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Cell Migration and Cell Adhesion Assays to Investigate Leishmania-Host Cell Interaction

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TITLE:

Cell Migration and Cell Adhesion Assays to Investigate *Leishmania*-Host Cell Interaction

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SUMMARY:

Here we study implications of *Leishmania*-host interaction by exploring *Leishmania*-infected dendritic cells migration. The differentiation and infection of dendritic cells, migration analysis, and the evaluation of adhesion complexes and actin dynamics are described. This method can be applied to other host cell migration studies when infected with *Leishmania* or other intracellular parasite species.

ABSTRACT

Leishmania is an intracellular protozoan parasite that causes a broad spectrum of clinical manifestations, ranging from self-resolving localized cutaneous lesions to a highly fatal visceral form of the disease. An estimated 12 million people worldwide are currently infected, and another 350 million face risk of infection. It is known that host cells infected by *Leishmania* parasites, such as macrophages or dendritic cells, can migrate to different host tissues, yet how migration contributes to parasite dissemination and homing remains poorly understood. Therefore, assessing these parasites' ability to modulate host cell response, adhesion, and migration will shed light on mechanisms involved in disease dissemination and visceralization. Cellular migration is a complex process in which cells undergo polarization and protrusion, allowing them to migrate. This process, regulated by actin and tubulin-based microtubule dynamics, involves different factors, including the modulation of cellular adhesion to the substrate. Cellular adhesion and migration processes have been investigated using several models. Here, we describe a method to characterize the migratory aspects of host cells

during *Leishmania* infection. This detailed protocol presents the differentiation and infection of dendritic cells, the analysis of host cell motility and migration, and the formation of adhesion complexes and actin dynamics. This *in vitro* protocol aims to further elucidate mechanisms involved in *Leishmania* dissemination within vertebrate host tissues and can also be modified and applied to other cell migration studies.

INTRODUCTION:

Leishmaniasis, a neglected tropical disease caused by protozoan parasites belonging to the genus *Leishmania,* results in a wide-ranging spectrum of clinical manifestations, from self-healing localized cutaneous lesions to fatal visceral forms of the disease. It has been estimated that up to one million new leishmaniasis cases arise annually, with a reported 12 million people currently infected worldwide[1]. Visceral leishmaniasis (VL), which can be fatal in over 95% of cases when left untreated, causes more than 50,000 deaths annually, affecting millions in South America, East Africa, South Asia, and the Mediterranean region[2]. The main etiological agent of VL in the new world, *Leishmania infantum*, is transmitted to humans by infected female sandflies during blood-feeding[3]. These parasites are recognized and internalized by phagocytes, e.g., macrophages and dendritic cells[3–5]. Inside these cells, parasites differentiate into their intracellular forms, known as amastigotes, which will then multiply and be transported *via* the lymphatic system and bloodstream to different host tissues[6,7]. However, the mechanisms by which *Leishmania* parasites are disseminated in the vertebrate host, as well as the role played by host cell migration in this process, remain unclear.

Cell migration is a complex process executed by all nucleated cells, including leukocytes[8]. According to the classic cycling model of cell migration, this process involves several integrated molecular events that can be divided into five steps: leading-edge protrusion; adhesion of the leading edge to matrix contacts; contraction of cellular cytoplasm; release of the rear edge of the cell from contact sites; and the recycling of membrane receptors from the rear to the front of the cell[9].

For cell migration to occur, protrusions must be formed and then stabilized through attachment to the extracellular matrix. Among the different receptor types involved in the promotion of cell migration, integrins are notable. Integrin activation results in migration-related signaling; intracellular signaling then occurs via focal adhesion kinase (FAK) and Src family kinases, in addition to talin, vinculin, and paxillin molecues[10-12]. The phosphorylation of paxillin by activated kinases, including FAK, leads to the recruitment of effector molecules, which transduces external signals that prompt cell migration. It has been shown that paxillin is an intracellular molecule that is crucial to cell adhesion, actin polymerization, and cell migration processes[13–15].

The actin cytoskeleton plays a central role in the polarization and migration of phagocytes[16]. During cell migration process, protrusions formed due to actin polymerization become stabilized through cell adhesion to the extracellular matrix. This process may be modulated by integrin receptors associated with the actin cytoskeleton[17–19]. Several actin-binding proteins regulate the rate and organization of actin polymerization in cellular protrusions[20]. Studies have shown that RhoA, Rac, and Cdc42 regulate actin reorganization after the stimulation of adherent cells by

extracellular factors[21, 22]. During migration, Rac1 and Cdc42 are located at the leading edge of the cell, controlling the extension of lamellipodia and filopodia, respectively, while RhoA, located at the rear of the cell, regulates the contraction of the actomyosin cytoskeleton[15, 23–25].

Studies have shown that *Leishmania* infection modulates host cell functions, such as adhesion to the cellular substrate and migration[26–31]. Immature DCs reside in peripheral tissues; upon interaction with PAMPS, these cells become activated and migrate to the draining lymph nodes, prompting antigen presentation to T cells. A previous study using a mouse model showed that *L. amazonensis* infection provokes a reduction in the migration of DCs to draining lymph nodes[29]. It was also demonstrated that the inhibition of the adhesion process reduced DC migration after infection with *L. major*[30]. Nonetheless, the impact of DC migration on parasite dissemination in the host, as well as the mechanisms involved in this process, remain poorly understood.

Here we present a compiled step-by-step protocol to perform an *in vitro* adhesion and migration assay involving human DCs infected by *Leishmania*. This method comprises not only the differentiation and infection of DCs, but also permits the analysis of host cell motility and migration, the formation of adhesion complexes, as well as actin dynamics. The presently described *in vitro* protocol allows researchers to further investigate the mechanisms involved in *Leishmania* dissemination within vertebrate host tissues and can also be manipulated and applied to other cell migration studies.

PROTOCOL

The procedures described herein were approved by the Institutional Review Board of the Gonçalo Moniz Institute (IGM-FIOCRUZ, protocol no. 2.751.345). Blood samples were obtained from healthy volunteer donors. Animal experimental procedures were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian law 11.784/2008 and were approved and licensed by the Ethical Committee for Animal Research of the Gonçalo Moniz Institute (IGM-FIOCRUZ, protocol no. 014/2019).

1. Isolation and differentiation of human dendritic cells

1.1 Pipette 10 mL of the density gradient medium in 50 mL centrifuge tubes.

1.2 Label the 50 mL tubes according to each donor's sample.

1.3 Collect ~ 50 mL of venous blood from healthy donors and after collection, follow the procedures described below in the flow cabinet.

1.4 Carefully transfer the collected blood into centrifuge tubes and dilute in saline solution (0.9% sodium chloride) in a ratio of 1:1 at room temperature.

1.5 Slowly overlay the diluted blood on the top of the density gradient medium.

1.6 Centrifuge tubes containing the blood and density gradient medium at 400 x *g* for 30 min at room temperature.

NOTE: Switch off the brake before centrifuging to prevent the mixing of gradient layers. After the 1st centrifugation, lower the centrifuge temperature to 4 °C.

1.7 Carefully remove the tubes from the centrifuge.

1.8 Identify the ring of peripheral blood mononuclear cells (PBMC) in the sample (buffy coat); gently remove the residual plasma with a pipette.

NOTE: After centrifugation, the following gradient layers will have formed: erythrocytes, density gradient medium, PBMC ring, and plasma. The PBMC ring is located between the density gradient medium and plasma layers.

1.9 Transfer the cloud-like PBMC layer to a new tube and bring up the volume to 30 mL with saline solution.

1.10 Centrifuge the tubes containing cell suspension at 250 x *g* for 10 min at 4 °C. Discard the supernatant and resuspend the pellet in 1 mL of saline solution.

1.11 Collect an aliquot for cell counting with the trypan blue exclusion method. First, dilute at a ratio of 1:1,000. Then, use 10 µL of the diluted cells for trypan blue staining and count cells using a Neubauer chamber to determine cell viability.

1.12 Centrifuge again at 200 x *g* for 10 min under 4 °C.

1.13 Resuspend the pellet in MACS buffer. Use 80 µL of the buffer for every 1 x 10^7 cells.

NOTE: MACS buffer composition: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA. Dilute the buffer solution at a 1:20 ratio with the rinsing solution. Keep the buffer cold and store at 2-8 °C.

1.14 Add CD14 microbeads to the cell suspension prepared in step 1.13. Use 20 µL of CD14 microbeads for every 1 x 10^7 cells.

NOTE: CD14 microbeads are used for the positive selection of human monocytes from PBMCs, as beads bind to CD14-positive cells expressed on most monocytes.

1.15 Homogenize the solution containing the pellet and microbeads using a pipette. Keep on ice for 15 min.

1.16 Centrifuge the suspension at 300 x *g* for 10 min under 4 °C.

1.17 Resuspend cells in MACS buffer. Use 1-2 mL for 1 x 10^7 cells in the cell microbead mixture.

1.18 Centrifuge the suspension at 300 x *g* for 10 min under 4 °C.

1.19 Aspirate the supernatant and resuspend the pellet in 500 µL of MACS buffer.

NOTE: This is the maximum volume of the cell suspension that can be processed through one column.

1.20 Assemble the magnetic column.

1.21 Wash the column once by adding 500 µL of MACS buffer. Allow buffer to flow under gravity through the column.

NOTE: Do not allow the column to dry, as any air that enters can obstruct the column.

1.22 Add 500 µL of the cell sample solution from step 1.19 per column. Allow the cell sample solution to flow under gravity through the column.

1.23 Wash the column by adding 500 µL of MACS buffer (2x). Only add the fresh buffer when the column reservoir is empty. Avoid drying.

1.24 Pipette 1 mL of the MACS buffer onto the column and place it in a new tube underneath. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

1.25 Centrifuge the CD14 enriched cells at 300 x *g* for 10 min under 4 °C.

1.26 Count cells using a Neubauer chamber.

1.27 Resuspend cells in 1 mL complete RPMI with interleukin-4 (IL-4) (100 µL/mL) + granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/mL).

NOTE: GM-CSF is a cytokine secreted by macrophages, T cells, mast cells, natural killer cells, endothelial cells, and fibroblasts that induces the differentiation and proliferation of myeloid progenitors in the bone marrow. GM-CSF and IL-4 are used to induce dendritic cell differentiation[32].

1.28 Seed cells in a 24 well plate at a concentration of 2×10^5 cells per well in 500 µL of complete RPMI medium containing and incubate the cells for 7 days in the cell culture incubator at 37 °C.

2. *Leishmania infantum* cultivation

NOTE: *Leishmania infantum* (MCAN/BR/89/BA262) parasites are used in this assay. Hamsters were intravenously infected with 20 µL of the solution containing 1 x 10^6 *L. infantum* promastigotes in sterile saline. After 1 to 2 months, animals were euthanized and the amastigote forms of *Leishmania* were recovered from their spleens and differentiated into promastigotes[33].

2.1 Grow *L. infantum* promastigotes isolated from previously infected hamster spleens in an inclined 25 cm^2 cell culture flask containing 3 mL of Novy-Nicolle-MacNeal medium (NNN) and 5 mL of supplemented Minimum Essential Medium (MEM).

NOTE: In this assay, MEM medium was supplemented with 10% fetal bovine serum (FBS) and 24.5 nM hemin bovine.

2.2 Incubate at 24 °C for 7 days in BOD (Bio-Oxygen Demand) incubator.

2.3 Pipette 100 µL of promastigote culture into a new 25 cm^2 cell culture flask containing 5 mL of supplemented MEM medium.

2.4 Incubate the promastigote culture at 24 °C in BOD incubator until promastigotes reach the stationary phase. Periodically count cultured promastigotes by transferring an aliquot of parasite suspension diluted in saline to a Neubauer chamber (i.e., hemocytometer) to determine the stationary phase of growth.

NOTE: It is not recommended to use first-passage parasites after cultivation in NNN medium to avoid residual traces of rabbit blood.

2.5 Transfer 1 x 10^5 of stationary growth phase culture of *L. infantum* promastigotes to a new 25 cm^2 cell culture flask and add 5 mL of MEM supplemented medium.

2.6 Monitor the growth of the culture periodically for approximately 7 days using a Neubauer chamber until the stationary phase is achieved. Parasites are now ready for use in experimental infection procedures.

NOTE: Promastigote cultures are considered viable for infection for up to 7 passages *in vitro;* additional passages may induce loss of virulence.

3. Human dendritic cell infection

3.1 Inside a biological laminar flow cabinet, transfer all the contents from the parasite culture flask to 50 mL centrifuge tubes.

3.2 Add cold saline solution to a final volume of 40 mL.

3.3 Centrifuge at 1,600 x *g* for 10 min under 4 °C.

3.4 Discard the supernatant following centrifugation and resuspend the pellet in 1 mL of cold saline solution (Repeat steps 3.3-3.4 three times).

3.5 After washing the cells to remove any non-viable parasites, resuspend the pellet in 1 mL of cold saline solution.

3.6 Pass the contents slowly through a 1 mL syringe with a 16 G needle 5 times to separate the parasite clusters.

3.7 Remove an aliquot to determine the parasite concentration in a hemocytometer (calculate the mean number of parasites in 4 quadrants x dilution factor x 10^4).

3.8 Wash dendritic cells by adding 1 mL of saline solution and centrifuging cells at 300 x *g* for 10 min at room temperature (3 times).

3.9 Calculate the quantity of *L. infantum* required for the experimental infection; ratio: 20 parasites per cell.

3.10 Place the required volume of *L. infantum* in each well of cell culture plates.

3.11 Incubate dendritic cells with parasites for 4 h in an incubator at 37 °C under 5% CO_2.

3.12 After step 3.11, centrifuge plates at 300 x *g* for 10 min under 4 °C.

4. Migration assay using cell culture inserts

4.1 After infection, wash dendritic cells (infected or not) 3 times with 1 mL of saline solution at room temperature to remove non-internalized parasites. After each wash, centrifuge plates at 300 x *g* for 10 min under 4 °C.

4.2 Once non-internalized parasites are removed, detach cells by adding 200 µL of the cell dissociation reagent in each well and incubate for 15 min. Keep the cells in an incubator at 37 °C under 5% CO_2.

4.3 Homogenize cells using a 1,000 µL pipette tip to allow cells to loosen.

4.4 Transfer the dissociated cells to a 50 mL centrifuge tube.

4.5 Centrifuge at 300 x *g* for 10 min under 4 °C.

4.6 Resuspend the pellet in 1 mL of RPMI medium supplemented with 10% FBS.

4.7 Pass the contents of each tube slowly through a 1 mL syringe with a 25 G needle 5 times to separate cells.

4.8 Remove an aliquot, dilute using trypan blue to determine the cell concentration using a hemocytometer (mean of viable cells in 4 quadrants × dilution factor × 10^4).

4.9 Preparation of cell culture microplate inserts:

NOTE: Cell culture inserts (5.0 µM pore polycarbonate membrane) are recommended for migration assays using dendritic cells, macrophages, and monocytes, as these inserts allow phagocytes to pass through the membranes.

4.9.1 Remove inserts with sterile tweezers and place in empty wells.

4.9.2 Add 600 µL of RPMI medium supplemented with 10% FBS to each well; add chemoattractant chemokine (C-C motif) ligand 3 (CCL3) (1 µL for every 1 mL of medium).

NOTE: CCL3 is a chemokine that regulates DC migration[34].

4.9.3 Using sterile tweezers, place inserts in wells containing the medium.

4.10 Add 2 x 10^5 dendritic cells (infected or not) in 100 µL of medium to each insert.

4.11 Incubate for 4 h to allow cells to migrate.

4.12 After 4 h, take the plate out and aspirate the medium. Add 100 µL of 4% paraformaldehyde and incubate for 15 min.

4.13 Remove paraformaldehyde and add 100 µL of saline solution.

NOTE: Plates can be kept at 4 °C for later assembly.

4.14 Collect the supernatant from inserts or from wells to count non-migrating cells or those that crossed the membrane, respectively. Incubate with paraformaldehyde 4% for 15 min.

4.15 Concentrate cells using a cytocentrifugation technique[35].

4.16 Add 10 µL of the mounting medium with DAPI to the membranes and place coverslips over the wells.

4.17 Assembly of the insert membrane.

NOTE: This step can be performed outside the biosafety cabinets.

4.17.1 Remove insert membranes from wells.

4.17.2 Scrape the surface of the insert with a swab to remove any cells that have not migrated.

4.17.3 Using a scalpel, remove the membrane from the insert.

4.17.4 Place the membrane on a slide, with cells facing up.

4.17.5 Add 10 µL of mounting medium with DAPI to each membrane and then place overlay coverslips.

4.17.6 Cover plates with aluminum foil.

4.17.7 Incubate plates at room temperature for 30 min, then store at -20 °C

4.18 To analyze cell migration, count the number of cells per field (no less than 10 fields) using a fluorescence microscope at a laser excitation wavelength of 405 nm.

5. Adhesion assay and evaluation of actin polymerization by immunofluorescence

NOTE: For this assay, use 24-well plates with coverslips.

5.1 After infecting cells for 4 h, wash each well 3 times with sterile saline solution at room temperature to remove any non-internalized parasites.

5.2 Centrifuge the plate at 300 x g for 10 min under 4°C. Add 500 µL of 4% paraformaldehyde to each well for 15 min.

5.3 Remove paraformaldehyde and add 1 mL of saline solution.

5.4 Prepare solutions as described in **Table 1**.

[Place **Table 1** here]

5.5 Immunostaining

NOTE: The following steps must be performed under agitation.

5.5.1 Wash coverslips 3 times with 1x PBS for 5 min.

5.5.2 Add 500 µL of ammonium chloride solution per well for 10 min.

5.5.3 Wash coverslips 3 times with 1x PBS for 5 min.

5.5.4 Permeabilize the membrane with Saponin 0.15% for 15 min.

5.5.5 Incubate cells with the blocking solution (PBS 1x / ABS 3% / Saponin 0.15%) for 1 h.

5.5.6 Wash coverslips 3 times with PBS 1x/Saponin 0.15% for 5 min.

5.5.7 Incubate cells with primary antibody diluted in PBS 1x/ABS 1% /Saponin 0.15% for 1 h.

5.5.8 Dilute primary antibodies in PBS 1x /ABS 1% /Saponin 0.15% as: Rabbit anti-FAK (pTyr397): 1:500 dilution, Rabbit anti-paxilin (pTyr118): 1:100 dilution. Mouse Anti-Rac1: 1:100 dilution. Rabbit Anti-Cdc42: 1:200 dilution. Rabbit Anti-RhoA: 1:200 dilution.

NOTE: FAK and Paxillin are key molecules involved in the formation of adhesion complexes[13-15]. The Rho GTPase family is responsible for the organization of the actin

cytoskeleton. RAC1 and Cdc42, located at the front edge of the cell, control the formation of lamellipodia and filopodia, respectively; RhoA is located at the rear of the cell and is involved in cytoskeleton contraction[15, 23-25].

5.5.9 Wash coverslips 3 times with 1x PBS/Saponin 0.15% for 5 min.

5.5.10 Dilute secondary antibody or phalloidin as follows: Anti-rabbit IgG, Alexa Fluor 568: 1:500 dilution; Anti-mouse IgG, Alexa Fluor 488: 1:500 dilution; Anti-mouse IgG, Alexa Fluor 594: 1:500 dilution; Phalloidin Alexa Fluor 488: 1:1200 dilution.

5.5.11 Incubate cells with secondary antibody or phalloidin diluted in 1x PBS/ABS 0.3% Saponin 0.15% for 45 min.

NOTE: Incubation of the secondary antibody or phalloidin should be performed in the absence of light. Cover plates with aluminum foil to protect from light during the following steps.

5.5.12 Wash coverslips 3 times with 1x PBS for 5 min.

5.5.13. Remove coverslips from wells.

5.5.14 In the absence of light, add 10 µL of mounting medium with DAPI onto clean microscopic glass slides.

5.5.15 Place coverslips with cells facing down to allow contact between cells and DAPI solution.

NOTE: Cover slides with aluminum foil to protect from light.

5.5.16 Incubate for 30 min at room temperature.

5.5.17 Store at -20 °C.

6. Confocal microscopy, image acquisition, and quantification using FIJI

NOTE: To acquire/capture immunofluorescence images, use a confocal laser scanning microscope. Oil-immersion 63x objective lens is recommended for optimal resolution.

6.1 Allow coverslips to reach room temperature, protected from light, for at least 30 min prior to image acquisition.

6.2 Clean the coverslips with absorbent tissue.

6.3 Add a drop of immersion oil to the objective and place each slide under the microscope.

6.4 Move the objective until the oil touches the slide.

6.5 Observe and adjust the microscope focus; select the **63x objective with oil**.

6.6 Turn on lasers at **488 nm**, **552 nm,** and **405 nm** wavelengths.

6.7 Select image resolution: **1024 x 1024**.

6.8 Click on the **Live** button, set the Z stack, and press **Begin**. Repeat the process and then press **End**.

6.9 After selecting the **Maximum Projection** option in the **Tool** menu, wait for the image to be acquired.

6.10 **Save** the experiment.

6.11 **Export** images in .lif format on a computer. Use FIJI open-source image analysis software to analyze images.

6.12 Select the images to be analyzed and select **Duplicate Image**.

6.13 To have the image in grayscale, select **Image | Adjust | Color Threshold**. Select **0** and **255** (saturation).

6.14 Select the threshold method: **Default**.

6.15 Select threshold color: **B and W** (black and white).

6.16 Do not select **Dark background**.

6.17 Select: **Process | Binary | Options** and then select the relevant data to be measured: **Area, Min**, **Max**, **gray value**, **integrate density**.

6.18 Select the free hands' tool in the Fiji toolbar and trace each cell manually carefully.

6.19 From the **Analyze** menu, select **Set measurements**. Make sure to have area integrated intensity and mean grey value selected. Repeat this process for each cell.

6.20 Select all data in the Results window and copy and paste into a spreadsheet file.

6.21 Calculate the corrected total cell fluorescence (CTCF): CTCF (Integrate density = Area of selected cell x Mean fluorescence of background readings).

6.22 Open the file containing data using statistical analysis software to perform statistical analysis.

7. Statistical analysis

7.1 Open a **New Project** when a welcome dialog appears.

7.2 Choose the type of graph and medium with SD.

7.3 Apply the values obtained from the experimental results to the table.

7.4 Select **Descriptive Statistics** and choose the option column **Statistics [all tests]** to analyze data distribution.

7.5 If data follow a Gaussian distribution, choose the t-test to analyze samples by comparing two pairs. If the distribution is non-Gaussian, analyze data using the Mann-Whitney test.

7.6 Choose the best graph option for optimal data representation.

REPRESENTATIVE RESULTS

This protocol described herein enables the evaluation of cell migration and its associated mechanisms, such as actin dynamics and adhesion, thereby providing a tool to determine the migration of *Leishmania*-infected host cells within the vertebrate host. The results presented here demonstrate that this *in vitro* assay provides rapid and consistent indications of changes in cellular adhesion, migration, and actin dynamics prior to *in vivo* experimentation.

First, cells were successfully cultured following aseptic techniques and lab protocols. Data generated via migration analysis using cell culture membrane inserts allowed us to evaluate the migration of *L. infantum*-infected or uninfected human dendritic cells. DAPI staining permitted the facile visualization of migratory cells, enabling us to discriminate between infected and non-infected cells as staining procedures incorporate both dendritic cell and parasite nuclei. Infected cells can be identified by visualizing the large macrophage nuclei and the number of smaller amastigote nuclei clustered around each macrophage nucleus. Next, infected cells (*L. infantum* infected group) and uninfected cells (control group) were then counted for each field of vision using a manual counter. Finally, the number of migratory infected cells was compared to the number of uninfected cells (control group) that migrated. Our results indicate higher rates of cell migration following *L. infantum* infection when compared to non-infected controls (**Figure 1**).

The evaluation of actin dynamics and the formation of adhesion complexes, factors critical to cellular migration, allows for an enhanced understanding of how infection may modulate host cell migration. To assess these mechanisms, we performed immunostaining for molecules involved in actin dynamics (phalloidin, Rac1, Cdc42, and RhoA) and adhesion complex formation (FAK and paxillin). The expression of each protein was evaluated using confocal microscopy. The differences in protein expression were assessed by comparing the fluorescence intensity between infected and uninfected cells for each protein analyzed. Our results demonstrate actin polymerization in infected and non-infected cells and the formation and localization of adhesion

complexes. DAPI staining enabled the identification of infected cells through the staining of parasite nuclei. Fluorescence analysis indicated increased FAK and paxillin expression in DCs following *L. infantum* infection (**Figure 2**). To evaluate the organization of actin filaments in DCs, actin was labeled with fluorescent phalloidin. The resulting images revealed more areas with actin polymerization, yet no differences in phalloidin staining comparing infected and non-infected cells (**Figure 3A**). However, considering that the structure of actin is highly dynamic, the evaluation of actin-associated molecules may provide additional insight into the organization of this structural protein. Thus, we evaluated the expression of Rho GTPase proteins. Although phalloidin staining yielded similar results in infected and non-infected cells, increases were noted in Rac1, Cdc42 and RhoA expression after *L. infantum* infection when compared to uninfected controls (**Figure 3B,C,D**). These results reinforce the need for further investigation of molecules involved in actin polymerization to gain a more comprehensive understanding of its associated dynamics.

[Place **Figure 1** here]

[Place **Figure 2** here]

[Place **Figure 3** here]

FIGURE LEGENDS:

Figure 1: Evaluation of dendritic cell migration in *L. infantum* infection. Dendritic cells were infected by *L. infantum* at a ratio of 20:1 for 4 h. At 6, 12, 24 or 48 h after infection, dendritic cells could migrate in the presence of CCL3 chemoattractant through the cell culture insert system for an additional 4 h. Migrating cells were washed, fixed, and stained with DAPI. Bars represent numbers of migratory cells after *L. infantum* infection from random counts in 10 fields using confocal microscopy. Each dot represents one cell. *p<0.05 (Student's *t*-test).

Figure 2: Evaluation of adhesion complex formation in *L. infantum*-infected dendritic cells. Dendritic cells infected or not with *L. infantum* were stained with anti-pFAK or anti-paxillin antibodies. (**A**) Fluorescence intensity of FAK expression. (**B**) Fluorescence intensity of paxillin expression. For each group, 30 cells were analyzed using FIJI software. Red: anti-pFAK, or anti-paxillin; Blue: DAPI; Grayscale: differential interference contrast (DIC). Scale bar = 0.18 inches. *p<0.05 (Student's *t*-test).

Figure 3: Evaluation of actin dynamics in *L. infantum*-infected dendritic cells. Dendritic cells infected or not with *L. infantum* were stained with anti-Rac1, anti-RhoA and anti-Cdc42 antibodies or fluorescent phalloidin. (**A**) Fluorescence intensity of phalloidin expression (green). (**B**) Fluorescence intensity of Rac1 expression (red). (**C**) Fluorescence intensity of Cdc42 expression (red) (**D**) Fluorescence intensity of RhoA expression (red). For each group, 30 cells were analyzed using FIJI software. Red: anti-Rac1 or anti-Cdc42; Green: anti-RhoA or phalloidin; Blue: DAPI; Grayscale: differential interference contrast (DIC). Scale bar = 0,18 inches. *, p<0,05 (Student's t-test).

Table 1: Buffer recipes.

DISCUSSION

The method described here for evaluating cell migration using the cell culture membrane inserts system allows researchers to study the migratory response of cells in a two-dimensional environment. In this technique, some steps are considered critical. Firstly, the differentiation of human DCs and infection with *Leishmania* are determinative since the infection rate is donor-dependent. Using more than one donor per experiment and healthy *Leishmania* cultures will allow for more consistent results. It is also crucial that parasites be maintained in host animals, which favors the selection and maintenance of virulent strains and the ability to readily colonize host cells. Following DC differentiation, we recommend checking the expression of surface markers CD80 and CD11c to verify that the cells being used in experimentation are, in fact, dendritic cells. Despite variability in infection rates, increased DC migration following *L. infantum* infection was observed in all experiments.

The cell culture membrane inserts system assay, employed herein, entails cell migration through a porous polycarbonate membrane from the upper to the lower compartment of the cell culture membrane inserts system. A chemoattractant is placed in the bottom of each well to direct cell migration through the porous membrane during incubation[36]. It is important to use an appropriate pore size for the cell type of interest. For DCs, we used a cell culture membrane inserts system with a 5μm pore size. Of note, after the fixation step, the surfaces of the insert membranes were scraped with a swab to remove cells that had not migrated. This step was implemented to ensure that only those cells that successfully migrated through the membrane were evaluated. Another critical point that warrants consideration is the use of DAPI staining, a rapid procedure that allows investigators to not only identify host cells but also parasite nuclei, thus enabling the convenient identification of infected cells.

Although the cell culture membrane inserts system has been extensively used as an effective tool for assessing migration[28, 36–39], there are some limitations associated with this technique. During cell washing and fixation steps, less-adherent cells may be lost when analyzing the membrane, resulting in an underestimation of the number of cells that migrated. Another limitation is time related. After long incubation periods, gradient loss may occur due to diffusion through the porous membrane. Thus, this system should be considered more efficient for shorter incubation periods[36]. On the other hand, the use of the cell culture membrane inserts system is advantageous compared to other methods such as the scratch assay or random migration since it allows the study of directional migration in the presence of a chemoattractant.

Another essential component of this protocol is the use of immunofluorescence to investigate mechanisms involved in cellular migration, such as adhesion complex formation and actin dynamics. This technique allows investigators to visualize specific targets in tissues or cells using specific antibodies for proteins of interest. In this protocol, to assess cellular adhesion, we evaluated the expression of phosphorylated FAK and paxillin, both crucial proteins involved in the formation of adhesion complexes in different cell types, including leukocytes[13], and consequently excellent tools for studying cell adhesion. Previous studies have shown that increased FAK signaling

promotes cellular motility[11]. Also, the use of this technique to evaluate cell adhesion does not require the use of more labor-intensive techniques, such as the use of inflamed connective tissue[26].

The phalloidin staining technique[40,41] provides information about the expression of polymerized actin, F-actin, and the regions of cells with higher polymerization rates. To gain further insight into actin dynamics in *L. infantum*-infected cells, we also evaluated the expression of Rac1, Cdc42, RhoA, and Rho GTPase, which participate in the polymerization of actin filaments[42]. The expression of these molecules was also performed using immunofluorescence. This technique is not only an alternative to the use of transgenic mice expressing fluorescent actin[28] but also provides further information about molecules modulated during the actin polymerization process.

Several factors can affect immunofluorescence quality and efficacy. Antibody dilution, for example, when not carefully determined, can impair the acquisition of images and lead to non-specific staining due to elevated levels of background signals. Samples must also always be protected from light to avoid any loss of cell fluorescence or staining[43].

In summary, here we describe an *in vitro* protocol that allows for the evaluation of cell adhesion and migration processes in the context of *Leishmania* infection. Our primary focus was on DCs, which are known to play a significant role in the immunopathogenesis of leishmaniasis; however, how these cells participate in parasite dissemination in the vertebrate host remains poorly understood. This protocol can also be modified to investigate cellular migration in other types of host cells for the investigation of other species of intracellular parasites. Also, extracellular matrix, such as collagen I or Matrigel, can be added to the cell culture membrane inserts system to evaluate 3D migration and cellular invasion in different fields of study.

DISCLOSURES

The authors declare they have no competing financial interests.

ACKNOWLEDGMENTS

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Figure 1

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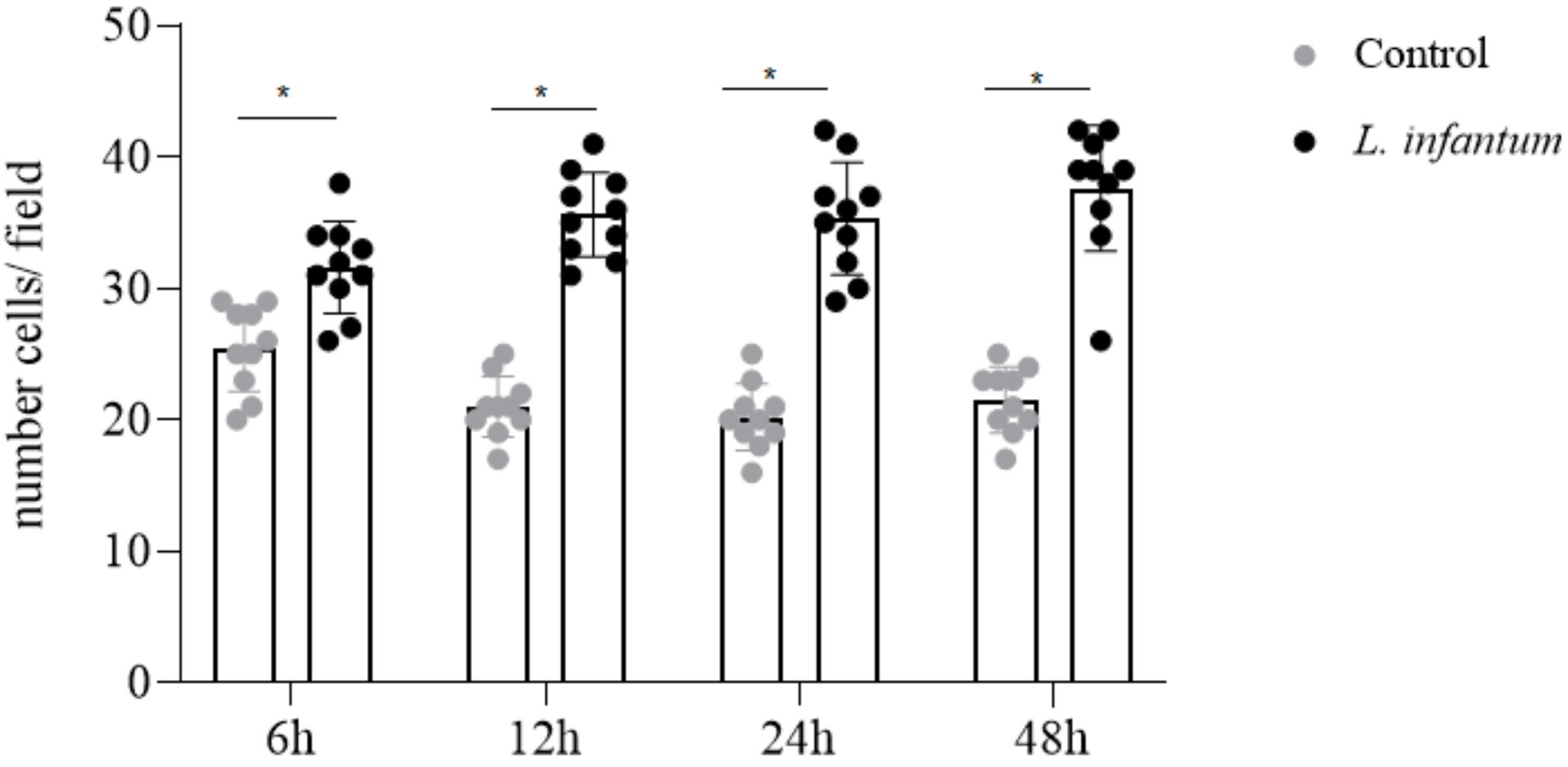

Figure 2

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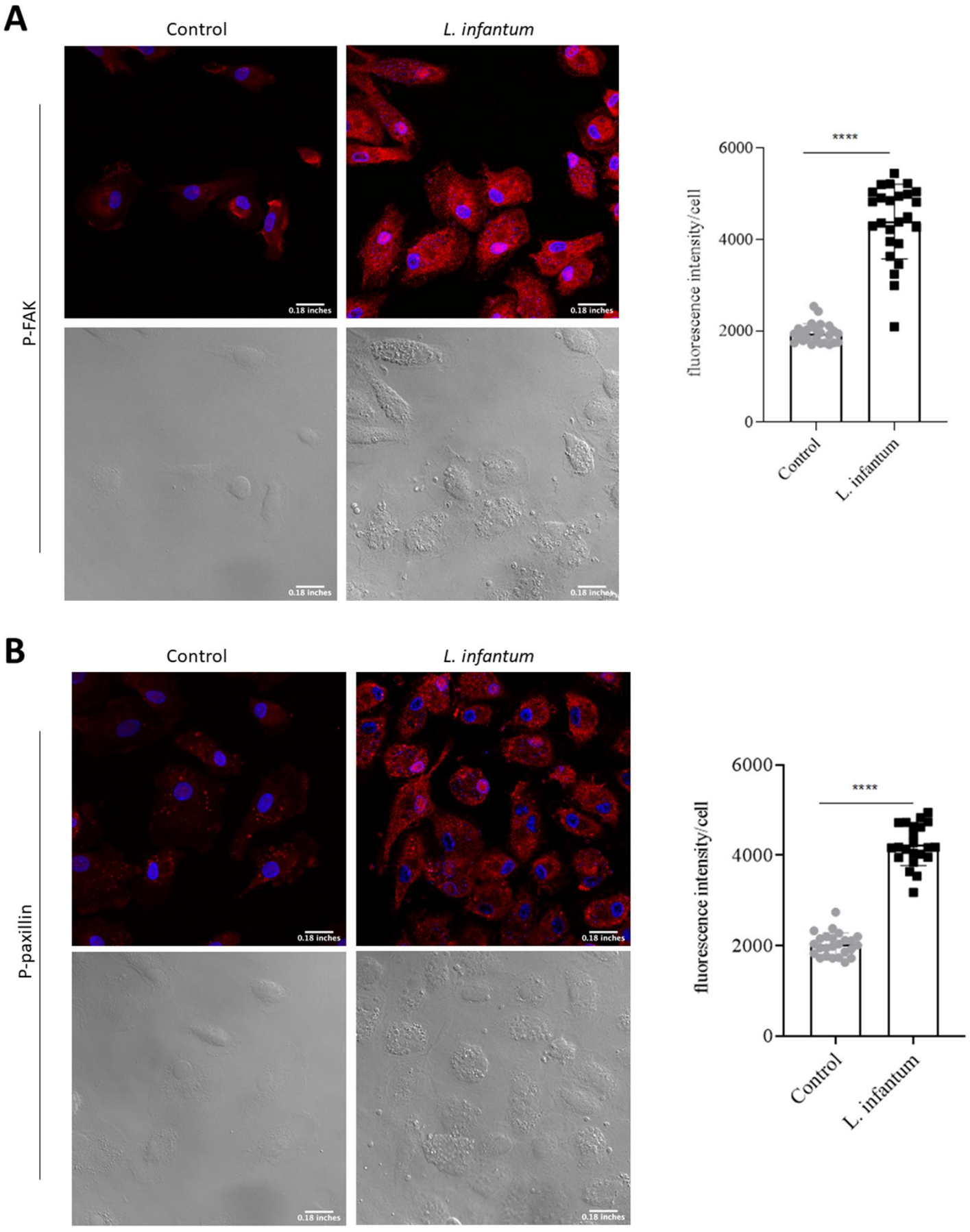

Figure 3

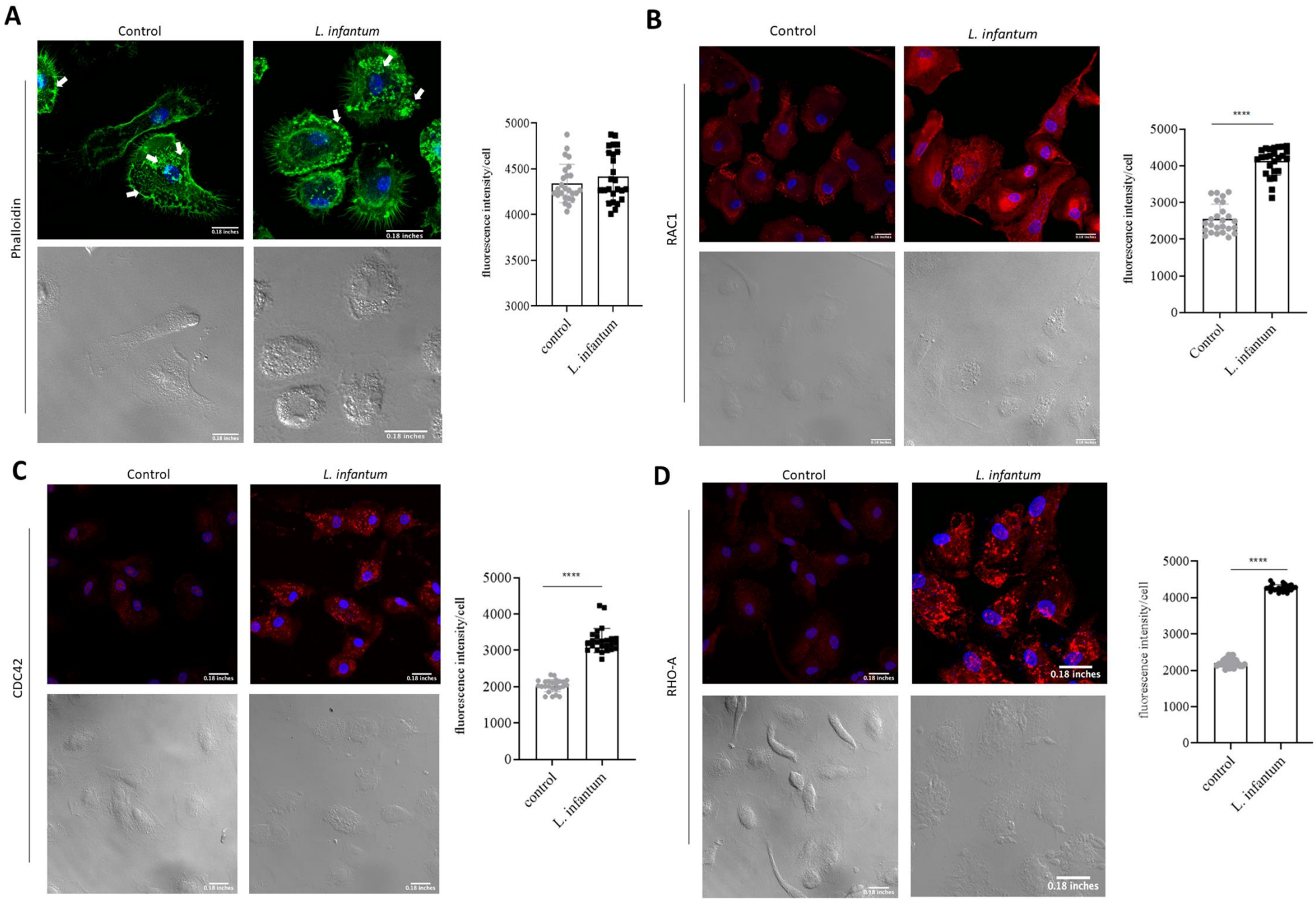

Table 1

Primary solutions	**Chemical compound**	**Diluent**
Ammonium chloride solution	0,134 g of NH_4Cl	50 ml of PBS 1X
Saponin 15%	150 mg of saponin	1 mL de PBS 1X
Albumin from bovine serum (ABS) 10%	1 g of ABS	10 mL of PBS 1X
Secondary solutions	**Component 1**	**Component 2**
Saponin 0,15%	1mL of saponin 15%	100 mL of PBS 1X
PBS 1X / ABS 3% / Saponin 0,15%	13,8 mL of PBS 1X	6 mL of ABS 10%
PBS1X / ABS 0,3% / Saponin 0,15%:	19,2 mL of PBS 1X	0,6 mL of ABS 10%
PBS 1X / ABS 1% / Saponin 0,15%	17,8 mL of PBS 1X	2 mL of ABS 10%

Component 3

-

200 µL of Saponin 15%

200 µL of Saponin 15%

200 µL of Saponin 15%

Applying cell migration and adhesion assays to investigate *Leishmania*-host cell interaction

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May 18th, 2021

Dear Editor,

We would like to thank you and the referees for the valuable contributions that have improved the present manuscript to be suitable for publication in JoVE. Please find enclosed a marked-up copy detailing the changes made to the previously submitted version of the manuscript, which has been revised to take the reviewers' comments into account. We would like to emphasize that all criticism has been carefully considered, and that every amendment and clarification has also been included in the authors' responses below, separated by the points raised by each referee. In addition, an extensive revision by a native English speaker has been made throughout the text to improve the overall clarity of the manuscript.

Sincerely,

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Editorial and production comments:
Editorial Changes
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer: We thank the editorial board and have submitted the manuscript to a native speaker to address these issues.

2. Please provide an email address for each author, and provide complete affiliation details.

Answer: This information has been included in the manuscript (lines 6-10).

3. Please include a Summary clearly describing the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Answer: We have added this information to the manuscript (lines 18-23).

4. Please revise the following lines to avoid previously published work: 33-37, 58-62, 64-66.

Answer: We agree with the referee's suggestion and have modified the manuscript as follows:

From (lines 33-37): "Leishmaniasis is a neglected tropical disease caused by set of protozoan parasites of the genus *Leishmania.* This parasite causes a broad spectrum of clinical manifestations ranging from self-resolving localized cutaneous lesions to a highly fatal visceral form of the disease. An estimated 0·7-1 million new leishmaniasis cases per year are reported, and 12 million people worldwide are currently infected with these parasites[1]."

To (lines 46-50): "Leishmaniasis, a neglected tropical disease caused by protozoan parasites belonging to the genus Leishmania, results in a wide-ranging spectrum of clinical manifestations, from self-healing localized cutaneous lesions to fatal visceral forms of disease. It has been estimated that up to one million new leishmaniasis cases arise annually, with 12 million people reportedly being currently infected worldwide."

From (lines 55-62): "Although several different receptors are involved in cell migration, integrins are a major family of receptors promoting migration. Upon integrin engagement, migration-related signaling molecules are activated, and intracellular signaling is initiated through focal adhesion kinase (FAK) and Src family kinases, as well as molecules such as talin, vinculin, and paxillin[9–11]. Phosphorylation of paxillin by activated kinases, such as FAK, recruits effector molecules, transducing external signals

into cell migration changes. Thus, paxillin is a crucial intracellular molecule that coordinates cell adhesion, actin polymerization, and cell migration[12–14].”

To (lines 69-76): “Among the different receptor types involved in the promotion of cell migration, integrins are notable. Integrin activation results in migration-related signaling; intracellular signaling then occurs via focal adhesion kinase (FAK) and Src family kinases, in addition to talin, vinculin and paxillin molecues9–11. The phosphorylation of paxillin by activated kinases, including FAK, leads to the recruitment of effector molecules, which transduces external signals that prompt cell migration. It follows that paxillin is an intracellular molecule that is crucial to cell adhesion, actin polymerization and, resultingly, cell migration processes.”

From (lines 64-67): “Cell protrusions observed during cell migration are formed as a result of actin polymerization and are stabilized by cell adhesion to the extracellular matrix and facilitated in many cases through integrin receptors that are linked to the actin cytoskeleton[16–18].”

To (lines 78-80): “During cell migration, protrusions formed due to actin polymerization become stabilized through cell adhesion to the extracellular matrix. This process may be modulated by integrin receptors associated with the actin cytoskeleton.”

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Consider combining some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Answer: We have modified the manuscript numbering to conform to the JoVE author instructions as requested.

6. Use “mL, µL” instead of “ml, uL/ul”. Add a single space between the quantity and its unit E.g. “37 oC” instead of “37oC”. Use decimal points instead of “,”. Follow these for the figures and figure legends as well.

Answer: We thank the editor for this criticism and have revised/corrected units and spacing, etc. throughout the text.

7. Line 96: How much blood was collected?

Answer: We have modified the manuscript to address the editor’s concern as follows:

From (line 96): “Collect blood from healthy donors and, after collection, follow the procedures in the flow cabinet.”

To (lines 110-111): “Collect venous blood (50 mL) from healthy donors and, after collection, follow the procedures in the flow cabinet.”

8. Line 99: Saline concentration?

Answer: This information has been included on line 112-113.

9. Line 106: How is the PBMC ring identified?

Answer: We have included the following information to clarify this point:

"NOTE: After centrifugation, the gradient layers are formed as follow: erythrocytes, density gradient medium, PBMC ring and plasma. The PBMC ring is located between the density gradient medium and plasma layers."(lines 122-124).

10. Lines 233, 234: Do not use symbols.

Answer: We have removed the use of symbols as requested. We also added a separate table as suggested.

11. Line 216: Specify the wavelengths used.

Answer: In accordance with the editor's suggestion, this information has been added:

From (lines 215-216): "To analyze cell migration, count the number of cells per field (at least 10 fields) using a fluorescence microscope."

To (lines 258-259): "To analyze cell migration, count the number of cells per field (at least 10 fields) using a fluorescence microscope with laser excitation at 405 nm."

12. Line 297, 298: Specify the units.

Answer: We have added this information as well.

From (line 297): "Open the Leica program and turn on lasers 488, 552, and 405."

To (line 324): "Turn on lasers 488 nm, 552 nm, and 405 nm wavelengths."

13. Consider adding the solution compositions (lines 224-247) as a separate table, and referencing it in the protocol.

Answer: We agree with the editor's suggestion and have included a table detailing the solution compositions to the manuscript.

14. Use L.infantum instead of Li to avoid confusion.

Answer: We agree with the editor's suggestion and have modified our use of this abbreviation throughout the text.

15. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an

instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Ficoll, Miltenyi biotech, Prolong Gold, Leica, GraphPad etc.

Answer: We have modified our use of commercial references throughout the text as suggested.

16. Please include a scale bar for Figure 1A and define the scale in the appropriate Figure Legend. Also specify what the arrows indicate.

Answer: We accept the editor's suggestion; however, a new image must be obtained. At the moment our DAPI filter is not working, so we have modified Figure 1 accordingly and will provide new images to represent the insert's membrane with migrating cells when the video is produced.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors, by using a method to characterize the migratory aspects of host cells during Leishmania infection, describe a detailed protocol to perform differentiation and infection of dendritic cells, and to evaluate host cell motility and migration analysis, as well a as the formation of adhesion complexes and actin dynamics analysis. This in vitro protocol includes novelty, and more than that, efficacy to evaluate their aim to evaluate migration a and host cell motility promoted by the Leishmania pathogen.

Major Concerns:

However, to guarantee its reproducibility, the authors must clarify several minor issues as herein listed.

Line 91: please specify how the samples were obtained from the donors (Venous blood, how many ml?)

Answer: We thank referee for his/her careful consideration of our manuscript. To address uncertainly regarding sample obtainment, we have modified the manuscript as follows:

From (line 96): "Collect blood from healthy donors and, after collection, follow the procedures in the flow cabinet."

To (lines 110-111): "Collect venous blood (50 mL) from healthy donors and, after collection, follow the procedures in the flow cabinet."

Line 103: please explain why the break of the centrifuge should be switched off.

Answer: We have included this information as suggested.

From (line 103): “NOTE: Switch-off the break before centrifuge. After the 1st centrifugation, lower the temperature of the centrifuge to 4°C.”

To (line 117-118): “NOTE: Switch-off the break before centrifuge to prevent mixing of gradient layers.”

Line 106: please specify what is PBMC, what do the authors mean by identification, can a picture be included?

Answer: We have clarified this information as follows:

From (lines 106-107): “Identify the PBMC ring in the sample and gently remove the residual plasma with a pipette.”

To (lines 120-121): “Identify the peripheral blood mononuclear cells (PBMC) ring in the sample and gently remove the residual plasma with a pipette.”

Line 119: please clarify what for CD14 microbeads are used? What are CD14 microbeads?

Answer: We have clarified this information as follows:

From (line 119): “Add 20 µl of CD14 MicroBeads for every 10^7 cells counted in the Neubauer chamber.”

To (lines 138-141): “Add 20 µL of CD14 MicroBeads for every 10^7 cells counted in the Neubauer chamber.
NOTE: CD14 microbeads can be used for positive selection of human monocytes from PBMCs. The beads bind to the CD14 positive cells, which are expressed on most monocytes.”

Line 133: please clarify why GM-CSF is used? What is GM-CSF?

Answer: We thank referee’s comment and have clarified this information in the manuscript as follows:

From (line 133): “Resuspend in 1 mL of complete RPMI with IL - 4 (100Ul /mL) + GM - CSF (50ng/mL).”

To (lines 157-162): “Resuspend in 1 mL of complete RPMI with interleukin-4 (IL-4) (100 µL/mL) + granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/mL).

NOTE: GM-CSF is a cytokine secreted by macrophages, T cells, NK cells, acting as a. GM-CSF and IL-4 are used to induce dendritic cell differentiation."

Line 148: please clarify why the first passage of parasites should not be used?

Answer: We thank the referee for this suggestion and have modified the text as follows:

From (line 148): "NOTE: Don't use the first passage post NNN Medium in experiments."

To (lines 183-184): "NOTE: We do not recommend using the first passage post NNN medium in experiments to avoid residues of rabbit blood."

Lines 155-173: please clarify the cells that are going to be infected, it is not clear in the text. Also, in line 172 it says that the incubation will be for 4 h while in figure legend 1 it says that the incubations was for 6, 12, 24 and 48 h. Please clarify (Now lines 241 and 245)

Answer: We apologize for the lack of clarity on this point and have revised information regarding the infection procedures throughout section 3 of the protocol.

From (line 172): "Incubate cells for 4 h in an incubator at 37 ºC, 5% CO_2."

To (line 209): "Incubate dendritic cells with parasites for 4 h in an incubator at 37 °C, 5% CO_2."

Line 188: please define the pore of the transwell plates and why this pore size was used.

Answer: We have included a description of the transwell inserts for clarification.

From (line 188): "Remove the insert with sterile tweezers and place it in an empty well."

To (lines 227-229): "NOTE: Transwell with 5.0 µm pore polycarbonate membrane insert is recommended for migration assays using dendritic cells, macrophages and monocytes, allowing phagocytes to pass through the membrane."

Line 191: please define what is CCL3

Answer: This information has been added to the text.

From (line 191): "Add 600 µL of RPMI medium supplemented with 10% fetal bovine serum in each well and add the chemoattractant CCL3 (1 µl for every 1 mL of medium)."

To (lines 231-233): "Add 600 µL of RPMI medium supplemented with 10% fetal bovine serum in each well and add the chemoattractant Chemokine (C-C motif) ligand 3 (CCL3) (1 µL for every 1 mL of medium)."

Line 194: please clarify which cells are added in this case

Answer: We have modified the text as follows:

From (line 194): “Add 100 µl of medium containing $2x10^5$ cells to each insert.”

To (lines 235-236): “ Add 100 µL of medium containing $2x10^5$ dendritic cells (infected or not) to each insert.”

Line 204-214: please correct the numbers, they should be 17.1 and so on

Answer: We have updated the text to correctly display decimal information in section 4.

Lines 260-264: please define these antibodies and what are they for

Answer: We have included this information in the manuscript as follows:

“NOTE: FAK and Paxillin are key molecules involved in the formation of adhesion complexes. The Rho GTPase family is responsible for the organization of the actin cytoskeleton. RAC1 and Cdc42 are at the front edge of the cell controlling the formation of lamelipodia and filopodia respectively, while RhoA is at the rear end of the cell and is involved in contraction of the cytoskeleton.” (lines 287-292)

Line 304: please clarify if the format of the file is lif or tif

Answer: Our experimental images are saved in .lif format.

Line 362: please see comment for lines 155-173

Answer: We thank referee’s suggestion and have modified this information (lines 193-210; lines 401-407).

Line 396: please comment on the potential influence of gravity in the end results of this assay

Answer: Although gravity could play a role in cell migration, the membrane pore size constitutes a physical barrier. As the migrating cells must change shape to pass through the pores, this reduces the effect of gravity on the migration process using the transwell technique. In addition, while all cells are subject to the effects of gravity, infected cells will experience greater migration.

Minor Concerns:

As a reviewer I feel that this thoroughly described method will allow a sound interpretation of the data, therefore I recommend to accept this protocol once the issues herein raised are amended.

Reviewer #2:

Manuscript Summary:

One important point still not clarified in Leishmania research is how parasites get to their final sites in organs. The present manuscript argues that the migratory pattern of infected cells, namely dendritic cells, is altered relatively to non-infected ones. Indeed there is now different publications suggesting that DC and macrophage motility is different if cells are infected. This has also been shown to be the case for other parasites. It is thus relevant to publish the present protocol so that different aspects of migration by parasites as important as Leishmania can be studied in different labs. I have no major concerns regarding publication, provided the concerns outlined below (most relatively minor) are taken into consideration by the authors.

Minor Concerns:

Line 33: Leishmaniasis would be better described as caused by a set of protozoan parasites of the genus Leishmania. Indeed, the genus includes different species with medical relevance and some of this are distinct is different aspects.

Answer: We thank the referee for his/her consideration of our manuscript, and accept the suggestion to modify the description of leishmaniasis as follows:

"Leishmaniasis, a neglected tropical disease caused by protozoan parasites belonging to the genus *Leishmania*." (lines 46-47)

Line 45: References outlined do not support the statement that is being made.

Answer: We agree with referee's criticism and have revised the references in question (line 58; lines 502-507). .

Line 75: Also relevant for the statement made in lanes 74/75 is the recent reference "Leishmania infantum Enhances Migration of Macrophages via a Phosphoinositide 3-Kinase γ-Dependent Pathway, ACS Infect Dis., 6:1643-1649. 2020. doi: 10.1021/acsinfecdis.0c00080". I suggest that the authors include it.

Answer: We accept the referee's suggestion and included this reference in the manuscript (line 88; lines 568-570)

Lane 133: When referring to GM-CSF, should this be recombinant product from a company or can this be recovered from cell supernatant within each lab?

Answer: To maintain homogeneity in our experiments, we have only used the recombinant product from Milteny Biotech company as cited in our materials table.

Lane 134: Is the medium changed be for infection?

Answer: We use complete RPMI medium with GM-CSF and IL-4 added for dendritic cell differentiation. After differentiation, we performed experiments using complete RPMI medium in the absence of these cytokines.

Lane 137: Perhaps the authors should give some more detail on how promastigotes are recovered from hamsters.

Answer: We are grateful to the referee for pointing this out and have included the following information as suggested:

"NOTE: *L. infantum* (MCAN/BR/89/BA262) parasite was used in this assay.
Hamsters were intravenously infected with $1x10^6$ *L.infantum* promastigotes, in a volume of 20 µL of sterile saline. After 1 to 2 months, animals were euthanized and the amastigote forms of *Leishmania* were recovered from the spleen and differentiated into promastigotes." (lines 165-169)

Lines 143/144: is the order of these steps correct or should it be the inverse?

Answer: We thank the referee for this observation and have modified the text as follows:

"Add 5 mL of supplemented MEM medium." (line 177-178)

Lines 151/154: Can you give an idea of the number of days you are referring to?

Answer: We have clarified this information in the text as follows:

From (line 151-154): "Monitor the growth of cultures periodically, using the Neubauer chamber, until it reaches a stationary phase. It may be then used in the experiments.
NOTE: Use promastigote cultures up to 7 passages *in vitro* to avoid the loss of virulence."

To (line 187): "Monitor the growth of cultures periodically for approximately 7 days…"

Lines 199/201 and further down: What exactly is done to count migration. Do you count cells on mebrane plus those that are already on the well? I assume this is done but it is not perceived from this text.

Answer: We thank the referee for pointing out the lack of clarity regarding migration assessment, and emphasize that counting is done through the membrane analysis. The upper surface of the membrane was scraped to remove any residual non-migrating dendritic cells. Moreover, when this protocol for dendritic cells was established we evaluated both cells in the membrane and also those in the wells and verified that the results were consistent.

Lane 200: Irrespectively of what I wrote above, please insert the word "respectively" at the end of the 1st sentence of step 14 (after membrane).

Answer: We have included this information in section 4 as requested.

Lines 367/8: what does each dot represent? A field? "cell" must be a mistake.

Answer: Each dot represents a cell as described in the figure legend.

Figure 1: Is fig 1A and 1B the same magnification? Nuclei on the left appear much larger than on B. also, what is the shadow that one can observe on the left panel? Do you know if the cells that crossed the membrane on panel B are infected?

Answer: The magnification used was not the same in the two panels, and because of that we have updated Figure 1. A new image must be obtained, but at the moment our DAPI filter is not working, so we have modified Figure 1 accordingly and will provide new images to represent the insert's membrane with migrating cells when the video is produced.

Regarding the visualization of infection, DAPI is also used to observe *Leishmania* nuclei.

Lines 364/365 reads that cells were washed and fixed, removed, and stained. This is difficult to understand because what you show on the panels are cells that are on the membrane, hence, why "removed". Please clarify.
Discussion: One important point would be to confirm infections and state what is

Answer: We would like to clarify what is meant by the removal of cells. To evaluate only migrating cells, after fixation we used a swab to remove cells on the upper side of the membrane and then only count cells on the lower part of the membrane. The legend of Figure 1 has been modified to clarify this point.

From (lines 361-368): "Figure 1. Evaluation of dendritic cell migration in *L. infantum* infection. Dendritic cells were infected by L. infantum at a ratio of 20:1. Six, 12, 24, or 48 hours after infection, cells were allowed to migrate in the presence of CCL3 chemoattractant through the transwell system for an additional 4 hours. Cells were washed, and fixed, removed, and stained with DAPI for nuclear staining. (A) Image of the membrane, showing DNA stained with DAPI. (B) Representative result of migratory cells at the membrane. Cells were counted (10 fields) randomly using fluorescence microscopy. Each dot represents one cell. *, p<0,05 (Student's t-test)."

To (lines 401-407): "Figure 1. Evaluation of dendritic cell migration in *L. infantum* infection. Dendritic cells were infected by L. infantum at a ratio of 20:1 for 4 hours. Six, 12, 24, or 48 hours after infection, dendritic cells were allowed to migrate in the presence of CCL3 chemoattractant through the transwell system for an additional 4 hours. Migrating cells were washed, fixed and stained with DAPI. Numbers of migratory cells after *L. infantum* infection. Cells were counted (10 fields) randomly using confocal microscopy. Each dot represents one cell. *, p<0,05 (Student's t-test)."

Video Produced by Author: Less than 50 MB. If your video is greater than 50 MB, click "offline" as the delivery method and our

This piece of the submission is being sent via mail.