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In Vitro Differentiation of Human Dendritic Cells and their Markers in *Leishmania* Infection

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Citation

Paixão, A.R., Tibúrcio, R., Carvalho, E., Silva, T., Veras, P.S.T., Tavares, N.M., de Menezes, J.P.B., Brodskyn, C.I. *In Vitro* Differentiation of Human Dendritic Cells and their Markers in *Leishmania* Infection. *J. Vis. Exp.* (182), e62794, doi:10.3791/62794 (2022).

Date Published

April 6, 2022

DOI

10.3791/62794

URL

jove.com/video/62794

Abstract

Leishmaniasis comprises a collection of clinical manifestations associated with the infection of obligate intracellular protozoans, *Leishmania.* The life cycle of *Leishmania* parasites consists of two alternating life stages (amastigotes and promastigotes), during which parasites reside within either arthropod vectors or vertebrate hosts, respectively. Notably, the complex interactions between *Leishmania* parasites and several cells of the immune system largely influence the outcome of infection. Importantly, although macrophages are known to be the main host niche for *Leishmania* replication, parasites are also phagocytosed by other innate immune cells, such as neutrophils and dendritic cells (DCs).

DCs play a major role in bridging the innate and adaptive branches of immunity and thus orchestrate immune responses against a wide range of pathogens. The mechanisms by which *Leishmania* and DCs interact remain unclear and involve aspects of pathogen capture, the dynamics of DC maturation and activation, DC migration to draining lymph node (dLNs), and antigen presentation to T cells. Although a large body of studies support the notion that DCs play a dual role in modulating immune responses against *Leishmania*, the participation of these cells in susceptibility or resistance to *Leishmania* remains poorly understood. After infection, DCs undergo a maturation process associated with the upregulation of surface major histocompatibility complex (MHC) II, in addition to costimulatory molecules (namely, CD40, CD80, and CD86).

Understanding the role of DCs in infection outcome is crucial to developing therapeutic and prophylactic strategies to modulate the immune response against *Leishmania*. This paper describes a method for the characterization of *Leishmania*-DC interaction. This detailed protocol provides guidance throughout the steps of DC differentiation, the characterization of cell surface molecules, and infection protocols, allowing scientists

to investigate DC response to *Leishmania* infection and gain insight into the roles played by these cells in the course of infection.

Introduction

Leishmaniasis constitutes a complex of neglected diseases caused by different species of the *Leishmania* genus[1]. *Leishmania* is an intracellular protozoan of the Trypanosomatidae family that infects humans and other mammals, causing a spectrum of diseases ranging from skin lesions to visceral forms[2]. The main clinical manifestations of this disease are tegumentary leishmaniasis (TL) and visceral leishmaniasis (VL). The World Health Organization (WHO) estimates that 700,000 to 1 million new cases occur annually, causing 70,000 deaths each year[2]. Worldwide, leishmaniasis affects approximately 12 to 15 million people, and 350 million are at risk of contracting the disease[3].

The genus *Leishmania* presents two evolutionary forms: the promastigote and the amastigote[4]. *Leishmania* promastigotes are characterized by the presence of flagella and high motility. These forms are found in the digestive tract of the sand fly, where they undergo differentiation into the infective form (metacyclic promastigotes)[5]. By contrast, amastigotes are found in the intracellular environment of infected mammalian cells. This evolutionary form, in turn, replicates in the phagolysosomes of phagocytic cells[6].

The transmission cycle of *Leishmania spp.* starts during blood-feeding, when sandflies inoculate metacyclic promastigotes into the host's skin[1]. Shortly after *Leishmania* inoculation, innate immune cells, including neutrophils and tissue-resident macrophages, phagocytize the parasites. Inside parasitophorous vacuoles, *Leishmania* differentiate into amastigotes and replicate, culminating in the rupture of the host cell membrane, which allows the infection of neighboring cells and parasite spread[4]. The cycle is completed when phlebotomines ingest amastigote-containing phagocytes, which differentiate into procyclic promastigotes and later into metacyclic promastigotes in the insect's intestinal tract[7].

Dendritic cells, professional antigen-presenting cells found in tissues and lymph nodes, act as a sentinel for the immune system[8]. These cells are found in peripheral tissues at immature stages, mainly involved in antigen capture and processing. After contact with pathogens, DCs undergo a maturation process that culminates in their migration to the lymph nodes, subsequently presenting antigens to naïve $CD4^+$ T cells. These cells are also essential in orchestrating the innate and adaptative immune responses that generate tolerance or inflammation[9]. The DC maturation process involves several aspects, including increased expression of MHC and costimulatory molecules, such as CD40 and CD86, as well as enhanced cytokine secretion. DCs express different markers, including CD11b and CD11c, and, in humans, the DCs that originate from $CD14^+$ monocytes (moDCs) express CD1a[10]. CCR7 is highly expressed on DCs and indicates the complex migratory process of these cells[12]. CD209 and CD80 also play an important role in the initial contact with DCs and lymphocytes[13].

In leishmaniasis, studies suggest that moDCs phagocytose parasites and deliver them to the draining lymph nodes (dLNs), where they present antigens to T cells[13]. The parasite capture mechanism is associated with cytoskeletal reorganization by actin filaments during phagocytosis, which

promotes the internalization of the parasite[14]. Most studies concerning the roles exercised by DCs in leishmaniasis have focused on *L. major*, *L. amazonensis*, and *L. braziliensis*[15]. Interestingly, *in vivo* studies of *Leishmania* infection have demonstrated that the impairment of DC function occurs in a parasite strain-specific manner.

It has been demonstrated that during the early stages of *L. amazonensis* infection, DCs exhibit a decreased ability to constrain parasite infection. Conversely, in an experimental model of *L. braziliensis* infection, DCs were shown to mount appropriate immune responses that restricted Leishmania survival[16]. The chief aspects known to be associated with differential responses to *Leishmania spp.* infection are the degree of DC maturation and activation. This paper describes a method to investigate the role human DCs play in *Leishmania* infection to further understand how these cells influence disease outcomes.

Protocol

NOTE: Cells were obtained from healthy donor volunteers. The procedure described herein was approved by the National Ethics Committee (number 2.751.345)-Fiocruz, Bahia, Brazil).

1. Differentiation of human dendritic cells

1. Pipette 10 mL of polysucrose-sodium triazoate mixture in 50 mL conical tubes.
2. Label the 50 mL conical tubes respectively for each donor.
3. Collect 30 mL of blood from healthy donors and perform all subsequent steps in the laminar flow hood.
4. Carefully transfer the blood into the conical tubes and dilute the blood in saline solution (0.9% sodium chloride) at a ratio of 1:1 at room temperature.
5. Overlay the slowly diluted blood onto the polysucrose-sodium triazoate mixture in the tubes (step 1.1). Centrifuge the tubes once at 400 × *g* for 30 min at 25 °C.
   NOTE: Turn off the brake before centrifugation to prevent the mixing of gradient layers. After the first centrifugation, decrease the temperature to 4 °C in the centrifuge.
6. Carefully take the tubes out from the centrifuge.
7. Look for the ring formed by peripheral blood mononuclear cells (PBMCs) in the sample (buffy coat); aspirate the residual plasma carefully with a pipette.
   NOTE: Centrifugation leads to the formation of the following gradient layers: erythrocytes, density gradient medium, PBMC ring, and plasma. The PBMC ring is between the density gradient medium and the plasma layers.
8. Transfer the cloudy PBMC layer to another tube and add saline to a final volume of 30 mL.
9. Centrifuge the tubes containing the cell suspensions at 250 × *g* for 10 min at 4 °C. Discard the supernatant and add 1 mL of saline to resuspend the pellet.
10. Collect an aliquot for cell counting and dilute it 1:1,000. Use 10 µL of the diluted cells for counting the cells by trypan blue exclusion method using a Neubauer chamber to determine cell viability.
11. Spin the suspension again at 200 × *g* for 10 min under 4 °C.
12. Resuspend the pellet in magnetic-activated cell sorting (MACS) buffer. Use 80 µL of the buffer per 1 × 10^7 cells.

NOTE: See **Table 1** for the MACS buffer composition. Keep the buffer cold and store it at 2-8 °C.

13. Add CD14 microbeads to the cell suspension prepared in step 1.9. Use 20 µL of CD14 microbeads per 1 × 10^7 cells.
    NOTE: CD14 microbeads are used to positively select human monocytes from PBMCs, as beads containing human anti-CD14 bind to $CD14^+$ cells expressed on the surfaces of most monocytes.
14. Pipette up and down to resuspend the pellets and microbeads uniformly. Keep on ice for 15 min.
15. Centrifuge the suspension at 300 × *g* for 10 min at 4 °C.
16. Resuspend the cells in MACS buffer. Use 1-2 mL per 1 × 10^7 cells in the cell-microbead mixture.
17. Centrifuge the suspension at 300 × *g* for 10 min at 4 °C.
18. Remove the supernatant and resuspend the pellet in 500 µL of MACS buffer.
    NOTE: This is the maximum volume of the cell suspension to be processed in one column.
19. Assemble the magnetic column.
20. Wash the column once with 500 µL of MACS buffer and allow the buffer to flow under gravity through the column.
    NOTE: Take care not to allow the column to become dry, as air can obstruct the column.
21. Add 500 µL of the cell-bead suspension (step 1.19) per column. Allow the cell suspension to flow under gravity through the column.
22. Wash the column with 500 µL of MACS buffer (2x). Add fresh buffer only when the column reservoir is empty. Do not let the column dry.
23. Place a new tube underneath the column, pipette 1 mL of the MACS buffer onto the column, and immediately flush the magnetically labeled cells out of the column by firmly pushing the plunger.
24. Centrifuge the CD14 enriched cells at 300 × *g* for 10 min at 4 °C.
25. Count the cells using a Neubauer chamber.
26. Resuspend the cells in 1 mL of complete RPMI with 100 µL/mL of interleukin-4 (IL-4) ((50 ng/mL) + granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/mL).
27. Seed cells on a 24-well plate at a concentration of 2 × 10^5 cells per well in 500 µL of complete RPMI medium containing the above cytokines and incubate for 7 days at 37 °C.

2. *Leishmania* culture

NOTE: *L. amazonensis* (MHOM/BR88/Ba-125) parasites were used in this assay.

1. Maintain promastigotes on Novy-Nicolle-MacNeal (NNN) blood agar medium with 5 mL of Schneider's complete medium or Minimum Essential Medium (MEM) in a 25 cm^2 culture bottle in a Bio-Oxygen Demand (BOD) incubator at 24 °C.
   NOTE: To cultivate the promastigote form of the *L. amazonensis* species, 5 mL of Schneider's complete medium (Schneider´s Insect Medium containing 10% inactivated fetal bovine serum (FBS) and gentamicin at a concentration of 50 µg/mL) was used.
2. Incubate for 7 days at 24 °C in the BOD incubator.
3. Pipette 100 µL of promastigote culture into a new 25 cm^2 cell culture flask.

4. Add 5 mL of the supplemented MEM.
5. Incubate at 24 °C in the BOD incubator and periodically count the promastigotes by transferring an aliquot of saline-diluted parasite cell suspension into a Neubauer chamber (i.e., hemocytometer) until stationary phase has been reached.
   NOTE: Do not use the first passage post-NNN Medium for experiments.
6. Transfer 1 × 10^5 of *Leishmania* stationary growth phase promastigotes into a new 25 cm^2 cell culture flask and add 5 mL of the supplemented MEM.
7. Periodically monitor the growth of cultures using a Neubauer chamber until the stationary phase is achieved.
   NOTE: Use promastigote cultures for up to 7 passages *in vitro* to avoid loss of virulence.

3. *Leishmania* infection

1. After verifying stationary-phase growth of cultured parasites, remove all contents from culture bottles and place them in 50 mL conical tubes. Add cold saline solution to achieve a final volume of 30 mL.
2. Centrifuge for 10 min at 4 °C at 1,600 × *g* three times. Discard the supernatant following centrifugation and resuspend the pellet in a cold saline solution.
3. Wash the cells to remove any non-viable parasites, and resuspend the pellet in 1 mL of cold saline. Pass the suspension 5 times slowly through a 1 mL syringe fitted with a 16 G needle to separate parasite clusters.
4. Remove an aliquot from the sample to count parasite concentration in a hemocytometer using Eq (**1**).

   Parasite concentration = mean of parasites in 4 quadrants × dilution factor × 10^4 (**1**)
5. Calculate the amount of *Leishmania sp.* required for the number of host cells to be plated to maintain a 10:1 parasite: host cell ratio (step 1.27).
6. Wash the DCs by adding 1 mL of saline solution and centrifuging the cells three times at 300 × *g* for 10 min at room temperature.
7. Place the required volume of *Leishmania* in each well of the 24-well cell culture plate (see step 3.5).
8. Incubate the DCs with parasites for 4 h in an incubator at 37 °C in a 5% CO_2 atmosphere.

4. Immunostaining for flow cytometry analysis

1. After infection, wash cells twice with 1 mL of saline solution to remove any non-internalized parasites.
   NOTE: The panel with antibody clones and fluorochromes is listed in the **Table of Materials**.
2. Dilute antibodies (1:50) in fluorescence-activated cell sorting (FACS) buffer (1x PBS with 1% BSA) at a final volume of 50 µL per experimental condition.
   NOTE: It is important to titrate antibodies before experimentation to ensure optimal staining concentrations. In this protocol, 1:50 was used after titration experiments.
3. Prepare a master mix (1x PBS with 1% BSA + antibody mix). Vortex and pipette 50 µL of the mix in wells containing cells.
4. Briefly incubate the DCs with the antibody mix (anti-human CD1a, anti-human CCR7, anti-human CD83, anti-CD11c, anti-human CD209, anti-human HLA-DR) on ice for 30 min, protected from light.

5. Wash cells twice with 1 mL of cold staining buffer (1% FBS in 1x PBS) and centrifuge at 300 × *g* for 5 min at 4 °C.
6. Aspirate the supernatant and resuspend the pellet in 200 µL of cold staining buffer.
7. Proceed with data acquisition on a flow cytometer.
   NOTE: To ensure optimal conditions for FACS analysis, data acquisition should be performed within 48 h after sample staining. Data were analyzed using FlowJo software.
   1. Open the FlowJo software program and create a new workspace. Add the flow cytometry files to be analyzed by dragging them into the workspace window.
   2. Click on the **tube name** to select side scatter (SSC) and forward scatter (FSC) parameters.
      NOTE: Flow cytometric analysis of DC maturation was based on the expression of CD1a, CD11c, CD80, CCR7, CD209, and HLA-DR.
   3. Using the **polygon gating** tool, draw a gate around the DCs. Double-click on the selected gated cells to display a new window. Perform doublet cleanup by selecting the FSC-A and FSC-H parameters. Using the **polygon** tool, draw a new plot around individual cells.
   4. Double-click on the gated cells and select SSC and CD11c parameters to generate a gate containing $CD11c^+$ cells.
   5. Export the cleaned $CD11c^+$ cell gate by selecting it. Right-click on the gate and select the **Select equivalent nodes** option. Right-click and export the gate along with all compensated parameters by saving it to a recently created folder.
   6. Create a new workspace by opening the recently saved files. Click on **Workspace** and select **plugins | Downsample | 30,000 to 50,000 events**; press **OK**.
      NOTE: As TSNE is both a time-consuming and computationally demanding process, this step is recommended to reduce the total number of analyzed events.
   7. Right-click on a downsampled file and select **equivalent nodes**.
   8. Right-click on a downsampled file and select **Export/ concatenate**.
   9. Click on the **concatenate** window and select **all compensated parameters**. Select the concatenated files and click on **workspace | plugin | tSNE**.
   10. Select the desired parameters (CD1a, HLA-DR, CCR7, CD80, CD209, and CD11c) to create clusters. Press **OK**.
   11. Click on the concatenated file to visualize the tSNE1 and tSNE2 parameters.

5. Actin immunostaining

1. Plate infected DCs on coverslips placed in 24-well plates. Centrifuge the plates at 300 × *g* at 4 °C for 10 min. Wash cells 3x with sterile saline after infection at room temperature to remove any extracellular parasites.
2. Incubate the DCs with 500 µL of 4% paraformaldehyde for 15 min at room temperature. Remove the paraformaldehyde and add 1 mL of saline (see **Table 1**).

6. Immunolabeling

NOTE: Perform the following steps under agitation.

1. Wash the coverslips with 1x PBS for 5 min; repeat 3x. Add 500 µL of ammonium chloride solution per well; incubate for 10 min at room temperature.
2. Wash the coverslips with 1x PBS for 5 min; repeat 3x.
3. Permeabilize the membrane with 0.15% Saponin for 15 min.
4. Block for 1 h with 1x PBS 1x/3% BSA/0.15% saponin. Wash the coverslips with 1x PBS/0.15% saponin for 5 min; repeat 3x.
5. Incubate the cells for 1 h with phalloidin (1:1,200] diluted in 1x PBS/1% BSA/0.15% saponin. Wash the coverslips with 1x PBS/0.15% saponin for 5 min; repeat 3x.
6. Place the coverslips with cells facing down on mounting medium with 4',6-diamidino-2-phenylindole (DAPI). Cover the slides with aluminum foil to protect them from light.
7. Acquire images using a confocal fluorescence microscope with a 63x/1.4 objective.

7. Confocal microscopy acquisition and Fiji quantification

1. Capture immunofluorescence images using a confocal laser scanning microscope (see the **Table of Materials**).
   NOTE: For best resolution, use a 63x objective oil immersion lens.
2. Protect the coverslips from light and leave them at room temperature for at least 30 min prior to acquisition.
3. Use absorbent tissue to clean the coverslips. Add a drop of immersion oil to the objective and place the slide on the microscope stage. Select the 63x objective with oil, raise the platform until the oil touches the slide, and adjust the focus on the microscope.
4. Open the program and activate the laser wavelengths of 488, 552, and 405 nm.
5. Set the image resolution to 1024 x 1024 pixels.
6. Click on **live bottom**, set the **Z stack**, and press **Begin**. Repeat this process and then press the **End** button.
7. Wait for the image acquisition to complete, and then select the option **Maximum Projection** in **tools**.
8. Save the experiment, and export the .lif format images to a computer.
9. Open the FIJI program, open the experiment in FIJI, and set the **view stack** to **Hyperstack**.
10. Select **open image files individually | stitch tiles**.
11. Select the **free hand** tool in the **Fiji** toolbar and trace each cell carefully by hand. Press **analyze | measure** to visualize fluorescence intensity. Repeat this process for each cell type per group.
12. Save the measurements and export them to a spreadsheet. Open the file containing data using statistical analysis software (see the **Table of Materials**) to perform statistical analysis.

8. Statistical analysis

1. Open a new project in the software.
2. For data with normal distribution, use Student's *t*-test; for nonparametric data, use the Mann-Whitney *U* test.
3. Paste the values obtained from the experimental results in the table.

4. Select **descriptive statistics** and choose the option **column statistics [all tests]** to analyze data distribution.
   NOTE: If the data follow Gaussian distribution, choose *t*-test to examine samples by comparing two pairs. If the distribution is non-Gaussian, analyze data using the Mann-Whitney *U* test with central tendency measures (means or medians) and measures of variation.
5. Choose the **best graph** option for optimal data representation.

Representative Results

This report investigates the role of DCs in *Leishmania* infection using flow cytometry and confocal microscopy. Initially, the phenotypic profile of the human monocyte-derived DC was established. Notably, the obtained $CD11c^{+}$ dendritic cell populations were positive for CCR7, CD209, CD80, CD1a, and HLA-DR. The results indicate that the expression of these markers in DC populations is profoundly impacted by *Leishmania* infection. Infected DCs exhibited augmented CD80, CD209, CCR7, and HLA-DR expression. However, downregulation of CD1a was also observed in *L .amazonensis*-infected DCs. Additionally, principal component analysis revealed substantial differences in the expression of these molecules (**Figure 1B-D**). The T-Distributed Stochastic Neighbor Embedding (t (tSNE) algorithm, a dimensionality reduction technique, was employed to better visualize how *Leishmania* infection affects the expression of maturation-related molecules.

Of note, the tSNE density plots show that infected DCs present differential HLA-DR and CD80 expression, which further suggests the upregulation of molecules involved in antigen presentation (**Figure 2**). To visualize DCs, F-actin immunostaining was performed by labeling the cells with fluorescent phalloidin. To observe *L. amazonensis* infection inside DCs, nuclear staining (DAPI) was used to compare *Leishmania*-infected cells with non-infected cells (**Figure 3**). The kinetic analysis of hDC infection involving *L. amazonensis* promastigotes (10:1) indicates the percentage of infected cells and the number of parasites per infected cell at 4 h after infection. Cells were then washed and re-incubated for 6, 12, or 24h. On average, 4-5 parasites were observed per DC in ~55% of the infected cells.

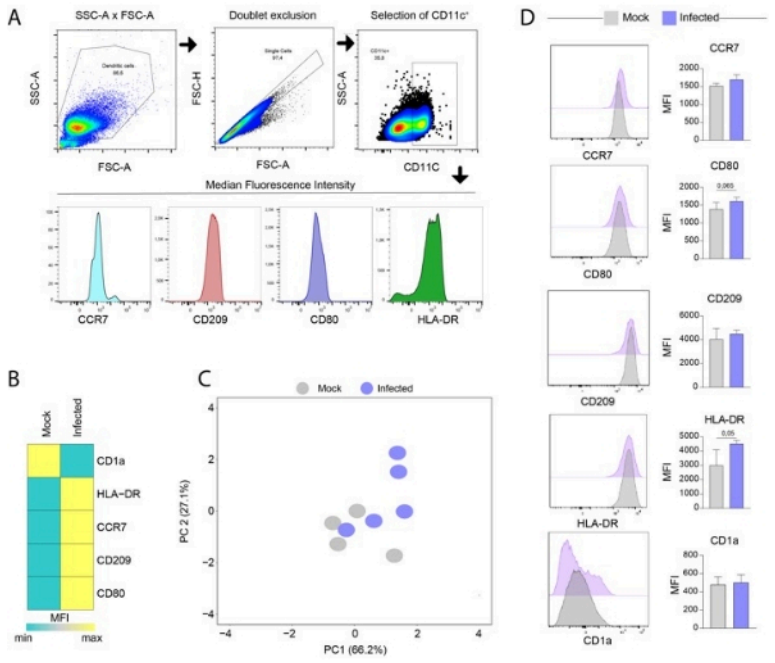

Figure 1: Marker-based human DC characterization after *Leishmania amazonensis* infection; gating strategy for selection of CD11c^{+}. (**A**) Assessment of costimulatory molecule MFI. (**B**) Heatmap and (**C**) principal component analysis of costimulatory molecule MFI values. (**D**) Representative histograms and scatter plots depicting MFI for each molecule. Abbreviations: DC = dendritic cell; MFI = median fluorescence intensity; SSC-A = side scatter area; FSC-A = forward scatter area; FSC-H = forward scatter height. Please click here to view a larger version of this figure.

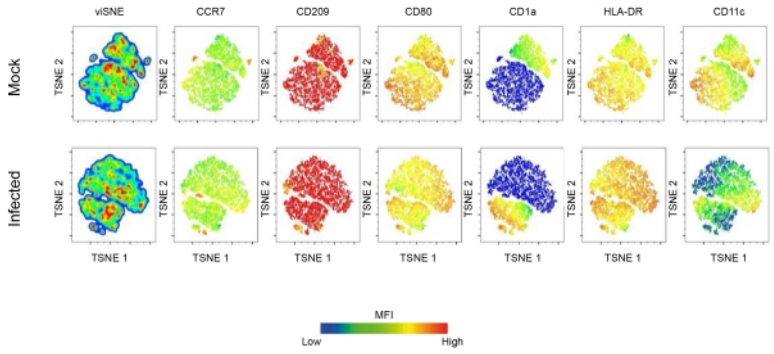

Figure 2: *Leishmania*-induced DC surface molecular expression. In-depth characterization of DC maturation landscape induced by *Leishmania* infection. Briefly, monocyte-derived DCs were obtained after 7 days of differentiation with GM-CSF and IL-4. Fully differentiated DCs were infected with *L. amazonensis* and then stained with a panel of maturation FACS antibodies. TSNE, a dimensionality reduction technique, was employed to perform a high dimensional analysis of flow cytometry data. Abbreviations: DC =dendritic cell; GM-CSF = granulocyte-macrophage colony-stimulating factor; FACS = fluorescence-activated cell sorting; IL-4 = interleukin-4; TSNE = T-Distributed Stochastic Neighbor Embedding. Please click here to view a larger version of this figure.

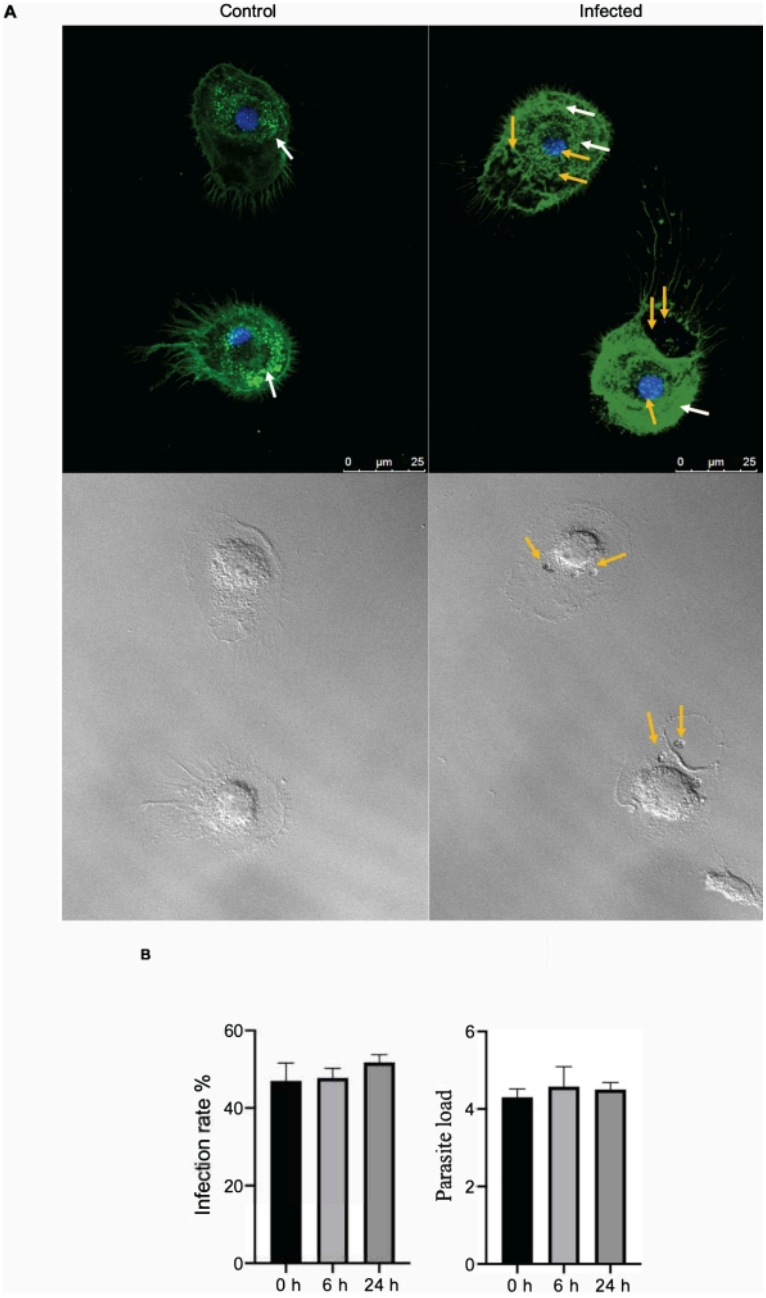

Figure 3: hDC infection with *Leishmania amazonensis.* Human dendritic cells were infected with *L. amazonensis* (10:1) for 4 h, washed, and labeled with phalloidin. (**A**) Fluorescence of F-actin labeling in infected or non-infected hDCs. Green, F-actin; blue, DAPI for nuclear staining of hDCs and nuclei of *Leishmania* parasites; yellow arrows represent amastigotes inside the cell; white arrows represent hDCs F-actin; greyscale, DIC. (**B**) Percentage of infection and parasitic burden in 400 cells randomly evaluated by fluorescence microscopy (analyzed using Anova). Scale bars = 25 µm. Abbreviations: hDC =

human dendritic cell; DAPI = 4',6-diamidino-2-phenylindole; DIC = differential interference contrast. Please click here to view a larger version of this figure.

MACS buffer
EDTA 2 mM
PBS 1x
BSA 0.5%
Phosphate-buffered saline (PBS) 1x
Ammonium chloride solution: 0.134 g of NH_4Cl
Saponin 15%
150 mg of saponin in 1 mL of 1x PBS
1 mL of 15% saponin in 100 mL of 1x PBS
10% Bovine serum albumin
100 mg – 1 mL → 10%
1 g – 100 mL → 1%
For 10% BSA - 1 g of BSA in 10 mL of 1x PBS
1x PBS/ 1% BSA / 0.15% Saponin: 20 mL of 1x PBS 2 mL of 10% BSA 200 µL of 15% Saponin
1x PBS/ 3% BSA / 0.15% Saponin: 20 mL of 1x PBS ; 6 mL of 10% BSA ; 200 µL 15% Saponin
1x PBS / 0.3% BSA / 0.15% Saponin: 20 mL PBS; 0.6 mL of 10% BSA; 200 µL of 15% Saponin

Table 1: Compositions of solutions used in this protocol.

Discussion

Leishmaniasis is a severe public health problem worldwide. The pathogenesis of this disease is quite complex, and the mechanisms favoring parasite survival in vertebrate hosts remain elusive[17]. DCs are professional antigen-presenting cells found throughout the body, including filtering and lymphoid organs. Following antigen capture and processing, immature DCs undergo a complex maturation process that culminates in their migration to lymph nodes, where these cells are responsible for presenting antigens to T lymphocytes[18]. This maturation process is characterized by increased MHC-class II molecular expression, notably CD11c, CD86, CD80, and CD1a[19]. In leishmaniasis,

monocyte-derived DCs phagocytose parasites and deliver the cargo to dLNs, inducing antigen presentation to T cells[13].

This paper describes a concise protocol that enables the investigation of the impact of *Leishmania* on the immunobiological functioning of DCs in an *in vitro* model of infection. This protocol encompasses the use of state-of-the-art immunological techniques, including the obtaining and differentiation of human monocytes into DCs, as well as DC characterization via flow cytometry and confocal microscopy[14]. Initially, monocytes were purified from the PBMCs of healthy donors. The use of a positive selection method, such as anti-human CD14 microbeads, ensures a high degree of purity (>95%) of differentiated moDCs. These cells are incubated with anti-human CD14 microbeads that bind the antibody to CD14-positive cells in the sample, which are then separated by a high-gradient magnetic field inside the column of a magnetic cell separator[20].

Notably, the *in vitro* differentiation of human monocytes into DCs requires the addition of GM-CSF and IL-4. GM-CSF is a pleiotropic growth factor that not only downregulates the expression of macrophage colony-stimulating factor (M-CSF) but also inhibits M-CSF-induced differentiation into macrophages. In addition, IL-4 acts by inhibiting macrophage colony formation, considered a suitable method for culturing moDCs *in vitro*[21]. The present protocol proposes the use of flow cytometry to assess DC differentiation by analyzing the expression of maturation-related markers, such as CCR7, CD209, CD80, CD1a, HLA-DR, and CD11c. Additionally, high-dimensional flow cytometry techniques (e.g., t-SNE) are used to precisely identify expression patterns influenced by *Leishmania* infection[22]. It is crucial to optimize the antibody dilution during immunostaining.

Confocal microscopy constitutes an excellent tool to analyze and quantify fluorescence intensity in human cells[23]. It is important to note that this assay requires careful manipulation before the image acquisition such as washing the coverslips gently and avoiding exposure to light. The main limitation of this method is the acquisition of suitable image resolution in confocal microscopy, which requires experience with microscopy manipulation. However, this method can be used to study the response of DCs to infection by different pathogens. Alternatively, the steps described in this protocol can also be extrapolated to other models of pathogen infection.

Disclosures

The authors have no conflicts of interest to disclose.

Acknowledgments

We thank the Gonçalo Moniz Institute (IGM-Fiocruz) (Bahia, Brazil) and the department of microscopy for assistance. The authors are grateful to Andris K. Walter for critical analysis, English language revision, and manuscript copyediting assistance.

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