Journal of Visualized Experiments

Investigating the Phagocytosis of Leishmania using Confocal Microscopy

--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE62459R1
Full Title:	Investigating the Phagocytosis of Leishmania using Confocal Microscopy
Corresponding Author:	Patricia Veras BRAZIL
Corresponding Author's Institution:	
Corresponding Author E-Mail:	patricia.veras@fiocruz.br
Order of Authors:	Amanda Paixão
	Beatriz Dias
	Luana Palma
	Natália Tavares
	Cláudia Brodskyn
	Juliana Menezes
	Patricia Veras
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please specify the section of the submitted manuscript.	Immunology and Infection
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	We would like to let you know that the video regarding the manuscript Investigating the Phagocytosis of Leishmania using Confocal Microscopy will be submitted in the near future.
Please indicate whether this article will be Standard Access or Open Access.	Open Access ($3900)

TITLE:

Investigating the Phagocytosis of *Leishmania* using Confocal Microscopy

AUTHORS:

Amanda R. Paixão[1†], Beatriz R. S. Dias[1†], Luana C. Palma[1], Natália M. Tavares[1], Cláudia I. Brodskyn[1], Juliana P. B de Menezes[1] and Patricia S. T. Veras[1,2*]

[1] Laboratory of Host-Parasite Interaction and Epidemiology, Gonçalo Moniz Institute, Candeal, Salvador, Brazil

[2] National Institute of Science and Technology of Tropical Diseases - National Council for Scientific Research and Development (CNPq), Brazil

[†]These authors have contributed equally to this work.

amandareboucas95@gmail.com
beatrizrsdias@gmail.com
luanabio@hotmail.com
nmachado302@gmail.com
cbrodskyn2009@gmail.com
julianaperrone@gmail.com

***Correspondence:**

Corresponding Author:

patricia.veras@fiocruz.br; pstveras@gmail.com

SUMMARY

The mechanism associated with phagocytosis in *Leishmania* infection remains poorly understood. Here, we describe methods to evaluate the early events occurring during *Leishmania* interaction with the host cells.

ABSTRACT

Phagocytosis is an orchestrated process that involves distinct steps: recognition, binding, and internalization. Professional phagocytes take up *Leishmania* parasites by phagocytosis, consisting of recognizing ligands on parasite surfaces by multiple host cell receptors. Binding of *Leishmania* to macrophage membranes occurs through complement receptor type 1 (CR1) and complement receptor type 3 (CR3) receptors and Pattern Recognition Receptors. Lipophosphoglycan (LPG) and 63 kDa glycoprotein (gp63) are the main ligands involved in macrophage-*Leishmania* interactions. Following the initial recognition of parasite ligands by host cell receptors, parasites become internalized, survive, and multiply within parasitophorous vacuoles. The maturation process of *Leishmania*-induced vacuoles involves the acquisition of molecules from intracellular vesicles, including monomeric G protein Rab 5 and Rab 7, lysosomal associated membrane protein 1 (LAMP-1), lysosomal associated membrane protein 2 (LAMP-2), and microtubule-associated protein 1A/1B-light chain 3 (LC3).

Here, we describe methods to evaluate the early events occurring during *Leishmania* interaction with the host cells using confocal microscopy, including (i) binding (ii) internalization, and (iii) phagosome maturation. By adding to the body of knowledge surrounding these determinants of infection outcome, we hope to improve the understanding of the pathogenesis of *Leishmania* infection and support the eventual search for novel chemotherapeutic targets.

INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania*, resulting in a broad spectrum of clinical manifestations in the vertebrate host, including cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis[1]. The World Health Organization (WHO) estimates that over one billion people are at risk, with more than one million new cases reported per year[2].

Leishmania spp. are obligate intracellular protozoans that survive inside host cells, including monocytes, macrophages and dendritic cells[3]. *Leishmania*-macrophage interaction is a complex process that involves multiple host cell receptors and parasite ligands either through direct interaction or by opsonization involving complement receptors[4,5]. Classical surface receptors, such as CR1, CR3, mannose-fucose, fibronectin, toll-like and scavenger receptors, mediate parasite attachment to macrophages[6-8]. These receptors recognize molecules on the surface of *Leishmania*, including the 63 kDa glycoprotein (gp63) and glycolipid lipophosphoglycan (LPG)[9]. These are the most abundant molecules on the surface of promastigotes and play an essential role in the subversion of host immune response, favoring the establishment of parasite infection in mammalian cells[10]. After parasite surface ligands bind to macrophage receptors, F-actin accumulates on mammalian cell surfaces, surrounding parasites as they are phagocytosed. Subsequently, this leads to the formation of a parasite-induced compartment termed a parasitophorous vacuole (PV), which presents phagolysosomal features[11]. Once inside these phagolysosomes, parasites undergo several alterations essential to survival and multiplication[3].

The biogenesis of PVs is a highly regulated membrane trafficking process critical to the intracellular survival of this pathogen[12]. The formation of this compartment results from sequential fusion events between phagosomes and compartments of the host endocytic pathway. Classical cell biology studies have revealed that the maturation of PVs involves the acquisition of monomeric G protein Rab 5 and Rab 7 proteins, which are mainly associated with early and late endosome maturation, respectively [13]. In addition, these compartments acquire lysosome-associated membrane proteins 1 and 2 (LAMP 1, LAMP 2), the principal protein constituents of the lysosomal membrane and microtubule-associated protein 1A/1B-light chain 3 (LC3), an autophagosome marker[14]. Despite apparent similarities, the kinetics of PV formation[15,16] and the morphology of these compartments vary depending on *Leishmania* species. For example, infection caused by *L. mexicana* or *L. amazonensis* induces the formation of large compartments containing a great number of parasites[17]. By contrast, other species, such as *L. braziliensis* and *L. infantum,* form smaller vacuoles that normally contain only one or two parasites in each vacuole[18].

Despite this knowledge surrounding host cell-*Leishmania* interaction, the initial events triggered

by contact between host receptors and parasite ligands have not been fully elucidated. These events are known to be determinants of the outcome of parasite infection and are dependent on parasite species, the type of host cell receptors recruited to recognize parasites and the activation of macrophage signaling pathways[19,20]. Therefore, it is essential to identify the molecules involved in the biogenesis of *Leishmania*-induced PVs and determine the role(s) played by these molecules in infection establishment and outcome. Here, we describe a method of monitoring early events occurring during the phagocytosis of *Leishmania*, including binding, internalization, phagosome formation and maturation. This work could aid in clarifying the participation of PLC, Akt, Rab5, Rab7 and LC3 in the formation of PVs induced by different *Leishmania* species. Importantly, this protocol can be used to investigate the participation of other proteins involved in PV maturation. Future studies will expand the knowledge surrounding mechanisms involved in *Leishmania*-host cell interaction and contribute to the design of novel chemotherapeutic strategies.

PROTOCOL

Cells were obtained from healthy donors following the approval of procedures by the National Research Ethics Committees (ID: 94648218.8.0000.0040).

1. Cell cultures

1.1. Human monocyte-derived macrophages

NOTE: To obtain human monocyte-derived macrophages for *in vitro* differentiation into macrophages, collect blood from healthy donors and purify peripheral blood mononuclear cells (PBMC) as described by D. English and B. R. Andersen [21].

1.1.1. After collecting peripheral blood (50 mL), pour it into a heparinized tube and then dilute the blood 1:1 in a phosphate buffer solution (PBS) at room temperature. Gently place diluted heparinized blood on top of previously distributed density gradient medium.

1.1.2. Centrifuge the tubes at 252 × *g* for 30 min at 24 °C to avoid hemolysis.

NOTE: Set centrifuge break-off to avoid mixing of gradient layers. After centrifugation, discontinuous gradient layers are formed from the bottom to the top: erythrocytes, density gradient medium, PBMC ring and plasma.

1.1.3. Transfer the PBMC ring, located between the density gradient medium and plasma layers, to a new tube and fill with PBS to wash out excess density gradient medium.

1.1.4. Wash cells once and centrifuge at 190 × g for 10 min at 4 °C.

1.1.5. Discard the supernatant and resuspend pellet in 1 mL of complete RPMI medium.

1.1.6. Count the cells and plate 2×10^6 cells in 500 mL of Roswell Park Memorial Institute (RPMI) supplemented with 25 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulfonic acid] (HEPES), 2 g/L sodium bicarbonate, 2 mM glutamine, 20 g/mL ciprofloxacin and 10% inactivated Fetal Bovine Serum (FBS) (complete RPMI medium) for 7 days at 37 °C under 5% CO_2 in a 24-well plate to allow monocytes to differentiate into macrophages by adhesion.

1.2. THP-1 cultures

1.2.1. Grow THP-1 cell line at a concentration of 2×10^5 cells in 10 mL of complete RPMI medium in 75 cm^2 culture flask."

1.2.2. Maintain cell cultures in an incubator at 37 °C under 5% CO_2 for 7 days.

1.2.3. Centrifuge cells at 720 × g for 10 min at 4 °C and resuspend the pellet in complete RPMI medium.

1.2.4. Count cells in a Neubauer chamber.

1.2.5. Plate cells on 13 mm glass coverslips at a concentration of 2×10^5 cells per well in 500 μL of complete RPMI medium containing 100 nM phorbol myristate acetate (PMA) at 37 °C under 5% CO_2 to allow differentiation of THP1 cells into macrophages.

1.2.6. After three days, wash twice cells with 0.9% NaCl solution to remove medium containing PMA.

1.2.7. Incubate differentiated THP-1 cells in PMA-free complete RPMI medium at 37 °C under 5% CO_2 for an additional 2 days before starting experimentation.

2. Parasite cultures and CellTracker Red staining

NOTE: To visualize parasites through fluorescence microscopy, perform staining using CellTracker Red fluorescent dye (CMTPX). Alternatively, other markers, including carboxyfluorescein can be used in accordance with manufacturer instructions or promastigotes constitutively expressing GFP, RFP, or other fluorescent reporter genes. Parasites used to infect cells are those at stationary phase of growth obtained from a promastigote axenic culture of no more than 7 passages.

2.1 Grow *Leishmania* spp. promastigotes at 1×10^5 parasites per 1,000 μL of medium in a cell culture flask containing 5 mL of Schneider's medium supplemented with 50 μg/mL gentamicin and 10% FBS.

2.2 After incubating parasite axenic cultures in a biochemical oxygen demand (B.O.D.) at 24 °C, perform daily counting in a Neubauer chamber. Check for parasite form (thin, elongated) and

mobility for 5 days. Parasites are considered in stationary phase of growth when two consecutive counts with 8 hours of interval display similar amounts.

2.3 Upon reaching the stationary phase of growth, incubate the parasites in 4 mL of 0.9% NaCl solution with 1 μM CMTPX for 15 min at 37 °C under 5% CO_2 avoiding contact with light.

2.4 Add FBS at a 1:1 proportion and incubate parasite suspension for an additional 1 min.

2.5 Wash parasites thrice with PBS, followed by centrifugation at 1,781 × g for 10 min.

2.6 Ressuspend parasite pellet in 1,000 μL of RPMI complete medium.

2.7 Count parasites in a Neubauer chamber.

3. Assessment of *Leishmania* binding to macrophages

3.1 Seed 2 × 10^5 THP-1 cells or human monocyte-derived macrophages in 500 μL of complete RPMI medium per well on a 24-well plate with 13 mm glass coverslips.

3.2 Cultivate cells at 37 °C under 5% CO_2 for 24 h.

3.3 Wash the cells twice with 0.9% NaCl solution and incubate in complete RPMI medium at 4 °C for 10 min.

3.4 Add stationary phase promastigotes as described by A. L. Petersen[22] at a 10:1 ratio to well plates, and then centrifuge at 720 × g for 5 min under 4 °C.

3.5 Incubate at 4 °C for 5 min.

3.6 Wash the cells twice with 0.9% NaCl solution to remove any non-internalized promastigotes.

3.7 Fix the cells in 4% paraformaldehyde for 15 min at room temperature.

3.8 Incubate the coverslips with 15 mM NH_4Cl for 15 min at room temperature.

3.9 Wash thrice with PBS 0.15% bovine serum albumin (BSA). Incubate with blocking solution (3% BSA in PBS) for 1 h at room temperature.

3.10 Wash thrice with PBS and then permeabilize with 0.15% PBS-Saponin for 15 min at room temperature.

3.11 Add phalloidin (diluted 1:1,200) for 1 h at room temperature and protect from light.

3.12 Mount coverslips using mounting media.

3.13 Acquire images via a confocal fluorescence microscope using a 63×/1.4 objective.

4. Assessment of *Leishmania* phagocytosis by macrophages

4.1 Seed 2 × 10^5 THP-1 cells or human monocyte-derived macrophages in 500 μL of complete RPMI medium per well on a 24-well plate with 13 mm glass coverslips.

4.2 Cultivate cells for 24 h at 37 °C under 5% CO_2.

4.3 Wash cells twice in 0.9% NaCl solution and incubate in complete RPMI medium in 24-well plate at 4 °C for 10 min.

4.4 Add stationary phase *Leishmania* spp. as described by A. L. Petersen[22] at a 10:1 (parasite:host cell) ratio, and then centrifuge at 720 × g for 10 min under 4 °C.

4.5 Incubate cells at 4 °C for 5 min.

4.6 Wash the cells twice with 0.9% NaCl solution to remove any non-internalized promastigotes.

4.7 Incubate the cells in supplemented RPMI medium at 37 °C for 1 h.

4.8 Fix the cells with 4% paraformaldehyde for 15 min.

4.9 Mount coverslips using preferred mounting media.

4.10 Count no less than 400 cells in random fields under a fluorescence microscope using a 100×/1.4 objective.

5. Evaluation of *Leishmania*-induced vacuole maturation

NOTE: THP-1 cell transfection should be performed as described by M. B. Maess, B. Wittig and S. Lorkowski [23]. Here we summarize this protocol, with minimal modifications. Nucleofection is a specific transfection method that requires a nucleofector. As an alternative method, cells can be transfected using lipofectamine[24] and lentivirus transduction[25].

5.1 To investigate the biogenesis of *Leishmania*-induced PV, transfect THP1 cells with PLC[26,27], Akt[26,27], Rab 5[28-30] or Rab 7[28,29,31] plasmids.

NOTE: This methodology can be used to transfect THP-1 cells with other genes than those listed above.

5.2 Seed THP-1 cells at 1.5 x 10^7 in 75 cm^2 tissue culture flasks containing 10 mL complete RPMI medium supplemented with 100 ng/mL PMA and 50 µM 2-mercaptoethanol for 48 h.

5.3 Wash cells once in 0.9% NaCl solution.

5.4 Detach cells using a non-enzymatic cell dissociation solution and centrifuge (250 × g) for 5 min at room temperature.

5.5 Resuspend THP-1 cells in 1 mL of RPMI medium and perform counts in a Neubauer chamber.

5.6 Centrifuge THP-1 cells again at 250 × g for 10 min at room temperature. Discard the supernatant.

5.7 Resuspend 2 × 10^6 cells in 100 µL of Nucleofector solution and incubate with 0.5 µg of the plasmid coding for the protein of interest, tagged with a fluorescent protein.

5.8 Transfer the suspension containing THP-1 cells and nucleic acid to the Nucleofector cuvette.

5.9 Transfect THP1 cells using Nucleofector Program Y-001.

5.10 Recover the transfected cells (2x10^6) and seed in 500 µL RPMI medium on 24-well plates with 13 mm glass coverslips

5.11 Incubate THP-1 cells in complete RPMI medium at 37 °C for 0.5, 2, 4, 6, 12 and 24 h.

5.12 Repeat steps 3.13 and 3.13.

6. Evaluation of the recruitment of LC3 to *Leishmania* spp. PVs

NOTE: The autophagic membrane marker LC3 can be used to investigate whether phagosomes present autophagic features. LC3 recruitment to *Leishmania*-induced PVs can be assessed during infection by immunolabelling cells with the anti-LC3 antibody, as previously described by C. Matte[32] and B. R. S. Dias[33].

6.1 Seed 2 × 10^5 THP-1 cells or human monocyte-derived macrophages in 500 µL complete RPMI medium on a 24-well plate with 13 mm glass coverslips.

6.2 Cultivate cells for 24 h at 37 °C under 5% CO_2.

6.3 Wash cells twice in 0.9% NaCl solution and incubate in complete RPMI medium.

6.4 Add stationary phase *Leishmania* spp. promastigotes as described by A. L. Petersen[22] at a 10:1 (parasite: host cells) ratio and centrifuge cells at 720 × g for 5 min under 4 °C.

6.5 Incubate at 37 °C for 30 min or 4 h. Then wash twice and fix the cells to evaluate the LC3 recruitment to *Leishmania*-induced PV membranes at the early stages of infection.

6.5.1 Alternatively, to assess LC3 recruitment to PV membranes at later stages of infection, wash twice another macrophage group at 4 h of infection to remove any non-internalized promastigotes. Incubate infected cells in complete RPMI medium for an additional 12 h and 24 h, to finally wash twice and fix.

NOTE: Fixed cells can be kept in PBS or 0.9% NaCl solution at 4 °C until labeling.

6.6 Simultaneously block and permeabilize the fixed cells in 0.1% Triton X-100, 1% BSA, 20% normal goat serum, 6% non-fat dry milk, and 50% FBS for 20 min at room temperature.

6.7 Incubate the cells with anti-LC3 antibody (1: 200) diluted in PBS for 2 h at room temperature.

NOTE: As a negative control of the immunostaining, a group of cells should be incubated with immunoglobulin G (IgG) from the animal of primary antibody origin in a concentration equivalent to that used for the primary antibody.

6.8 Wash the cells thrice with 0.9% NaCl solution at room temperature.

6.9 Incubate the cells with AlexaFluor 488-conjugated goat anti-rabbit IgG (1:500) or the preferred fluorescent-dye conjugated secondary antibodies for 1 h at room temperature.

6.10 Wash the cells thrice with 0.9% NaCl solution at room temperature.

6.11 Mount coverslips using preferred mounting media.

6.12 Acquire images via confocal fluorescence microscope using a 63x/1.4 objective.

7. Confocal microscopy acquisition and Fiji quantification

NOTE: Acquiring immunofluorescence images should be performed using a confocal laser scanning microscope. To reach a better resolution, use an oil-immersion 63x objective lens.

7.1 Leave the 13 mm glass coverslips at room temperature and protect them from the light at least 30 min before the acquisition.

7.2 Clean the coverslips with an absorbent tissue.

7.3 Add a drop of immersion oil to the objective and add the slide.

7.4 Move the objective up until the oil touches the slide.

7.5 Observe and adjust the focus on the microscope and choose the option 63x objective with oil.

7.6 Open the Leica program and adjust the lasers in the 488, 552, and 405 wavelengths.

7.7 Select the image resolution 1,024 x 1,024.

7.8 Click on the **Live** button, set the Z stack, and press the **Begin** option. Then, do it again and press the **End** button. We recommend 20 μm for slice thickness to get confocal images with good resolutions.

7.9 Wait for the image acquisition, and then select the option "Maximum Projection" in the Leica tools.

7.10 Save the experiment.

7.11 Export the lif or tiff format images to a computer and open the FIJI program.

7.12 Open the experiment and set the view stack with the hyper stack. Then select open files individually and stitch tiles.

7.13 Select the free hands tool in the Fiji toolbar and trace the cell carefully by hand.

7.14 Press the **Analyze** button and measure to visualize the fluorescence intensity.

7.15 Repeat this process to each cell per group.

7.16 Save the measurements and export them to a spreadsheet editor.

7.17 Add this data to a statistical analysis program and do the statistical analysis.

8. Statistical analysis

NOTE: For data analysis and graphics, use a statistical analysis program.

8.1 Open the program.

8.2 Insert the obtained data and test the normality parameters.

8.3 For data with normal distribution, use the Student *t*-test and for nonparametric tests, Mann-Whitney test.

8.4 Consider data with a statistically significant difference when the *p-value* is less than 0.05.

8.5 Prepare graphics representing the data, with central tendency measures (mean or median) and variation measures.

REPRESENTATIVE RESULTS

This report aims to evaluate the early events occurring during the phagocytosis of *L. braziliensis* isolated from patients presenting *L. braziliensis*-LCL or *L. braziliensis*-DL form of CL. Using confocal microscopy, we investigated the main events associated with parasites' phagocytosis: binding, internalization, and phagosome maturation. We first evaluated the *L. braziliensis*-LCL or *L. braziliensis*-DL binding and phagocytosis by human monocyte-derived macrophages. The data show that both *L. braziliensis*-LCL and *L. braziliensis*-DL similarly bind to macrophages (**Figure 1**). Also, no differences were observed regarding *L. braziliensis*-LCL and *L. braziliensis*-DL phagocytosis by host cells (**Figure 2**). Finally, we compared the recruitment of LC3 to the PVs induced by *L. braziliensis*-LCL or *L. braziliensis*-DL in infected cells. After 4 h of infection, we observed similar percentages of LC3 decorated PVs in *L. braziliensis*-LCL and *L. braziliensis*-DL-infected macrophages (**Figure 3**). These representative results showed that *L. braziliensis*-LCL and *L. braziliensis*-DL similarly interact with macrophages during binding, phagocytosis, and biogenesis of PVs, concerning the LC3 recruitment.

Microscopic images representing THP-1 cells efficiently transfected with PLC-GFP, Rab5-GFP, Rab7-GFP plasmids are shown in **Figure 4**.

FIGURE AND TABLE LEGENDS

Figure 1. **Evaluation of *L. braziliensis-LCL* and *L. braziliensis*-DL binding to human macrophages.** Human monocyte-derived macrophages were infected with *L. braziliensis*-LCL- or *L. braziliensis*-DL. After 10 min at 4 °C, the binding was assessed by confocal microscopy. (**A**) Confocal microscopy images of *L. braziliensis*-LCL or *L. braziliensis*-DL (labeled with CMTPX, red) binding to macrophages (labeled with phalloidin, green). For confocal microscopy, cell nuclei were labeled with DAPI (blue). Arrows depict *Leishmania*-macrophage binding. (**B**) Percentage of *Leishmania* binding to the macrophages. A total of 30 cells per group were analyzed. Data represent each replicate of one experiment performed in quintuplicate (unpaired *t* test, $p > 0.05$).

Figure 2. Evaluation of *L. braziliensis*-LCL and *L. braziliensis*-DL phagocytosis by human macrophages. Human monocyte-derived macrophages were incubated with *L. braziliensis*-LCL or *L. braziliensis*-DL for 10 min at 4 °C followed by additional 1 h at 37 °C. Cells were then analyzed by fluorescence microscopy by counting a total of 400 cells. (**A**) Confocal microscopy images of human macrophages infected by *L. braziliensis*-LCL or *L. braziliensis*-DL. For confocal microscopy, cell nuclei were labeled with DAPI (blue). Arrows depict *Leishmania* parasites nuclei. (**B**) Percentage of *Leishmania* phagocytosis. Circles represent data from each replicate of one experiment performed in triplicate (unpaired *t* test, $p > 0.05$).

Figure 3. Assessment of LC3 recruitment to PVs induced by *L. braziliensi*s-LCL or *L. braziliensis*-*DL* in macrophages. Human monocyte-derived macrophages were infected and then stained with anti-LC3 antibody for 30 min, 4 and 12 h. (**A**) Confocal microscopy images of *L. braziliensi*s-LCL or *L. braziliensis*-*DL*-infected macrophages labeled with anti-LC3 followed by the secondary anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (green). For confocal microscopy, cell nuclei were labeled with DAPI (blue); (**B**) Percentage of *L. braziliensi*s-LCL or *L. braziliensis*-DL-induced PVs decorated with LC3-II. A total of 30 cells per group were analyzed. The circles correspond to each randomly selected field analyzed (unpaired *t* test, $p > 0.05$).

Figure 4. THP-1 cells expressing PLC, Rab5 or Rab7. After differentiating into macrophages, THP-1 cells were subjected to nucleofection with each gene of interest coupled to GFP fluorescent probes: PLC, Rab5 and Rab7. Subsequently, these cells were fixed, had the nucleus stained with DAPI (blue) and were observed under a confocal microscope using a 63x/1.4 objective.

DISCUSSION

Leishmania-macrophage interaction is a complex process and involves several steps that can influence disease development[5]. To better understand the mechanisms involved in the interaction of unopsonized *Leishmania* and host cells, we have described a protocol that employs confocal fluorescence microscopy to assess phagocytosis from early to late stages of *Leishmania* infection. The use of fluorescence techniques involving two or more fluorophores to investigate cell biology mechanisms, including immunolabeling and the expression of fluorescent-labeled proteins, allows us to analyze the location of several proteins, as well as to simultaneously evaluate cell morphology. The advantages offered by these methods make them the best tools to monitor pathogen-host cell interaction[34].

To better understand the phagocytic process involving different particles, it is crucial to analyze this highly dynamic process at the molecular level[35]. Confocal-fluorescence microscopy has been used for decades to this end and has been shown to be an excellent tool for quantifying phagocytosis through the determination of numbers of internalized particles, or the types of proteins known to be involved in early stages of host-pathogen interaction[34]. The present study proposed the use of confocal microscopy to analyze events occurring during the phagocytosis of *L. braziliensis* isolated from patients with different clinical forms (LCL and DL). This technique enables us to study cells expressing specific fluorescent proteins, including PLC, Akt, Rab 5, and Rab 7, and subsequently evaluate the participation of these proteins in the phagocytosis of *Leishmania* isolates to identify elements relevant to different infection outcomes.

The present study employed primary macrophages and THP1 cells to assess *L. braziliensis* phagocytosis at early stages of infection. The presently described protocol can also be used to study phagocytosis in *Leishmania* spp. by other phagocytes, including dendritic cells, monocytes, macrophage cell-lines, and neutrophils derived from human peripheral blood. During the parasite internalization process, a dynamic change in F-actin occurs at the cell membrane surface[11]. We then labeled proteins located in the cell membrane using a specific marker of phagocytosis[36], such as fluorescent PLC, which allowed us to observe the binding stage of *Leishmania* to host

cells, as shown in **Figure 4**. Staining parasites with fluorescent markers, such as CMTPX or CSFE, is also crucial to assess parasite binding to host cells by immunofluorescence. It is worth noting that this assay requires careful execution: i) wash coverslips gently using washing solutions at room temperature (25 °C), otherwise, samples can be damaged; ii) prepare reagent dilutions precisely; and iii) protect the samples from light[34].

A confocal microscope configured to the optimal laser excitement wavelength is capable of obtaining a high-quality sample image. Labeled cells can be stored for weeks in the dark at 4 °C or frozen until the time of analysis. The use of confocal microscopy to evaluate phagocytosis is limited by prolonged times of exposure and high intensity laser beams, which can damage samples, and, in some cases, lead to high levels of background detection in images[35,37].

In the present study, instead of using live imaging to follow the phagocytosis of *Leishmania* spp., we performed a kinetic study by fixing cells at several early times of infection (30 min, 4 h, and 12 h). It must be considered that live imaging offers some advantages, such as the potential to analyze the spatial and temporal dynamics of myriad cellular processes, including phagocytosis, and capturing details that are not observable in static images[34]. However, live imaging requires that cells be healthy throughout the entire experimentation process, including controlling temperature, pH and oxygen conditions in a microscopic chamber. It is important to note that this cannot be reliably performed at several laboratories around the world.

The nucleofection protocol described has demonstrated efficacy in the transfection of THP-1 cells, as previously reported by M. B. Maess, B. Wittig and S. Lorkowski [23]. In this process, it is crucial to gently detach cells to avoid cell damage or loss in cell viability. Based on our experience, we recommend using a non-enzymatic cell dissociation solution to detach cells from plates prior to performing transfection. The authors of the original protocol[23] state that the main limitations of this procedure are the need for cells to be in suspension during the nucleofection process, and the fact that inadequate detachment can cause stress. Despite these limitations, the protocol does allow for reliable transfection, reaching a 90% successful transfection rate without losing cell viability.

The characterization of PVs using a set of endocytic markers, including PLC, Akt, Rab5, and Rab7, is essential to improving our understanding of *Leishmania* phagocytosis. Identifying new proteins that participate in PV biogenesis and comprehensively characterizing these compartments can clarify differences in macrophage response during *Leishmania* spp. infection. The contribution of our results to the body of knowledge surrounding *Leishmania* infection outcome will undoubtedly advance our understanding of the pathogenesis of *Leishmania* infection and support the eventual search for novel chemotherapeutic targets. It is worth noting that this technique can also be extended to other types of studies, including infection by bacteria, yeasts or bead engulfment by many types of cells[38,39].

ACKNOWLEDGMENTS

We thank Gonçalo Moniz Institute, Fiocruz Bahia, Brazil and the department of microscopy for assistance. This work was supported by INOVA-FIOCRUZ number 79700287000, P.S.T.V. holds a

grant for productivity in research from CNPq (305235/2019-2). Plasmids were kindly provided by Mauricio Terebiznik, University of Toronto, CA. The authors would like to thank Andris K. Walter for English language revision and manuscript copyediting assistance.

DISCLOSURES

The funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

1 Goto, H., Lauletta Lindoso, J. A. Cutaneous and mucocutaneous leishmaniasis. *Infectious Disease Clinics of North America.* **26** (2), 293-307 (2012).

2 World Health, O. Control of the leishmaniases. *World Health Organization Technical Report Series.* (949), xii-xiii, 1-186, back cover (2010).

3 Alexander, J., Russell, D. G. The interaction of Leishmania species with macrophages. *Advances in Parasitology.* **31**, 175-254 (1992).

4 Mosser, D. M., Rosenthal, L. A. Leishmania-macrophage interactions: multiple receptors, multiple ligands and diverse cellular responses. *Seminars in Cell Biology.* **4** (5), 315-322 (1993).

5 Awasthi, A., Mathur, R. K., Saha, B. Immune response to Leishmania infection. *Indian Journal of Medical Research.* **119** (6), 238-258 (2004).

6 Blackwell, J. M. Role of macrophage complement and lectin-like receptors in binding Leishmania parasites to host macrophages. *Immunology Letters.* **11** (3-4), 227-232 (1985).

7 Mosser, D. M., Edelson, P. J. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of Leishmania promastigotes. *Journal of Immunology.* **135** (4), 2785-2789 (1985).

8 Gough, P. J., Gordon, S. The role of scavenger receptors in the innate immune system. *Microbes and Infection.* **2** (3), 305-311 (2000).

9 Russell, D. G., Wilhelm, H. The involvement of the major surface glycoprotein (gp63) of Leishmania promastigotes in attachment to macrophages. *Journal of Immunology.* **136** (7), 2613-2620 (1986).

10 Handman, E., Goding, J. W. The Leishmania receptor for macrophages is a lipid-containing glycoconjugate. *EMBO J.* **4** (2), 329-336 (1985).

11 Holm, A., Tejle, K., Magnusson, K. E., Descoteaux, A., Rasmusson, B. Leishmania donovani lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKCalpha and defective phagosome maturation. *Cellular Microbiology.* **3** (7), 439-447 (2001).

12 Vergne, I. *et al.* Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America.* **102** (11), 4033-4038 (2005).

13 Courret, N., Lang, T., Milon, G., Antoine, J. C. Intradermal inoculations of low doses of Leishmania major and Leishmania amazonensis metacyclic promastigotes induce different immunoparasitic processes and status of protection in BALB/c mice. *International Journal for Parasitology.* **33** (12), 1373-1383 (2003).

14 Gutierrez, M. G. *et al.* Autophagy induction favours the generation and maturation of the Coxiella-replicative vacuoles. *Cellular Microbiology.* **7** (7), 981-993 (2005).
15 Dermine, J. F., Scianimanico, S., Prive, C., Descoteaux, A., Desjardins, M. Leishmania promastigotes require lipophosphoglycan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis. *Cellular Microbiology.* **2** (2), 115-126 (2000).
16 Desjardins, M., Descoteaux, A. Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. *Journal of Experimental Medicine.* **185** (12), 2061-2068 (1997).
17 Antoine, J. C., Prina, E., Lang, T., Courret, N. The biogenesis and properties of the parasitophorous vacuoles that harbour Leishmania in murine macrophages. *Trends in Microbiology.* **6** (10), 392-401 (1998).
18 Alexander, J. *et al.* An essential role for IL-13 in maintaining a non-healing response following Leishmania mexicana infection. *European Journal of Immunology.* **32** (10), 2923-2933 (2002).
19 Aderem, A., Underhill, D. M. Mechanisms of phagocytosis in macrophages. *Annual Review of Immunology.* **17**, 593-623 (1999).
20 Olivier, M., Gregory, D. J., Forget, G. Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view. *Clinical Microbiology Reviews.* **18** (2), 293-305 (2005).
21 English, D., Andersen, B. R. Single-step separation of red blood cells. Granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. *Journal of Immunology Methods.* **5** (3), 249-252 (1974).
22 Petersen, A. L. *et al.* 17-AAG kills intracellular Leishmania amazonensis while reducing inflammatory responses in infected macrophages. *PLoS One.* **7** (11), e49496 (2012).
23 Maess, M. B., Wittig, B., Lorkowski, S. Highly efficient transfection of human THP-1 macrophages by nucleofection. *Journal of Visualized Experiments.* 10.3791/51960 (91), e51960 (2014).
24 Berges, R. *et al.* End-binding 1 protein overexpression correlates with glioblastoma progression and sensitizes to Vinca-alkaloids in vitro and in vivo. *Oncotarget.* **5** (24), 12769-12787 (2014).
25 Franco, L. H. *et al.* The Ubiquitin Ligase Smurf1 Functions in Selective Autophagy of Mycobacterium tuberculosis and Anti-tuberculous Host Defense. *Cell Host & Microbe.* **22** (3), 421-423 (2017).
26 Corbett-Nelson, E. F., Mason, D., Marshall, J. G., Collette, Y., Grinstein, S. Signaling-dependent immobilization of acylated proteins in the inner monolayer of the plasma membrane. *Journal of Cell Biology.* **174** (2), 255-265 (2006).
27 Yeung, T. *et al.* Receptor activation alters inner surface potential during phagocytosis. *Science.* **313** (5785), 347-351 (2006).
28 Romano, P. S., Gutierrez, M. G., Beron, W., Rabinovitch, M., Colombo, M. I. The autophagic pathway is actively modulated by phase II Coxiella burnetii to efficiently replicate in the host cell. *Cellular Microbiology.* **9** (4), 891-909 (2007).
29 Vieira, O. V. *et al.* Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. *Molecular and Cellular Biology.* **23** (7), 2501-2514 (2003).
30 Roberts, R. L., Barbieri, M. A., Ullrich, J., Stahl, P. D. Dynamics of rab5 activation in endocytosis and phagocytosis. *Journal of Leukocyte Biology.* **68** (5), 627-632 (2000).

31 Vitelli, R. *et al.* Role of the small GTPase Rab7 in the late endocytic pathway. *Journal of Biological Chemistry.* **272** (7), 4391-4397 (1997).
32 Matte, C. *et al.* Leishmania major Promastigotes Evade LC3-Associated Phagocytosis through the Action of GP63. *PLoS Pathogens.* **12** (6), e1005690 (2016).
33 Dias, B. R. S. *et al.* Autophagic Induction Greatly Enhances Leishmania major Intracellular Survival Compared to Leishmania amazonensis in CBA/j-Infected Macrophages. *Frontiers in Microbiology.* **9**, 1890 (2018).
34 Babcock, G. F. Quantitation of phagocytosis by confocal microscopy. *Methods in Enzymology.* **307**, 319-328 (1999).
35 Sanderson, M. J., Smith, I., Parker, I., Bootman, M. D. Fluorescence microscopy. *Cold Spring Harbor Protocols.* **2014** (10), pdb top071795 (2014).
36 Lennartz, M. R. Phospholipases and phagocytosis: the role of phospholipid-derived second messengers in phagocytosis. *International Journal of Biochemistry & Cell Biology.* **31** (3-4), 415-430 (1999).
37 Rashidfarrokhi, A., Richina, V., Tafesse, F. G. Visualizing the Early Stages of Phagocytosis. *Journal of Visualized Experiments.* 10.3791/54646 (120) (2017).
38 Ramarao, N., Meyer, T. F. Helicobacter pylori resists phagocytosis by macrophages: quantitative assessment by confocal microscopy and fluorescence-activated cell sorting. *Infection and Immunity.* **69** (4), 2604-2611 (2001).
39 Bain, J., Gow, N. A., Erwig, L. P. Novel insights into host-fungal pathogen interactions derived from live-cell imaging. *Seminars in Immunopathology.* **37** (2), 131-139 (2015).

Figure 1

Click here to access/download;Figure;Fig 1) Binding.tif

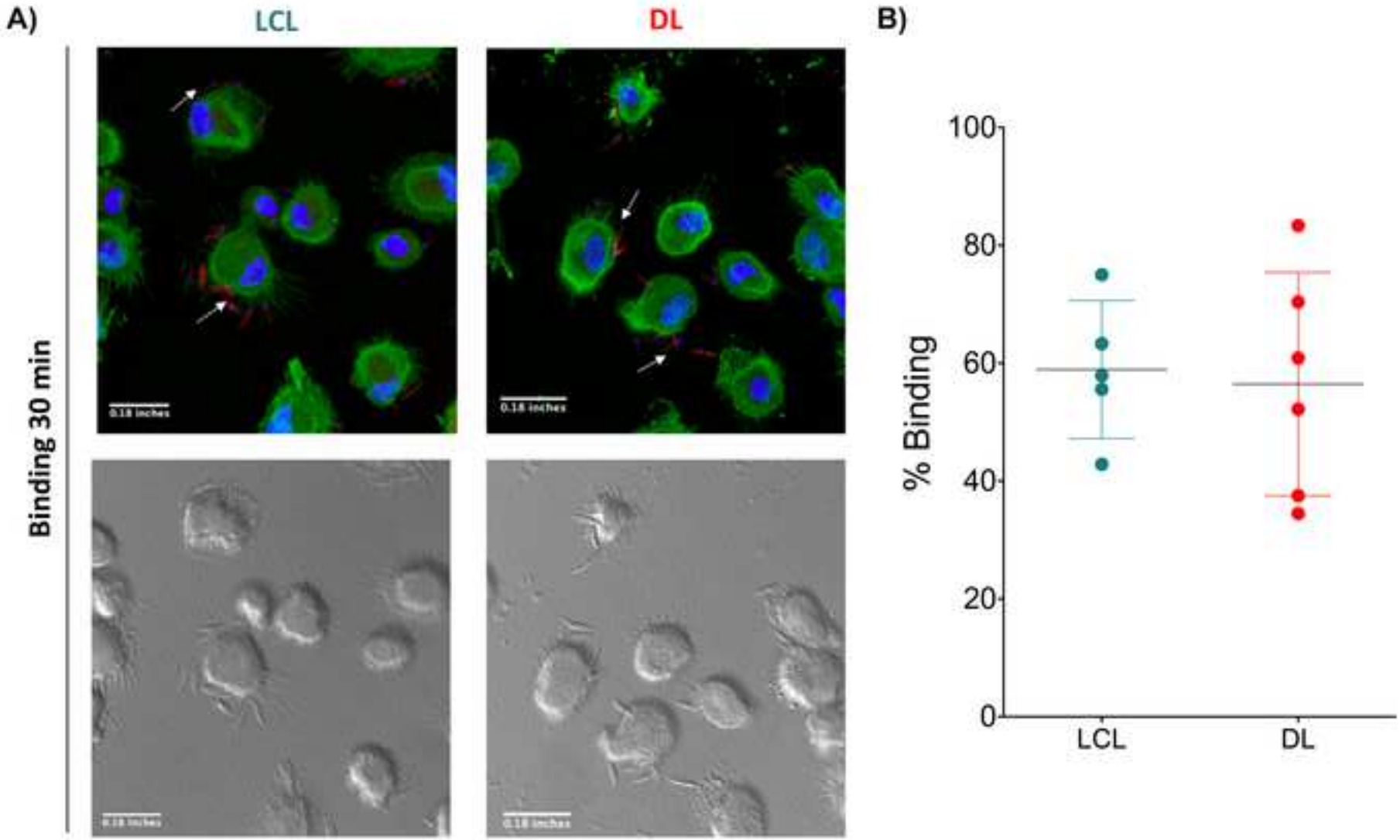

Figure 2

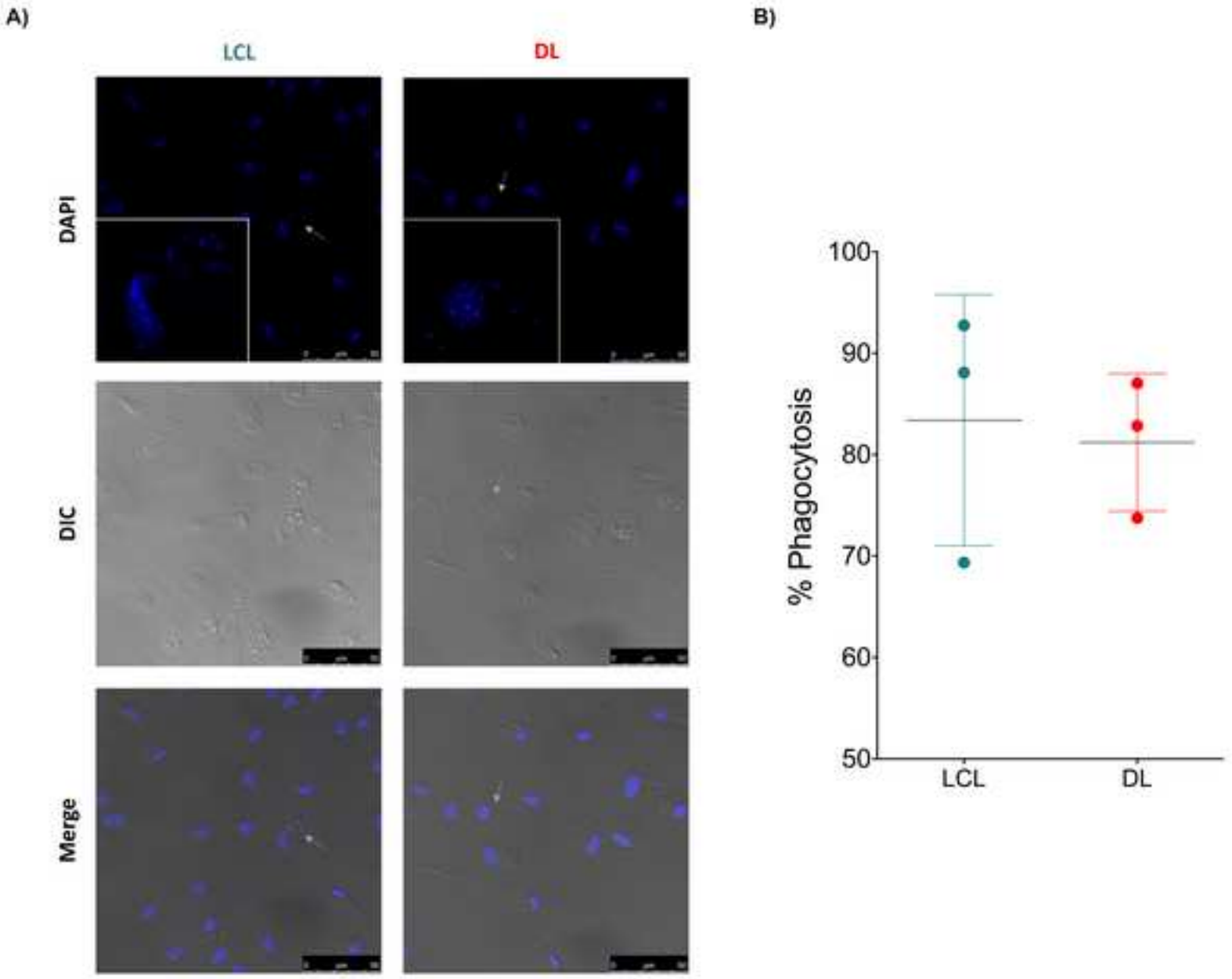

Figure 3

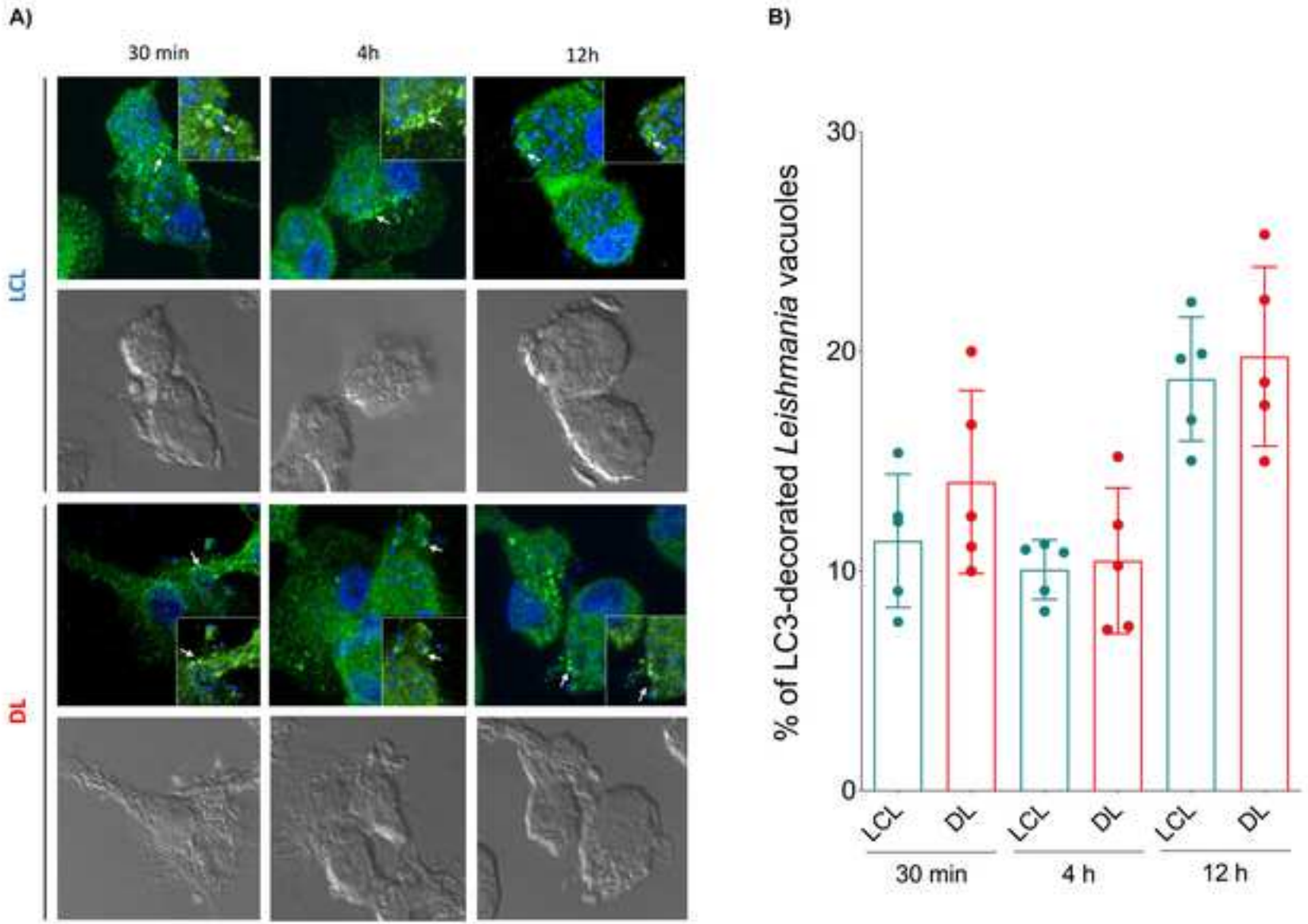

Figure 4

Click here to access/download;Figure;Fig 4) Nucleofection.tif

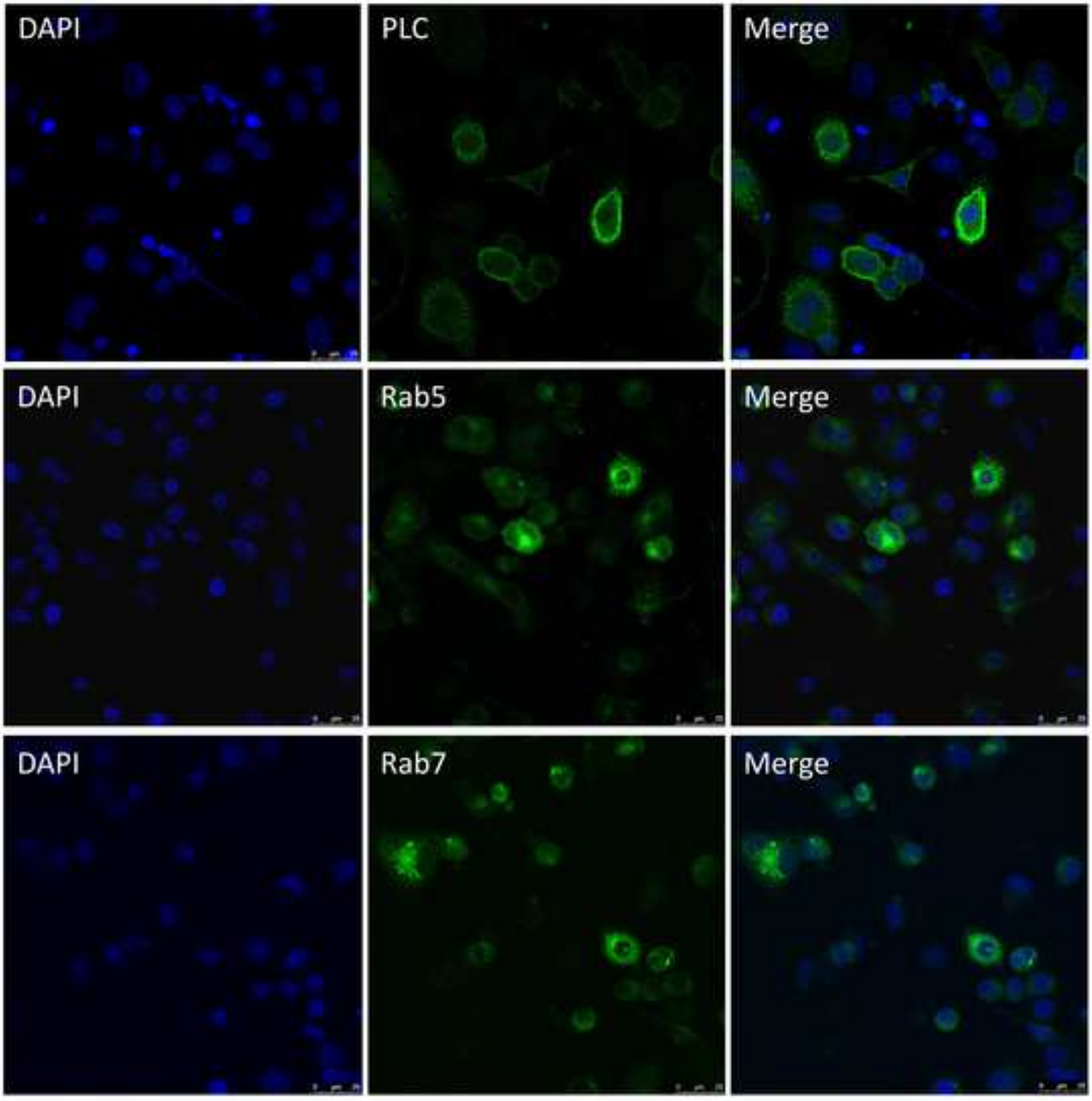

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_PAIXAO_AR_2020.xls

Name of Material/ Equipment	Company	Catalog Number
2-mercaptoethanol	Thermo Fisher Scientific	21985023
AlexaFluor 488-conjugated goat anti-rabbit IgG	Thermo Fisher Scientific	Tem varios no site
anti-LC3 antibody	Novus Biologicals	NB600-1384
Bovine serum albumin (BSA)	Thermo Fisher Scientific	X
CellStripper	Corning	25-056-CI
CellTracker Red (CMTPX) Dye	Thermo Fisher Scientific	C34552
Centrífuga	Thermo Fisher Scientific	
Ciprofloxacin	Isofarma	X
CO2 incubator	Thermo Fisher Scientific	X
Confocal fluorescence microscope (Leica SP8)	Leica	Leica SP8
Fetal Bovine Serum (FBS)	Gibco	10270106
Fluorescence microscope (Olympus Lx73)	Olympus	Olympus Lx73
Gentamicin	Gibco	15750045
Glutamine	Thermo Fisher Scientific	35050-061
HEPES (N- 2-hydroxyethyl piperazine-N'-2-ethane-sulfonic acid)	Gibco	X
Histopaque	Sigma	10771
M-CSF	Peprotech	300-25
NH_4Cl	Sigma	A9434
Normal goat serum	Sigma	NS02L
Nucleofector 2b Device	Lonza	AAB-1001
Nucleofector solution	Lonza	VPA-1007
Paraformaldehyde	Sigma	158127
Phalloidin	Invitrogen	A12379
Phorbol myristate acetate (PMA)	Sigma	P1585
Phosphate buffer solution (PBS)	Thermo Fisher Scientific	10010023
ProLong Gold Antifade kit	Life Technologies	P36931
Roswell Park Memorial Institute (RPMI) 1640 medium	Gibco	11875-093
Saponin	Thermo Fisher Scientific	X
Schneider's Insect medium	Sigma	S0146

Sodium bicarbonate	Sigma		S5761
Sodium pyruvate	Sigma		S8636
Triton X-100	Sigma	X	

Comments/Description

Investigating the Phagocytosis of *Leishmania* using Confocal Microscopy

Amanda R. Paixão[1†], Beatriz R. S. Dias[1†], Luana C. Palma[1], Natália M. Tavares[1], Cláudia I. Brodskyn[1], Juliana P. B de Menezes[1] and Patricia S. T. Veras[1,2*]

[1] Laboratory of Host-Parasite Interaction and Epidemiology, Gonçalo Moniz Institute

[2] National Institute of Science and Technology of Tropical Diseases - National Council for Scientific Research and Development (CNPq)

[†]These authors have contributed equally to this work

***Correspondence:**
Corresponding Author:
patricia.veras@fiocruz.br; pstveras@gmail.com

April 26, 2021

Dear Editor,

We would like to thank you for yours and referees' contributions that will undoubtedly improve the present manuscript to be fitted for publication at JoVE. We have carefully considered the criticisms and suggestions that have been made and would like to let you know that every amendment and clarification are included (in blue) in this rebuttal letter. In addition, an extensive revision by a native English speaker has been made throughout the text to improve manuscript clarity.

Sincerely,

Patrícia Sampaio Tavares Veras, PhD

Corresponding author:

Patricia Sampaio Tavares Veras

Address: Laboratory of Parasite – Host Interaction and Epidemiology, Gonçalo Moniz Institute, FIOCRUZ. Rua Waldemar Falcão, 121, Candeal - Salvador/BA, Brazil. Zip Code: 40296-710
Phone: +55 71 8122-0451
Email: patricia.veras@fiocruz.br; pstveras@gmail.com

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript entitled "Investigating the phagocytosis….." gives methodology for studying binding, and phagocytosis of leishmanial by macrophages. It also provides pointers to study the involvement of various macrophage compartments/processes or molecules in phagocytosis & maturation of PVs. While it provides methodology to explore is a very much required field of pathogen-host immune cell interaction and phagocytosis, there are a large number of concerns or issues which must be addressed before it can be published.

Major Concerns:

1. First major concern is that though this manuscript describes the methods for visualization of *Leishmania*-macrophage interactions by confocal fluorescence microscopy, the authors have not described the methods for fluorescent labelling of *Leishmania* by some dye like CFSE or by transfecting with GFP-containing plasmid. This is a very vital part of this methodology & should be included.

Authors answer (AA): We thank the referee for this suggestion and would like to emphasize that our experiments labeled parasites with the fluorescent membrane marker Cell tracker RED CMTPX dye; this is now properly described in section 2 (lines 141-160) of the protocol (Dagley, 2015). In the NOTE (lines 142-145), we included the alternative procedures to visualize *Leishmania*-macrophage interactions by confocal fluorescence microscopy.

NOTE: To visualize parasites through fluoresce microscopy, perform staining using CellTracker Red fluorescent dye (CMTPX). Alternatively, other markers, including carboxyfluorescein can be used in accordance with manufacturer instructions or promastigotes constitutively expressing GFP, RFP, or other fluorescent reporter genes.

Dagley MJ, Saunders EC, Simpson KJ, McConville MJ. 2015. Highcontent assay for measuring intracellular growth of Leishmania in human macrophages. Assay Drug Dev Technol 13:389 –401.

2. In Protocol no. 5, the authors have mentioned nucleofection as method of transfection into THP-1 cells. This is a very specific method which requires a very specific apparatus the Nucleofector, which may not be available to most researchers. Hence, it is important to provide some alternative general transfection protocol in NOTE, which can be utilized by most researchers.

AA: We agree with referee's suggestion and have included a NOTE (lines 203-205) indicating that researchers can alternatively use lipofectamine (Berges et al, 2014) and lentivirus transduction (Franco et al, 2017) as a method of cell transfection.

Berges, R. et al. End-binding 1 protein overexpression correlates with glioblastoma progression and sensitizes to Vinca-alkaloids in vitro and in vivo. *Oncotarget*. 5 (24), 12769-12787, (2014).

Franco, L. H. et al. The Ubiquitin Ligase Smurf1 Functions in Selective Autophagy of Mycobacterium tuberculosis and Anti-tuberculous Host Defense. *Cell Host Microbe*. 22 (3), 421-423, (2017).

3. Also the authors have described transfection with PLC, Rab, LAMP etc plasmid to visualize their involvement in phagocytosis-related pathways. I stead of using transfections, labelling endogenous proteins with fluorescently-tagged specific antibodies may be a better approach. This will provide a more authentic/physiologically relevant picture of phenomenon inside the host cell during phagocytosis, as GFP-tagged transfected proteins can many times be mistargeted, and hence expressed ectopically inside the cell.

AA: With all due respect, we disagree with the reviewer's criticism and submit that many well-respected studies have successfully employed this approach. Our transfection of cells with plasmids containing gene sequences (PLC [22,23], Akt [22,23], Rab 5[24-26]or Rab 7[24,25,27]) tagged with GFP or RFP genes have been widely used to follow the dynamics of protein recruitment in phagosome formation and maturation phases.

22 Corbett-Nelson, E. F., Mason, D., Marshall, J. G., Collette, Y. & Grinstein, S. Signaling-dependent immobilization of acylated proteins in

the inner monolayer of the plasma membrane. *J Cell Biol*. 174 (2), 255-265, (2006).
23 Yeung, T. et al. Receptor activation alters inner surface potential during phagocytosis. *Science*. 313 (5785), 347-351, (2006).
24 Romano, P. S., Gutierrez, M. G., Beron, W., Rabinovitch, M. & Colombo, M. I. The autophagic pathway is actively modulated by phase II Coxiella burnetii to efficiently replicate in the host cell. *Cell Microbiol*. 9 (4), 891-909, (2007).
25 Vieira, O. V. et al. Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. *Mol Cell Biol*. 23 (7), 2501-2514, (2003).
26 Roberts, R. L., Barbieri, M. A., Ullrich, J. & Stahl, P. D. Dynamics of rab5 activation in endocytosis and phagocytosis. *J Leukoc Biol*. 68 (5), 627-632, (2000).
27 Vitelli, R. et al. Role of the small GTPase Rab7 in the late endocytic pathway. *J Biol Chem*. 272 (7), 4391-4397, (1997).

4. In Representative Results Figure 3, the confocal microscope images for phagocytosis are not shown. They should be added along with the quantitation data.

AA: We are grateful to the referee for pointing this out, and have included phagocytosis image in Figure 2 to better reflect the information displayed.

5. In Representative Results Figure 4 is not very clear or convincing. The data shown is of 3 different time points, but only one time point images are shown. Even in these the points made are not very clear. A proper zoom in & highlight of the area showing LC3 recruitment should be properly represented & pointed out.

AA: The referee raises a valid point, and we have now included images of the 3 different time points and appropriate images zoomed in on the LC3 recruitment area in figure 3 .

Minor Concerns:

Minor Points

1. In abstract & elsewhere in manuscript many abbreviations are used without providing full forms. This should be rectified.

AA: We thank the referee for calling our attention to this oversight and have clarified all abbreviations with full forms throughout the text (lines 30-32; 36-38; 51, 59, 65, 73-75; 104-105; 108, 120-123; 135; 143).

2. CR1 and CR2 are complement receptors, so do the author's mean to imply, that *Leishmania* binding to macrophage occurs through complement. Also, they should clearly discuss if they are talking about phagocytosis of *Leishmania* through opsonization.

AA: Phagocytosis in *Leishmania* occurs either through direct interaction with complement receptors, or by opsonization involving complement receptors. Our study focused exclusively on direct interaction between parasites and host receptors in general, including complement receptors.

This has been clarified in the Introduction and Discussion (lines 55-56) sections.

Introduction (lines 55-56): "…either through direct interaction or by opsonization involving complement receptors4,5.

Discussion (lines 359-362): "To better understand the mechanisms involved in the interaction of unopsonized *Leishmania* and host cells,..".

3. In line 22 change assessment to phagocytosis or interaction.

AA: The authors thank the referee for pointing out this inconsistency and have modified the manuscript as follows:

From (line 22): ”Here we described methods to evaluate the early events occurring during *Leishmania* assessment to the host cells”.

To: (lines 22-23): “Here we described methods to evaluate the early events occurring during *Leishmania* interaction with the host cells”.

4. In line 106, not clear what the NOTE means. Modify/clarify

AA: For clarification purposes, this NOTE has been modified from: “Activate the break-off”

To: “Set centrifuge break-off to avoid mixing of gradient layers” (line 112).

5. In line 107, explain where the ring forms (it's position)

AA: This is a valid point, and we have described the ring position following PBMC centrifugation using a Ficoll gradient as follows:

From: (line 107) “Transfer the PBMC ring formed during centrifugation to a new tube and fill up with PBS”.

To (Lines 113-114): NOTE: “After centrifugation, discontinuous gradient layers are formed from the bottom to the top: erythrocytes, density gradient medium, PBMC ring and plasma”.
(Lines 116-117) ‘’Transfer the PBMC ring, located between the density gradient medium and plasma layers, to a new tube and fill with PBS to wash out excess density gradient medium.

6. In line 109 and many other places where WASH is mentioned, the authors should mention how many washes are recommended at this step.

AA: We have updated the proper washing procedures throughout the manuscript, i.e. “WASH” has been replaced by 1, 2 or 3 washes, accordingly, in lines 118, 137, 158, 167,172, 176, 178, 187, 192, 213, 236, 244, 253, 256).

7. In line 111, 119, mention how many cells are seeded per ml, and in what culture dish/flask or plate.

AA: We thank the reviewer for this comment and have added this information in lines 120-125 and 161-162 as follows:

Line 120-125: “Count the cells and plate 2 × 10^6 cells in 500 mL of Roswell Park Memorial Institute (RPMI) supplemented with 25 mM N-[2-hydroxyethyl] piperazine-N′-[2-ethane sulfonic acid] (HEPES), 2 g/L sodium bicarbonate, 2 mM glutamine, 20 g/mL ciprofloxacin and 10% inactivated Fetal Bovine Serum (FBS) (complete RPMI medium) for 7 days at 37 °C under 5% CO_2 in a 24 well plate to allow monocytes to differentiate into macrophages by adhesion”.

Line 128-129: “Grow THP-1 cell line at a concentration of 2 × 10^5 cells in 10 mL complete RPMI medium in 75 cm^2 culture flask.”

8. In Section 2 of protocols, culture conditions of *Leishmania* are not very clear. Also they have not mentioned how to check the viability or phase of growth of Leishmania.

AA: For clarification, culture conditions and information to check cell viability are now included in the text (lines 151-154):

“After incubating parasite axenic cultures in a biochemical oxygen demand (B.O.D.) 24 ^oC, perform daily counting in a Neubauer chamber, checking for parasite form (thin, elongated) and mobility during 5 days. Parasites are considered in stationary phase of growth when two consecutive counts with 8 hours of interval display similar amounts”.

9. In Section 3 Protocols, Line 151, Medium, volume & culture conditions are not mentioned.

We are grateful for the referee's criticism and this information is now included in the manuscript (Line 164-165):

"Seed 2 × 10^5 THP-1 cells or human monocyte-derived macrophages in 500 μL complete RPMI medium per well on a 24-well plate with 13 mm glass coverslips".

10. Line 157, 177 not clear if in this step cells are in tube or plate.

AA: In accordance with the referee's suggestion, this information has been added in lines 169-170:

"Add stationary phase promastigotes as described by A. L. Petersen, et al. [21] at a 10:1 ratio to well plates, and then centrifuge at 720 × g for 10 min under 4 °C."

Line 187-188 : "Wash cells twice in 0.9% NaCl solution and incubate in complete RPMI medium in 24-well plate at 4 °C for 10 min."

11. In Section 3 Protocols, in later steps the temperature at which processing is done is not mentioned.

AA: The temperatures at which processing was performed have been added to the text in lines 166, 167, 170, 174, 175, 177, 178, 180.

12. According to Section 3 Protocols, to study binding of *Leishmania* to macrophages total time for co-culture of *Leishmania* & macrophages is 15 minutes (10 min in centrifuge + additional mins) but in Figure 2 showing binding, the images were captured at 30 mins. There is a discrepancy in the protocol & the representative figure in results.

AA: To clarify protocol procedure, the authors replaced 30 min for 10 min (5 min in centrifuge + 5 min additional incubation) at 4°C, the correct period of time that the parasites established the contact with the macrophages:

(lines 169-170): Add stationary phase as described by Petersen et al, 2012.[21] at a 10:1 ratio to well plates, and then centrifuge at 720 × g for 10 min under 4 ^{o}C.

Petersen A.L.D.O.A., Guedes C.E.S., Versoza C.L., Lima J.G.B., de Freitas L.A.R., Borges V.M., Veras P.S.T. 17-AAG Kills Intracellular *Leishmania amazonensis* while Reducing Inflammatory Responses in Infected Macrophages.*PLoS ONE.* 2012.

13. In Protocol 4, line 174, media used and volume is not mentioned.

AA: This information has been included in the manuscript (line 184-185).

14. In line 176 temperature & conditions of cultivation are not mentioned

AA: We have updated the manuscript to include all relevant temperature and culture conditions as requested.

15. All the media used should be clearly described (complete medium, supplemented medium etc.)

AA: All media used in the experiments have been properly described throughout the manuscript: lines 119, 123, 129,132, 135, 137, 139, 149, 159, 165, 167, 185,187,194, 212, 216,225, 234, 236, 243.

16. Line 191, Objective is missing at the end.

AA: We have specified information relevant to the “objective” in the text as follows:

From (line 191-192): “Count no less than 400 cells in random fields under a fluorescence microscope (Olympus Lx73) using a 100x/1.4”

To (line 197-198): “Count no less than 400 cells in random fields under a fluorescence microscope using a 100×/1.4 objective.”

17. Line 210, appropriate references should be quoted for full details about the plasmids used

AA: We agree with the referee’s suggestion and have modified the manuscript as follows:
From (line 206 - 210): NOTE: To investigate the biogenesis of *Leishmania*-induced PV, transfect the THP1 cells at least with PLC, Akt, Rab 5, Rab 7, LAMP,

or LC3 plasmid (kindly provided by Mauricio Terebiznik, University of Toronto, CA).

To (line 206-207): “To investigate the biogenesis of Leishmania-induced PV, transfect THP1 cells with PLC [22,23], Akt [22,23], Rab 5[24-26] or Rab 7[24,25,27] plasmids.

18. In line 215, 230 the amount of medium is not written.

AA: We have included this information on lines 211 and 225.

From (line 215): **“**Recover the transfected cells and seed them in RPMI medium in 24-well plates containing the 12 mm glass coverslips (4 wells/transfection).”

To (line 223-224) : “Recover the transfected cells ($2x10^6$) and seed in 500 μL RPMI medium on 24-well plates with 13 mm glass coverslips”.

From (line 230): “Seed $2x10^5$ THP-1 cells or human monocyte-derived macrophages in a 24-well plate containing 13 mm glass coverslip.”

To (line 184-185) : “Seed 2×10^5 THP-1 cells or human monocyte-derived macrophages in 500 μL complete RPMI medium on a 24-well plate with 13 mm glass coverslips.”

19. Line 245-247, 252 temperatures for these steps is not mentioned.

AA: Temperature and condition have been included on lines 247-251.

From (line 245- 247): :Simultaneously block and permeabilize the fixed cells in 0.1% Triton X-100, 1% BSA, 20% normal goat serum, 6% non-fat dry milk, and 50% FBS for 20 min. “

“Incubate the cells with anti-LC3 antibody (1: 200) diluted in PBS for 2 h.”

To (line 246-249): “Simultaneously block and permeabilize the fixed cells in 0.1% Triton X-100, 1% BSA, 20% normal goat serum, 6% non-fat dry milk, and 50% FBS for 20 min at room temperature.

"Incubate the cells with anti-LC3 antibody (1: 200) diluted in PBS for 2 h at room temperature."

20. Line 251, not mentioned with what to wash.

AA: We have clarified the washing solution to use and modified the manuscript as follows on line 253: "Wash the cells thrice with 0.9% NaCl solution at room temperature".

21. Line 254, Is it required to fix cells again after staining with different antibodies?

AA: No, these cells were fixed only once.

22. Line 273, What slice thickness is recommended for Z-sectioning to get good confocal images of macrophages and Leishmania?

AA: In our experiments, we performed a slice of 20 μm thickness to obtain confocal images with good resolution. This information has been added to the protocol (lines 272-273).

23. In Figure-2 the *Leishmania* appears red in color. There is no mention of this staining in protocols or figure legend. What does the arrow point to in the Figure? This should be mentioned in the legend.

AA: We have included a description of parasite staining with CMTPX in section 2 subitem "Parasite culture and Cell Tracker Red staining". We have updated the Figure legend to reflect that the arrow is pointing to *Leishmania*-macrophage binding.

24. In Discussion line 375, Figure 1 is mentioned, but this Figure does not show any phalloidin staining as is mentioned.

AA: We thank the reviewer for pointing out this inconsistency. Cells expressing PLC fluorescent protein were indeed tagged with GFP. We have replaced this sentence as follows:

From (lines 374-375): "Therefore, the cell membrane labeling with phalloidin, a specific marker of F-actin[27], seems to be essential to follow the binding stage of parasite-*Leishmania* interaction as shown in **Figure 1."**

To (lines 382-284) "We then labeled proteins located in the cell membrane using a specific marker of phagocytosis[29], such as fluorescent PLC, which allowed us to observe the binding stage of *Leishmania* to host cells, as shown in **Figure 4**."

Reviewer #2:

Manuscript Summary:
The authors describe protocols for the assessment of *Leishmania* binding and phagocytosis on human macrophages. Also, protocols for the maturation process of Leishmania-induced vacuoles and LC3 recruitment are displayed. They finalize the manuscript by presenting the protocol for confocal acquisition and further analysis.

Major Concerns:
None.

Minor Concerns:
The introduction and discussion are appropriate to this manuscript. A few details on the protocols could be better described.

Line 105 and 109: Why centrifuge the tubes at 24ºC and then at 4ºC?

AA: Centrifugation procedures are first performed at room temperature to avoid hemolysis in the PBMC separation process. For reference, a seminal work has been cited in line 102: “To obtain human monocyte-derived macrophages, collect blood (50 mL) from healthy donors and purify peripheral blood mononuclear cells (PBMC) and described by English and Andersen, 1974 [20] for *in vitro* differentiation into macrophages.”

English, D. & Andersen, B. R. Single-step separation of red blood cells. Granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. *J Immunol Methods*. 5 (3), 249-252, (1974).

Line 110: The cells are kept in PBS during counting. Why not already in complete RPMI?

AA: Have updated the text to reflect that RPMI medium was used for ressuspend cells; unfortunately, this information was omitted from the text. Accordingly, the proper procedure now states:

From (line 110): “Discard the supernatant and resuspend in 1 mL of PBS.”

To (lines 119): “ Discard the supernatant and resuspend pellet in 1 mL of complete RPMI medium.”

Line 132: describe free RPMI.

AA: For clarification, the authors have replaced “free RPMI” with “ PMA-free RPMI” (line 145-146).

From: (line 132): “Reincubate the cells with free RPMI medium for additional two days before starting the experiment.”

To: (lines 139-140): “Reincubate differentiated THP-1 cells in PMA-free RPMI complete medium at 37 °C under 5% CO_2 for an additional 2 days before starting experimentation.”

Line 136 and 142: correct the units.

AA: Units have been corrected throughout the text in lines 120, 135, 148, 149,159, 184, 218, 223, 233.

Line 137: define BOD

AA: BOD has been defined in line 151 as biochemical oxygen demand incubator.

Line 140: NOTE: The authors should be more precise on the promastigote's culture day for infection protocol.

AA: In accordance with the referee’s suggestion, this NOTE has been modified as follows:

From (line 140): “NOTE: Use parasites at the stationary phase of growth.”

To (lines 146-147): NOTE: Parasites used to infect cells are those at stationary phase of growth obtained from a promastigote axenic culture of no more than 7 passages.”

Line 157 and 180: Present protocol for metacyclic isolation or add references for the different species of *Leishmania*.

AA: As suggested, the authors have made it clear that promastigote forms in the stationary phase of growth were used to infect cells. This information has been included in the text (lines 169-170)

(Lines 169-170): “Add stationary phase promastigotes as described by A. L. Petersen*, et al.* [21] at a 10:1 ratio to well plates, and then centrifuge at 720 × g for 5 min under 4°C.”

Line 219-220: Which fluorochromes are present on each plasmid? And could it be performed similarly by using monoclonal antibodies to these proteins?

AA: In most of the experiments, we did not use antibodies labelled with fluorochromes; rather, we employed cells expressing proteins tagged with GFP or RFP (red fluorescent protein) that were previously transfected with plasmids containing gene sequences of fusion proteins expressing GFP (green fluorescent protein) or RFP and the protein of interest. This approach has been largely used to characterize protein participation in phagocytosis and the biogenesis of pathogen-induced vacuoles. Although it is possible to use monoclonal antibodies to label the proteins of interesting, employing cell expressing fluorescent fusion proteins has the advantage of using non-functional mutant proteins as experiment controls ([26]).

Romano, P. S., Gutierrez, M. G., Beron, W., Rabinovitch, M. & Colombo, M. I. The autophagic pathway is actively modulated by phase II Coxiella burnetii to efficiently replicate in the host cell. Cell Microbiol. 9 (4), 891-909, (2007).