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# Intracellular and extracellular effector activity of mouse neutrophils in response to cutaneous and visceral *Leishmania* parasites

Ana Valério-Bolas[a], Maria Pereira[a,b], Graça Alexandre-Pires[c], David Santos-Mateus[a], Armanda Rodrigues[a], Mariana Rafael-Fernandes[a], Aurea Gabriel[a], Felipe Passero[d], Gabriela Santos-Gomes[a,*]

[a] Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, IHMT, Universidade Nova de Lisboa, UNL. Rua da Junqueira 100, 1349-008 Lisboa, Portugal

[b] Escola Superior Agrária de Elvas, Edifício do Trem Auto, Avenida 14 de Janeiro s/n Apartado 254, 7350-903 Elvas, Portugal

[c] Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, Av. Universidade Técnica, 1300-477 Lisboa, Portugal

[d] Biosciences Institute, São Vicente Unit, Paulista Coast Campus, Universidade Estadual Paulista Júlio de Mesquita Filho, Praça Infante Dom Henrique, s/n, 11330-900 São Vicente, SP, Brazil

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ABSTRACT

Neutrophils are short-lived phagocytic cells equipped with several receptors for pathogen recognition and phagocytosis and have intracellular and extracellular effector mechanisms that can inactivate pathogens. Leishmaniases are diseases caused by different species of *Leishmania* that mainly afflicts poorer populations of tropical and subtropical regions and immunocompromised individuals. Thus, the present study aims to investigate the effector response of murine neutrophils to species of *Leishmania* causing American cutaneous leishmaniasis and zoonotic visceral leishmaniasis by evaluating pattern recognition receptors (PRR) and intracellular and extracellular effector microbicide activity. When exposed to *Leishmania* parasites, mouse neutrophils produced superoxide, released enzymes in the extracellular space and generated neutrophil extracellular traps, although PRR gene expression is negatively regulated. *L. infantum*, *L. guyanensis*, and *L. shawi* inhibited enzymatic activity, whereas *L. amazonensis* reduced the emission of extracellular structures. These findings indicate that although neutrophils trigger several microbicide mechanisms, *Leishmania* parasites can manipulate extracellular effector mechanisms. The present study also provides evidence that neutrophils can internalize parasites by coiling phagocytosis.

## 1. Introduction

Leishmaniases designates a group of diseases with different clinical presentation caused by the protozoan parasites of the genus *Leishmania.* These diseases that affect human beings and domestic and wild animals are considered an important public health issue. Based on parasite development within the sand fly gut, *Leishmania* is classified into two subgenera according to the location in the gut where parasite replication occurs [1]. Parasites of the subgenus *Leishmania* have a foregut development whereas for the subgenus Viannia, parasite replication occurs in the hindgut. In humans, the disease can be clinically classified into cutaneous and visceral leishmaniasis. Cutaneous leishmaniasis can be caused by species of subgenus *Viannia*, such as *L. shawi*, *L. guyanensis,* and *L. braziliensis* among many other species or by parasites of the subgenus *Leishmania* as is the case of *L. amazonensis*. *L. infantum,* the agent of zoonotic visceral leishmaniasis that causes canine leishmaniasis and affects children and immunosuppressed patients is included in the *Leishmania* subgenus.

Innate immunity plays a crucial role in the initial phase of *Leishmania* infection. During a blood meal, sand flies inject promastigote forms into the host dermis, generating an influx of leukocyte cells at the site of infection. Neutrophils are the first cells to be recruited, followed by a macrophage wave [2–4]. Parasite recognition and phagocytosis by neutrophils and macrophages can occur *via* two distinct

*Abbreviations:* CatG, Catepsin G; MΦ, Macrophages; NBT, Nitroblue tetrazolium; NE, Neutrophil elastase; NET, Neutrophil extracellular traps; NOD, Nucleotide-binding leucine-rich repeat-containing proteins like receptor; $O_2^-$, Superoxide anion; PAMP, Pathogens associated molecular patterns; PMA, Phorbol myristate acetate; PMN, Polymorphonuclear cells; PRR, Pattern recognition receptors; TLR, Toll-like receptor

* Corresponding author.
*E-mail address:* santosgomes@ihmt.unl.pt (G. Santos-Gomes).

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mechanisms that can be dependent or independent of opsonization. Parasite sensing can be mediated by direct recognition of pathogens associated molecular patterns (PAMP) *via* the pattern recognition receptors (PRR) [5] that include toll-like (TLR) and NOD (nucleotide-binding leucine-rich repeat-containing proteins)-like receptors. TLRs are transmembrane receptors that recognize antigens shared by different pathogenic agents and participate in the innate immune response. NOD-like receptors are cytosolic proteins able to detect pathogenic agents by recognizing intracellular ligands. Upon activation, these innate sensors recruit signaling molecules that drive the NF-κB-/AP-1-dependent expression of proinflammatory cytokines [6–8].

Phagocytosis culminates in the generation of an intra-cytoplasmic phagosome containing the parasite (parasitophorous vacuole). Neutrophil cytoplasmic primary granules, enriched in the serine protease neutrophil elastase (NE) and cathepsin G (CatG) fuse with the phagosome membrane and discharge granule cargo into the parasitophorous vacuole, leading to the subsequent activation of the enzyme NADPH (nicotinamide adenine dinucleotide phosphate-oxidase). The assembly of different subunits of NADPH oxidase and activation converts molecular oxygen into superoxide anion ($O_2^-$) [9] that can promote intracellular parasite killing. In addition, the content of primary granules released by exocytosis [10] can contribute to extracellular parasite elimination. Neutrophil intracellular and extracellular mechanisms can be independently activated or associated, ensuring microbial killing.

The generation of neutrophil extracellular traps (NETs) is another effector mechanism triggered by activated neutrophils aiming to contain and eliminate extracellular pathogens. NETs are mainly constituted by DNA fibers, histones and enzymes [11] and exhibit a significant potential microbicide that might negatively interfere with the phagocytosis of viable parasites. Macrophages (MΦ) are recognized as the definitive host cells for Leishmania, being able to phagocyte free parasites, apoptotic infected-neutrophils, or parasites that abandoned neutrophils; thus, either supporting parasite replication and dissemination or, in contrast, contributing to the control of the infection through the efficient activation of antileishmanial mechanisms.

Previous studies indicate that neutrophils can provide a transient safe shelter for *Leishmania* parasites prior to being uptake by macrophages where the parasite can replicate. Apoptotic neutrophils release the chemokine MΦ inflammatory protein (MIP-1β) recruiting MΦ that phagocyte apoptotic neutrophils with intracellular parasites. In this model of intracellular infection, parasites use neutrophils as "Trojan horses" to invade their definitive host cells, preventing the activation of its effector mechanisms [3,12,13]. Further studies showed viable *L. major* parasites being released from mouse apoptotic neutrophils in the vicinity of surrounding MØ [3], favoring parasite phagocytosis by MØ. This mechanism was called "Trojan rabbit" [14].

Furthermore, neutrophils contribute to the initiation of inflammation, a process which is recognized as essential in launching immunity. The importance of neutrophils as decision shapers on the development of immune response is recent since these cells have long been considered as short-lived and non-dividing cells of poorest interest. However, this view is changing and neutrophils currently appear not only as key components of the inflammatory response, but also as cells that display important immune regulatory roles in different microbial infections.

Thus, given the recognized importance of innate immune response, especially of neutrophils, the present study aims to investigate the activity of BALB/c mouse neutrophils against visceral and cutaneous species of *Leishmania*, exploring the relative importance of cell sensors, the activation of oxidative and non-oxidative pathways and the extracellular release of NETs. In parallel, a cell line of mouse MΦ was also used to compare parasite sensing.

## 2. Methods

### 2.1. Neutrophil primary cells and macrophage-like cell line

*Mus musculus* mice were used to isolate polymorphonuclear cells (PMN) according to Marques *et al* [15]. Animals were purchased from Institute Gulbenkian Ciência (Lisbon, Portugal) and maintained in the IHMT animal facility, in sterile cabinets with sterile food and water *ad libitum*. Animal care and handling were in conformity with the institutional guidelines and in compliance with EU (2010/63/EU) and national requirements (Law 113/2013). Mice were injected intraperitoneally with 0.8 ml of thioglycolate 10% (Sigma-Aldrich, Germany) to induce PMN migration. Animals were sacrificed by $CO_2$ inhalation and posterior cervical dislocation 18 h later. Cells were harvested by peritoneal lavage using 10 ml of cold phosphate buffered solution (PBS) 1×, pH 7.2 (Lonza, Germany). The cell suspension was washed with PBS at 300 × *g*, for 10 min at 4 °C, carefully loaded on a discontinue Hystopaque® gradient assembled with 2 ml of Hystopaque®-1077 (Sigma-Aldrich) layered onto 2 ml of Hystopaque®-1119 (Sigma-Aldrich) and then centrifuged at 340 ×*g* for 30 min, at room temperature. After centrifugation, the opaque ring in the interface of Hystopaque®-1077 and Hystopaque®-1119 enriched in PMN was collected and washed. Cells were resuspended in Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich) and viability and concentration were assessed by trypan blue exclusion in Neubauer-counting chamber (Marienfeld, Germany). Morphological evaluation of purity of neutrophil cell suspension was determined after cytocentrifugation on glass slides at 55 ×*g* for 4 min (StatSpin 2 Cytofuge ®, USA) followed by methanol (VWR International) fixation, treatment with fetal bovine serum (FBS, Sigma-Aldrich) and staining with Giemsa (Fisher Scientific). Slides were then analyzed by optical microscopy (Olympus BX51). The methodology used to isolate and purify murine neutrophils allowed obtaining 95–97% cell purity and a mean viable neutrophil (primed neutrophils) yield of $10^4$ cells.ml$^{-1}$.

Monocyte/MΦ mouse cell line (P388D1, ATCC, USA) was maintained in RPMI (Sigma-Aldrich) supplemented with 10% (v/v) of heat-inactivated FBS in a humid atmosphere at 37 °C with 5% $CO_2$. Every two days medium was replaced by a fresh one assuring a constant cell concentration of approximately 2 × $10^6$ cells.ml$^{-1}$.

### 2.2. Parasites

*L. amazonensis* (MHOM/BR/1973/M2269), *L. shawi* (MHOM/BR/96/M15789), *L. guyanensis* (M19663) and *L. infantum* (MHOM/PT/89/IMT151) promastigotes were maintained in Schneider medium (Sigma-Aldrich, Germany) supplemented with 10% (v/v) of heat-inactivated FCS, 100 U.ml$^{-1}$ penicillin and 100 µg.ml$^{-1}$ streptomycin (Biochrom, Germany) at 24 °C.

### 2.3. Exposure of neutrophils and macrophages to Leishmania promastigotes

*L. infantum*, *L. amazonensis*, *L. shawi,* and *L. guyanensis* promastigotes were incubated with 1 × $10^5$ neutrophils at a ratio of neutrophil-parasite of 1:5 in a final volume of 300 µl, at 37 °C in a humidified atmosphere containing 5% $CO_2$. MΦ were also incubated with *L. infantum*, *L. amazonensis*, *L. shawi* and *L. guyanensis* promastigotes at a ratio of 5 parasites per cell. Non-infected MΦ and primed neutrophils were used as controls.

Cells incubated with 0.2 µg.ml$^{-1}$ of phorbol myristate acetate (PMA, Sigma-Aldrich) were used as a positive control of gene expression, $O_2^-$ production and NET emission. Neutrophils incubated with 1 µg.ml$^{-1}$ of *Escherichia coli* lipopolysaccharide (LPS, Sigma-Aldrich) were used as a positive control of granule exocytosis.

**Table 1**
Forward (FW) and reverse (RV) primer sequences, base pair (bp) of amplified fragments and the annealing temperature of mouse target genes.

| Target genes | Sequence forward (FW) and reverse (RV) | Amplification product (bp) | Annealing temperature (°C) |
|---|---|---|---|
| ***NOD1*** | FW - 5′ TCAGCGTCAACCAGATCACC 3′<br>RV - 5′ AACCCAGGAACGTCACGATC 3′ | 84 | 54.5 |
| ***NOD2*** | FW - 5′ GTCAGCGCTCCTCAGGAAG 3′<br>RV - 5′ GGTACAGTTGGATGCCCTCT 3′ | 77 | 56 |
| ***TLR2*** | FW - 5′ AAC CTC AGA CAA AGC GTC AAA TC 3′<br>RV - 5′ ACC AAG ATC CAG AAG AGC CAA A 3′ | 65 | 64 |
| ***TLR4*** | FW - 5′CGC TTT CAC CTC TGC CTT CAC TAC AG 3′<br>RV - 5′ACA CTA CCA CAA TAA CCT TCC GGC TC 3′ | 109 | 61.5 |
| ***TLR9*** | FW - 5′ ACTTCGTCCACCTGTCCAAC 3′<br>RV - 5′ TCATGTGGCAAGAGAAGTGC 3′ | 93 | 53 |

Parasite internalization by neutrophils and MΦ was registered, and infection levels were assessed.

### 2.4. Parasite internalization by neutrophils and macrophages

Neutrophils and MΦ were incubated with promastigotes of *L. infantum*, *L. amazonensis, L. shawi,* and *L. guyanensis* for 3 h and 5 h, respectively. In the particular case of *L. shawi* and *L. guyanensis*, the time of incubation with MΦ was extended to 24 h to assure parasite internalization. Cell suspension (100 µl) was cytocentrifuged, and slides were stained with Giemsa. Parasite morphology and internalization were observed under an optical microscope, and the level of infection was estimated by counting infected cells *per* a total of 100 cells.

### 2.5. NOD and TLR gene expression

To indirectly evaluate parasite effect on innate cell sensors, gene expression of NOD1, NOD2, TLR2, TLR4, and TLR9 were quantified by real-time PCR. Primer sequences for *TLR2* [16] and *TLR4* [17] were previously described. *NOD1*, *NOD2*, and *TLR9* primers (Table 1) were designed using Primer-Blast and produced by STAB VIDA (Portugal). The conditions of amplification were optimized by conventional polymerase chain reaction (PCR), ensuring that the amplification of the *Leishmania* genetic material did not occur.

Total RNA was extracted from neutrophils and MΦ exposed to *L. infantum*, *L. amazonensis, L. shawi,* and *L. guyanensis,* non-activated neutrophils, unprimed MΦ, PMA-activated neutrophils, and PMA-stimulated MΦ, using the RNeasy Mini kit (Qiagen, Germany) and following the manufacturer's instructions. RNA integrity was checked by running in 1% agarose gel, and RNA purity was determined by optical density (OD) at 230 nm, 260 nm and 280 nm in a spectrophotometer (NanoDrop 100). Was only used RNA samples presenting a 260/280 ratio of 1.9–2.1.

After RNA extraction, synthesis of cDNA was done using the kit CDNA NZYTech (NZYTech), according to manufacturer's instructions.

Quantitative real-time PCR was performed in the CFX Connect™ Real-Time PCR Detection System thermal cycler (Bio-Rad, USA) using SYBR® Green. Amplifications were carried out in a total volume of 20 µl containing: 2 µl of cDNA sample and 10 µl SYBR Green PCR Master Mix (Applied Biosystems), which included SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, dNTPs, optimized buffer and specific primers (20 $pmol.ml^{-1}$). Each PCR reaction was performed in duplicate wells, using the following conditions: 5 min at 95 °C for chain denaturation and 40 cycles with the following thermal profile: 30 s at annealing temperature (Ta) specific for each gene (Table 1), 30 s at 95 °C, hybridization, and extension for 10 s. A melting curve was also performed for assay quality control purposes, starting at an initial temperature of 50 °C and followed 90 cycles with 0.5 °C increments *per* cycle.

For each gene, the concentration of external cDNA standards obtained as previously described [18] was determined by OD measurement at 260 nm. Serial dilutions from the resulting clones were used as standard curves, each containing a known amount of input copy number [19]. Final results were expressed as the copy number of each sensor per 1000 cells. Efficiencies of amplification were greater than 90%.

### 2.6. Superoxide production by neutrophils

To assess the oxidative activity of neutrophils, production of $O_2^-$ was measured using colorimetric nitroblue tetrazolium (NBT) assay. Yellow-colored NBT absorbed by cells is reduced to water-insoluble blue formazan particles by intracellular $O_2^-$.

Hanks 0.4% NBT (Sigma-Aldrich) was added to neutrophils separately exposed to *L. infantum*, *L. amazonensis*, *L. shawi,* or *L. guyanensis* parasites for 3 h. After 90 min of incubation, cells were washed with warm PBS to remove extracellular NBT and solubilized with 100 µl of 10% (w/v) sodium dodecyl sulfate (SDS, Sigma-Aldrich) and 100 µl of 0.1 N HCl. Absorbance was measured at 550 nm using a microplate reader (Anthos 2010). Supernatants of non-activated neutrophils and PMA-stimulated neutrophils were also evaluated.

### 2.7. Neutrophilic elastase and cathepsin G exocytosis

To evaluate the activity of non-oxidative pathways exocytosis of NE and CatG was measured in neutrophil supernatants, using specific colorimetric substrates [15]. In a 96-well plate, 1 mM of NE substrate *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-pNA (Sigma-Aldrich) or 2.5 mM of CatG substrate *N*-Succinyl-Ala-Ala-Pro-Phe-pNA (Sigma-Aldrich) diluted in 100 µl of 25 mM of Tris-HCl pH 7.5 was added to 100 µl of cell supernatants. Absorbance was immediately read (~0 min) at 405 nm in a fluorometer and then after 15 min and 30 min of incubation at 37 °C with 5% $CO_2$. Absorbance was also evaluated in supernatants of non-activated neutrophils and LPS-stimulated neutrophils. It was assumed that the intensity of the color was proportional to the activity of the enzyme under study.

### 2.8. Extracellular structures

To evaluate the release of extracellular structures, neutrophils exposed to *L. infantum*, *L. amazonensis*, *L. shawi,* or *L. guyanensis* parasites were observed by scanning electron microscopy (SEM). In parallel, PMA-stimulated neutrophils were also observed.

Sample preparation for SEM was performed according to the protocol established by Brinkmann et al. [20]. Neutrophils ($2 \times 10^5$ cells) were seeded on sterile 22 mm round glass coverslips placed in a 24-well plate and, *L. infantum*, *L. amazonensis*, *L. shawi,* or *L. guyanensis* promastigotes were added at a ratio of 5 promastigotes/cell. The plate was then incubated for 90 min at 37 °C in a humidified atmosphere with 5% $CO_2$.

After the incubation period, the coverslips were fixed with 4% paraformaldehyde for 8 h and then post-fixed for 30 min with a solution of 2.5% glutaraldehyde (Sigma-Aldrich).

After washing, the coverslips were incubated with 0.5% osmium tetroxide (Sigma-Aldrich) for 30 min and further incubated with 1% tannic acid (Sigma-Aldrich). Then, cells were dehydrated by successive passages in a graded ethanol series (30%, 50%, 70%, 80% and 90%) and stored at 4 °C with 100% ethanol before the critical point drying and metallization with gold palladium. Cells were observed under a scanning electron microscope (JEOL 5200-LV, Japan) and images were acquired and used for the identification of NETs, and to estimate the number of neutrophils showing extracellular activity. To determine the NET frequency, neutrophil emitting NETs were register and data was normalized to 100 neutrophils.

### 2.9. Statistical analysis

Non-parametric Wilcoxon test was used to compare variables of two dependent samples in relation to all studied parameters. Differences were considered significant with a 5% significance level ($p < 0.05$). Statistical analysis was performed with the SPSS Statistics version 16.0 (IBM, USA), using values of three independent experiments and three replicates *per* sample.

## 3. Results

### 3.1. Murine neutrophils uptake Leishmania promastigotes and tolerate amastigote-like differentiation

Promastigotes of *L. infantum* (Fig. 1A), *L. amazonensis* (Fig. 1B), *L. shawi* (Fig. 1C) and *L. guyanensis* (Fig. 1D) were internalized by murine neutrophils, evidencing intracellular differentiation into amastigote-like parasites. After 3 h of exposure, *L. guyanensis* and *L. shawi* were responsible for the largest number of infected neutrophils (~11%) followed by *L. infantum* (~5%). However, in the case of *L. amazonensis* only 3% of neutrophils were parasitized, pointing towards a low phagocytic activity (Fig. 1). When compared with the visceralizing *L. infantum* species, the percentage of *L. shawi* or *L. guyanensis* infected neutrophils were significantly different ($p = 0.0313$).

After 5 h of exposure, the maximum of infected MΦ was obtained by *L. amazonensis* followed by *L. infantum,* being these results significantly different ($p = 0.0078$, Fig. 2A). *L. guyanensis* and *L. shawi* infected MΦ were only observed after 24 h of incubation (Fig. 2B). The lower level of infection of these cutaneous species associated with the higher time needed to be internalized can be related to the recognition process of MΦ receptors.

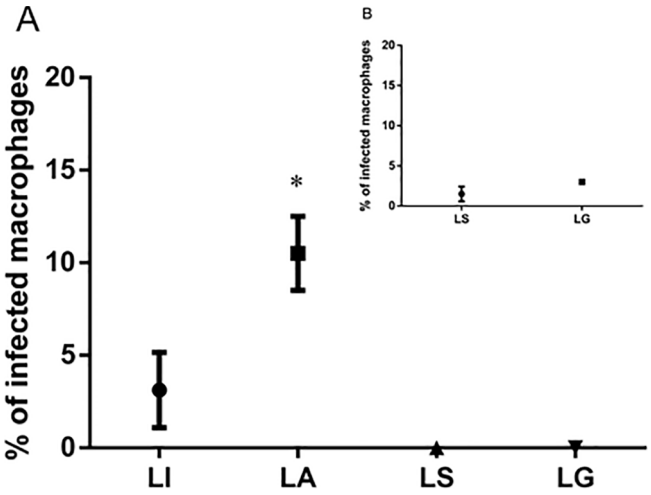


**Fig. 2.** Macrophages infected by *Leishmania* spp. MΦ were incubated with *L. infantum* (LI), *L. amazonensis* (LA), *L. shawi* (LS) and *L. guyanensis* (LG) (5 parasites per cell) for 5 h (A) and also for 24 h (B) in the case of *L. shawi* (LS) and *L. guyanensis* (LG). The frequency of infected MΦ was estimated by optical microscopy. Results are expressed by the median, maximum and minimum values of at least three independent experiments and of three replicates per sample. * ($p < 0.05$) denotes statistical differences when comparing *L. infantum vs* the cutaneous species.

### 3.2. Neutrophil innate sensors are not disturbed by Leishmania parasites

Gene expression of cell receptors NOD1, NOD2, TLR2, TLR4, and TLR9 was quantified by real time-PCR in MΦ and neutrophils exposed to *L. infantum*, *L. amazonensis*, *L. shawi,* and *L. guyanensis*.

Neutrophils exposed to parasites exhibited mRNA accumulation similar to primed neutrophils (negative control, Supplementary Table). Nevertheless, when PMA was added to neutrophils a significant upregulation of TLR2 was observed (6 017 copies per 1000 cells) when compared with primed neutrophils ($p = 0.0156$), indicating that cells were viable and responsive, able to generate mRNA.

When compared with non-infected MΦ, *L. infantum*-infected MΦ exhibited significant upregulation of TLR2 ($p = 0.0313$, Fig. 3C) and TLR9 ($p = 0.0469$, Fig. 3D) gene expression. Higher accumulation of NOD1 (Fig. 3A) and TLR9 (Fig. 3D) mRNA was found in *L. amazonensis*-infected MΦ ($p = 0.0313$). On the other hand, *L. shawi* induced a significant down-regulation of NOD2 gene expression ($p = 0.0313$, Fig. 3B) and *L. guyanensis* did not cause a considerable effect on gene

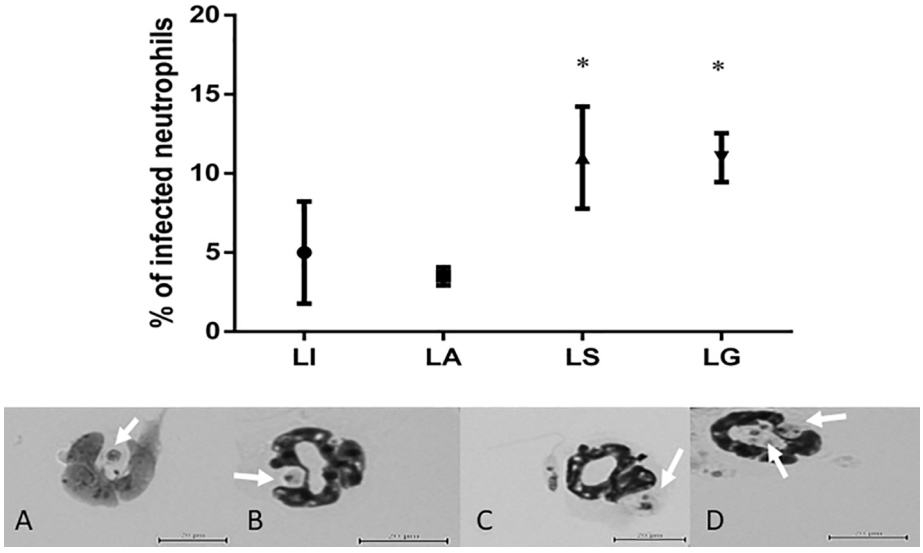


**Fig. 1.** Neutrophils internalize *Leishmania* spp. BALB/c mouse neutrophils were incubated with *L. infantum* (LI), *L. amazonensis* (LA), *L. shawi* (LS) or *L. guyanensis* (LG) parasites (5 parasites *per* cell) for 3 h. The amount (%) of infected neutrophils was estimated by optical microscopy and images (× 1000 magnification) were acquired. Results are expressed by the median, maximum and minimum values of at least three independent experiments and three replicates *per* sample. * ($p < 0.05$) denotes statistical differences when comparing *L. infantum vs* the cutaneous species. Intracellular amastigotes (arrows) of *L. infantum* (A), *L. amazonensis* (B), *L. shawi* (C) and *L. guyanensis* (D) can be observed.

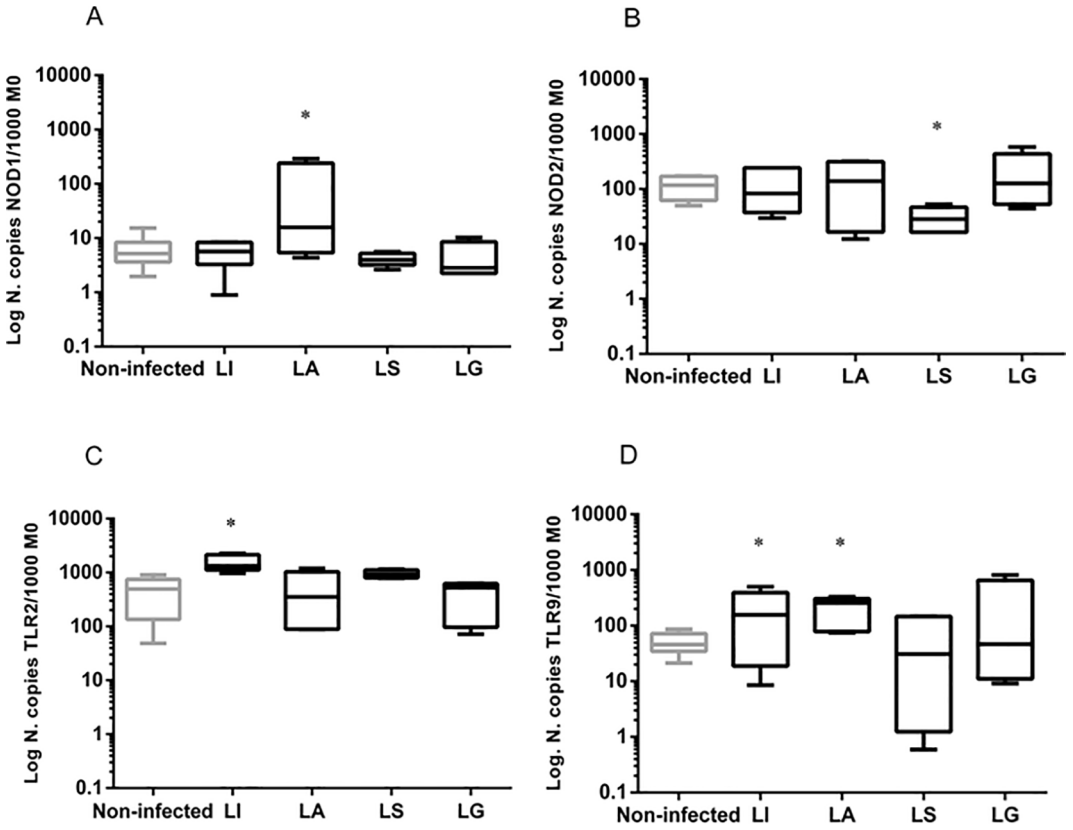


**Fig. 3.** Gene expression of NOD1, NOD2, TLR2, TLR4, and TLR9 by MΦs infected with cutaneous and visceral species of *Leishmania*. After 5 h of infection with *L. infantum* (LI), *L. amazonensis* (LA), *L. shawi* (LS) and *L. guyanensis* (LG), RNA was extracted and the number of copies per 1000 MΦ of *NOD1* (A), *NOD2* (B), *TLR2* (C) and *TLR9* (D) were determined by real-time PCR. In parallel, gene expression of innate receptors of non-infected MΦs was also evaluated. Results are expressed as median and maximum from at least three independent experiments and three replicates per sample. * ($p < 0.05$) denotes statistical differences between non-infected MΦs and infected-MΦs.

expression of MΦ sensors. Furthermore, parasites did not induce major changes in TLR4 gene expression.

### 3.3. Neutrophils produce superoxide when activated by Leishmania parasites

Production of $O_2^-$ by neutrophils was evaluated in the presence of PMA and after 3 h of exposure to *L. infantum, L. amazonensis, L. shawi,* and *L. guyanensis*.

PMA-stimulated neutrophils (positive control) significantly increased $O_2^-$ production when compared with primed-neutrophils (negative control) ($p = 0.0020$), indicating that the cells were viable and responsive. Significant high levels of $O_2^-$ were also detected in neutrophils activated by *L. infantum* ($p = 0.0098$), *L. amazonensis* ($p = 0.0039$), *L. shawi* ($p = 0.0049$), and *L. guyanensis* ($p = 0.0020$) compared with primed-neutrophils. Of the *Leishmania* species analyzed, the higher production of $O_2^-$ was induced by *L. shawi* ($p = 0.0195$) and by *L. amazonensis* ($p = 0.0068$) when compared with *L. infantum* (Fig. 4). Therefore neutrophils generate reactive oxygen species (ROS) against visceral and cutaneous species of *Leishmania*.

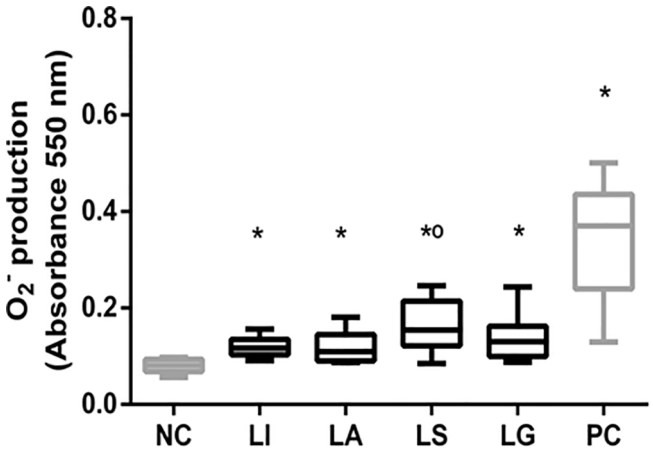


**Fig. 4.** Superoxide production by neutrophils exposed to different species of *Leishmania.* Primed neutrophils (negative control, NC), neutrophils exposed to *L. infantum* (LI), *L. amazonensis* (LA), *L. shawi* (LS) or *L. guyanensis* (LG) and PMA-stimulated neutrophils (positive control, PC) were incubated with NBT. Results are presented by median, and minimum and maximum values of three independent experiments and three replicates per sample. * and ° ($p < 0.05$) indicate significant differences when compared NC or LI, respectively *vs* the other conditions.

### 3.4. Neutrophils release granule content when activated by Leishmania parasites

The activity of NE and CatG was quantified in supernatants of neutrophils exposed to *L. infantum*, *L. amazonensis*, *L. shawi,* or *L. guyanensis* immediately after the addition of colorimetric substrates specific for each enzyme (~0 min) and, after 15 min and 30 min of incubation.

LPS-stimulated neutrophils (positive control) presented high enzymatic activity ($p_{NE\ 0\ min,15\ min,\ 30\ min}$, $p_{CatG\ 15\ min} = 0.0156$, $p_{CatG\ 0\ min,\ 30\ min} = 0.0313$) when compared with primed neutrophils (negative control), indicating that cells were able to exocytosis granules rich in NE (Fig. 5A) and CatG (Fig. 5B).

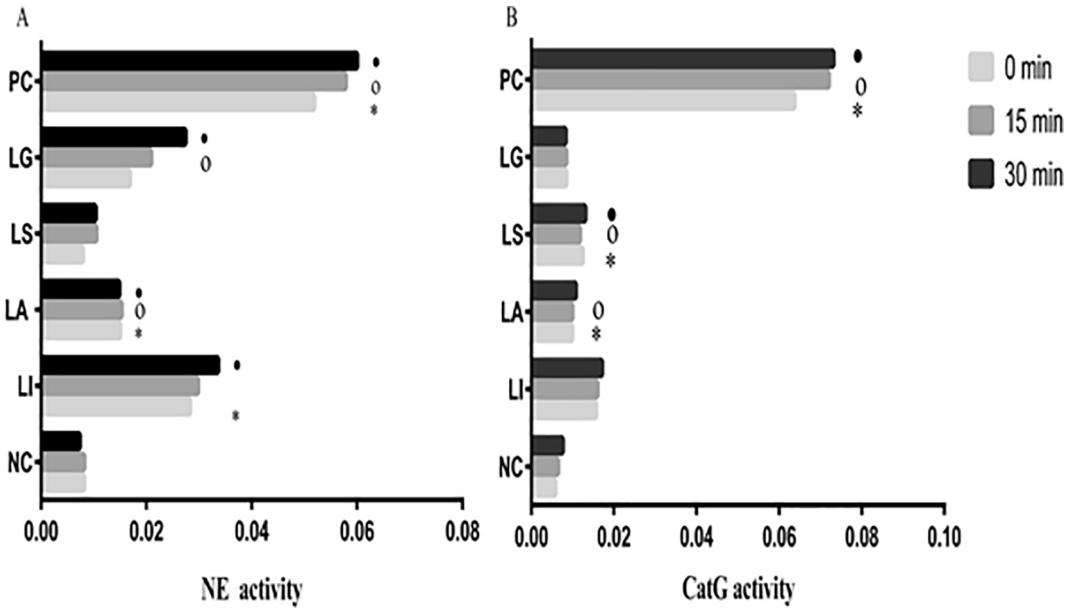


**Fig. 5.** Granule exocytosis by neutrophils exposed to different species of *Leishmania*. Enzymatic activity of neutrophil elastase (NE) (A) and cathepsin G (CatG) (B) from primed neutrophils (negative control, NC), neutrophils exposed to *L. infantum* (LI), *L. amazonensis* (LA), *L. shawi* (LS) and *L. guyanensis* (LG), and LPS-stimulated neutrophils (positive control, PC) was evaluated through a colourimetric enzyme-substrate reaction at ~ 0 min, 15 min, and 30 min following the substrate addition. Results are expressed by means from at least three independent experiments and three replicates per sample. $^{*}$, $^{\circ}$ and $^{\cdot}$ ($p < 0.05$) indicate significant differences at 0 min, 5 min and 30 min when comparing NC *vs* the other conditions.

Neutrophils exposed to *L. infantum* ($p_{0\ min,\ 30\ min} = 0.0391$), *L. amazonensis* ($p_{0\ min\text{-}30\ min} = 0.0156$), or *L. guyanensis* ($p_{15\ min} = 0.0391$) showed significant increases of NE activity when compared with primed-neutrophils (Fig. 5A). Significant CatG activity was only observed in neutrophils exposed to *L. amazonensis* ($p_{0\ min} = 0.0078$, $p_{15\ min} = 0.0234$) and *L. shawi* ($p_{15\ min} = 0.078$, $p_{30\ min} = 0.0391$) (Fig. 5B).

Although all species of *Leishmania* included in the present study were able to stimulate murine neutrophils to exocytosis granules, *L. amazonensis* is the only species promoting both NE and CatG enzymatic activity. Exposure of neutrophils to *L. shawi* promastigotes only promoted CatG activity and, *L. infantum* and *L. guyanensis* induced NE activity. Thus, the extracellular activity of neutrophil enzymes seems to be dependent on parasite species.

### 3.5. Neutrophils emit NETs against Leishmania parasites

The extracellular activity of mouse neutrophils exposed to visceral and cutaneous species of *Leishmania* was evaluated by SEM (Fig. 6). *L. infantum* (Fig. 6A, B, and C), *L. amazonensis* (Fig. 6D and E), *L. shawi* (Fig. 6F), and *L. guyanensis* (Fig. 6G and H) promastigotes promoted the release of extracellular structures by murine neutrophils. These cells emitted fiber-, web- and tube-like structures similar to the extracellular activity exhibited by PMA-activated neutrophils (Fig. 6I), which is known as a NET inductor. Promastigotes of cutaneous and visceral species of *Leishmania* were often trapped by NETs. The close contact of the parasite with the extracellular fibers (Fig. 6C) emitted by neutrophils suggests a strong interaction that could stick and entraps the promastigotes and, progressively cause the loss of parasite viability. On the other hand, the tubular structures can represent pseudopods responsible for the coiling phagocytosis of *L. infantum* promastigotes (Fig. 6B).

*L. shawi* ($p = 0.0117$) and *L. guyanensis* ($p = 0.0195$) induced neutrophils to exhibit higher extracellular activity than PMA-stimulate neutrophils. Although not different of PMA-stimulate neutrophils, *L. amazonensis* is the parasite that least promoted the release of extracellular structures when compared with *L. shawi* ($p = 0.0210$) and *L. guyanensis* ($p = 0.0425$) (Fig. 7).

Despite the differences in NET intensity, mouse neutrophils emit NETs against cutaneous and visceral species of *Leishmania*, probably controlling parasite uptake and restraining the viability of extracellular parasites.

## 4. Discussion

*Leishmania* parasites are introduced into the vertebrate skin and then taken up by phagocytes where can persist and replicate, being dispersed to other host cells, tissues and organs, according to the species of *Leishmania* and the immune background of mammal hosts. To defend against parasite invasion, cells of mammal innate immunity must be recruited to the site of promastigote inoculation, recognize, phagocyte, kill the parasite or, ultimately induce the functional activation of acquired immunity, preventing parasite replication, migration to other cells and spread over the reticuloendothelial system. A recent study of *L. donovani* transmission to mice by sand fly bites [21] showed that the interaction of sand fly saliva and gut microbiota with mouse tissues have a crucial role in promoting neutrophil infiltration by inducing a fast release of interleukin-1β. Thus, neutrophils that are the first immune cells to encounter the parasite at the infection site play a key role in directing the infection outcome by inactivating, transiently hosting or delivering the parasite to the definitive host cell [3,12–14]. Fully understanding the processes used by *Leishmania* parasites to induce neutrophil activity at the initial infection steps is crucial to the design and validation of prophylactic approaches that could lead to leishmaniasis reduction or even elimination. Hence, this in vitro study examined neutrophil activation drove by visceralizing *Leishmania* parasites (*L. infantum*) and by American cutaneous species of *Leishmania* (*L. amazonensis*, *L. shawi*, and *L. guyanensis*).

The present study confirms that murine neutrophils internalize *Leishmania* parasites that originates cutaneous and visceral leishmaniasis as already described in previous studies [2,3,5,22]. However, it seems that *Leishmania* spp. belonging to *Viannia* subgenus were more efficiently taken up by mouse neutrophils. In comparison, MΦ also internalize the parasites, but in this case, the parasites of *Leishmania*

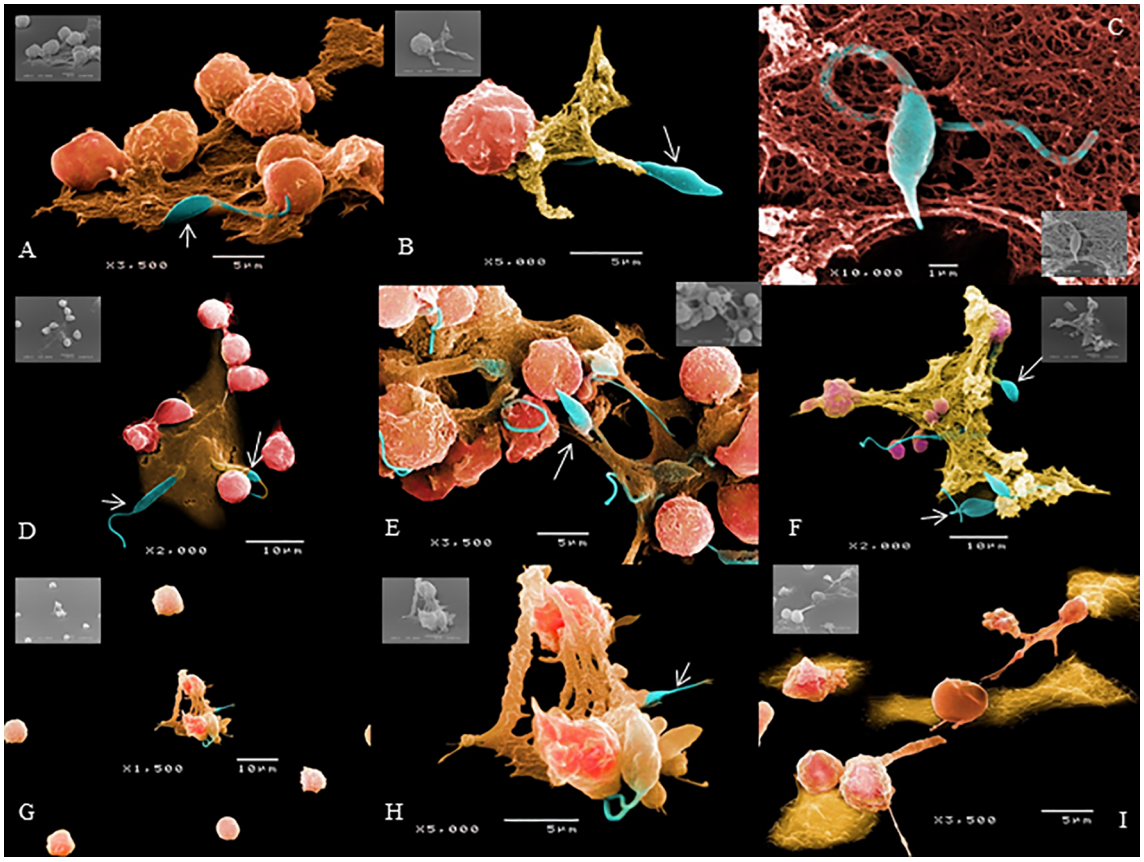


**Fig. 6.** The extracellular activity of neutrophils exposed to *Leishmania* spp. Neutrophils incubated with *L. infantum* (A, B, and C), *L. amazonensis* (D and E), *L. shawi* (F) and *L. guyanensis* (G and H) were observed by scanning electron microscopy and images were acquired. PMA-stimulated neutrophils were also observed (I). Promastigotes captured by netting-like structures emitted by a neutrophil cluster (A, E, F), promastigote trapped by a tube-like structure (B), detail of a promastigote stuck into a fiber network (C), promastigotes trapped in web-like structures (D), neutrophils surrounding activated cells releasing tubular-like structures (G) and a detail of trapped promastigotes (H) are shown. PMA-activated neutrophils emitting web-, fiber- and tube-like structures (I) can be also observed. Images were artificially colored using the GIMP2.10.0. Promastigotes are in light blue and indicated by arrows.

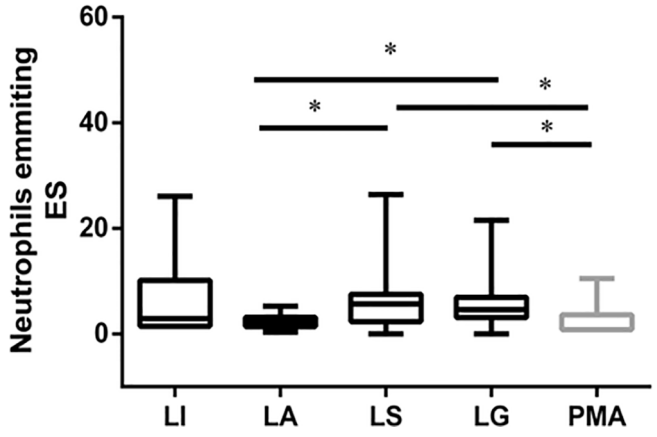


**Fig. 7.** The extracellular activity of neutrophils exposed to *Leishmania* spp. Neutrophils incubated with *L. infantum* (LI), *L. amazonensis* (LA), *L. shawi* (LS) and *L. guyanensis* (LG) were observed by scanning electron microscopy and images were used to estimate the frequency of neutrophils that emitted extracellular structures (ES). In parallel, PMA-stimulated neutrophils (PMA) releasing extracellular structures were also estimated. Results are expressed by the median, maximum and minimum values of at least three independent experiments. * ($p < 0.05$) denotes statistical differences.

subgenus easily and faster infect these cells than the parasites of *Viannia* subgenus. These findings suggest that the cells used in this study, BALB/c mouse neutrophils and MΦ-like mouse cells recognize and engage the parasites differently, possibly pointing towards different host invasion strategies.

Rapidly and in large numbers, neutrophils are the first cells recruited to infection site [2–4,22–24], being the innate cells that primary sense the parasite through PRR. Phagocytes recognize pathogen molecules by innate receptors in the cytoplasm or associated with membranes. In the present study, visceral and cutaneous species of *Leishmania* do not disturb the gene expression of the highly conserved TLR2, TLR4, TLR9, NOD1 and NOD2 of BALB/c mouse neutrophils, at least during an initial short time (3 h) of parasite exposure. These findings indicate that extracellular and intracellular parasites do not signaling through sensors, avoiding the early activation of PRR dependent pathways or that a longer contact parasite-neutrophil is needed to promote receptor activation. Nevertheless, parasites seem to be sensed by MΦ through different innate receptors. TLR9 is associated with endocytic compartment membranes and recognize unmethylated cytosine-phosphate-guanine (CpG) dinucleotide sequences, which are relatively common in pathogen DNA. In the present study, the upregulation of MΦ-TLR9 point towards the recognition of CpG from internalized parasites of both *Leishmania* subgenus. Activation of TLR9 downstream pathway leads to increased levels of co-stimulatory molecules that can initiate the expansion of the T cell population and the differentiation of B cells, potentially endorsing the development of an acquired immune response. Furthermore, it is possible that the visceral species *L. infantum* engage TLR2, a transmembrane receptor expressed on the cell surface associated with chemotaxis and phagocytosis, probably assuring its dispersion throughout the host. Glycosylphosphatidylinositol (GPI) and lipophosphoglycan (LPG) present in *Leishmania* have been identified as

TLR2 ligands. The cutaneous species *L. amazonensis* also seem to be signaling through NOD1, an intracellular receptor that senses pathogenic microorganisms living and replicating inside the host cell. Once activated, this sensor triggers intracellular pathways that could lead to the expression of inflammatory genes. The two species *L. infantum* and *L. amazonensis* appear to have ligands recognized by two PRR of different localization, unveiling the complexity of parasite-host interaction. On the other hand, *L. guyanensis* does not seem to signal through the sensors evaluated in the present study, and *L. shawi* downregulates NOD2 gene expression, perhaps preventing ROS production. Furthermore, the steady TLR4 mRNA level reinforces previous reports indicating that this sensor seems to be indifferent or is not induced by *Leishmania* parasites [25–27]. TLR4 signaling is required for efficient iNOS activation [28,29] and nitric oxide (NO) production, leading to parasite death. The non-activation of TLR4 downstream pathway drives to urea formation and reduces NO production [30,31].

Neutrophils have a considerable antimicrobial arsenal able to contain the pathogen dispersion in the intracellular and extracellular space, which includes respiratory burst, granule exocytosis, and NET emission.

Neutrophil oxidative burst is induced by all the species of *Leishmania*, promoting their own intracellular destruction. *L. shawi* promotes the highest $O_2^-$ production levels.

*Leishmania* parasites cause neutrophil degranulation, releasing enzymes for the extracellular space that could compromise the integrity of parasite membrane, promoting parasite death. However, the enzymatic activity seems to differ with the parasite species. CatG evidence low activity when neutrophils are exposed to *L. infantum* and *L. guyanensis* parasites and NE enzymatic activity seem to be unchanged when facing *L. shawi*. Inhibitors of serine peptidases were identified in *L. major* and *L. donovani* [32,33] and can play a role in parasite survival by regulating host serine proteases. Thus, it is possible that *L. infantum, L. guyanensis* and *L. shawi* also produce inhibitors of serine proteases directed to the specific inactivation of CatG and NE. Furthermore, when exposed to *L. amazonensis* NE and CatG exhibit high enzymatic activity, suggesting that this parasite, in particular, is unable to promote enzyme inactivation, being more susceptible to neutrophil activity.

Upon contact with pathogens or under the influence of inflammatory stimuli, neutrophil internal organization changes, generating the extracellular release of NET. The space covered by the NET web-like structures usually is bigger than the neutrophil volume, and its constitution by nuclear DNA, histones, and antimicrobial proteins confers the advantage of trapping and kill microorganisms. In the present study, extracellular structures emitted by neutrophils exposed to *Leishmania* parasites are consistent with the NET release. *L. amazonensis* appears to minimize NET emission while the species of *Viannia* subgenus caused an accentuated NET release. Comparatively with parasites of subgenus *Viannia* investigated in the present study, the subgenus *Leishmania* seems to be better tolerated by neutrophils, favoring parasite phagocytosis by the host cell, and further dispersion of *L. infantum* to internal organs and development of localized skin lesions by *L. amazonensis* [34]. Despite the scarce available information on *L. guyanensis* and *L. shawi* infectivity and host immune response, the present findings underline the possible influence of neutrophils in reducing the parasite availability at the early phase of infection, hampering disease evolution.

Coiling phagocytosis, described as a projection of a unilateral pseudopod able to enwrapping live and death pathogens, seems to be an active mechanism used by phagocyte cells for the uptake of pathogenic spirochetes [35–38]. However, there are only sparse references on trypanosomatid coiling phagocytosis [39–41]. The present study also gives evidence of *in vitro* coiling phagocytosis of *L. infantum* promastigotes by mouse neutrophils, suggesting that this unconventional mechanism could be involved in parasite internalization. Further studies are needed to understand how this process is induced and its importance for parasite survival or by the contrary to ensure early *Leishmania* inactivation by neutrophils.

## 5. Conclusions

The development of visceral or cutaneous diseases depends on a complex interplay established by parasite species with the different components of the host immune system. *Leishmania* parasites, the sand fly derived molecules, and the microbiota transmitted to the host together with the individual biological characteristics of the host are key factors that can influence the immune response by favoring infection establishment and directing disease development. A proper immune response at the early phase of infection, shortly after promastigote inoculation in the dermis by the sand fly, can determinate the disease outcome. The innate immune response and in particular neutrophils appear to have a non-negligible role in parasite infection. Despite the regular high effectiveness of neutrophil processes in fighting pathogens, it seems that the success of neutrophil leishmanicidal mechanisms is dependent on parasite species. These findings indicate that neutrophil activity, which is an innate immune response crucial for pathogen inactivation, can be counteracted by *Leishmania* parasites.

## Conflict of interest

The authors declare no conflict of interest. The sponsors that have found the study had no intervention in the design of the study, in sample collection, in data interpretation or in manuscript writing.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.11.003.

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