Dynamic imaging reveals surface exposure of virulent *Leishmania* amastigotes during pyroptosis of infected macrophages

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ABSTRACT

*Leishmania* spp. are obligate intracellular parasites that infect phagocytes, notably macrophages. No information is available on how *Leishmania* parasites respond to pyroptosis of their host cell, which is known to limit microbial infection. Here, we analyzed the pyroptotic process and the fate of intracellular amastigotes at the single-cell level using high-content real-time imaging. Bone marrow–derived macrophages were infected with virulent *Leishmania amazonensis* amastigotes and subsequently treated with lipopolysaccharide and ATP to induce pyroptosis. Real-time monitoring identified distinct pyroptotic phases, including rapid decay of the parasitophorous vacuole (PV), progressive cell death and translocation of the luminal PV membrane to the cell surface in 40% of macrophages, resulting in the extracellular exposure of amastigotes that remained anchored to PV membranes. Electron microscopy analyses revealed an exclusive polarized orientation of parasites, with the anterior pole exposed toward the extracellular milieu, and the parasite posterior pole attached to the PV membrane. Exposed parasites retained their full infectivity towards naïve macrophages suggesting that host cell pyroptosis may contribute to parasite dissemination.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: *Leishmania*, Macrophage, Pyroptosis, High-content imaging, Single-cell imaging, Real-time imaging, Virulence

INTRODUCTION

*Leishmania amazonensis* is a protozoan parasite, causing localized cutaneous and anergic diffuse cutaneous leishmaniasis in South America (Barral et al., 1991; Silveira et al., 2004). Restriction of intracellular *L. amazonensis* replication *in vitro* and *in vivo* has been shown to depend on the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome (Zamboni and Sacks, 2019). This intracellular sensor is induced in response to a ‘priming signal’ represented by cytokines or ligands of Toll-like receptors, and is further activated by damage-associated molecular patterns (DAMPs), such as ATP. Inflammasome activation triggers caspase-1 activity, which cleaves pro-IL-1β and pro-IL-18 into mature cytokines – promoting an anti-microbial, pro-inflammatory immune response (Swanson et al., 2019) – and cleaves gasdermin D, which forms pores inside the macrophage plasma membrane, sustaining IL-1β release (Lieberman et al., 2019) and leading to pyroptosis (Bergsbaken et al., 2009; Fink and Cookson, 2006; Jorgensen et al., 2017; Shi et al., 2015). Importantly, pyroptotic cell death contributes to a protective response against intracellular pathogens by removing the niche for infection (Bergsbaken et al., 2009), by exposing microbes to the immune system (Jorgensen and Miao, 2015) and by rendering pathogens more susceptible to anti-microbial agents (Jorgensen et al., 2016) or direct microbial killing (Liu et al., 2016).

Even though the interaction of *L. amazonensis* with the NLRP3 inflammasome has attracted considerable attention (Lecoeur et al., 2020; Lima-Junior et al., 2013), how pyroptosis affects parasite survival and virulence remains to be elucidated. Here, we deployed a real-time, high-content single-cell analysis that uncovers unique features of *Leishmania* amastigotes during host macrophage pyroptosis.

RESULTS AND DISCUSSION

A high-content imaging protocol for dynamic single-cell analysis of pyroptotic macrophages infected by *L. amazonensis* amastigotes

To analyze the dynamics of pyroptosis in *L. amazonensis*-infected macrophages, we established a new imaging protocol combining high-content and single-cell analyses using the OPERA™ QEH5 confocal plate reader (Fig. 1A). Bone marrow–derived macrophages (BMDMs) were seeded into 96-well plates (macrophage settlement). They were subsequently infected for 3 days with mCherry transgenic virulent amastigotes isolated from lesions of infected Nude mice, and allowed to form characteristic communal parasitophorous vacuoles (PVs; parasite settlement). Then, lipopolysaccharide (LPS) was added for 225 min (NLRP3 priming) followed by staining with fluorescent reporters. Pyroptosis was triggered after NLRP3 activation by treatment with ATP (pyroptosis induction). Dynamic cellular changes were monitored at high-content and single-cell levels by using Hoechst 33342 (to determine cell number; Fig. 1B1), YO-PRO-1 [to determine loss of plasma membrane (PM) integrity, Fig. 1B2] (Adamczak et al., 2014), LysoTracker green (LTG, to determine PV acidity, Fig. 1B3) (Aulner et al., 2013) and mCherry (to determine parasite localization, Fig. 1B4) (Aulner et al., 2013). Macrophage morphology was analyzed by transmission light microscopy (TL, Fig. 1B5). High-content assay (HCA) analyses were carried out in real-time up to 240 min post ATP addition. Analyses at the population level were performed using segmentation procedures (Fig. S1A,B). The analysis of fluorescent read-outs at 240 min documented that (1) no macrophages were lost during the analyses (Fig. 1C1), (2) pyroptosis induction was successful (Fig. 1C2), and the PV integrity was lost in all pyroptotic cells (Fig. 1C3). The induction of pyroptosis after LPS and ATP treatment in *L. amazonensis*-infected macrophages was validated by monitoring...
cell morphology (Fig. S2A), caspase-1 cleavage and release (Fig. S2B), and IL-1β secretion (Fig. S2C). Single-cell analyses permitted to determine macrophage PV area, and parasite location (Fig. 1D).

Rapid decay of PV precedes amastigote extracellular exposure during macrophage pyroptosis

Pyroptosis dynamics, PV integrity and parasite localization were analyzed every 5 min after ATP addition for a duration of 240 min in infected LPS-primed macrophages. HCA analysis of YO-PRO-1 incorporation revealed that pyroptosis was asynchronous, as judged by the progressive increase in the number of dead cells (Fig. 2A, black curve). Pyroptosis occurred with a constant rate (+18.2±4.2% decrease of LTG fluorescence intensity, (2) an intermediate rate (45–90 min, stage 1) leading to a 47.6% decrease of LTG fluorescence intensity, (2) an intermediate rate (45–90 min, stage 1) leading to a 47.6% decrease of LTG fluorescence intensity, (3) a slow rate (90–120 min, stage 2), and (4) a slow rate (120–240 min, stage 3). This decay may result from ATP-induced changes in the vacuolar pH and of the PV integrity that could be caused by osmotic changes similar to those described for lysosomes (Guha et al., 2013; Takenouchi et al., 2009). Single-cell analyses revealed differences in the PV decay kinetics between macrophages (Fig. S3A–D) as well as between PVs inside a same macrophage (Fig. 2B1–B3). Monitoring individual PVs revealed progressive reduction of PV area and loss of about half of the PVs at the final time point of 240 min (Fig. S3B). Importantly, the decrease of PV area is more pronounced in large PVs (area of more than 2000 square pixels) than in smaller ones (area less than 1000 square pixels) (Fig. S3C).

HCA analysis revealed that amastigotes either remained intracellular (62% of macrophages) or were exposed at the cell surface (38% of macrophages), as shown in representative cells (Fig. S2A,B; Fig. S3A). Importantly, different decay rates and parasite localizations were observed for individual PVs, even inside the same macrophage as indicated in Fig. 2B2, showing three independent vacuoles (a, b, and c) that rapidly lost LTG fluorescence (stage 1). PVs a and b displayed a moderate decrease in size and retained intracellular amastigotes (Fig. 2B2,B3), whereas PV c collapsed (at 60 min) and the luminal side of its membrane was exposed to the extracellular milieu (at 120 min, stage 2). This PV lumen exposure could be triggered by ATP, as judged by previous reports implicating ATP in the

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**Fig. 1. Description of assay and readouts to monitor L. amazonensis-infected BMDMs undergoing pyroptosis.** (A) Experimental flow chart. Mouse BMDMs (Mφ) were differentiated from bone marrow progenitors in the presence of rm-CSF1 and seeded in 96-well plates. After 5 h, mCherry lesion-derived amastigotes of L. amazonensis (L. am) were added to BMDMs (multiplicity of infection (MOI)=4:1, 72 h, 34°C). NLRP3 was primed by adding 500 ng/ml LPS for 3 h 45 min. Then fluorescent reporters Hoechst 33342 (Ho 33342), LysoTracker Green DND-26 (LTG) and YO-PRO-1 were added. Finally, pyroptosis was triggered by NLRP3 activation with 5 mM ATP. Real-time analyses were performed using the OPERA™ plate reader at 34°C, 7.5% CO2 for 240 min. (B) HCA read-outs. Image acquisition was performed every 5 min. A representative field image is displayed for each channel [time=0 (T0) for Ho 33342, LTG, mCherry and transmitted light (TL); T=120 min (T120) for YO-PRO-1 staining]. Macrophage nuclei were stained with Ho 33342 (blue channel, panel B1). Nuclei of pyroptotic macrophages were stained with YO-PRO-1 (green channel, panel B2). PV integrity and acidity were evaluated by the LTG staining (green channel, panel B3). Parasite location was determined by the mCherry fluorescence signal (red channel, panel B4) in relation to the macrophage body (TL, panel B5). Macrophage area was evaluated on TL pictures. Scale bars: 20 µm. (C) HCA analysis quality controls. HCA analyses were performed at the population level analyzing 1000 cells per well. Three quality controls were performed by: (1) monitoring macrophage numbers to control that no cells were lost during the analysis, (2) analyzing the percentage of dead macrophages (YO-PRO-1 positive) 4 h post ATP addition to control for efficient pyroptosis induction (at least 70% of death must be observed at this time point), and (3) assessing LTG fluorescence at T0 and T240 to monitor PV presence (T0) and pyroptosis associated with the complete loss of PV staining (T240). Results in C2 are mean±s.e.m. (one representative experiment of three experiments). For C3, the box represents the 25–75th percentiles, and the median is indicated. The whiskers show the 5th and 95th percentiles, and outliers are indicated (one representative experiment of three experiments). (D) Single-cell analyses. Merged pictures (Ho 33342, LTG, mCherry and TL images) of representative infected macrophages (T0). Identification and quantification of areas of PVs a, b and c, and parasite location. The unit of PV area is square pixels.
extracellular discharge of secretory exosomes (Andrei et al., 2004; Bergsbaken et al., 2011), in exosome exocytosis (Qu et al., 2007), or in the release of auto-phagolysosomes and phagocytosed intracellular particles (Bergsbaken et al., 2011; Takenouchi et al., 2009). Amastigote exposure occurred preferentially in macrophages displaying a rapid PV decay (Fig. 2; Fig. S3). Additionally, parasite exposure does not correlate with PV size, PV number or amastigote number per vacuole (data not shown). In contrast, PVs that display a high area value and that rapidly expose parasites – such as PV c (Fig. 2B2) – decayed faster than other PVs (Fig. S3C,D).
Ultrastructural analysis of amastigote attachment zone and surface exposure

Pyroptosis has been recognized as an anti-microbial strategy during bacterial infection that is capable of trapping pathogens inside dying host cells (Jorgensen et al., 2016) and directly killing bacteria (Liu et al., 2016). Our observation that amastigotes remain attached on the surface of pyroptotic cells primed us to investigate parasite integrity and the molecular structures underlying parasite surface retention.

Scanning electron microscopy analyses showed that 38.8% of pyroptotic macrophages displayed intact parasites at the cell surface (Fig. 3A1,A2; Fig. S3E,F), thus confirming our results obtained with the OPERA™ system (Fig. 2). Ultrastructural analysis demonstrated that exposed amastigotes remained attached at macrophage membranes. Significantly, exposed amastigotes showed a highly polarized orientation with only the anterior pole (flagellar side) being exposed toward the extracellular milieu. This feature was also observed for pyroptotic macrophages infected with *L. donovani* amastigotes that reside in individual PVs (Fig. S3G). We next investigated the posterior attachment zone by transmission electron microscopy (TEM), which revealed an electron-dense membranous junction formed between parasite and PV membranes both in non-stimulated and pyroptotic macrophages (Fig. 3B1,B2). This junction corresponds to a defined attachment site – showing features similar to gap junctions – that permits amastigotes of communal *Leishmania* species to be anchored to PV membranes (Benchimol and de Souza, 1981). These results demonstrate that amastigotes remain strongly attached to pyroptotic macrophages through their attachment site (see Movie 1), which represents a new type of interaction observed in pyroptotic cells, different from the one previously described in the literature (Jorgensen et al., 2016).

*Leishmania amazonensis* amastigotes are resistant to host cell pyroptosis and retain full infectivity

Since bacteria can be damaged during host cell pyroptosis (Liu et al., 2016), we next investigated whether parasite viability and infectivity were affected during macrophage pyroptosis. Amastigotes isolated from pyroptotic macrophages (A-pm) were compared to amastigotes isolated from unstimulated control macrophages (A-cm). Macrophage pyroptosis did not reduce parasite viability (99% of A-pm remained YO-PRO-1 negative; Fig. 4A1). In addition, when pyroptotic macrophages were cultured in promastigote-specific medium (48 h, 27°C), amastigotes rapidly transformed into ovoid cells with a long flagellum, motile and proliferating promastigotes that detached from macrophage remnants (Fig. 4A2). These promastigotes showed normal growth (visual parasite counting, Fig. 4A3) and metabolic activity (resazurin reduction data, Fig. 4A4). Finally, A-pm maintained their capacity to establish efficient infection of new macrophages (Fig. 4B). In addition, amastigotes associated to macrophage remnants were efficiently phagocytosed by naïve BMDMs and established normal infection levels as shown by the formation of typical large communal PVs housing numerous parasites after a 3-day co-culture (Fig. 4C3).

**Conclusion**

We analyzed dynamic interactions between *Leishmania* amastigotes and PVs in pyroptotic BMDMs at the population, single-cell and ultrastructural levels. Real-time HCA analysis uncovered three distinct stages of the pyroptotic process in our experimental system (Fig. S4): During stage 1, the PV membrane decays (rapid loss of PV acidity and reduced PV size). In stage 2, the PV lumen is externalized in 38% of cells, resulting in exposure of membrane-anchored amastigotes. In stage 3, cellular alterations are further sustained (80% of cell death). *L. amazonensis* amastigotes retained their viability and infectivity during macrophage pyroptosis. We therefore can speculate that parasites attached to pyroptotic cell debris *in vivo* may favor parasite spreading – as shown for other forms of host cell death – allowing for uptake by macrophages newly recruited to inflammatory infection sites (de Menezes et al., 2016). On the other hand, the extracellular exposure of amastigotes could expose parasites to complement-dependent cytotoxicity, which is known to control parasite load *in vivo* (Laurenti et al., 2004), or to anti-leishmanial antibodies that could promote cell-mediated cytotoxicity via Fc receptor-dependent phagocytosis. Our results will stimulate future studies designed to assess the role of macrophage pyroptosis in *Leishmania* dissemination, transmission, and immuno-pathology.

**MATERIALS AND METHODS**

**Ethics statement**

Animals were housed at the Institut Pasteur animal facilities accredited by the French Ministry of Agriculture for performing experiments on live rodents. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, February 6th, 2013). All experiments were approved by the Ethic Committee for animal experimentation (CETEA#89) and authorized by the French ministry of...

![Fig. 3. Amastigotes are exposed during macrophage pyroptosis.](image)
higher education, research and innovation under the reference 2013-0092 in accordance with the Ethics Charter of animal experimentation that includes respect of the 3Rs principles, appropriate procedures to minimize pain and animal suffering.

Bone marrow-derived macrophage cultures
Female C57BL/6 mice were obtained from Janvier (Saint Germain-sur-l’Arbresle, France). Bone marrow cell suspensions were recovered from tibias and femurs as described previously (Courret et al., 1999). Bone marrow cells were plated at 1.5×10^7 cells/ml in hydrophobic Petri dishes (#664161, Corning Life Science) and cultured for 6 days at 37°C in a 7.5% CO2 atmosphere in complete Dulbecco’s modified Eagle’s medium (DMEM; #P04-03500, Pan Biotech) containing 4.5 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate and 3.7 g/l NaHCO3 and supplemented with 15% fetal calf serum (FCS; #A3160801, Gibco), 10 mM HEPES (# 15630080, Gibco), 50 µg/ml penicillin/streptomycin (#P4333, Sigma-Aldrich), and with 75 ng/ml recombinant mouse CSF-1 (#234311, ImmunoTools). Adherent bone marrow-derived macrophages (BMDMs) were recovered and seeded in complete DMEM supplemented with 30 ng/ml recombinant mouse CSF-1 (rmCSF-1, #12343115, ImmunoTools) into various culture-treated supports, including (1) flat bottom 96-well black µClear plates (# 655090, Greiner Bio-One) for OPERA analyses, (2) 24-well plates (# 353047, Comin. Falcon®) with glass coverslips inside for scanning electron microscopy analyses, and (3) 6-well plates (#353046, Dutscher, Falcon®) for amastigote isolation from infected BMDMs.

Macrophage infection and activation
*Leishmania amazonensis* amastigotes (LV79 strain, MPRO/BR/72/M1841) expressing mCherry (Lecoeur et al., 2020) were isolated from footpad lesions of infected Swiss nu/nu mice and purified as described previously (Courret et al., 1999). Infections were carried out at 34°C at a ratio of four amastigotes per macrophage. *Leishmania donovani* amastigotes (Ld1S2D strain, MHOM/SD/62/1S-CL2D) were isolated from infected spleens of female RjHan:AURA Golden Syrian hamsters and purified as described previously (Pescher et al., 2011; Prieto Barja et al., 2017). Infections were carried out at 37°C at a ratio of eight amastigotes per macrophage.

All pyroptosis experiments were performed after 3 days of infection using a sequential treatment of 500 ng/ml LPS (# LP511-1, Alpha Diagnostic) for 4 h and 5 mM ATP (# A26209, Sigma-Aldrich) for different periods up to 4 h.

Real-time analysis of pyroptosis by confocal microscopy
The following fluorescent reporters were added to the cell cultures 15 min before ATP stimulation: Hoechst 33342 (10 µg/ml) (# H3570, Invitrogen), LysoTracker Green DND-26 (1 µM) (LTG, # L7526, Invitrogen) and YO-PRO-1 (1 µM) (# Y3603, Invitrogen). The pyroptotic process was monitored at 34°C and 7.5% CO2 using a fully automated spinning disk confocal microscope OPERA™ quadruple Excitation High Sensitivity (QEHS, PerkinElmer Technologies) with a 40x water immersion objective (Aulner et al., 2013). Image acquisition was performed every 5 min after ATP addition using the following sequential acquisition settings: (1) 405 nm laser line excitation, filter 450/50 for Hoechst 33342 detection,
Scanning and transmission electron microscopy analyses
Pyroptotic BMDMs were fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C and post-fixed in 0.1 M cacodylate buffer (pH 7.2) containing 1% OsO4. After serial dehydration, samples were critical-point dried (Emitech K850 or Balzers Union CPD30) and coated with gold using a sputter coater (Gatan Ion Beam Coater 681). Scanning electron microscopy observations were made with the JEOL 7600F microscope. Images were colorized using Adobe Photoshop CS software.

Western blotting
Cells were lysed in RIPA buffer (# R0278, Sigma-Aldrich) supplemented with a cocktail of anti-protease and anti-phosphatase inhibitors (MS-SAFE, Sigma-Aldrich). Proteins were resolved by SDS-PAGE on NaPAGe gels (4–12% Bis-Tris) in MOPS buffer and electroblotted onto polyvinylidene difluoride (PVDF) membranes in transfer buffer (Lecoeur et al., 2020). Membranes were blocked with 5% fat-free milk in 1× Tris-buffered saline containing 0.25% Tween 20 and then probed overnight at 4°C with anti-mouse-IgG peroxyside conjugate secondary antibodies. Membrane signals were revealed by SuperSignal West Pico reagent (#10481945, Thermo Fisher Scientific) in a high-resolution PXI machine (Syngene).

Amastigote isolation from pyroptotic and control macrophages
Supernatants of BMDM cultures (pyroptotic and control BMDMs) were carefully removed with fresh medium without LPS or ATP. After detaching cell remnants, isolated amastigotes were centrifuged (1500 g for 10 min), washed in PBS, counted and used to assess parasite viability, the capacity of amastigotes to differentiate into proliferating promastigotes in situ, and amastigote virulence by infection of naïve BMDMs.

Flow cytometric analysis of parasite death by YO-PRO-1 staining
Amastigotes isolated from Leishmania-infected, pyroptotic and control BMDMs were seeded in a 96-well plate (# 353072, Dutcher, Falcon®) at a final concentration of 5 × 10^6 parasites per ml and incubated for 10 min with YO-PRO-1 (10 µM, final concentration (# Y3603, Thermo Fisher Scientific). Samples were then immediately analyzed on the CytoFLEX cytometer (Beckman Coulter) in a BSL2 containment to evaluate YO-PRO-1 incorporation. Data were analyzed using the Kaluza 1.5 software (Beckman Coulter Life Sciences). Lesion-derived amastigotes were treated with 70% ethanol for 10 min and were included as a positive control for cell death and YO-PRO-1 incorporation.

Analysis of parasite differentiation, growth and metabolic status
Amastigote to promastigote differentiation was analyzed by scanning electron microscopy (as described above) directly on amastigotes exposed on pyroptotic macrophages after 24 h incubation in 1 ml of promastigote culture medium at 27°C. For growth analysis in culture, purified amastigotes were incubated at 27°C in promastigote culture medium at 10^5 parasites/ml. Promastigote culture density was determined daily during 6 consecutive days by visual counting using a Malassez chamber. The parasite metabolic status was analyzed at days 2 and 3 through a resazurin assay. Briefly, 200 µl of parasite culture was transferred into wells of a 96-well plate (#655090, Sigma-Aldrich) and incubated with 2.5 µg/ml of resazurin (#R7017, Sigma-Aldrich) for 4 h at 27°C. The fluorescence intensity of the resazurin-derived resorufin was determined using a Tecan Safire2 plate reader (558 nm excitation, 585 nm emission) (Lamotte et al., 2019).


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