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# A new insight into the cellular mechanisms of envenomation: Elucidating the role of extracellular vesicles in Loxoscelism

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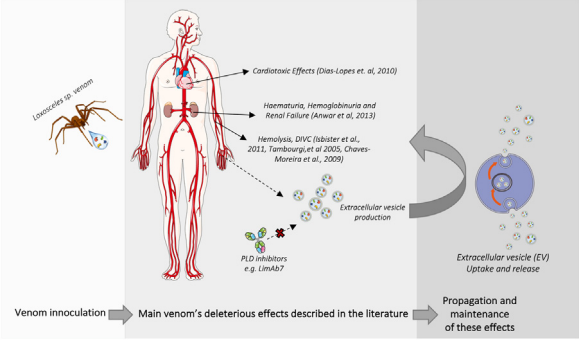
## HIGHLIGHTS

- Venoms of Loxosceles spiders induce extracellular vesicle (EVs) production in different cell lines.
- Phospholipases D (PLDs) present in *L. intermedia* venom have crucial toxic effects on the induction of EV production.
- EVs carrying toxins can contribute to the progression of the envenomation process.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Envenomation by the *Loxosceles* genus spiders is a recurring health issue worldwide and specially in the Americas. The physiopathology of the envenomation is tightly associated to the venom's rich toxin composition, able to produce a local dermonecrotic lesion that can evolve systemically and if worsened, might result in multiple organ failure and lethality. The cellular and molecular mechanisms involved with the physiopathology of Loxoscelism are not completely understood, however, the venom's Phospholipases D (PLDs) are known to trigger membrane injury in various cell types. Here, we report for the first time the *Loxosceles* venom's ability to stimulate the production of extracellular vesicles (EVs) in various human cell lineages. Components of the *Loxosceles* venom were also detectable in the cargo of these vesicles, suggesting that they may be implicated in the process of extracellular venom release. EVs from venom treated cells exhibited phospholipase D activity and were able to induce *in vitro* hemolysis in human red blood cells and alter the HEK cell membranes' permeability. Nonetheless, the PLD activity was inhibited when an anti-venom PLDs monoclonal antibody was co-administered with the whole venom. In summary, our findings shed new light on the mechanisms underlying cellular events in the context of loxoscelism and suggest a crucial role of EVs in the process of envenomation.



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## 1. Introduction

Envenomation can be widely defined as the result of the exposure to a venom or toxin caused by the bite or sting of a venomous animal. The great majority of envenomation accidents is usually caused by serpents and invertebrates, the latter being mostly arthropods belonging to the Arachnida class. It is estimated that, worldwide, about 1.8 to 2.7 million cases of snakebite occur every year, with a mortality rate of about 10 % (Gutiérrez et al., 2017). Moreover, envenomation cases provoked by members of the Arachnida class (spiders and scorpions) are also numerous, with at least 1 million scorpion accidents reported annually (Chippaux, 2015; Dias-Lopes et al., 2018).

*Loxosceles* spider envenomation can produces a potentially deleterious injury, representing a major public health issue in countries around the globe, and the Americas in particular (Vetter, 2018). More than 7990 cases were reported in Brazil in 2017 (Oliveira-Mendes et al., 2020). The venom can cause a wide range of pathophysiological effects to the victims, such as hemolysis, complement overactivation and intense local inflammatory responses that can evolve into cell death, dermonecrosis and in some cases renal and multiple organ failure, as a consequence of reported hematological disorders (Chaves-Moreira et al., 2017a, 2017b, 2017c).

The severity of an envenomation *per se* is highly influenced by the toxicity and concentration of certain components present in the venoms (Utkin, 2015). It is known to be a complex mixture of biologically active compounds, such as hyaluronidases, serineproteases, metalloproteases and phospholipases D. These toxic molecules affect different cells through their distinct and synergic proteolytic activities, potentially leading to the activation of signaling cascades that will implicate surrounding cells (Wille et al., 2013; Gremski et al., 2014; Corrêa et al., 2016) and could be involved with the most serious pathological effects observed approximately 72 h post-bite. Amongst these components, the phospholipases D (PLDs) are the most thoroughly characterized and associated with the venom's main deleterious effects. Previous studies have elucidated the role of PLDs in membrane remodeling, cell death and red blood cell (RBCs) clearance, in either physiological or pathological contexts (Chaves-Moreira et al., 2009, 2011; Kuo et al., 2017; Sorkin et al., 2018; Nguyen and Pandey, 2019). However, the cellular communication environment in the envenamation episode is poorly described.

In the context of cell signaling and communication, recent interest has been given to extracellular vesicles (EVs), and their role in molecular basis of various chronic and infectious diseases (van Niel et al., 2018). EVs are defined as membranous cell-derived structures that involve exosomes produced through the multivesicular bodies (MVBs) secretion pathway, and microvesicles, originated from the plasmatic membrane by a myriad of molecular processes. All these events are strongly influenced by cell membrane composition (Egea-Jimenez and Zimmermann, 2018; Ramirez et al., 2018; Pollet et al., 2018).

Considering that PLDs are major Loxosceles venom components and responsible for various cell membrane directed effects, we questioned whether extracellular vesicles could be involved in these processes. Henceforth, the present study focused on the investigation of the production, composition and phenotypic effects of EVs in RBCs and secondary cells lines stimulated with *Loxosceles intermedia* venom, aiming to better understand the mechanisms underlying cellular communication that might partake in the envenomation process.

We hereby report for the first time the involvement of extracellular vesicles (EVs) in the context of Loxoscelism. We have shown that when in contact with different cell lineages, *Loxosceles intermedia* venom augmented EV production. We were also able to detect components of the *Loxosceles* venom in the cargo of these vesicles, suggesting that they may be implicated in the process of venom extracellular release. Altogether, our findings shed new light on the mechanisms underlying important cellular events in the context of loxoscelism and suggest a crucial role of EVs in the process of envenomation.

## 2. Materials and methods

### 2.1. Venoms, antibodies and reagents

Vacuum dried venoms from *L. intermedia*, *L. gaucho*, *L. laeta* spiders were kindly supplied by Dr. João Carlos Minozzo from the Centro de Produção e Pesquisa de Imunobiológicos (CPPI, Piraquara, PR, Brazil) and resuspended in a 10 mM $Na_2HPO_4$ phosphate saline buffer containing 137 mM NaCl and 2.7 mM KCl pH 7.4 to a final concentration of 0.8–1 mg.mL$^{-1}$). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (Sigma–Aldrich Chemical Co., St. Louis, MO, USA). DAPI (40, 6-diamidino-2-phenylindole, dihydrochloride) and CFSE (Carboxyfluorescein succinimidyl ester) were obtained from Molecular Probes (Invitrogen, Camarillo, CA, USA). PMA (Phorbol myristate acetate) was obtained from Sigma-Aldrich. All chemicals were of standard grade and acquired from Sigma Aldrich (Saint-Louis, MO, USA) or equivalent.

The LimAb7 hybridoma was previously produced by Alvarenga et al. (2003) from mouse immunization with *L. intermedia* venom. It secretes a monoclonal $IgG_{1k}$ capable of neutralizing the dermonecrotic activity of *L. intermedia* venom by binding to its several PLD isoforms. In the present study, LimAb7 was purified from the supernatant of thawed and cultivated hybridomes through IMAC (Immunoaffinity Chromatography) with a protein A-Sepharose CN-Br column (Sigma Aldrich) (Alvarenga et al., 2003). Horse hyperimune sera (SALOX) reactive to *L. intermedia*, *L. laeta*, and *L. gaucho* venoms was provided by CPPI and horse IgG F $(ab)'_2$ anti-*Loxosceles* venom conjugated to HRP was prepared as previously reported (Chávez-Olórtegui et al., 1998).

### 2.2. Cell culture

Human embryonic kidney cells (HEK-293) and Human monocyte THP-1 cells, (Tsuchiya et al., 1980) were grown as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) and Rosewell Park Memorial Institute (RPMI) 1640 medium, respectively, 10 % fetal bovine serum (FBS), 1% streptomycin (100 mg.mL$^{-1}$) and penicillin (100 U. mL$^{-1}$) (Sigma–Aldrich). The cultures were kept at 37 °C in a humidified atmosphere with 5% $CO_2$. HEK-293 cells were fed every three days and passaged using 0.025 % trypsin with 0.22 mM EDTA when 80–90 % confluent.

THP-1 monocytes in exponential growth ($5-8 \times 10^5$ cells.mL$^{-1}$) were treated with 15 ng.mL$^{-1}$ of differentiation inducing agent PMA, diluted in RPMI cell culture media. Cells were seeded in a 24-well plate ($2 \times 10^5$ cells/well) and incubated at 37 °C, 5% $CO_2$, for 48 h (Lund et al., 2016). 2 days after PMA administration, media containing PMA was removed and adhering macrophage-like cells were washed 2 times with sterile PBS and once with FBS-free RPMI. After 24 h, cells were utilized in cytokine dosage assays and their supernatant was collected and stored at -80 °C.

### 2.3. Animals

6- to 8-week-old female Swiss mice were purchased and maintained at the Vivarium of the Biological Sciences Department of the Federal University of Parana (UFPR, Curitiba, PR, Brazil). The animals received water and food under controlled environmental conditions. All experimental procedures were performed according to the guidelines for the use of laboratory animals and approved by the Ethics Committee of Animal Experimentation of

*Setor de Ciências da Saúde do Universidade Federal do Paraná* (Curitiba, PR, Brazil), under the reference number 409.

### 2.4. Preparation of the erythrocytes (RBCs)

Blood from healthy human donors was collected in tubes containing sodium citrate buffer (BD Plastipak, Franklin Lakes, NJ USA). The platelet-rich plasma and buffy coat were removed by aspiration after centrifugation at 200*g* for 15 min. Erythrocytes (RBCs) were washed three times with Ringer's Lactate Solution (125 mM NaCl, 5 mM KCl, 1 mm $MgSO_4$, 32 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$, pH 7.4, 300 mOsm/kg $H_2O$) and resuspended at a final concentration of $1 \times 10^8$ cells. $mL^{-1}$. This study has been approved by the Human Research Ethics Committee from *Setor de Ciências da Saúde do Universidade Federal do Paraná* (Curitiba, PR, Brazil) under the certificate number CEP/SD2911004.

### 2.5. Extracellular vesicle (EVs) production and determination of cell viability

EVs were obtained by collecting the supernatant from THP-1 cells, HEK-293 cells, and healthy human donors' RBCs after stimulation. All assays were carried out in FBS-free media to eliminate EV contaminants. To stimulate EV release, HEK-293 and THP-1 cells ($1 \times 10^5$. $mL^{-1}$) were incubated in the presence of 10 $\mu g.mL^{-1}$ of *Loxosceles intermedia* venom or only in SBF-free media for 3, 12 and 24 h, at 37 °C, on a linear shaker. In the case of the HEK-293 lineage, cells were previously trypsinized, resuspended in serum-free DMEM and the assay was carried out. RBCs obtained from the blood of human donors were also incubated ($10^8$ cells. $mL^{-1}$) with 2.5., 5 and 10 $\mu g.mL^{-1}$ of *L. intermedia, L. gaucho* and *L. laeta* venom diluted in Ringer's solution, 2 mM of $CaCl_2$ (EVs-$CaCl_2$), a known inducer of EV production (Kim et al., 2019) diluted in Ringer's solution or Ringer's solution alone for 3, at 37 °C, on a linear shaker. Next, EVs were isolated from the cells media by differential centrifugation as follows: 160 xg for 5 min; twice at (4000 xg for 30 min) and 100,000×*g* for 90 min, following the International Society of Extracellular Vesicles (ISEV) guidelines (Lötvall et al., 2014; Théry et al., 2018). All centrifugation steps were conducted at 4 °C and the ultracentrifugation was done in a Beckman Coulter Optima MAX-XP ultracentrifuge: (MLS-50 aluminum swinging-bucket rotor). EV pellets were resuspended in 100 µl of cold sterile filtered PBS or HEPES buffer (10 mM HEPES Buffer, 140 mM NaCl, pH 7.4). 10−20 µl of the sample were used for protein quantification and the rest was kept at 4 C° for 5 days' maximum, for further use in functional assays. Cell viability was assessed using the Trypan Blue dye exclusion test. For RBCs, cells were counted before and after each treatment for viability determination. EV production and viability values obtained for untreated cells were subtracted from those of treated cells for data representation.

### 2.6. In vivo *stimulation of EV production*

Mice were subcutaneously injected with 1 $\mu g.g^{-1}$ *Loxosceles intermedia* venom diluted in PBS, pH 7.4, to a final volume of 100 µL. Venom injected animals were divided into two groups (n = 2/group); the former treated for 1 h and the latter, 4 h. A separate group of mice (n = 2) was injected only with the vehicle and evaluated after 4 h. Subsequently, their blood was drawn, and the animals were euthanized.

Blood was collected from the orbital plexus in the absence of anticoagulant and its fractions were separated after centrifugation at 400 *g*, 5 min at RT. The serum was aspired and centrifuged at 160 *g*, 5 min at RT, in order to further eliminate cellular debris. Next, serum EVs were isolated following the same differential centrifugation protocol mentioned in the EV production section.

### 2.7. Inhibition of EV production in vitro

Healthy human donors' RBCs were simultaneously pre-treated with 2.5, 5 and 10 $\mu g.ml^{-1}$ of *L. intermedia* venom +10 $\mu g.ml^{-1}$ Limab7, or +10 $\mu g.ml^{-1}$ horse anti-*Loxosceles* polyclonal antibodies. *L. intermedia* venom treated RBCs +10 $\mu g.ml^{-1}$ of a non-neutralizing monoclonal antibody were considered as a negative control for production inhibition. Next, EVs were isolated and quantified as previously described in the EV induction section.

### 2.8. Characterization of EVs

**Protein Quantification:** The total protein concentration of EVs was determined by the Bradford Reagent for protein quantification following the method described by Bradford (Bradford, 1976). **Nanoparticle Tracking Analysis (NTA):** Size and quantity of isolated EVs was measured using a Nanosight NS300 instrument (Malvern Instruments Ltd, Malvern, UK). Data were analyzed using the NTA software (version 2.3 build 0017). To perform the measurements, samples were diluted in filtered PBS and readings were performed at room temperature, in triplicates sampled for 60 s each, at 10 frames per second. **Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for venom detection in EVs:** 96-well plates were coated with 10 $\mu g.mL^{-1}$ of LimAb7 or horse IgG F(ab)'$_2$ anti-*Loxosceles* venom, diluted in sodium bicarbonate buffer (pH 9.6), for 16 h at 4 °C and the saturated with 2% casein for 1 h at 37 °C. Next, plates were incubated for 1 h with *L. intermedia* venom at a serial dilution of 1 $\mu g.mL^{-1}$ to 0.001 $\mu g.mL^{-1}$ or with isolated lysed EVs from venom stimulated RBCs (EVs-Li) (10, 20 or 40 $\mu g.mL^{-1}$) for 1 h, at 37 °C. Subsequently, plates were incubated with polyclonal anti-*L. intermedia*-HRP antibodies for 1 h, at 37 °C. The ortho-phenylenediamine substrate solution (OPD) was added and after 20 min, $H_2SO_4$ (1:20) was added to quench the reaction. The $A_{490nm}$ was read (Microplate Reader 550, BIORAD). Between each intermediate step, plates were washed four times with PBS containing NaCl 150 mM and Tween 20, 0.05 %. Negative controls were performed using EVs obtained from cells treated with $CaCl_2$ (EVs-$CaCl_2$). All assays were performed in duplicate. A reactivity curve was constructed from the $A_{490}$ values obtained from the detection of known venom concentrations by horse IgG F(ab)'$_2$ anti-*Loxosceles* venom. Interpolation of EV $A_{490}$ values allowed estimation of the amount of *L. intermedia* venom in EVs (Chávez-Olórtegui et al., 1998; Alvarenga et al., 2003). **Western Blotting for venom detection in EVs:** 2 µg of EVs-Li and EVs-$CaCl_2$, previously quantified by the Bradford method, were resolved in a 12 % SDS-PAGE polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with PBS/Tween-20 (0.05 %) and 5% skimmed milk. Subsequently, membranes were incubated with the purified LimAb7 antibody 10 $\mu g.mL^{-1}$ or with 1: 20,000 anti-*Loxosceles* horse polyclonal F(ab)'2, diluted in PBS/Tween 0.05 % and 0.5 % skimmed milk. Lastly, membranes were incubated with anti-mouse IgG HRP (1:2000, Sigma-Aldrich) or anti-horse Ig HRP (1: 30,000, Sigma-Aldrich). Antibody–antigen complexes were detected with ECL™ reagents (Thermo Fisher Scientific, Rockford, IL, EUA) by chemiluminescence. All incubations were performed for 1 h at 37 °C, under agitation, and the membranes were washed five times with PBS, pH 7.4, containing 0.05 % Tween 20, between each intermediate step. The assay was revealed as previously described in the Karim-Silva et al. (2016).

### 2.9. Phospholipase D activity of EVs

*Loxosceles intermedia* venom EVs (EVs-Li) were assessed using the Amplex® Red Sphingomyelinase Assay Kit, which provides a sensitive method for measuring sphingomyelinase activity *in vitro*. Briefly, *Loxosceles intermedia* venom (1−0.0625 $\mu g.mL^{-1}$) and 166,

33 and 17 μg EVs-Li obtained from RBCs, (equivalent to 5−0.05 μg. $mL^{-1}$ of Li venom, as determined by ELISA) were added to the Amplex Red reagent mixture. EVs obtained from Ringer's lactate solution treated RBCs (EVs-Lr) were utilized in the same quantities as EVs-Li (166, 33 and 17 μg) as a negative control. Reactions were performed at 37 °C for 30 min before measuring the excitation/emission fluorescence at 550/590 nm in a fluorimeter (Spectramax M2-Molecular Devices). All points were carried out in triplicates (Magalhães et al., 2013).

### 2.10. Hemolytic activity of EVs

In adaptation to the hemolysis protocol described by Karim-Silva et al. (2016), the hemolytic ability of RBCs EVs produced by cells treated with *Loxosceles intermedia* venom (EVs-Li) was evaluated. EV production was induced as aforementioned and its hemolytic activity in the concentrations of 43 μg.$mL^{-1}$ and 21.5 μg.$mL^{-1}$ (equivalent to 1.25 and 2.5 μg.$mL^{-1}$ of *Li* venom, as determined by ELISA) was assayed and compared to the incubation of *Loxosceles intermedia* crude venom alone (0.625–5 μg. $mL^{-1}$). EVs-Lr (43 μg. $mL^{-1}$) as well as bovine serum albumin (43 μg. $mL^{-1}$) were used as negative controls and Triton X-100 0.1 % as a positive hemolysis control. O.D. values were converted to percentage of hemolysis using the O.D. value of the positive control (Triton X-100 0.1 %) as representative of 100 % lysis.

For comparative purposes, the hemolysis protocol described by Karim-Silva et al. (2016) was also employed for the venoms of *L. intermedia, L. gaucho, L. laeta* (2.5 μg.$mL^{-1}$, 5 μg.$mL^{-1}$, 10 μg.$mL^{-1}$) as means to evaluate the differential hemolytic activity of these venoms and their ability to stimulate EV production.

### 2.11. Screening of cytokine profile by ELISA (Enzyme-Linked Immunosorbent Assay)

For cytokine detection, the supernatant of $2 \times 10^5$ macrophage-like cells (derived from THP-1 cells) was collected 2 and 24 h after treatment with 2, 20 and 50 μg.$mL^{-1}$ of RBCs EVs-Li and 2 and 50 μg.$mL^{-1}$ of RBCs EVs-Lr. Cytokine (IFN-γ, TNF-α, IL-6 and MCP-1/CCL2) production from the treated cells was evaluated by the Human standard (IFN-γ, TNF-α, IL-6 and MCP-1/CCL2) TMB ELISA Development kits (PeproTech, EUA), following the manufacturer's guidelines and in adaptation to the methods described by Gvirtz et al. (2020).

### 2.12. EV uptake assay

HEK-293 cells were plated on circular coverslips of 13 mm in diameter ($1.5 \times 10^5$ cells/ coverslip) (TPP, Trasadingen, Switzerland) and maintained in culture (37 °C, 5% $CO_2$) for 72 h. EVs were pre-labeled with CFSE for 1 h, then re-ultracentrifuged at 100,000 *g* for 90 min and suspended in HEPES. Cells were exposed to the venom of *L. intermedia* (20 μg.$mL^{-1}$), and treated with the labelled RBC EVs-Li or EVs-Lr (20 μg.$mL^{-1}$), for 3 h, at 37 °C. Next, cells were washed with PBS and fixed for 10 min in a 2% formaldehyde solution (Sigma, USA). 3 additional washing steps were performed with PBS and slides were stained with DAPI (4′, 6-diamidino-2-phenylindole, dihydrochloride) and Phalloidin Alexa 594 (diluted 1:500) for 20 min. Lastly, cells were washed with PBS and mounted on histological laminae with Fluoromount G (Thermo Fischer). Slides were then analyzed in a scanning confocal microscope (Nikon-Eclipse E800).

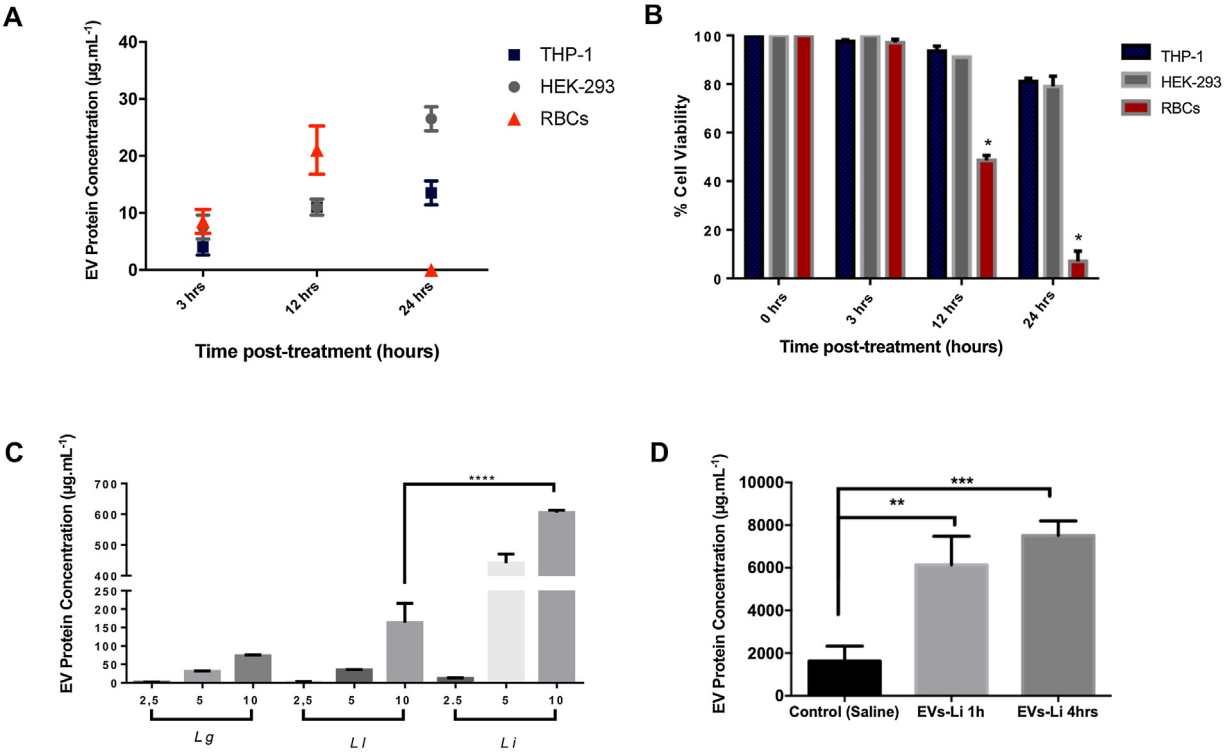


**Fig. 1.** Induction of EV production by *Loxosceles* venom in different cell lineages and cell viability analysis. **A)** Induction of EV production through the stimulus of different concentrations of *Loxosceles intermedia* venom in cell lineages THP-1, HEK-293 and human RBCs. **B)** Evaluation of the cell viability of THP-1, HEK-293 cells and human RBCs in different time points. **C)** RBCs were treated with increasing concentrations of 3 distinct *Loxosceles sp*, venoms: *L. intermedia* (Li), *L. gaucho* (Lg) *and L. laeta (Ll)* venoms (2.5, 5 and 10 μg.mL-1) and their ability to induce EV production and hemolysis in RBCs (data not shown) was quantified. **D)** Mice (n = 6) were injected with either *Loxosceles intermedia* venom (1 μg/g) or saline and after 1 and 4 h, their blood was collected, RBC EVs were isolated and quantified.

### 2.13. Analysis of EV effects on cell membrane integrity and LimAb7 protective effects

HEK-293 cells were plated on circular coverslips of 13 mm in diameter ($1.5 \times 10^5$ cells per coverslip) (TPP, Trasadingen, Switzerland) and maintained in culture (37 °C, 5% $CO_2$) for 72 h. The next day, cells were washed and incubated with serum-free DMEM and exposed to 2 μg.ml$^{-1}$ and 20 μg.ml$^{-1}$ of *L. intermedia* venom, RBCs EVs-*Li* or Ringer induced EVs-Lr for 1 h at 37 °C. After the treatment, cells were washed with PBS and fixed for 10 min in a 4% formaldehyde solution (Sigma, USA). Following fixation, three washes with PBS were performed for 10 min each, at room temperature, and propidium iodide was incubated at a concentration of 1 μg.mL$^{-1}$, for 10 min. For the positive control, cells were permeabilized with 0.005 % Triton X-100 for 15 min before treatment with propidium iodide. Lastly, cells were incubated with DAPI (3 mM, diluted in PBS) for 5 min, samples were mounted with Fluoromount-G and observed using a confocal fluorescence microscope (Nikon-Eclipse E800).

In order to assess the protection of membrane integrity granted by LimAb7, cells were incubated for 1 h with *L. intermedia* venom (20 μg.ml$^{-1}$) + EGTA (2 mM.mL$^{-1}$), *L. intermedia* venom (20 μg. ml$^{-1}$) + Limab7 (10 μg.ml$^{-1}$) and RBC EVs-*Li* 20 μg/mL + Limab7 (10 μg.ml$^{-1}$) for 1 h (37 °C, 5% $CO_2$). Next, cells were incubated with DAPI (3 mM, diluted in PBS) for 5 min, mounted with Fluoromount-G and observed using a confocal fluorescence microscope (Nikon-Eclipse E800).

## 3. Results

### 3.1. Induction of EV production by Loxosceles intermedia venom

Aiming to assess the Loxosceles sp. venom's ability to induce the production of EVs, three distinct cell lines were incubated for up to 24 h *L. intermedia* venom, and EVs release was observed in all cell types (Fig. 1A). Moreover, cell viability was not compromised by the venom in the first 3 h in all evaluated cell lineages, yet only HEK-293 and THP-1 cells have maintained viability over time (Fig. 1B). A considerable loss of viability at the 12 and 24 -h time points was detected for RBCs.

Additionally, when treated with increasing quantities of different *Loxosceles* species venoms for 3 h, RBCs showed a dose-dependent increase in EV production. We have also observed that in comparison to *L. gaucho* and *L. laeta*, the *L. intermedia* venom induces a two to three times higher EV production (Fig. 1C).

To assess the production of EVs in an *in vivo* model, mice were challenged with the *L. intermedia* venom for 1 and 4 h. From the

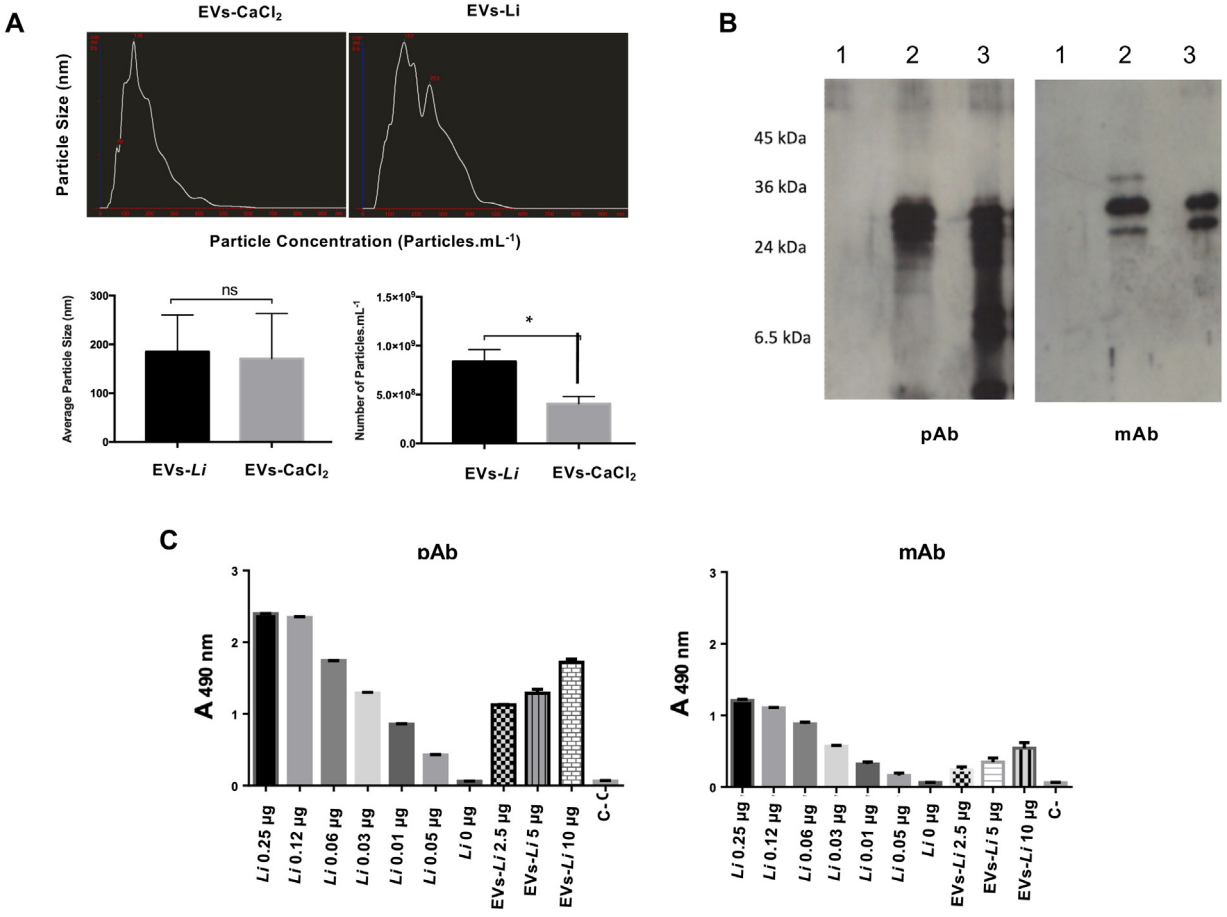


**Fig. 2.** EV Quantification, Population and Cargo Characterization. **A)** Nano Tracking Analysis (NTA) of EV population distribution and quantity: Graphic representation obtained by the Nanosight™ software shows that RBC derived EVs are around 120-220 nm in size. The graphs below depict the size distribution and EV concentration of analyzed samples. In the Y axis the EV average size (0-1000 nm) or concentration (in particles. mL$^{-1}$) are shown. **B)** Western Blot for venom detection in EVs: Samples of RBC derived EVs were loaded into a 12,5% polyacrylamide gel, resolved, and transferred to a PVDF membrane. 1. EVs-$CaCl_2$ (1 μg), 2. EVs-Li (1 μg), 3. *L. intermedia* venom (0,5 μg). For venom detection in the EVs, pAb anti-*Loxosceles intermedia* -HRP was used (1: 1500 – left panel) and on the right panel LimAb7 (10 μg. mL$^{-1}$) and its binding detected by an anti-mouse HRP-conjugate (1:4000). **C)** ELISA venom quantification inside EVs: densitometry of the resulting of the reactivity of Polyclonal horse Anti-*Loxosceles* (Fab')$_2$ (left panel) and monoclonal antibody LimAb7 (right panel) against the L. intermedia venom (0,25 μg. mL$^{-1}$, 0,12 μg. mL$^{-1}$, 0,06 μg. mL$^{-1}$, 003 μg. mL$^{-1}$); THP-1 derived EVs-Li (2.5, 5 and 10 μg. mL-1) and EVs-$CaCl_2$, the latter used as a negative control.

collected blood samples, a venom associated, and time-dependent EV production was observed (Fig. 1D).

### 3.2. EVs carry biologically active venom in their cargo

EVs produced during Venom-RBC interaction were quantified and analyzed by nanotracking analysis (NTA). The vesicle population sizes were homogeneous between positive controls ($CaCl_2$) and venom induced EVs, however, the latter has a higher amount of protein than the EV production control ($CaCl_2$), corroborating the results obtained for EV protein dosage (Fig. 1A and 2A). The average EV size observed ranged from 150–200 nm, suggesting a predominance of microvesicles (MVs) in the EV population produced upon venom stimuli. For venom detection, a polyclonal antibody produced against *L. intermedia* venom and a monoclonal antibody (LimAb7) reactive against the venom's Phospholipases D were employed and enabled the visualization of venom components in the EVs-Li (Fig. 2B-C).

Moreover, we have estimated the presence of *L. intermedia* venom components, contained in EVs by utilizing a quantitative sandwich-format ELISA assay (Fig. 2 C) finding that the *L. intermedia* venom should represent approximately 1–3 % of all EVs-Li protein cargo.

### 3.3. Phenotypic effects of EVs in eukaryotic cells

To evaluate the possible deleterious effects induced by EVs-Li, we were able to demonstrate through the carrying out of a Hemolysis Assay that the same antibodies used to identify the venom's toxins within EVs, inhibited EV production in human RBCs (Fig. 3A). Once venom components were detected in EVs by ELISA and Western Blot (Fig. 2B-C), we decided to investigate the phospholipase D enzymatic activity of these EVs-Li and found they retain significant hydrolytic activity over sphingomyelin (Fig. 3B). Therefore, the same EVs-Li population was assayed for their hemolytic activity and compared to the crude venom's described capacity of hemolytic induction (Fig. 3C) and we detected a dose-dependent relationship between the EV venom concentration and the percentage of hemolysis similar to that observed for the crude venom (Fig. 3C).

### 3.4. EVs as immunomodulators

Macrophage-like differentiated THP-1 cells were assayed for their cytokine production when treated with RBC derived EV-Li. No alterations were observed in the production of IFN-γ, TNF-α, IL-6 and MCP-1 after 4 h (data not shown). However, after 24 h an

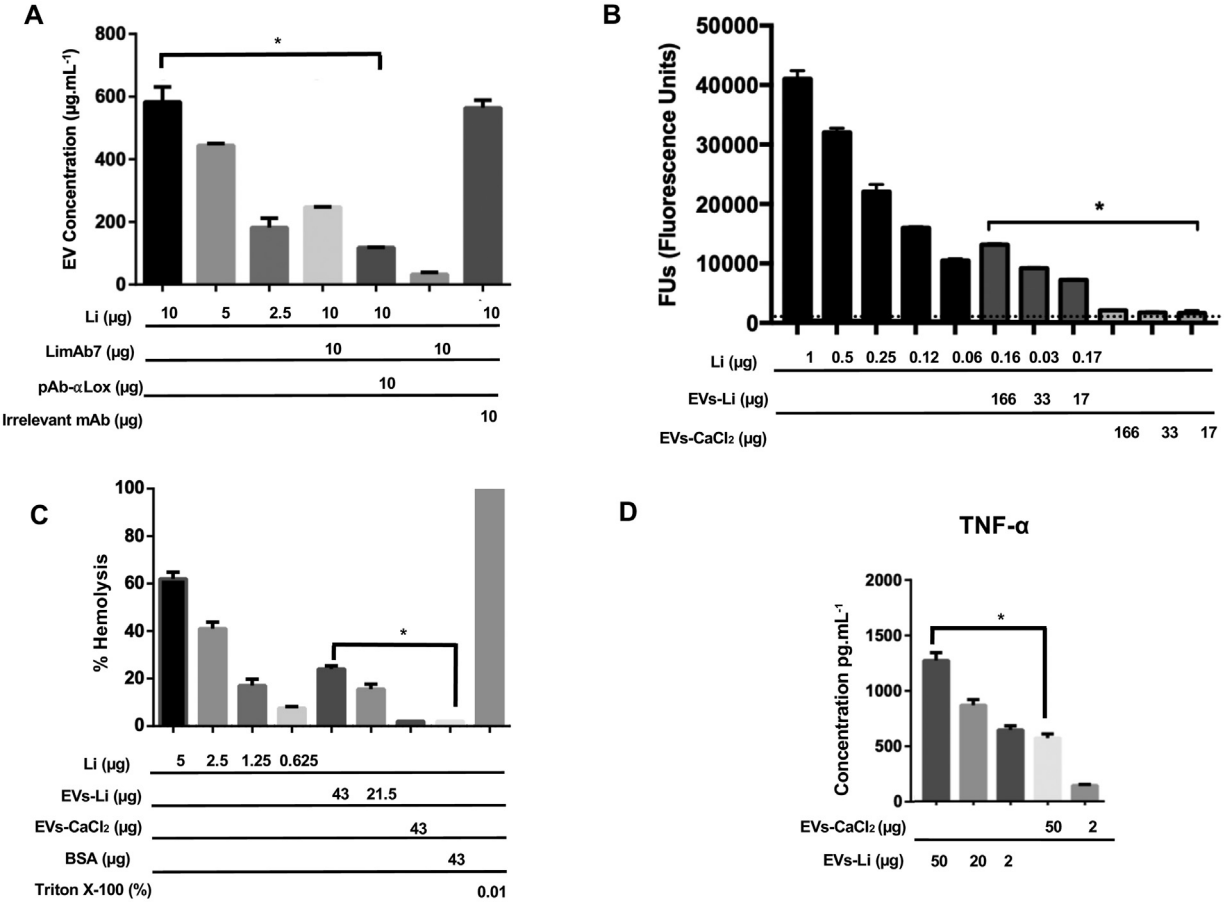


**Fig. 3.** Phenotypic Effects of EVs in Eukaryotic Cells. **A)** Inhibition of EV production in RBCs in the presence of 10 µg LimAb7, an anti-*L. intermedia* PLD monoclonal antibody and 10 µg of a pAb anti-*Loxosceles sp.* whole venom. **B)** Determination of the EVs-Li Phospholipase D activity by the Amplex Red Phospholipase D Assay Kit. *L. intermedia* venom was tested in crescent concentrations (2.5, 5 and 10 $\mu g.mL^{-1}$) as well as equivalent venom concentrations of EVs-Li. EVs-Lr were also assayed and showed no significant PLD activity. Positive and negative controls were employed based on the commercial assay guidelines. EVs-Li show PLD activity significantly above than the negative control and corresponding to an estimate between 0.125 and 0.0625 µg of Li venom. **C)** Hemolytic Assay in the presence of EVs: RBCs treated with crescent concentrations of *L. intermedia* venom (5, 2.5, 1.25 e 0.625 $\mu g.mL^{-1}$) and with equivalent venom concentrations in EVs-Li show around 20 % of hemolysis when treated with EVs-Li. Optical density values were converted to percentage of hemolysis considering the O.D. value of the positive control (Triton X-100 0,1%) as representative of 100 % of lysis. The O.D values were read at the wavelength of 570 nm. EVs-$CaCl_2$ were assayed as negative control and show no induction of hemolysis. **D)** Induction of TNF-α production by EVs: THP-1 cells were harvested and differentiated to macrophage-like cells with PMA. After differentiation, cells were stimulated with either EVs-$CaCl_2$ or EVs-Li for 2 and 24 h and TNF-α release was measured in $pg.mL^{-1}$.

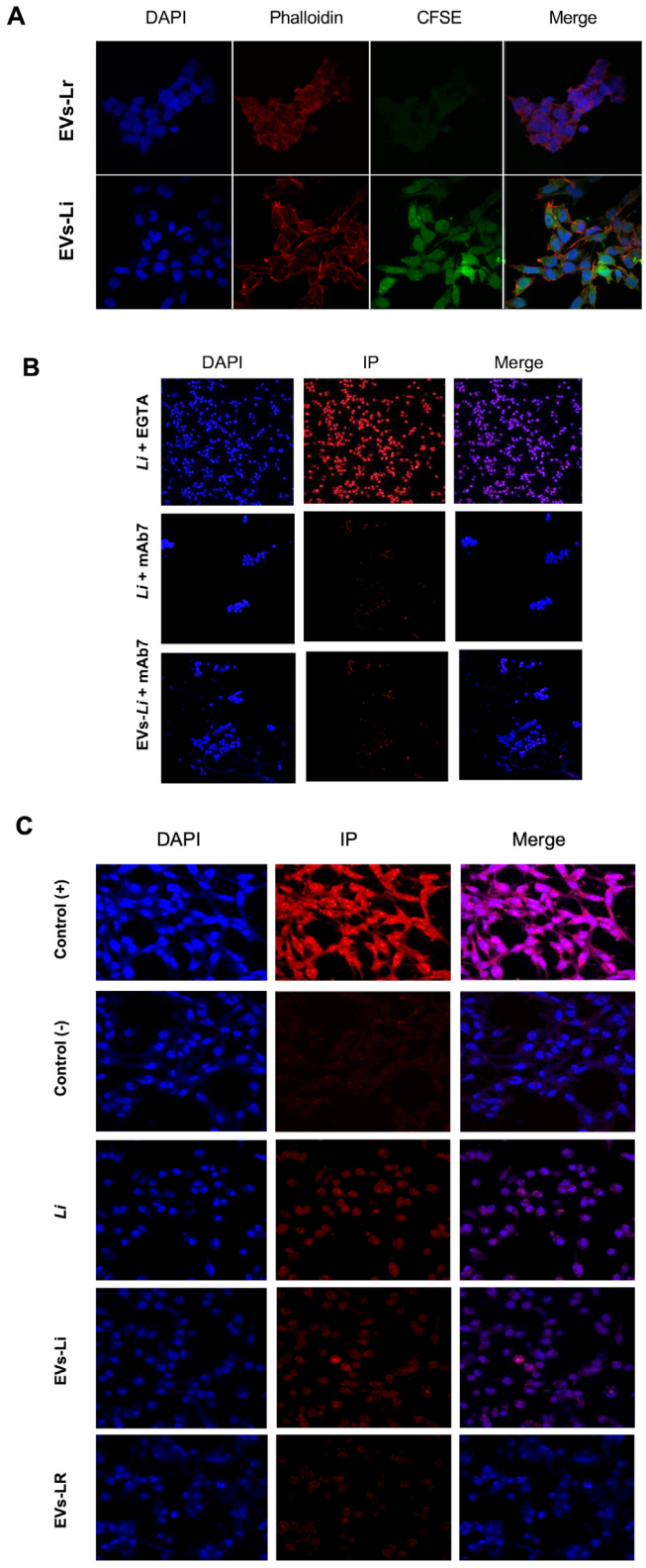


**Fig. 4.** EVs-Li undergo uptake by HEK-293 cells and are able modify the cell membrane's integrity. **A)** EVs-Li and EVs-Lr Uptake Assay: RBC derived EVs-Li and EVs-Lr were isolated, labelled with CFSE and incubated (20 μg.ml$^{-1}$) with harvested HEK-293 cells (1 × 10$^5$/well density), for 3 h and a preferential uptake of EVs-Li was observed, in comparison to EV-Lr. **B)** Evaluation of EV and *L. intermedia* whole venom effects on membrane integrity: whole Li venom, RBC EVs-Li and EVs-Lr were separately incubated with HEK-293 cells (1 × 10$^5$/well,), for 3 h. As a permeability control, cells were treated with Triton X-100 (0.005 %) for 15 min. Cells were then stained with Propidium Iodide (PI) (1 μg.ml$^{-1}$) for 10 min. and visualized in a scanning confocal microscope (Nikon-Eclipse E800). **C)** Assessment of LimAb7's protection of membrane integrity: 20 μg.ml$^{-1}$ of Li venom +2 mM.mL EGTA$^{-1}$ (a vesiculation inhibitor), 20 μg.ml$^{-1}$ of Li venom + 10 LimAb7 (10 μg.ml$^{-1}$) and EVs-Li (20 μg.ml$^{-1}$) + LimAb7 (10 μg.ml$^{-1}$) were incubated with HEK-293 cells (1 × 10$^5$/well), for 3 h. Cells were then stained with Propidium Iodide (PI) (1 μg.ml$^{-1}$) for 10 min. and visualized in a scanning confocal microscope (Nikon-Eclipse E800).

increase of TNF-α following EV-Li administration was detected (Fig. 3D).

### 3.5. L. intermedia *venom and EVs-Li's effects on the cell membrane's integrity*

Purified EVs were labeled with CFSE and placed in contact with HEK-293 monolayer cells for 3 h. Next, the internalization of the EVs was investigated by confocal and immunofluorescence microscopy and a more efficient internalization of EVs-Li to HEK-293 cells (as compared to EVs-Lr) was observed (Fig. 4A). We also verified that cells treated solely with *L. intermedia* venom or with EVs-Li showed an alteration in the cell membranes' permeability, demonstrated by an increase in the internalization of propidium iodide, thus suggesting that the venom has an important impact on the membrane's stability (Fig. 4B). Additionally, PI internalization was inhibited by adding LimAb7, a monoclonal antibody specific to *L. intermedia* PLDs (Fig. 4C). This deleterious effect was increased when cells were treated with EGTA, a calcium chelate and inhibitor of lysosome-dependent membrane repair mechanisms (Fig. 4B).

## 4. Discussion

Recent studies have elucidated the biogenesis and presence of extracellular vesicles (venisomes) as components of snake and wasp venoms *per se,* demonstrating that they play an important role in the venoms' cytotoxic properties enriching their composition (Carregari et al., 2018; Wan et al., 2019). Aiming to investigate whether *Loxosceles intermedia* venom would induce the release of EVs upon contact with human cells, three human cell lineages were chosen in order to represent the venom-eukaryotic cell interaction. Human red blood cells (RBCs), in which the venom's ability of inducing lysis has been extensively described (Chaves-Moreira et al., 2011; Karim-Silva et al., 2016; Lopes et al., 2020), human embryonic kidney cells (HEK-293), representative of the epithelial lining of the main injured tissue in systemic loxoscelism (Luciano et al., 2004; Nag et al., 2014) and human monocyte THP-1 cells, whose recruitment and migration by the action *Loxosceles* PLDs has been previously described (Rojas et al., 2017) were employed in this study.

Significant decreases in the cell viability of the mammalian lineages have been described when treated with quantities ranging from 20 to 50 μg of *Loxosceles* venom (Paludo et al., 2006; Nowatzki et al., 2012; Wille et al., 2013; Dantas et al., 2014). In this study, we treated cells with lower venom concentrations aiming to observe cytotoxic effects that precede cell death. The loss of viability observed at 12 and 24 -hs for RBCs is in accordance with the sensibility of these cells to lysis provoked by the *Loxosceles* venom, a phenomenon widely described by different groups (Tambourgi et al., 2000; Chaves-Moreira et al., 2009; Tambourgi et al., 2010; Chaves-Moreira et al., 2011). The lower RBC EV concentration at 12 and 24 -hs corroborates with the loss in RBC viability, thus suggesting the quantity of detected EVs is smaller given cell lysis.

The *L. intermedia*, *L. gaucho* and *L. laeta* venoms' ability to induce RBC lysis is already well documented (Fernandes Pedrosa et al., 2002; de Oliveira et al., 2005; Magalhães et al., 2013; Karim-Silva et al., 2016). Interestingly, we have also observed that in comparison to *L. gaucho* and *L. laeta*, the *L. intermedia* venom

induces a two to three times higher EV production. Significant interspecies variations in the composition, PLD hydrolytic activity and toxicity of *Loxosceles sp*. venoms have already been described (de Oliveira et al., 1999, 2005; Gomez et al., 2001; Paludo et al., 2006; Gremski et al., 2014; Chaves-Moreira et al., 2017a, 2017b, 2017c). Thus, the differential EV production herein observed for the different species' venoms might be related to the differential substrate preferences of these venoms' PLDs and, as a consequence, their different ability to remodel RBCs membranes (Chaves-Moreira et al., 2009, 2011). Moreover, our initial *in vivo* studies showed a time-dependent EV production associated with the venom's presence. This could possibly enable venom propagation to other cells by EVs, as it has already been demonstrated for EVs from bacterial CNF-1 pre-infected epithelial cells (de Oliveira et al., 1999). However, these studies must be carried out in a higher number of animals and alongside a thorough proteomic evaluation of EV content, as means to assess them for the presence of venom components and cellular by-products associated to the envenomation process (Fabbri et al., 2015).

After investigating our EV-Li population, we observed the average EV size observed ranged from 150−200 nm, suggesting a predominance of microvesicles (MVs) (van Niel et al., 2018). Microvesicle production has recently been described as a resulting event from membrane injury by bacterial pore forming toxins (PFTs) on various eukaryotic cell lineages (Romero et al., 2017; Ray et al., 2019). Perhaps the venom's PLD catalytic activity on found on EV samples could be related to the MV biogenesis mechanisms triggered upon *Loxosceles* venom injury in eukaryotic cells.

Various proteomic studies in the EV field have investigated the complexity of EV protein cargo, especially in the scope of malignant cell derived-EV modulation of healthy cells' phenotypes (Atanassoff et al., 2014; Leoni et al., 2015; Romero et al., 2017). Here, we investigated the presence of venom components in the cargo of venom induced EVs through the ELISA and Western blot assays. The employment of the LimAb7 antibody (Alvarenga et al., 2003) enabled the detection of venom components in the EVs-Li; This finding suggests that cells might be expelling the venom through EVs, in some sort of exocytosis repair mechanism, similar to the mechanism described for eukaryotic cells undergoing injury by bacterial PFTs (Fabbri et al., 2015; Romero et al., 2017), in order to repair their membrane or to even communicate with neighboring cells.

The detection, quantification, and characterization of EV cargo is already employed in the diagnosis and prognosis of diseases (Théry, 2015; Amiral and Seghatchian, 2016; Pang et al., 2020). This might also allow new perspectives in envenomation diagnosis, which remains a great challenge in the field (Dias-Lopes et al., 2018). Finally, we hypothesized that cells might be physiologically expelling the venom from their insides as a reactive repair mechanism and thus systemically propagating the venom and decided to investigate the phospholipase D enzymatic activity of these EVs-Li finding they retained significant hydrolytic activity over sphingomyelin.

PLDs are the most thoroughly studied *Loxosceles* venom components and have been vastly associated to the main deleterious venom effects (Gremski et al., 2020). Hence, the detection of venom PLDs, confirmed by a specific monoclonal antibody used (Alvarenga et al., 2003), as functional components in the cargo of EVs reinforces their cytotoxic role of in the process of envenomation. Moreover, amongst the various deleterious effects studied in loxoscelism, hemolysis has great relevance, as it is often clinically escribed in envenomed patients and also successfully reproduced *in vitro* (Chaves-Moreira et al., 2009; Nowatzki et al., 2012; Kodali et al., 2014; Karim-Silva et al., 2016)

Aiming to detect the loss of stability in membrane integrity during contact with venom and/or EVs, we verified that cells treated solely with *L. intermedia* venom or with EVs-Li showed an alteration in the cell membranes' permeability. This deleterious effect was increased when cells were treated with EGTA, a calcium chelate and inhibitor of lysosome-dependent membrane repair mechanisms, therefore suggesting that the venom has important deleterious capacities alone and when contained inside EVs (Fig. 4B). The biological activity detected in the EVS obtained from the erythrocytes in addition to the protection conferred when vesiculation inhibitors were used, supports the idea that EVs were produced by the venom's action.

Over the last few years, the knowledge on *Loxosceles* venoms has improved allowing for the *in vivo* and *in vitro* characterization of the cytotoxic and dermonecrotic features of venom components (Tambourgi et al., 1998; Tambourgi et al., 2000, 2007; Meeterenvan et al., 2004; Chaves-Moreira et al., 2009, 2011; Gremski et al., 2014). Nowatzki et al. (2010) described the venom's effect on epithelial cells, showing they internalized the venom, yet that did not provoke alterations in their viability, suggesting the existence of additional cellular and molecular events consequential from the cell-venom interaction. Moreover, important mechanisms following cell injury, such as membrane remodelling and repair, have been studied to a great extent in various cell lineages (Atanassoff et al., 2014; Leoni et al., 2015; Romero et al., 2017) and more recently, Lopes et al., 2020 demonstrated that these toxins can interfere in the architecture of lipid rafts, either in the expression of caveolin-1 or in the increase of flotilin-1 (Lopes et al., 2020). In this respect, our findings suggest that the *L. intermedia* venom, and consequently EVs-Li, alter HEK-293 membrane's integrity, possibly due to the activity of PLDs, thus permitting venom incorporation by the treated cells as visualized through PI internalization, which was inhibited in the presence LimAb7, an antibody specific to *L. intermedia* PLDs.

In the scope of toxin-induced immunomodulation, the production of cytokines as enhancers and direct consequences of inflammation has been well characterized in Loxoscelism as well as in scorpion, bee and snakebite envenomation (Petricevich, 2004; Voronov et al., 1999; de Souza et al., 2008; Horta et al., 2013; Rojas et al., 2017; Reis et al., 2019). Aiming to correlate our *in vitro* activity and cell communication data, we decided to evaluate whether EVs had the ability to stimulate macrophage cytokine release and detected a significant increase of TNF-α production by THP-1 cells following EV-Li administration. This finding corroborates previous studies on TNF-α production and consequent neutrophil recruitment elicited by *Loxosceles* venoms in eukaryotic cells and therefore indicates EVs-Li are as capable of inducing cytokine production as the venom alone (Domingos et al., 2003; Málaque et al., 1999; Rojas et al., 2017). Interestingly, it has been also shown that EVs containing virulence factors from fungi and parasites are capable of modulating the immune system and increasing the production diverse cytokines, including TNF-α, hence supporting the possibility of EVs acting as important immune modulators in Loxoscelism (Barbaro et al., 2010; Barbosa et al., 2018; Groot Kormelink et al., 2018; Ikeda et al., 2018). Moreover, EVs have been identified as important players in many host-parasite interactions (Coakley et al., 2015; Kuipers et al., 2018; Wu et al., 2019). Exosomes released from teratocytes of some species of endoparasitoid Hymenoptera larvae, have shown to carry molecules such as a fatty acid binding protein, crucial for the parasite-host interaction in their cargo (Salvia et al., 2019).

The biological activity of the EVs from venom-treated cells exhibited phospholipase D activity and induced hemolysis in human RBCs. This activity was inhibited when a monoclonal anti-*Loxosceles intermedia* PLD antibody (LimAb7) (Alvarenga et al., 2003) was administered alongside with the whole venom.

## 5. Conclusion

Extracellular vesicles (EVs) are a heterogeneous group of cell-derived membranous structures, which possess a key role in cellular crosstalk. We have shown that *L. intermedia* venom has a crucial toxic effect on the induction of MV production, particularly in the scope of PLDs, as confirmed by our results when specific inhibitors were employed. Our findings demonstrate, for the first time, human cells' ability to respond to *Loxosceles sp*. venom as a stimulus for EV release. EVs retaining venom in their cargo could potentially be associated to venom's systemic propagation and the maintenance of its deleterious effects in Loxoscelism . Yet, the involvement of EVs in the envenomation dynamics and even in specific systemic effects, such as renal failure, remains unclear.

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## Author contributions

LMA and MIR contributed to the outline and design of the study. GACC and IGJ performed the functional analysis. LMA, IGJ and MIR wrote the first version of the manuscript. All authors read and approved the submitted version.

## Declaration of Competing Interest

The authors report no declarations of interest.

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