtainment and cloning into pENTR™/D-TOPO®

In order to obtain the sequence encoding LALLT, the venom gland total RNA from thirty adults *L. intermedia* spiders was extracted by using the TRIzol® reagent [30]. The total RNA purified was converted to cDNA by reverse transcription reaction by using the oligo(dT)₁₇ primer (5'-CGGTACCATGGATCCTCGAGTTTTTTTTTTTTTTT-3') and the Improm-II Reverse Transcriptase Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cDNAs were submitted to ethanol precipitation in the presence of ammonium acetate and then were used as template to the amplification of the specific sequence encoding LALLT by conventional PCR. For this amplification specific primers were designed and synthesized based on a sequence related to allergens which was identified in the study of the transcriptome of the venom gland of *L. intermedia* [9]. Four nucleotide residues were added to the 5' extremity of the forward primer (5'-CACCATGTCTGCG ATGTAT-3') as a requirement for directional cloning into pENTR™/D-TOPO® vector. The reverse primer (5'-AAAACAGCTTCCGATTTC ACG-3') was designed to lack the stop codon sequence so that the C-terminal tags (V5-6xHis fusion tag) were expressed. The amplified product was then analyzed by agarose gel electrophoresis, gel-purified and cloned into the pENTR™/D-TOPO® vector. Next, the construction LALLT-pENTR™/D-TOPO® vector obtained was transformed into electrocompetent DH5α cells and positive clones were analyzed by agarose gel electrophoresis. The constructions extracted from the positive clones were sequenced on both strands using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Reactions were analyzed by an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). The M13 Forward (5'-GTAAAACGACGGCCAG-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3') primers were used for priming sequencing reactions. Sequences were analyzed and the deduced protein products from these sequences were used to search the GenBank protein databases at NCBI.

2.3. Screening for LALLT orthologues in venom glands from *L. laeta* and *L. gaucho*

Twenty spiders from each species (*L. gaucho* and *L. laeta*) were kindly provided by the Production and Research Center of Immunobiological Products, State Department of Health (CPPI). First, a venom extraction by electric shock was conducted aiming to stimulate the production of toxin-encoding mRNAs in spiders venom glands. Five days later, the venom glands were excised and stored instantly in a microtube placed on dry ice. The purification of total RNA was carried

out using TRIzol reagent, followed by precipitation with isopropanol and resuspension in ultrapure water. The cDNAs were obtained by reverse transcription reaction using an oligo-(dT)₁₇ primer (5'-CGGTACCATGGATCCTCGAGTTTTTTTTTTTTTTTTT-3'). In order to screen for LALLT orthologues sequences, PCR was performed using the reverse transcription reaction products as templates with the same primers used to amplify LALLT, previously described. The resulting PCR products were gel-purified using a Gel Band Purification Kit (GE Healthcare Life Sciences), cloned into the pGEM-T Easy vector (Promega) and transformed into *E. coli* DH5a. Randomly selected colonies were used to perform a colony PCR to confirm positive clones. Three positive clones for both *L. gaucho* and *L. laeta* were individually inoculated into LB broth containing 100 µg/mL ampicillin, then the culture was grown overnight at 37 °C under constant agitation. Recombinant plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, USA) and the cloned cDNAs were sequenced on both strands as previously described. The T7 and SP6 promoter regions were used to prime the sequencing reactions using the universal primers T7 promoter (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3'), respectively. Sequencing results were analyzed using the software FINCHTV v. 1.4.0 (Geospiza, Inc.). The resulting nucleotide sequences were analyzed for verification of the correct open reading frame and translated to amino acid sequences by means of the online tools Open Reading Frame Finder [31] and ExPASy Translate [32], respectively. Multiple sequence alignments were performed using the online tool Clustal Omega [33] based on the deduced amino acid sequences which were determined. Visual alignments were executed using the Boxshade Server v. 3.21 tool (https://embnet.vital-it.ch/software/BOX_form.html). Percentage identities of *L. gaucho* and *L. laeta* sequences in relation to LALLT were determined using the BLASTp algorithm [34].

2.4. Construction of the recombinant baculovirus and transfection of Sf9 cells

Recombination reaction was performed at 25 °C for 18 h by combining 300 ng of the purified entry vector with the coding sequence of LALLT, 300 ng of the BaculoDirect Linear DNA and 4 µL of LR Clonase® II Enzyme mix. The following procedures were carried out according to the User Guide of BaculoDirect™ Baculovirus Expression System kit, with some minor modifications. Briefly, lipid-mediated transfection of insect cells was performed with Cellfectin® II Reagent in six well plates. Each well received 8×10^5 Sf9 cells in Grace's Unsupplemented Insect Medium. Transfection mixture was prepared with the recombination reaction, Cellfectin® II Reagent and Grace's Unsupplemented Insect Medium, and incubated at room temperature for 30 min. After attachment of cells, the medium was replaced by Grace's Unsupplemented Insect Medium containing the transfection mixture and incubated for 5 h in an incubator at 27 °C. After incubation, the transfection mixture was replaced with 2 mL of complete growth medium and 100 µM of ganciclovir was added to each well. Next, the reactions were incubated at 27 °C for 72 h, when signs of viral infection were observed. This was designated as P1 viral stock. High-titer viral stocks were then prepared initially using 25 and 50% of previous stocks (designated as P2 and P3, respectively). Higher volumes of viral stocks were then prepared using 1–5% of previous stocks.

2.5. Recombinant protein expression and purification

The expression was performed in Sf900™ II insect cell medium with 2.5×10^6 Sf9 infected cells/mL with a fresh high-titer viral stock (5% v/v) and maintained on agitation (110 rpm) for 144 h at 27 °C. Next, supernatant was harvested (7000 ×g for 15 min), 0.22 µM filtered, and recombinant protein was purified by metal affinity chromatography on Äkta pure system (GE Healthcare Life Sciences). Briefly, the supernatant was applied in a HisTrap™ Excel 1 mL previously equilibrated with A buffer (20 mM NaH₂PO₄, 500 mM NaCl, pH 7.5), washed with 4% of B

buffer (20 mM NaH₂PO₄, 500 mM NaCl, 500 mM Imidazole, pH 7.5) and elution was performed with B buffer in 1 mL fractions. The solution containing the recombinant protein was then dialyzed with Phosphate-Buffered Saline (PBS) and further analyzed.

2.6. SDS-PAGE and western blotting

Purified recombinant protein samples were analyzed by 10% SDS-PAGE under reducing conditions, following Laemmli [35]. Gels were stained with Coomassie Blue Dye. Protein content was determined by the Coomassie Blue method (BioRad), based on Bradford [36]. For immunoblotting, recombinant LALLT (5 µg) or crude venom (100 µg) was submitted to 10% SDS-PAGE and then transferred to nitrocellulose filters using Trans-Blot® SD (20 V for 20 min) (Bio Rad). Immunodetection was performed using hyperimmune sera which react to *L. intermedia*, *L. laeta* or *L. gaucho* crude venoms or hyperimmune serum raised against the recombinant allergen (LALLT). Finally, detection used secondary alkaline phosphatase-coupled anti-IgG 1:5000 (Sigma Aldrich) and visualization of immunoreactions used the BCIP/NBT substrate reaction (Promega). Control of primary antibodies specificity was performed with pre-immune serum. The hyperimmune serum that recognizes LALLT was produced using mice according to Harlow and Lane (1988) [37] and the Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (Statement number 1297); briefly, pre-immune serum was collected and then, animals received a first intraperitoneal injection of 10 µg of the recombinant LALLT together with aluminium hydroxide adjuvant (Sigma Aldrich); two additional booster injections of 20 µg and 25 µg of LALLT emulsified also in aluminium hydroxide were administered at 3-week intervals; blood samples were withdrawn 10 days after the third injection, and the presence of polyclonal antibodies against LALLT was analyzed by immunoblotting. The same basic protocol described above with some modifications was used in order to obtain the hyperimmune sera that recognize the whole venom toxins (*L. intermedia*, *L. laeta* or *L. gaucho*); briefly, after collection of the pre-immune sera, New Zealand rabbits (~3 kg) received an initial injection of 15 µg of the specific whole venom emulsified in complete Freund's adjuvant (Sigma) distributed in three different injection sites (two intramuscular and one subcutaneous); three further booster injections of 30, 40 and 100 µg of whole venom together with incomplete Freund's adjuvant were administered at 3-week intervals; blood samples were withdrawn 21 days after the latter injection, and the presence of polyclonal antibodies against the whole venom toxins was detected by immunoblotting assays.

2.7. ELISA

The ELISA antibody capture assay was performed in order to detect if conformational epitopes from LALLT were present in the venom of *L. intermedia*, *L. laeta* and *L. gaucho*. For this, ELISA plates were coated overnight with 10 µg/mL of LALLT at 4 °C. The plate was washed with Tween-PBS (0.1%), blocked with PBS-BSA (1%) for 1 h and then incubated with the primary antibodies: antisera against *L. intermedia*, *L. laeta*, *L. gaucho* or pre immune serum (dilutions 1:500, 1:1000, 1:2000). After 1 h, the plate was washed with Tween-PBS (0.1%), incubated for 2 h with secondary antibodies IgG-Rabbit (1:5000) conjugated with horseradish peroxidase (Sigma Aldrich). The reaction was revealed using OPD (ortho-phenylenediamine) (Sigma Aldrich) in citrate buffer (50 mM of Na₂HPO₄ and 24 mM citric acid, pH: 5.0). The plate was read in 490 nm in spectrophotometry (Meridian ELX 800). The samples were performed in triplicate for each treatment with primary antibodies.

2.8. Animals

Adult Swiss mice (25–30 g) were taken from the Central Animal House of the Federal University of Paraná. Adult New Zealand rabbits

(~3 kg) were purchased from the Canguiri Experimental Farm of Federal University of Parana and maintained at CPPI. All experimental protocols using animals were performed according to the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching Purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation. The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Parana (Statement number 1297) certified that the procedures using animal in this work are approved.

2.9. LALLT effects on vascular permeability and paw edema-inducing activity

Changes in capillary permeability were estimated by the extravasation of plasma protein-bound dye into the extravascular compartment of mice skin [8]. Evans blue dye diluted in PBS was inoculated (I.V.) (30 mg/kg) 5 min prior to sample injections. Groups of 5 mice were injected subcutaneously (S.C.) into dorsal skin with one of the following treatments: 10 µg of LALLT, 10 µg of recombinant Serpin (a serinoprotease inhibitor from *L. intermedia* expressed and purified using the same methods as LALLT, used in order to discard the presence of contaminants) and PBS (negative control). An additional group of animals was pretreated with a mast cell degranulation inhibitor (cromolyn, 30 mg/kg) during 3 days before the inoculation of LALLT. After 60 min, the dorsal skin was removed for visualization of dye leakage. The paw edema-forming activity was evaluated at different intervals using the methods described by Sharma et al. (2004) [38]. Groups of five mice were injected subcutaneously into the right hind paw with LALLT or Serpin (10 µg dissolved in 33 µL of sterile PBS). Animals of control group were injected with 33 µL of sterile PBS. Edema was estimated by the change of paw thickness using a digital paquimeter (Digimess, São Paulo, SP, Brasil).

2.10. Macroscopic edema in rabbit skin induced by LALLT and histopathological analysis

In order to evaluate formation of edema on rabbit skin model, PBS (negative control) or LALLT 20 µg were injected subcutaneously in shaved areas on the dorsal region of the rabbits. For this experiment two animals were used. The macroscopic edema formed was observed and photographed at 3 and 24 h after the injection. For the purpose of histopathological analyses, the animals were euthanized by injections of ketamine (240 mg/kg) and xylazine (27 mg/kg) and skin samples were collected, which were fixed in ALFAC (ethanol 85%, formaldehyde 10% and glacial acetic acid 5%) for 16 h at room temperature. After that, the samples were submitted to successive dehydrations in ethanol solutions before paraffin embedding (58 °C, for 2 h). Thin sections of 5 µm thickness were obtained and sequentially stained with hematoxylin and eosin. Finally, the sections were analyzed in microscope Olympus BX41 with DP 72 camera for image capture (Olympus, Tokyo, Japan).

2.11. LALLT effects on rat mesenteric mast cells

In order to assay the activity of the recombinant LALLT on mesenteric mast cells, rats were initially anesthetized with ketamine (30 mg/kg) and xylazine (5 mg/kg). Part of the mesentery of the rats was removed and stretched on a Petri dish filled with paraffin. After that, PBS (negative control) or LALLT (10 µg and 20 µg) were directly applied on the surface of the collected mesentery. After 30 min, the treated mesentery regions were fixed with formol 10%, followed by the excision of tissue sections, which were placed on microscope slides and air-dried. Finally, after 24 h, the collected mesentery samples were stained with toluidine blue for 12 min and analyzed in microscope Olympus BX41.

2.12. Evaluation of in vitro mast cell degranulation induced by LALLT

Granular beta-hexosaminidase enzyme content released from lysed RBL-2H3 cultured cells was measured in order to confirm mast cell degranulation-inducing activity by the recombinant LALLT. Briefly, 3×10^4 cells were plated (96 wells) in DMEM medium (Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) (Gibco) and antibiotics penicillin (10,000 U/mL) (Sigma Aldrich) and streptomycin (10 mg/mL) (Sigma Aldrich). After 16 h, cells were washed and the medium was replaced by Tyrode's buffer (TGB: 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.2 mM Na₂HPO₄, 5.5 mM Glucose, 10 mM HEPES, and 0.1% BSA) containing LALLT (50 and 100 µg/mL) with or without cromolyn (10 µM), 48/80 compound (100 µg/mL) (used as positive control) and PBS for 3 h. From each experimental sample assayed (treatments and controls), one aliquot (50 µL) of the supernatant was collected and placed in another microwell plate. The cells incubated only with the TGB were lysed with 0.5% Triton X-100 (200 µL) (Sigma Aldrich) to evaluate the total enzyme content as 100% reference. To all samples, 50 µL of the substrate solution containing 1.3 mg/mL of *p*-nitrophenyl-Nacetyl-b-D-glucosamine (Sigma Aldrich) in 0.1 M citrate solution, pH 4.5, were added and, subsequently, the plates were incubated for 30 min at 37 °C, in a humidified 5% CO₂ incubator. Reactions were terminated by addition of 100 µL of 0.2 M glycine solution, pH 10.7, and the optical density was measured at 405 nm. The extent of release of the enzyme was expressed as the percentage of the total beta-hexosaminidase activity present in the wells after subtracting the activity in the supernatant of unstimulated cells [39].

2.13. Evaluation of calcium influx induced by LALLT in cultured RBL-2H3 cells

RBL-2H3 cells were removed from culture and washed with PBS. Next, 10^8 cells were loaded with Fluo-4 AM (10 µM) (ThermoFischer) in TGB with Pluronic F-127 (0.01%) for 30 min, at 37 °C. Then, the cells were washed twice with TGB in the absence of calcium and equilibrated for de-esterification for 30 min at room temperature. Then, 5×10^5 cells/well were incubated in 96 wells black plates with PBS (negative control), LALLT (50 e 100 µg/mL) and LALLT (100 µg/mL) combined with cromolyn (10 µM) for 5, 15, 30 and 60 min. The fluorescence intensity was recorded on a Tecan Infinite M200 spectrofluorometer (Tecan, Männedorf, Switzerland), at excitation wavelength of 485 nm and emission wavelength of 535 nm.

2.14. Statistical analysis

Statistical analyses of ELISA and paw edema in vivo assay were performed using two-way analysis of variance (ANOVA) followed by post-hoc Tukey test for average comparisons using the GraphPad Prism 6 program (GraphPad Software, San Diego, CA, USA), considering *p* values < 0.01 and <0.1 as significant, respectively. Statistical analyses of calcium influx and mast cell degranulation (beta-hexosaminidase) assays were assessed using one-way ANOVA with post-hoc Tukey test for average comparisons using the GraphPad Prism 6 program and statistical significance was established at *p* < 0.1.

3. Results

3.1. Molecular cloning of the allergen toxin from *L. intermedia* venom gland (LALLT) and screening for LALLT-like toxins in the venom glands of *L. laeta* and *L. gaucho*

The workflow regarding the molecular biology experiments herein carried out is depicted in Fig. 1A and B. First, a sequence encoding an allergen toxin from the venom of *L. intermedia* was screened from the total RNA extracted out of the venom glands by means of RT-PCR. After amplification, the cDNA encoding LALLT was successfully cloned

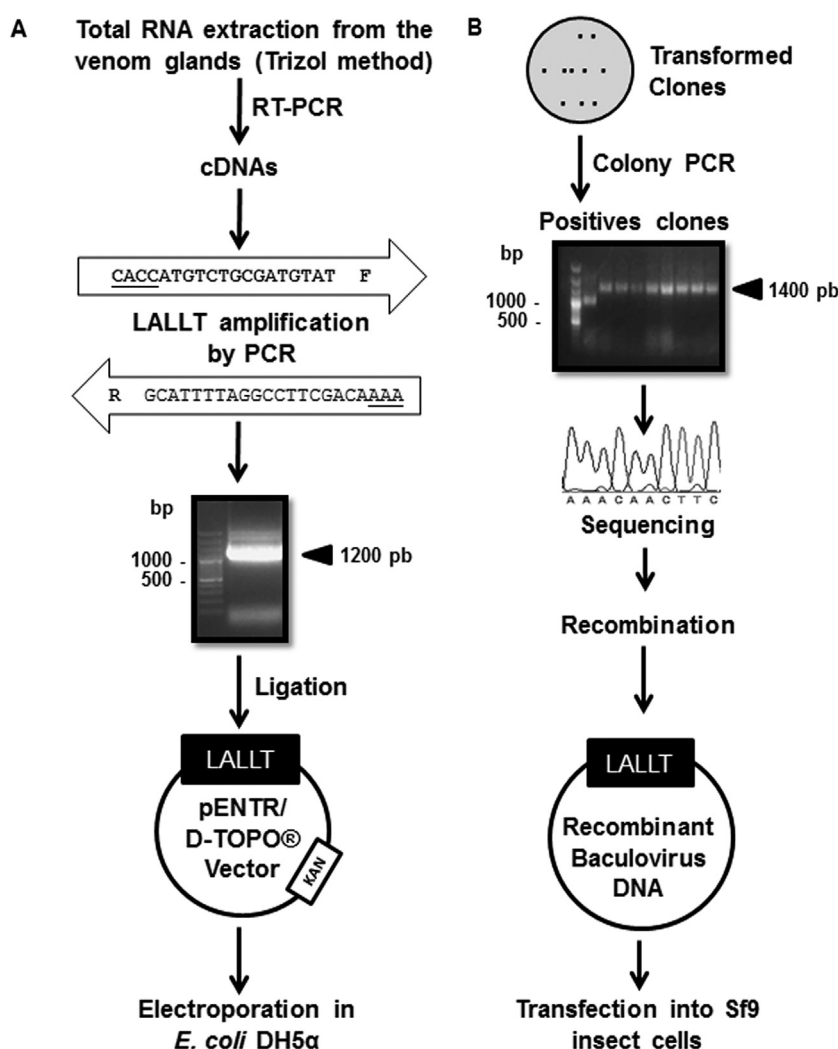


Fig. 1. Schematic diagram outlining the molecular biology approaches used in order to obtain the recombinant baculovirus for transfection of Sf9 cells. (A) Total RNA was extracted from *L. intermedia* venom glands and a reverse transcriptase PCR (RT-PCR) was performed in order to obtain the cDNAs. Then, primers with adaptors designed according to the manufacturer instructions (forward primer: CACC; reverse primer: AAA with removing of the stop codon for His and V5 TAG expression) were used to amplify the LALLT sequence (1200 bp approximately), which was analyzed by agarose gel electrophoresis. After, LALLT sequence was cloned into pENTR/D-TOPO® vector. The obtained construction was transformed into DH5α electrocompetent bacteria strain and the positive clones (1400 bp) were screened by colony PCR. (B) Positive clones were sequenced and one clone was chosen for the transfection that consists in the insertion of the LALLT into the linear baculovirus DNA by using the Gateway recombination system. The recombinant baculovirus DNA was then transfected into Sf9 cells.

into the vector pENTR™/D-TOPO®, followed by DNA sequencing analysis and prediction of the amino acid sequence, which are shown in Fig. 2A. The complete cDNA sequence of LALLT comprises 1248 bp and its deduced amino acid sequence revealed a complete mature protein of 392 amino acids, preceded by a putative signal peptide with 24 amino acids. Two sites for N-glycosylation (NWS and NIT) are found in the deduced mature protein sequence (Fig. 2A) and LALLT has a calculated molecular mass of 42 kDa, with a theoretical pI of 8.4. The deduced amino acid sequence of LALLT also shows the presence of eighteen cysteine residues in the mature protein, which are expected to establish disulfide bridges. These cysteine residues are displayed in Fig. 2A in bold and numbered in duplicate according to the theoretically deduced disulfide bridges established. Additionally, these predicted disulfide bridges are shown in Fig. 2B according to the analysis performed using the online tool DiANNA 1.1 Web Server.

We also performed a searching of additional members of the allergen family of toxins in other two South America medically important spiders, *L. laeta* and *L. gaucho*. By screening LALLT-related sequences from the total RNA expressed in the venom glands of the mentioned species and prediction of their amino acid sequences, we found the existence of one orthologue sequence for *L. laeta* (identified as LALLT2)

and two orthologue sequences for *L. gaucho* (identified as LALLT3 and LALLT4) as shown in Fig. 2C. These three new screened sequences present high identity with LALLT, corresponding to approximately 99%, diverging in only 1 or 3 amino acid residues (Fig. 2C).

3.2. Multiple alignment analysis and sequence relationship of LALLT to other allergen family members

To study the sequence relationship of LALLT with other members of allergen families, we performed a protein BLAST analysis using deposited sequences in GenBank database. The results (Fig. 3A) pointed high identity amongst LALLT and other allergen-like cDNAs from different spiders and from other animals as scorpion, mite and tick. The highest scores supported a great similarity of LALLT to allergens from the spiders *Periegops suteri* (QHA25178.1) with identity of 53,73%, *Loxosceles reclusa* (QHA25179.1) with identity of 53,10%, and *Grammostola rosea* (BAN13538.1) with an overall identity of 48,17%. Moreover, LALLT sequence presented significant identity with allergens from the scorpion *Centruroides sculpturatus* (41,30%) (XP_023241578.1), from the mite *Dinotrombium tinctorium* (35,35%) (RWS13422.1) and from the tick *Ixodes scapularis* (35,36%)

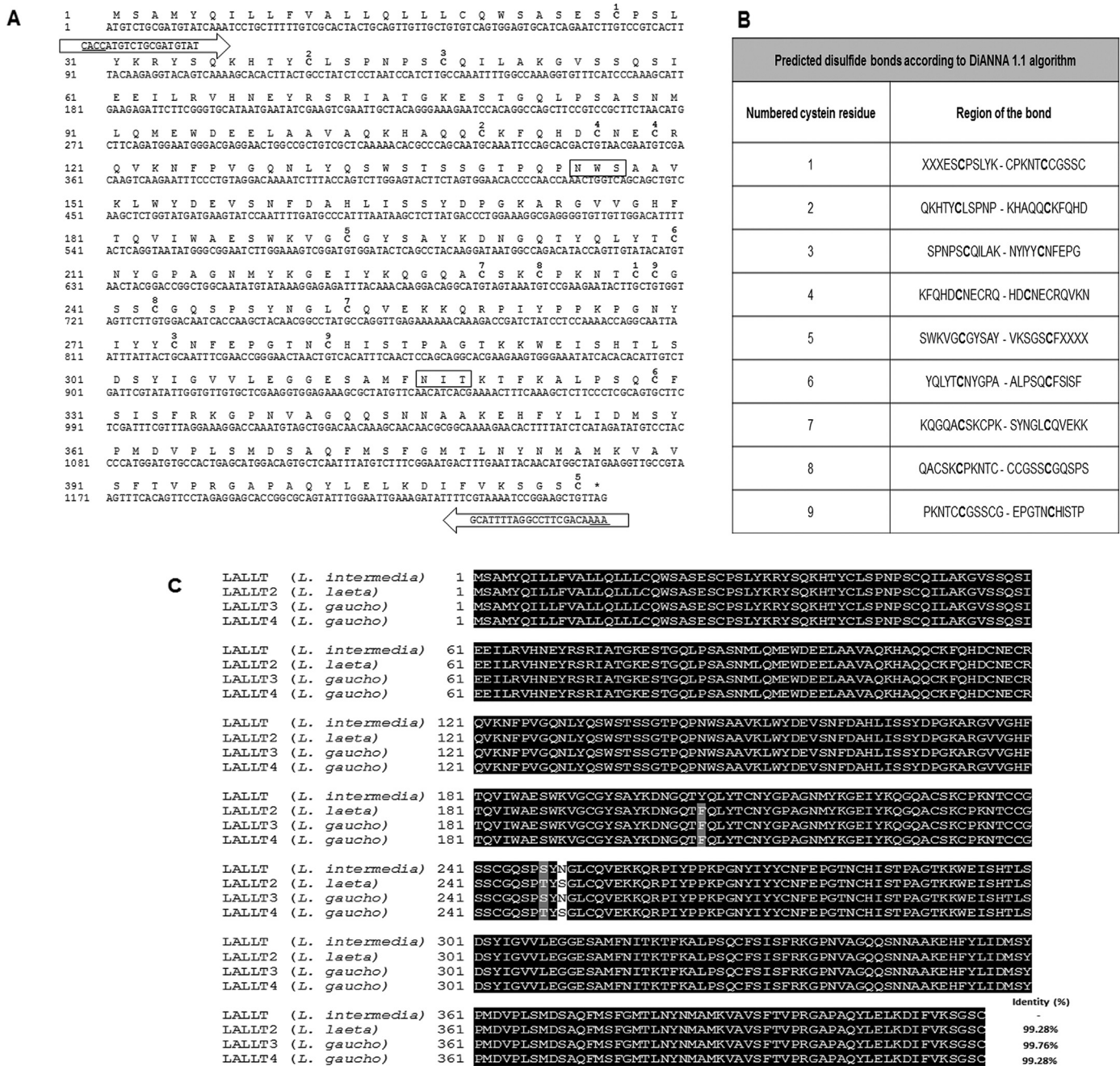


Fig. 2. Molecular cloning of the recombinant *Loxosceles* Allergen-Like Toxin (LALLT) from *Loxosceles intermedia* venom gland and comparison of LALLT cloned sequence to allergen-like sequences screened from *L. gaucha* and *L. laeta* venom glands. (A) Nucleotide and deduced amino acid sequences of cloned cDNA for LALLT from *L. intermedia* venom gland are shown. Annealing positions for primers used for cloning into the pENTR™/D-TOPO® vector are indicated by arrows. The predicted signal peptide containing 24 amino acid residues is underlined in the protein sequence. The 18 cysteine residues along the primary structure of toxin are shown in bold and are numbered from 1 to 9 according to the disulfide bridge connectivity prediction shown by the algorithm DiANNA 1.1 Web Server. The putative N-glycosylation sites (NWS and NIT) are highlighted within the boxes. (B) Table showing the predicted disulfide bridges for LALLT by the algorithm DiANNA 1.1 Web Server (<http://bioinformatics.bc.edu/clotelab/DiANNA/>). (C) Multiple alignment analysis of the cDNA-deduced amino acid sequences predicted for LALLT orthologues from *L. laeta* (LALLT2) and *L. gaucha* (LALLT3 and LALLT4). Amino acid identities are shaded in black and conservative substitutions are shown in gray.

(XP_002403125.2) (Fig. 3A). In silico analysis using the online databases Pfam (<https://pfam.xfam.org/>) and PROSITE (<https://prosite.expasy.org/>) indicated that LALLT contains signatures (CRISP1 and CRISP2) shared by proteins belonging to the CAP superfamily (Cysteine-rich secretory proteins (CRISP), proteins related to the Antigen 5, and Pathogenesis-Related (Pr-1) proteins). All the mentioned sequences, which present significant identities with LALLT, also seem to contain the CAP superfamily signatures in their primary structure and present all their cysteine residues aligned with LALLT and amongst them (Fig. 3A). As shown in the guide tree (Fig. 3B), LALLT sequence is grouped closer to

allergens from other spiders than to allergens from scorpions, ticks or mites, which is related to the higher identities amongst the sequences.

3.3. Expression and purification of LALLT

The experimental approach used to obtain, analyze and then perform biological/immunological tests in order to characterize LALLT is shown in Fig. 4A. The recombinant LALLT was expressed containing a C-terminal tag of six histidine residues and a V5-tag. The expression was performed using *Spodoptera frugiperda* (Sf9) cells transfected with

recombinant baculovirus containing the sequence of LALLT (Fig. 4B). LALLT expression in Sf9 cells was optimal after 6 days of culture (144 h), and the soluble recombinant protein was purified from culture supernatant using Ni^{2+} -chelating chromatography (Fig. 4C), resulting in a final yield of approximately 0.5 mg/L. As shown in Fig. 4D, the SDS-PAGE mobility of recombinant allergen under reduced conditions following β -mercaptoethanol treatment was approximately 45 kDa. The difference between the deduced molecular mass and the SDS-PAGE mobility for LALLT is a consequence of the presence of histidine and V5 tags, which increases the molecular mass of the recombinant LALLT in about 3 kDa.

3.4. Immunological cross-reactivity of recombinant LALLT and venom native toxins from different *Loxosceles* venoms

To evaluate the relationship amongst the recombinant LALLT and native crude venom toxins of *Loxosceles* spiders, antigenic cross-reactivity assays were performed using polyclonal antisera against crude venom toxins of *L. intermedia*, *L. laeta* and *L. gaucho* produced in rabbits and the recombinant LALLT. For this purpose, both ELISA antibody capture, which detects conformational and non-denatured antigen/epitopes, and western blot assays, which shows the recognition of linear or denaturation-resistant antigen/epitopes were performed. Recognition of the immobilized LALLT by all three polyclonal antisera in the ELISA assay was titer-dependent and significant when compared to pre-immune sera control reactions (Fig. 5A). Additionally, aiming to confirm cross-reactivity amongst the recombinant and native LALLTs, we produced polyclonal antibodies in mice against the recombinant LALLT and checked by western blot the immunoreactivity by crude venoms of *L. intermedia*, *L. laeta* and *L. gaucho* (as previously discussed, three medically important *Loxosceles* species). As depicted in Fig. 5B, when compared to pre-immune sera control reactions, antibodies for crude venom toxins cross-reacted with purified recombinant LALLT, and as shown in Fig. 5C, antibodies against purified LALLT cross-reacted with proteins with the expected mass of approximately 40–42 kDa in three different *Loxosceles* crude venoms tested.

3.5. Effect of recombinant LALLT on blood vessel permeability and paw edema

Sequentially, the Miles Assay (vessel permeability test) was performed in order to check the participation of LALLT on the increasing of blood vessel permeability. As shown in Fig. 6A, recombinant LALLT caused a dye extravasation compared to negative controls PBS and Serpin (recombinant protein produced in the same heterologous system), supporting the functionality of recombinant LALLT and pointing out LALLT involvement on biological activities of the crude venom. Additionally, the vessel permeability assay was repeated, but now incorporating cromoglycate (30 mg/kg), an inhibitor of mast cell degranulation [16]. As shown in Fig. 6B, cromoglycate reduced the effect of LALLT on vessel permeability to residual activity, indicating the LALLT effect as dependent of mast cell degranulation. Additionally, aiming to confirm the functionality of recombinant LALLT, mice received intradermal injection of the purified toxin into their paw and edema forming activity was measured with the time from 5 to 720 min and compared to values previously to injections (baseline values for paw thickness). As pointed in Fig. 6C, LALLT was able to induce paw edema in a time-dependent manner strengthening the biological effects and functionality for the obtained recombinant toxin.

3.6. Macroscopic and histopathological changes induced by recombinant LALLT following injections on rabbit skin

To demonstrate the involvement of the allergen in the harmful effects induced by the venom, rabbits were exposed to intradermal injections with purified recombinant LALLT (20 μg) for 3 and 24 h, which

correspond to the time of appearance of allergenic events following accidents. Phosphate buffer saline (PBS) was used as negative control. As can be seen in Fig. 7A, by macroscopic visualization, the injection of purified recombinant LALLT caused erythema and edema in the skin 3 h after injection, which correspond to the redness and swelling observed near the injection site, respectively. Erythema and edema signals decreased or disappeared 24 h after LALLT administration. Histopathological analyses (Fig. 7B) confirmed edema in the skin visualized by extracellular matrix collagen fibers disorganization, as well as the presence of a diffuse leukocyte infiltrate into the dermis.

3.7. Recombinant LALLT induced degranulation of rat mesenteric mast cells and triggered mast cell degranulation and calcium influx on cultured cells

Finally, with the aim to study the cellular mechanism by which LALLT can cause its biological effects, we exposed rat mesentery (a mast cells-rich tissue) to recombinant LALLT (10 and 20 μg) diluted in PBS for 30 min, using treatment with PBS under the same laboratory conditions as negative control for setting the baseline mast cell degranulation. As shown in Fig. 8A, mesenteric mast cells treated with PBS displayed a dark staining, which is the normal pattern for non-degranulated mast cells stained with toluidine blue. In contrast, treatments with LALLT caused mast cell degranulation, which can be observed by the diffuse and/or lighter staining of those cells when compared to PBS control, suggesting the rupture of the plasma membrane and the release of intracellular granules containing inflammatory mediators such as histamine. The effect of LALLT on inducing cultured RBL2H3 cells degranulation was also assessed by the beta-hexosaminidase activity assay (Fig. 8B). Beta-hexosaminidase is an acid hydrolase that characterizes lysosomal-derived secretory granules released during degranulation. The enzyme activity was quantified by product generation using p-nitrophenyl N-acetyl-beta-D-glucosamine as colorimetric substrate. The degranulation inducing-activity was observed after the recombinant toxin was incubated for 3 h with the cells. LALLT induced the degranulation effect in a concentration-dependent manner: the activity of beta-hexosaminidase after the treatment with 50 and 100 $\mu\text{g}/\text{mL}$ of LALLT was increased 1.8-fold and 2.0-fold, respectively, when compared to the negative control (PBS) (Fig. 8B). It has been reported that calcium induces the extrusion of secretory granules (exocytosis) in mast cells. Therefore, a calcium influx assay was performed in order to assess the involvement of LALLT on inducing calcium influx on cultured RBL2H3 cells. As shown in Fig. 8C we could observe a positive effect of LALLT in the Ca^{2+} influx, which was time and concentration-dependent when compared to the control (PBS). Additionally, cromolyn, which blocks or reduces the mediators released from mast cells, abrogated LALLT effects on Ca^{2+} levels.

4. Discussion

Allergenic toxins of venomous animals have been reported as playing a major role in the noxious effects observed after accidents such as edema, itching, exanthema, urticaria, cutaneous rash, erythema, papular reactions and even severe systemic hypersensitivity reactions such as anaphylaxis [40,41]. The presence of allergen-like factors in *Loxosceles* spider venom was previously suggested from clinical findings in humans, which were represented by skin rash, edema, exanthema and urticaria triggered by bites [2–5]. Some of these major signals can be observed in Supplementary Fig. S1, which points out the macroscopic appearance of a skin lesion caused by a Brown spider bite in a human injured patient. In addition to the clinical observations in humans, the allergenic potential of *Loxosceles* venom is supported by animal experiments, which showed after crude venom exposure paw edema, skin edema and increase of vessel permeability [18,42]. Moreover, the ability of *Loxosceles* venom to cause inflammatory reactions was reduced in animals pretreated with compound 48/80, pointing out the

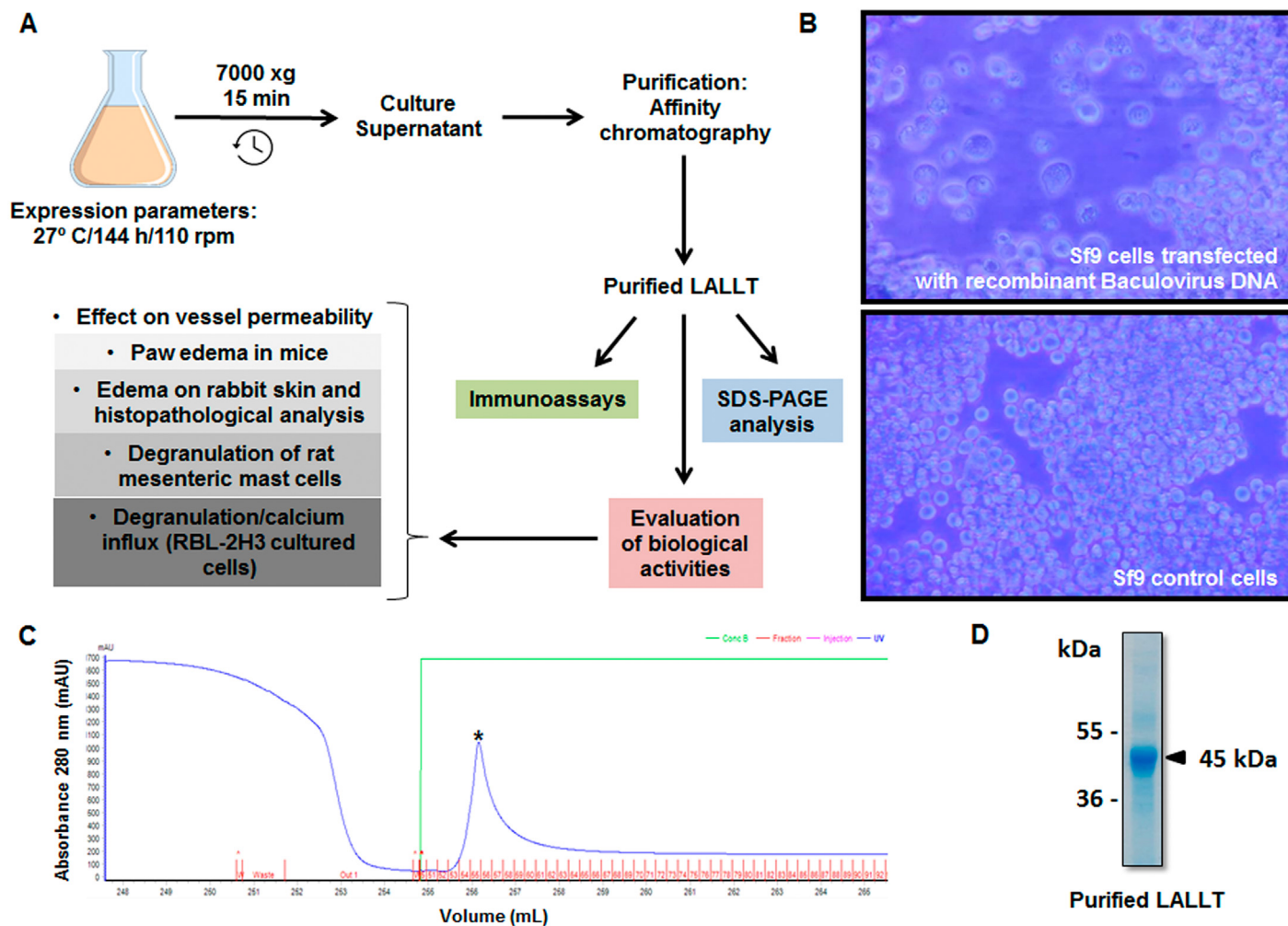


Fig. 4. Expression and purification of LALLT. (A) Schematic diagram outlining the expression and purification strategies of the recombinant LALLT in order to perform the biological and immunological assays. (B) Comparison of Sf9 control cells and Sf9 cells transfected with the recombinant baculovirus DNA containing LALLT sequence; major distinctions are their different sizes and membrane changes (400× magnification). Successive viral amplifications were carried out in cell culture in order to obtain a high-titer viral stock, which was used to transfect Sf9 cells for the expression step. After intracellular expression of LALLT and secretion by the Sf9 cells, the culture supernatant was used to purify the recombinant toxin. (C) Recombinant LALLT (approximately, 45 kDa) was purified from culture supernatant as shown in the elution profile resulting from the affinity chromatography (Ni^{2+} -NTA agarose column His Trap Excel). The fractions containing the purified LALLT were pooled and dialyzed against PBS. Purified protein appears as a peak indicated by an asterisk. (D) Electrophoretic profile of the purified LALLT analyzed in the 10% SDS-PAGE under reducing conditions and stained with Coomassie Blue. Molecular mass markers are shown on the left of the figure.

involvement of mast cells in these responses. In addition, pretreatment of mice with histamine receptor antagonists, including promethazine and cetirizine, or the serotonin receptor antagonist methysergide significantly decreased venom induced edema and vascular permeability [42]. The first direct evidence of allergenic toxins as self components of *Loxosceles* venom comes from transcriptome analysis from venom glands transcripts of *L. laeta* and *L. intermedia*. These transcripts represent 0.2% of venom toxins annotated in the transcriptome of *L. intermedia* venom glands and showed similarity with sequences of other venom allergens, including the fact they are cysteine-rich molecules [9]. Moreover, also based on a transcriptome study from venom glands, it was reported the presence of partial transcripts encoding

allergen toxins in the venom of *L. laeta* which present conserved cysteine residues when compared to other allergens from ant, wasps and mosquito [43]. Additionally, proteomic studies using crude venom of *L. intermedia* also reported toxins with characteristics of allergen molecules, which supports the idea of specific molecules in the *Loxosceles* venoms directly responsible for clinical signals such as erythema, tissue swelling and other allergic reactions [44]. Finally, a direct evidence of the existence of an allergenic factor in the venom of *Loxosceles* spiders was determined by cloning, heterologous expression, and functional evaluation of a toxin characterized as a member of the Translationally-controlled tumor protein (TCTP) family. The recombinant form of this TCTP from *L. intermedia* venom was able to increase vascular

Fig. 3. Multiple alignment analysis of the cDNA-deduced amino acid sequences and similarity relationship amongst the recombinant toxin LALLT and other allergen-like molecules. Sequences were aligned using the online algorithm Clustal Omega (www.ebi.ac.uk/CLUSTAL). (A) Multiple alignment analysis of LALLT deduced-amino acid sequence and other allergen-family related members available on GenBank. Amino acid identities are shaded in black, conservative substitutions are shown in gray and conserved cysteine residues are pointed out by asterisks. CRISP1 and CRISP2 indicate the predicted signatures of CAP superfamily proteins. The percentages of sequence identity with LALLT were also generated by Clustal Omega and are shown at the end of each sequence. The names and accession numbers of the sequences aligned are *Ixodes scapularis* (CRISP/Allergen/PR-1 - XP_002403125.2), *Dinotrothium tinctorium* (GTx-VA1-like protein - RWS13422.1), *Centruroides sculpturatus* (venom allergen 5-like - XP_023241578.1), *Grammostola rosea* (GTx-VA1 - BAN13538.1), *Periegops suteri* (venom allergen/CRISP - QHA25178.1) and *Loxosceles reclusa* (venom allergen/CRISP - QHA25179.1). (B) Guide tree of allergen-like toxins from several animal groups. The dendrogram and the percentage of sequence identity were generated by Clustal Omega online algorithm.

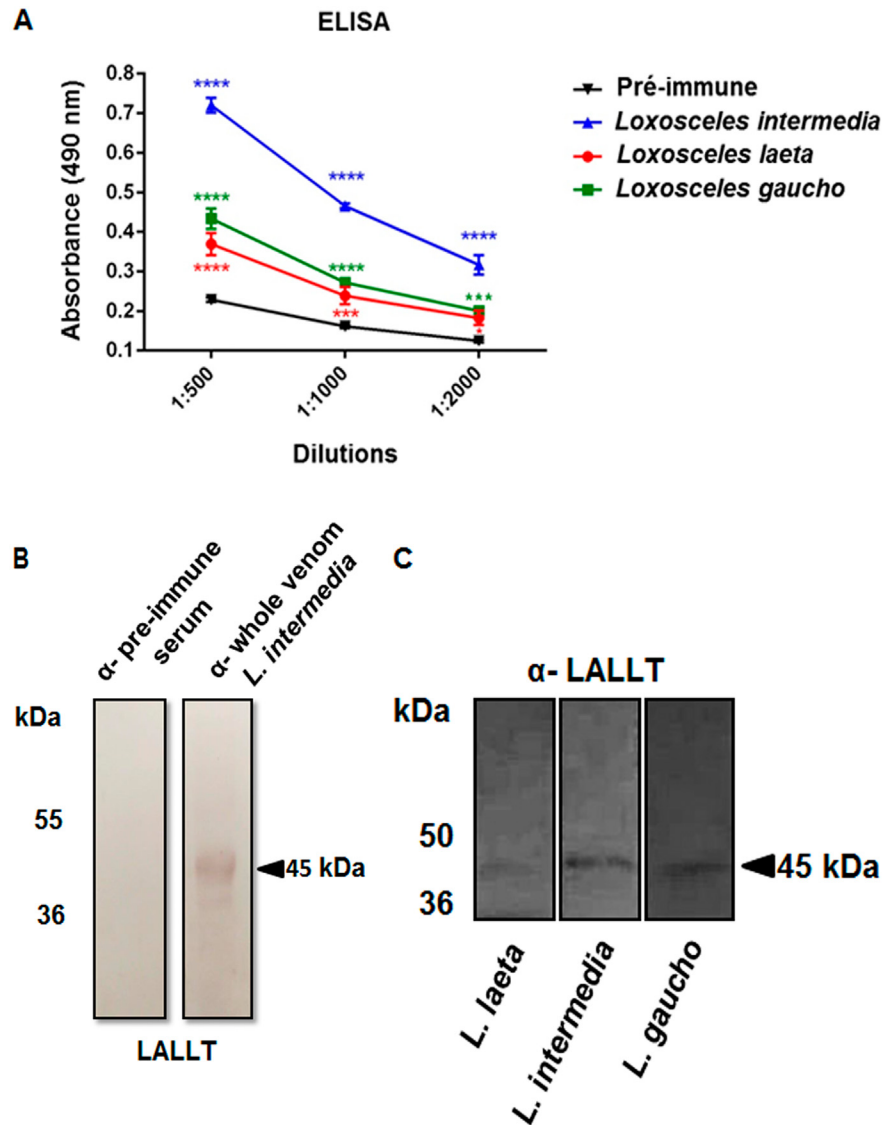


Fig. 5. Immunological cross-reactivity between recombinant LALLT and native *Loxosceles* venom toxins. (A) Immunotest of ELISA for antibody capture was performed by using the recombinant LALLT at concentration of 10 µg/mL to sensitize plate bottoms that were reacted with antisera against *L. intermedia*, *L. laeta* and *L. gaucho* crude venoms or pre-immune serum. Each point represents the mean $\pm$ s.d. Significantly different from control values: * $p < 0,01$, *** $p < 0,0001$, and **** $p < 0,00001$. Additionally, the cross-reactivity was analyzed by western blotting assays as shown in (B) and (C). (B) Purified recombinant toxin LALLT (5 µg) was separated by 10% SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes that were exposed to antiserum against *L. intermedia* crude venom toxins or pre-immune serum. Molecular mass markers are shown on the left of the figure. (C) Crude venoms (100 µg) of three different species of Brown spiders (*L. laeta*, *L. intermedia* and *L. gaucho*) were separated by 10% SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes and immunoblotted with a serum produced against purified LALLT. Molecular mass markers are shown on the left of the figures.

permeability and edema [14], besides acting as an agent that in synergism increases the inflammatory response caused by members of the dermonecrotic toxin family (Phospholipases-D) [16].

The cutaneous rash seen in loxoscelism is an early sign that appears a few minutes after the accident and is characterized by the presence of diffuse red spots on the skin of the injured victims (Fig. S1). This rash is not always restricted to the spider bite site, which differentiates it from the inflammatory response that begins some hours after the accident at the bite site and spreads in the direction of gravity, causing dermonecrosis, a hallmark of loxoscelism. It is also reported by patients a persistent itching in the regions of the skin where the cutaneous rash is located. Because they represent different signs and are located in different regions on the skin of victims, it is reasonable to believe that cutaneous rash and dermonecrosis have different etiologies and can be caused by different toxins. It is also consistent with this hypothesis that dermonecrosis evolves in almost all targeted victims, progressing over the time after envenoming and resulting from the action of toxins belonging to the phospholipase-D family, as previously reported so far

[4,5]. On the other hand, the cutaneous rash is restricted to a small number of patients, appears a few minutes after accidents and tends to disappear over the time, being its origin currently unknown.

In this report we described the identification, cloning of the full-coding sequence, heterologous expression using insect cells/baculovirus technology, purification, immunological cross-reactivity analysis and biological evaluation of a toxin of 42 kDa, characterized as an allergen factor present in the *Loxosceles* venoms, which was named LALLT.

As demonstrated by the screening of sequences encoding allergen-like toxins in the venom glands, LALLT presents biological conservation, since molecules with high identity and containing the main sequence features (conserved cysteine residues and sequence signatures for CAP family members) (Figs. 2C and 3A) are found in the venom of the three species with major clinical involvement in South America, which are *L. intermedia*, *L. laeta* and *L. gaucho* (Fig. 2C). Based on transcriptome analyses from venom glands and a presence of a non-prominent band near the electrophoretic mobility of LALLT when crude venom is analyzed by SDS-PAGE it is possible to assert that venom toxins related to

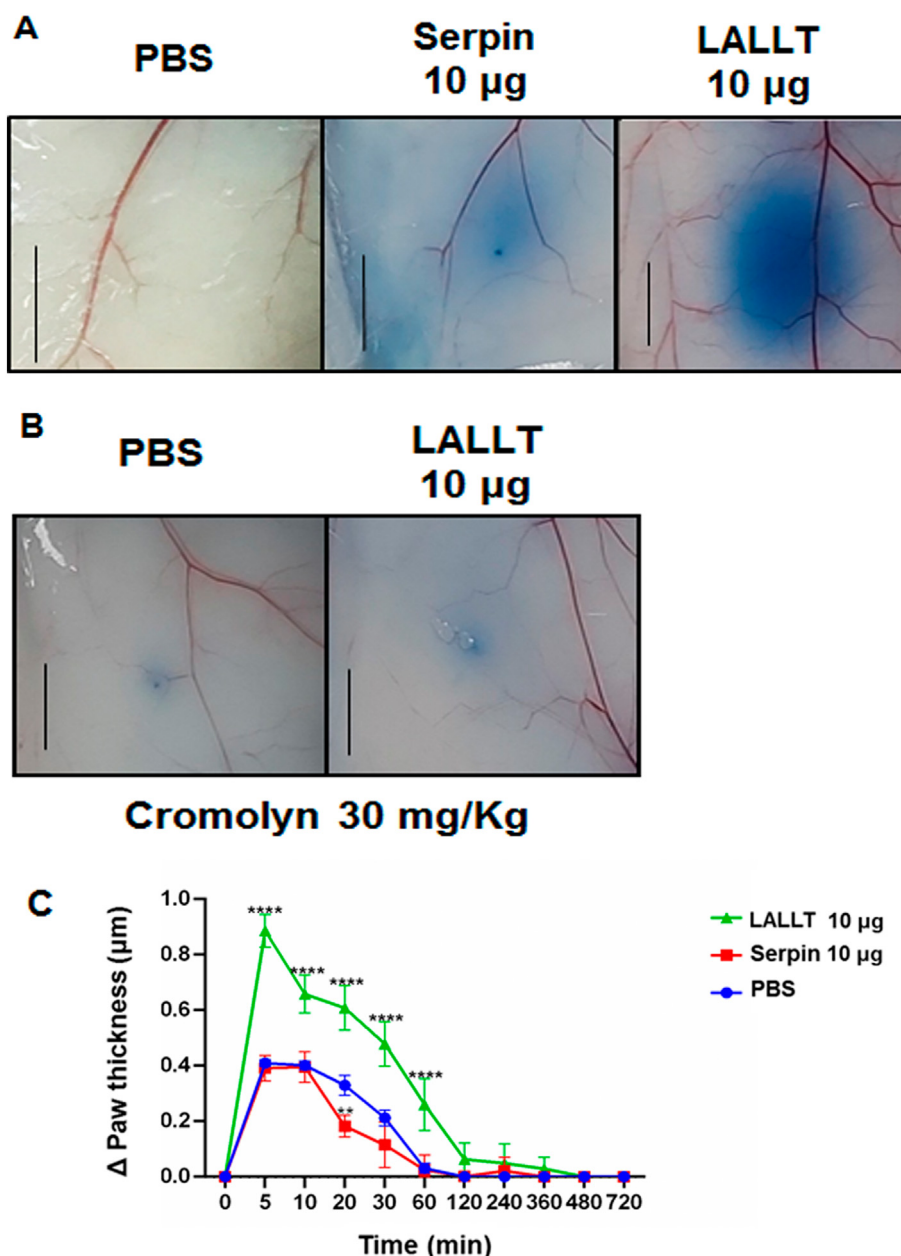


Fig. 6. Effect of recombinant LALLT on induction of vascular permeability of skin vessels and paw edema. (A) Mice ($n = 5$) were given intradermal injections of 10 µg of recombinant LALLT or 10 µg of Serpin (serine protease inhibitor protein) expressed and purified under the same laboratory conditions for LALLT (negative control used in order to discard the presence of contaminants). PBS was used as control for baseline permeability level. The photographs show dye leakage after exposure to LALLT as compared to the minimal vessel permeability due to the negative controls PBS and Serpin. Scale bars correspond to 0.5 cm. (B) Evans blue leakage was also checked for animals that were pretreated for three days with cromolyn (30 mg/kg) and received injection of LALLT under the same conditions as previously described. (C) Mice ($n = 5$) received intradermal injections of LALLT (10 µg) into the paw and edema was observed from 5 to 720 min after injections. As negative control for vehicle volume was used PBS and the recombinant Serpin obtained under the same laboratory conditions as previously described was used as control of the expression and purification methods. The paw edema was measured using a digital caliper. Final values were obtained subtracting edema measurements after sample administrations at respective times from paw thickness value prior to injections (baseline). Each point represents the mean $\pm$ s.e.m. for five animals. Significantly different from control values: ** $p < 0.01$, **** $p < 0.0001$. The colored version of this figure can be accessed to web version of this article.

LALLT are present in a small concentration, belonging to the group of low-expressed toxins in the *Loxosceles* venoms [4,9]. Together with individual factors of injured patients and unknown to date, perhaps the low concentration of these toxins in the crude venom may explain why the cutaneous rash is restricted to a small number of patients exposed to the venom.

Bioinformatic analysis pointed out that LALLT deduced-amino acid sequence displays significant identity to other allergenic proteins from animals, which belongs to the superfamily CAP of molecules (Fig. 3A). The CAP superfamily of proteins [cysteine-rich secretory proteins (CRISPs), antigen5 (Ag5), and pathogenesis-related 1 (Pr-1)] is constituted by structurally related proteins found in a wide group of animals,

from mammals and reptiles to arthropods such as spiders and ants. They are related to reproduction, immune function, cancer biology and venom toxicology [45,46]. Proteins from the CAP superfamily are present in the venoms of several animals such as snakes, scorpions and spiders [9,47,48]. In snakes, these proteins have been associated to the modulation of inflammatory responses after the envenoming: natrin, a native CRISP protein (CAP superfamily) purified from the venom of the snake *Naja atra* enhanced adhesion of monocytic cells to cultured endothelial cells by induction of ICAM-1, VCAM-1, and E-selectin expression on these endothelial cells [49].

The sequence features shared by LALLT and several different allergenic toxins from other animals highlighted by the multiple alignment

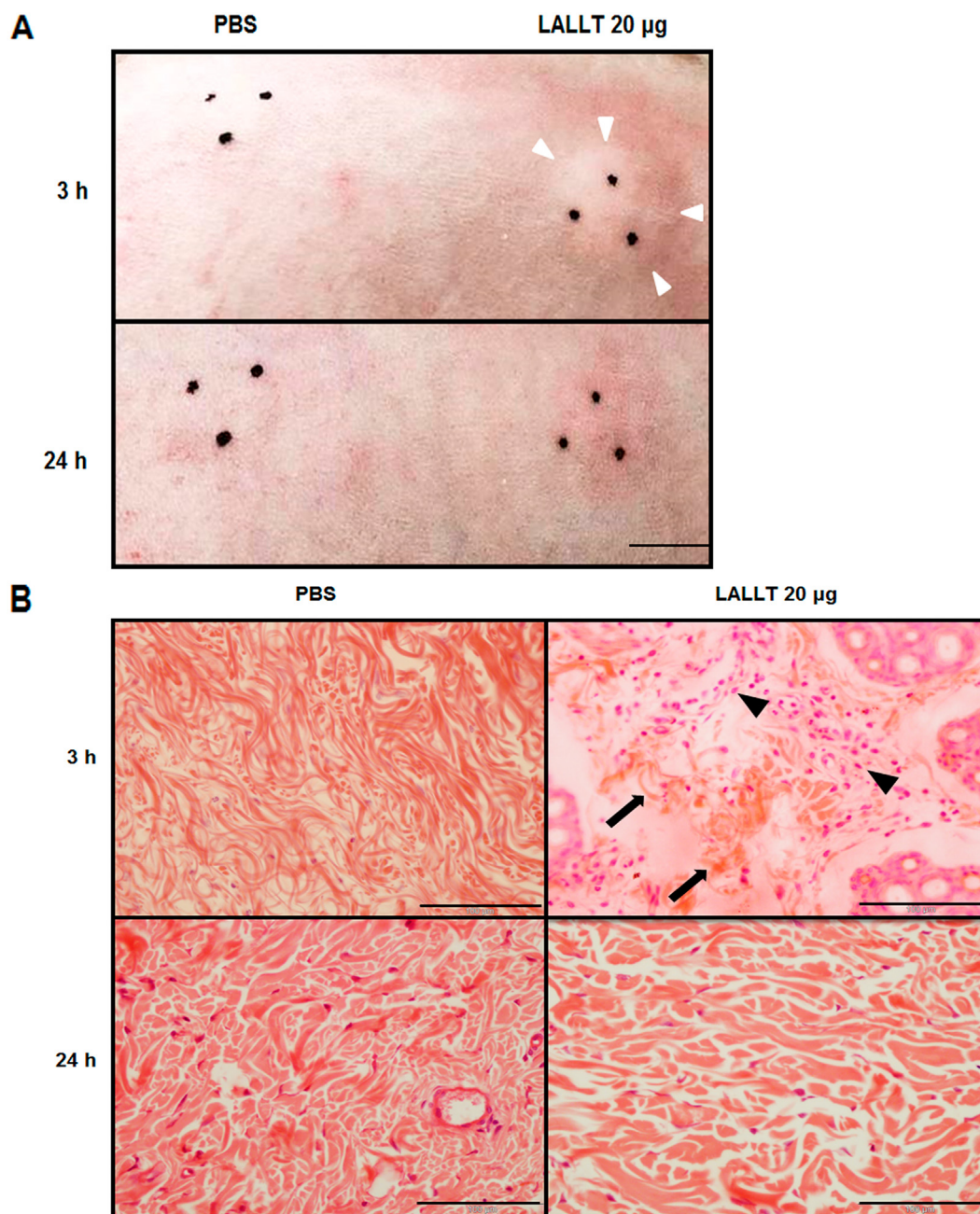


Fig. 7. Macroscopic and histological alterations followed LALLT injection on rabbit skin. (A) Rabbits received intradermal injections of purified LALLT (20 µg) and PBS (negative control). Macroscopic lesions were evaluated after 3 h and 24 h post toxin exposure. The black dots show the local of the injection and the scale bar correspond to 1 cm. As shown, exposure to recombinant LALLT compared to control caused erythema and edema in the skin after 3 h of toxin exposure (white arrows). The mentioned signals decreased or disappeared after 24 h of toxin injection. (B) Materials collected from skin were processed for histopathological analysis, stained by hematoxylin and eosin (HE) and observed by light microscopy. Scale bars correspond to 100 µm. As observed, samples collected and processed from animals which received the negative control treatment (PBS) showed normal tissue architecture represented by usual aspect of the extracellular matrix structures and absence of inflammatory cells into the dermis. On the other hand, injection of LALLT induced histopathological changes, which were evidenced by disorganization of extracellular matrix components (collagen fibers) (arrows), and presence of leukocytes radially diffused into the dermis (arrowheads) after 3 h of toxin exposure. Samples from animals treated for 24 h with LALLT showed decreased signals of extracellular matrix changes or inflammatory cells infiltration.

analysis strongly support the idea that these molecules are related and reinforce that LALLT is an allergen family member (Fig. 3A and B). Both two signatures of proteins grouped in the CAP superfamily are present in the LALLT sequences, as well as the several cysteine residues typical of CAP family members (Figs. 2A and 3A). Additionally, the sequence of LALLT closely resembles the allergen from the spiders *Periegops suteri*, *Loxosceles reclusa*, *Tritrame loki*, *Grammostola rosea*, and *Cupiennius salei* (Fig. 3A and B). On the other hand, LALLT is less similar to allergens from other animal groups such as scorpion, tick and mite, which may reflect the diverging phylogenetic proximity

amongst the animals. (Fig. 3A and B). These results indicated that LALLT theoretically could have similar functions in *Loxosceles* venoms to that presented by allergen-family members in other organisms.

Full-length LALLT was heterologously obtained in the Sf9 cells expression system and was purified to homogeneity from culture supernatant by Ni^{2+} -affinity chromatography (Fig. 4C–D). Unlike bacteria expression systems, in which the absence of post-translational modifications of proteins is a limiting step for the production of active recombinant molecules, insect cells allow protein modifications such as N- and O-glycosylation, signal peptides processing and disulfide

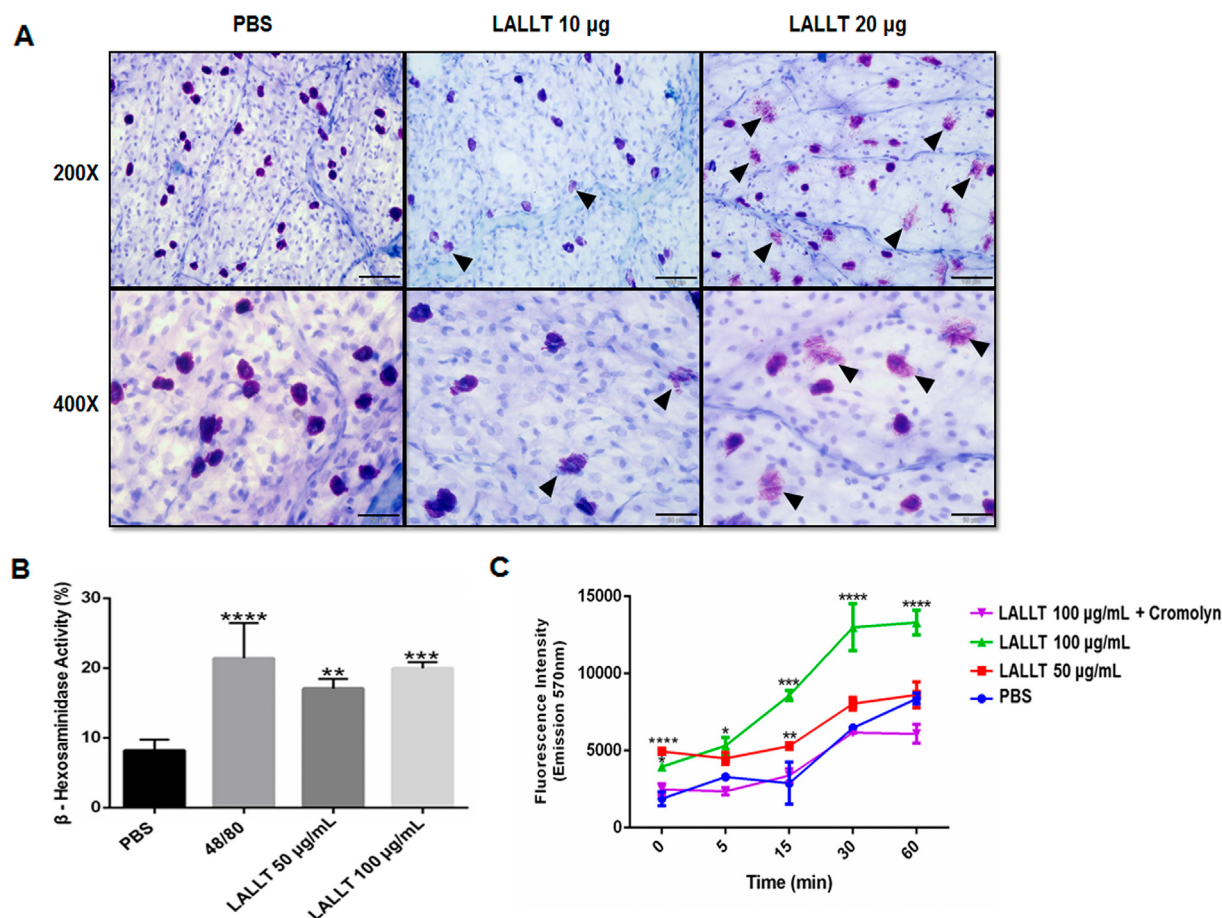


Fig. 8. Degranulation activity and calcium influx induced by LALLT. (A) Rat mesenterium obtained from rats previously anesthetized was exposed to LALLT treatments (10 and 20 µg) for 30 min (PBS was used as negative control for baseline mast cells degranulation evaluation). After this, tissue was washed with PBS, fixed in formalin 10% and stained by toluidine blue dye. The degranulation activity of LALLT on mast cells was determined by observation on light microscope Olympus BX 51 with DP 72 camera for image capture. Scale bars correspond to 100 µm (200× magnification) and 50 µm (400× magnification). Degranulated mast cells were observed after LALLT treatment as weakly stained cells, suggesting plasma membrane rupture and degranulation (arrowheads). (B) Alternatively RBL-2H3 cells were treated under culture conditions with LALLT (50 and 100 µg/mL), PBS (negative control) and 48/80 (100 µg/mL) (positive control) for 3 h and had beta-hexosaminidase activity evaluated in the culture medium. As shown, the activity of beta-hexosaminidase after 50 and 100 µg/mL LALLT treatments was increased 1.8-fold and 2.0-fold, respectively, when compared to PBS. (C) Calcium influx was assessed after treatment with PBS (negative control), LALLT (50 and 100 µg/mL) and LALLT combined with cromolyn (mast cell degranulation inhibitor). A dose-dependent positive effect of LALLT in the Ca^{2+} influx can be observed. In (B) and (C), the results represent the mean $\pm$ s.d. of each treatment in quadruplicate for one representative experiment of three independent experiments that were carried out. Significantly different from control values: * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

bond formation. These modifications enable the folding of recombinant molecules and therefore increase the ability of cells to process, fold and secrete recombinant molecules [50,51]. Through this methodology, recombinant LALLT (with theoretically nine disulfide bridges) could be specifically obtained in a pure and soluble form, as displayed by the elution profile of the affinity chromatography and further supported by the electrophoretic pattern visualized on the SDS-PAGE (Fig. 4C–D). The yield of the purified LALLT was about 0.5 mg/L, which is consistent to other allergens from animal venoms expressed in Sf9 cells, such as the allergens Api m 2 (from the honeybee *Apis mellifera*) and Ves v 2a (from the wasp *Vespa vulgaris*), both obtained in concentrations as of 0.2–1 µg/mL [52].

Antiserum produced against crude venoms from *Loxosceles intermedia*, *L. laeta* and *L. gaucho* toxins showed a high cross-reactivity with recombinant LALLT, and an antiserum produced against recombinant LALLT from *L. intermedia* venom cross-reacted with native toxins with molecular masses expected and present in crude venoms from *L. intermedia*, *L. laeta* and *L. gaucho* (Fig. 5A–C). The cross-reactivity corroborates the idea that epitopes present in the recombinant LALLT are strong antigenic determinants and, together with the finding of orthologue sequences encoded in the venom glands of *L. laeta* and *L. gaucho* (Fig. 2C), reinforces the hypothesis for the presence of

LALLT-like toxins in the venoms of different Brown spider species. This conservation of a family of toxins throughout the *Loxosceles* genus has already been observed for other molecules as phospholipases-D, metalloproteases and ICK peptides [53–55].

In addition, and in accordance with the structural and biochemical characteristics of LALLT, we further analyzed the functionality of the recombinant LALLT. The biological and functional effects of recombinant toxin were demonstrated by its activity on increasing vessel permeability by using murine model (Fig. 6A and B), increasing paw edema in mice (Fig. 6C), and macroscopic and histopathological changes in the skin of rabbits (Fig. 7A and B). These data not only proved the existence of an allergenic toxin in the *Loxosceles* venoms, but opened the possibility of future studies on the mechanisms by which this venom is able to induce deleterious effects. In addition, it included a novel molecule described as an allergen in the current literature that could be involved in reactions evolved due to Brown spider accidents such as cutaneous rash, pruritus, erythema, urticaria and angioedema. The degranulation-inducing activity of the purified LALLT on mast cells from rat mesentery (Fig. 8A) and toxin activity under culture conditions on RBL-2H3 cells (a rat mast cells-like cell line) that caused releasing of beta-hexosaminidase to culture supernatant (Fig. 8B) and increased calcium influx (Fig. 8C) additionally supported a putative cellular model

for the mechanism of action of this toxin, by inducing mast cells degranulation: the release of the content of mast cells granules increases the vascular permeability, causes edema and allergenic activities and activates host cells as neutrophils or other immune cells in order to protect [56].

The presence of an allergenic toxin in the Brown spider venoms, although expressed in low levels, as previously indicated by the transcriptome analysis from venom glands of *L. intermedia* [9] and from *L. laeta* [43] must be taken in account specially when patients are re-infected. In these cases, an allergenic reaction would develop as previously described to accidents involving insects such as those of *Hymenoptera* species in which allergy reactions are dependent on the frequency by which the individual is stung or bitten [41]. Other question that must be addressed is based on the clinical findings reported after Brown spider bites, in which patients can develop pruritic exanthema, erythema, edema and cutaneous rash but apparently no systemic hypersensitivity reactions [2–4,57,58]. The low concentration of expressed and secreted LALLT in the crude venom could explain such signals. During the accidents the presence of LALLT near the bite site could increase the possibility of allergenic reactions as local swelling and urticaria. Moreover, since LALLT has amino acid similarity to different allergens from several arthropods (demonstrated by multiple sequence alignment) and allergenic reactions are dependent on the immunity of patients, a special attention must be done to previously sensitized individuals, who by cross-reactivity based on common epitopes may elicit allergenic reactions after accidents [41,59]. This is especially important for patients exposed to mites, which are very common organisms in household environments, and as shown in Fig. 3A and B, have allergenic molecules with significant identity to LALLT.

Currently, by means of molecular biology technologies, it has been possible to use toxins from Brown spider venom to study molecular, cellular and pathophysiological mechanisms of *Loxoscelism*, basic biochemistry and cell biology protocols. *Loxosceles* venom toxins putatively could be used as tools in biochemical lipid research protocols (as is the case of recombinant phospholipase-D isoforms) [60] or as a recombinant antigen for serum therapy applications (phospholipase-D, hyaluronidase and metalloprotease) [61] and finally to determine tridimensional structures of different toxins using crystallization techniques and X-ray diffraction procedures (phospholipase-D) [4,62,63]. The production of the recombinant LALLT reported in this work will be of great value especially for future skin testing, cross-reactivity based on common epitopes studies, determination of specific IgE antibodies or mechanisms of allergy. Additionally, it may be used as a tool for efficient desensitization protocols as previously reported for other allergenic molecules and eventually to supply enough material to be used for crystallization and X-ray diffraction for further studies of allergenic epitopes [41,64]. Finally, two important questions remain concerning LALLT. What is the mechanism by which LALLT can trigger allergy? Is it an allergy dependent of IgE production with the ability to sensitize the victims or an adaptive response associated with local inflammation but independent of IgE production (an allergic contact dermatitis) as previously classified for allergic disorders [65]? We have no answer for these questions for today but, based on the direct ability of LALLT to induce degranulation of mast cells, we can hypothesize the involvement of this toxin/cells in the allergenic effects of Brown spider venom and in a near future with the use of recombinant molecule we hope to bring some insights for this topic.

5. Conclusion

In summary, we have identified and characterized for the first time in the specialized literature a Brown spider venom toxin characterized as a novel allergen. Our findings showed that LALLT belongs to a conserved family of toxins in the *Loxosceles* genus and has similarity to the allergens of the spiders *Periegops suteri*, *Loxosceles reclusa*, *Trittame loki*, *Grammostola rosea*, and *Cupiennius salei*. Moreover, we cloned,

heterologously expressed this toxin using a eukaryote expression system (baculovirus-infected insect cells) and purified this recombinant molecule. Recombinant LALLT induced biological effects such as increased vessel permeability and paw edema in mice, macroscopic and histological changes in rabbit skin, and rat mesentery and cultured mast cell degranulation. Together, these results bring insights into *Loxoscelism* and can contribute to further understanding allergic reactions. Moreover, new studies can be carried out to fully understand the mechanism by which allergenic molecules induce their toxic effects, in addition to exploring potential biotechnological or diagnostic applications.

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CRedit authorship contribution statement

Hanna Câmara da Justa: Investigation, Data curation, Writing - original draft, Visualization, Project administration. **Fernando Hitomi Matsubara:** Writing - original draft, Writing - review & editing, Visualization, Investigation. **Elidiana de-Bona:** Conceptualization, Investigation. **Zelinda Schemczsen-Graeff:** Investigation. **Nayanne Louise Costacurta Polli:** Investigation. **Thiago Lopes de Mari:** Investigation. **Marianna Boia-Ferreira:** Investigation, Writing - original draft. **João Carlos Minozzo:** Resources. **Ana Carolina Martins Wille:** Investigation, Resources. **Andrea Senff-Ribeiro:** Writing - review & editing. **Luiza Helena Gremski:** Conceptualization, Project administration. **Silvio Sanches Veiga:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

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Declaration of competing interest

None.

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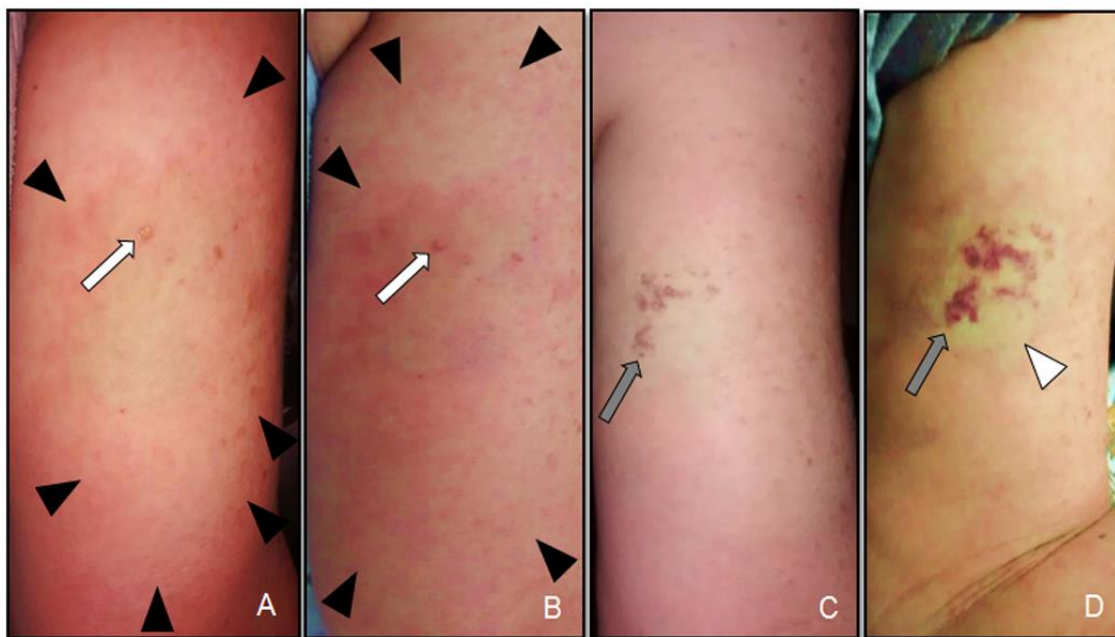
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LALLT (*Loxosceles* Allergen-Like Toxin) from the venom of *Loxosceles intermedia*: recombinant expression in insect cells and characterization as a molecule with allergenic properties

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Supplementary Figure



Supplementary Figure S1: Initial allergic reactions after a *Loxosceles* spider bite. The pictures shown depict signs developed by a 53-year-old patient which was bitten by a *Loxosceles* spider in Curitiba, Paraná, Brazil. The patient took the spider to specialized unit (Unidade Básica de Saúde Boa Vista, Curitiba) in order to have the correct spider identification. Images (A) and (B) point out the cutaneous rash developed in the first hours after a *Loxosceles* bite, highlighting the presence of a diffuse exanthema on the skin (black arrowheads), which spreads radially away from the bite site (white arrows). Images (C) and (D) show the aspect of the lesions triggered by the accident after 48 and 96 h of its occurrence, respectively. It is noticed the appearance of a marble plaque (white arrowhead) and dermonecrosis with gravitational spreading (grey arrows), which are both hallmark features of the cutaneous loxoscelism. A decreasing of the diffuse allergic exanthema when compared to (A) and (B) is also observed.