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TITLE:

Obtainment of macrophages from human monocytes to assess *Leishmania braziliensis* infection rate and innate host immune response

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SUMMARY

This protocol describes the process for obtaining human macrophages from monocytes for infection with *Leishmania braziliensis*. It also allows researchers to evaluate infection rate and parasite viability, ROS production by fluorescence microscopy, and the production of inflammatory mediators in culture supernatants to investigate macrophage response to infection.

ABSTRACT

Macrophages are multifunctional cells essential to the immune system function, and the primary host cell in *Leishmania braziliensis* (Lb) infection. These cells are specialized in microorganism recognition and phagocytosis, but also activate other immune cells and present antigens, as well as promote inflammation and tissue repair. Here, we describe a protocol to obtain mononuclear cells from peripheral blood (PBMC) of healthy donors to separate monocytes that then differentiate into macrophages. These cells can then be infected *in vitro* at different Lb concentrations to evaluate the ability to control infection, as well as evaluate host cell immune response, which can be measured by several methods. PBMCs were first isolated by centrifuging with Ficoll-Hypaque gradient and then plated to allow monocytes to adhere to culture plates; non-adherent cells were removed by washing. Next, adherent cells were cultured

with macrophage-colony stimulating factor (M-CSF) for 7 days to induce macrophage differentiation. We suggest plating 2 x 10^6 cells per well on 24-well plates in order to obtain 2 x 10^5 macrophages. Fully differentiated macrophages can then be infected with Lb for 4 or 24 hours. This protocol results in a significant percentage of infected cells, which can be assessed by optical or fluorescence microscopy. In addition to infection index, parasite load can be measured by counting the numbers of parasites inside each cell. Further molecular and functional assays can also be performed in culture supernatants or within the macrophages themselves, which allows this protocol to be applied in a variety of contexts and also adapted to other intracellular parasite species.

INTRODUCTION

The intracellular protozoan parasite of the genus *Leishmania* is the causative agent of a neglected disease complex known as leishmaniasis[1]. These tropical diseases have a wide range of clinical manifestations that can range from skin lesions to complications arising from the visceral form of the disease, which can be fatal if not treated. Cutaneous leishmaniasis (CL) is the most frequent form of leishmaniasis and is characterized by a single or few ulcerated skin lesions with exacerbated chronic inflammation[2]. The development of disease is dependent on the *Leishmania* species, in addition to a combination of factors associated with host immune response, which both define clinical outcomes[3,4]. *Leishmania braziliensis* is the main species that causes CL in Brazil, with cases reported throughout all states of the country[5]. The immune response against *L. braziliensis* is considered protective, since it restricts the parasite to the inoculation site, and involves several immune cell types, such as macrophages, neutrophils e lymphocytes[4,6,7].

Macrophages are multifunctional cells essential for the immune system, since they are specialized in the detection and phagocytosis of microorganisms, and can present antigens and activate other cell types. Macrophages are able to regulate processes from inflammation to tissue repair and the maintenance of homeostasis[8,9]. These cells play an essential role in the early immune response against intracellular parasites, such as *Leishmania*, being important for their elimination[10–12].

During *L. braziliensis* infection, macrophages can respond through different mechanisms to eliminate the parasite, such as the production of reactive oxygen species (ROS) and inflammatory mediators[13,14]. Immune responses can be guided by the production of proinflammatory or anti-inflammatory cytokines, which contribute to an exacerbated inflammatory state or tissue repair processes[6,15,16]. The plasticity of macrophages is fundamental to the immunopathogenesis of CL, as well as to parasite-host interaction, and these cells are considered crucial to the elucidation of disease mechanisms and to the development of new therapeutic approaches.

As CL is a complex disease, investigations require researchers to explore cell types that mimic those found in humans. The immune responses observed in different experimental models can vary and produce results that do not reflect the immune response observed in naturally infected humans. Thus, the protocol presented herein was designed to enable the study of human macrophages and their immune responses during CL caused by *L. braziliensis*.

PROTOCOLS

The Institutional Review Board for Ethics in Human Research at the Gonçalo Moniz Institute (Oswaldo Cruz Foundation-IGM-FIOCRUZ, Salvador, Bahia-Brazil), approved this study (protocol number: CAAE 95996618.8.0000.0040).

1. Isolation of human PBMCs

1.1. Ensure that the blood samples, 1.077 g/mL density gradient (e.g., Ficoll-Histopaque), and saline solution are at room temperature.

1.2. Dilute blood samples with saline solution at 1:1 ratio.

1.3. Transfer 10-12 mL of density gradient to 50 mL tubes.

1.4. Carefully overlay up to 40 mL of the diluted blood sample on top of density gradient. Separate the blood and density gradient layers.

1.5. First centrifugation: centrifuge tubes containing blood and density gradient layers at 400 x *g* for 30 min at 24 ºC.

NOTE: Switch-off the brake before starting the centrifuge.

1.6. Remove the plasma above the PBMC ring with a pipette (the buffy coat layer is located between the plasma and density gradient layers; below that are red blood cells/granulocytes pellet).

1.7. Transfer the cloud-like PBMC layer (buffy coat layer) with a pipette to a 15 mL tube and fill with cold saline (kept on ice or at 4 °C).

1.8. Second centrifugation: centrifuge tubes at 300 x *g* for 10 min at 4 °C with the brake switched-on.

1.9. Discard the supernatant and resuspend the pellet by filling the tube with cold saline.

1.10. Third centrifugation: centrifuge tubes at 250 x *g* for 10 min at 4 °C with the brake switched-on.

1.11. Discard the supernatant and resuspend the pellet, filling the tube with cold saline.

1.12. Fourth centrifugation: centrifuge the tube at 200 x *g* for 10 min at 4 °C with the brake switched-on.

1.13. Discard the supernatant and resuspend the pellet with 1 mL of cold RPMI medium.

1.14. Count cells to determine the number of cells obtained.

2. Differentiation into human macrophages

NOTE: For a 24-well plate, calculate the amount of total cells needed to plate 2 x 10^6 cells per well, which will yield 2 x 10^5 macrophages. This yield is based on an average of 10%

monocytes in human blood. Alternatively, monocytes can also be released by non-enzymatic methods and then counted for plating.

2.1. On a 24-well plate, place 13 mm round glass coverslips at the bottom of each well. Plate the equivalent of 2×10^6 cells in 1 mL of RPMI incomplete per well.

NOTE: Limit the number of cells in each well, since overestimated amounts can hinder cell adhesion and compromise culturing. In addition, all coverslips must be clean and sterile.

2.2. Incubate plates for 30 min at 37 ºC under 5% CO_2 for cell adhesion.

2.3. Remove the supernatants and wash once with 0.9% saline at room temperature to remove any non-adherent cells.

2.4. After washing, remove the saline and add 250 µL of supplemented RPMI medium at room temperature (10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 ng/mL M-CSF) to each well.

2.5. Incubate the cells for 7 days at 37 ºC under 5% CO_2.

2.5.1. Add 125 µL of supplemented RPMI medium to each well every two days. At the end of cell differentiation, the final volume will be 500 µL per well.

2.5.2. To analyze cell viability, perform another culture in parallel on a 96-well plate (2×10^5 per well).

2.5.3. After differentiation into macrophages (7 days), add 20 µL of AlamarBlue reagent.

2.5.4. After 7 hours of incubation, read plates on a spectrophotometer at wavelengths of 570 nm and 600 nm.

3. *Leishmania* culture and infection

NOTE: *L. braziliensis* promastigotes from two different strains (MHOM/BR/01/BA788 and MHOM/BR88/BA-3456) were used in this assay.

3.1. Count *Lb* parasites and calculate the volume to obtain 5×10^5/mL parasites.

3.2. Prepare supplemented Schneider's Insect medium (10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin).

3.3. Incubate parasites in a total volume of 5 mL in a 24 ºC incubator until the stationary phase is reached (4 to 6 days).

NOTE: Count parasites every day to assess growth.

3.4. After reaching the stationary phase, centrifuge Leishmania cultures at 100 x *g* for 10 min at 4 ºC to remove any dead parasites (precipitated at the bottom of the tube).

3.5. Transfer the supernatant to a new tube and centrifuge at 1,800 x *g* for 10 min at 4 ºC to recover viable parasites. Discard the supernatant.

3.6. Resuspend the pellet with 1 mL of supplemented RPMI medium to count parasites.

3.7. Calculate the volume of parasites to obtain a 10:1 parasite:cell ratio. Transfer parasites to culture plates containing macrophages previously cultured in supplemented RPMI medium (~300 µL/well) at room temperature.

3.8. Remove the supernatant from each well containing differentiated macrophages.

NOTE: As soon as possible, replace the medium in each well to avoid cells spending extended periods without medium. We suggest removing and replacing medium in three wells at a time.

3.9. Transfer the calculated amount of Lb to each well containing differentiated macrophages.

3.10. Infect cells for 4 or 24 hours at 37 ºC under 5% CO_2.

3.10.1. After 4 hours, wash macrophages 3 times with saline at room temperature to remove any non-internalized parasites.

3.10.2. For the 24-hour infection period, add 300 µL of supplemented RPMI medium to each well after washing, and then reincubate for another 20 hours at 37 °C under 5% CO_2.

3.11. Remove the supernatant to measure inflammatory mediators using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions.

3.11.1. Centrifuge the collected supernatant at 1,800 x *g* for 10 min at room temperature. Transfer the supernatant to a new tube. This procedure is performed to remove any non-internalized parasites. Supernatants can be frozen and kept at -80ºC until the time of future analysis.

NOTE: Cells may be used to assess infection rate, parasite viability or ROS production.

4. Evaluation of infection

4.1. Quantification of infection rate

4.1.1. Add 300 µL of methanol to each well after removing the supernatant. Allow 15 min to fix cells adhered to coverslips.

4.1.2. Remove the coverslips from the wells and place on a support to soak in the cell staining solution (e.g., Quick Panoptic 2) for 1 min.

4.1.3. Submerge coverslips twice in cell staining solution (e.g., Quick Panoptic 3) and wait until dried.

3.12. Place 15 µL of mounting medium (e.g., Entellan) on slides, and then place coverslips over the medium.

NOTE: All macrophages attached to coverslips should be in contact with the mounting medium.

4.1.4. Count 100 cells at random under an optical microscope using an 100x objective to quantify infection rate and number of internalized amastigotes.

4.2. Parasite viability

4.2.1. After 4 hours of infection, wash cells with saline at room temperature 3 times.

4.2.2. Add 300 μL of supplemented Schneider's Insect medium.

4.2.3. Incubate in a 24 ºC incubator.

4.2.4. Quantify parasite growth after 48, 72, 96 and 120 hours.

5. Evaluation of ROS production by fluorescence microscopy

5.1. After the infection period, remove the supernatant and wash cells with 500 μL of saline.

5.2. Add 300 μL of the fluorogenic probe reagent (e.g., CellROX Green Reagent at 5 μM) to each well.

5.3. Incubate cells for 30 min at 37 ºC and 5% CO_2.

5.4. Wash cells 3x with 500 μL of phosphate buffered saline (PBS).

5.5. Fix cells with 300 μL of 3.7% formaldehyde and let sit for 15 min.

5.5.1. Measure the florescence signal within 24 hours.

5.6. Add 5 μL of DAPI staining agent (e.g., DAPI ProLong Gold antifade) for cell staining. Place the coverslip with the surface containing macrophages in direct contact with the DAPI staining agent.

5.7. Analyze the florescence signal by fluorescence microscopy at an excitation wavelength of 485/520 nm.

5.8. Calculate the Corrected Total Cell Fluorescence (CTCF) from 30 cells for each coverslip using ImageJ.
CTCF = Integrated Density (Area of a selected cell x Mean fluorescence of background readings)

6. Statistical analysis

6.1. Use the Mann–Whitney test to compare two groups with unpaired samples. Perform the statistical analyses using GraphPad Prism. Consider differences statistically significant when $p < 0.05$.

REPRESENTATIVE RESULTS

The comprehension of parasites and host cells interaction is crucial to elucidate mechanisms involved in the pathogenesis of several diseases. Although cultured human cells are less used due to limitations of cell culture compared to cell lineages, the protocol presented herein shows a robust and reproducible differentiation of human macrophages. This protocol enables the analysis of several aspects of the immune response and cell biology, from the production of inflammatory mediators up to the susceptibility of an infectious agent in human macrophages. The first evidence that cellular differentiation is taking place is macrophage morphology (**Figure 1A**). On the plating day, cells are rounded and small when compared to the morphology after seven days of culture. The cellular spreading is observed when cultures are treated with macrophage colony-stimulating factor (M-CSF). In the absence of M-CSF, cell differentiation takes more time and results in an heterogenous population of macrophage-like cells (data not shown). After 7 days of differentiation, macrophages were incubated with the Alamarblue reagent for 24 hours until reading. This method allows the quantification of the cellular capacity to reduce resazurin to resorufin, thus differentiating viable from dead cells. The "ctrl" group refers to macrophages cultured for 7 days in supplemented medium, while the "dead" group refers to macrophages submitted to osmotic lysis during differentiation, which serves as a control for the technique. Once the differentiation in complete (for seven days), the macrophages derived from human monocytes remain viable and prompt to further assays that can last up to 24 hours after differentiation (**Figure 1B**) or few days (data not shown).

The first moments of interaction between *Leishmania* and a phagocyte is marked by close contact that will culminate in phagocytosis and internalization of the parasite. To understand the process of infection will help to explain the mechanisms involved in parasite killing or susceptibility to a certain pathogen. Based on the results, the first four hours of infection present the highest infection rate of *L. braziliensis* (both BA788 and BA3456 tested strains). After 24 hours of infection, there is a reduction in the infection rate of both strains, but we found statistical significance only for BA788 (**Figure 2A, D**). Considering longer periods of infection, no internalized parasites were found inside the cells after 72 hours (data not shown), suggesting that human macrophages are able to control *L. braziliensis* infection *in vitro*. The infection rate is measured by the count of 100 cells and, among those, the infected ones. This estimates the percentage of infection, which can vary due to the immune response of the cell donor, the amount of *Leishmania* parasites in the stationary phase and also due to the experimenter bias. **Figure 2H** shows representative images of a low infection rate (left) and a higher rate (right) by optical microscope.

Another data that can be assessed in cultured human macrophages is the parasite load, which is important to indicate the ability of macrophages to control the infection. It is measured by the average of internalized parasites in each cell and, after different time points, it is possible to determine whether the number of parasites has increased or reduced (**Figure 2B, E**). Finally, results of parasite viability further assemble information about the infection control. It is measured by the count of viable parasites in the culture after replacing RPMI to Schneider medium. Regarding *L. braziliensis* infection, we have already tested different time points, such as 48 h, 72 h, 96 h and 120 h to quantify viable parasites. The results indicate that 72 hours is recommended to evaluate parasite viability in these conditions (**Figure 2G**).

Based on our protocol, it is also possible to measure the inflammatory response against *L. braziliensis* infection in culture supernatant as soon as 4 hours after the infection. We were able to detect IL-6, TNF-α and LTB_4 (**Figure 3A-C**), but IL-10 and IL-1β production was below the detection level (data not shown).

Another important aspect of the immune response against *L. braziliensis* is the production of reactive oxygen-derived species (ROS) by macrophages. This is one of the main mechanisms for parasite killing. The protocol presented herein show that ROS production from macrophages is significantly increased after 4 hours of infection (**Figure 4A**). The quantification of ROS based on corrected total cell fluorescence (CTCF) allowed the detection of almost twice the ROS production between infected and uninfected macrophages (**Figure 4B**).

Together, the results show that macrophage derived from human monocytes allows the study of several aspects of the immune response against *L. braziliensis* infection *in vitro*. Thus, this protocol enables research groups to further explore the role of human macrophages in leishmaniasis, minimizing the bias of lineage or murine cell models.

FIGURE LEGENDS

Figure 1. Cell morphology during human macrophage differentiation *in vitro*. (**A**) Representative images of the morphology of adherent cells at first day of culture (left) and after seven days of differentiation. (**B**) Cell viability after culture for seven days for differentiation. Ctrl = macrophages in culture with supplemented medium; Dead = macrophages subjected to osmotic lysis; Objective 60x; n = 3.

Figure 2. Several parameters of infection caused by two strains of *Leishmania braziliensis* can be assessed by optical microscopy. (**A, D**) Infection rate (**B, E**), parasite load (four and 24 hours of infection) and (**C, F**) representative images of macrophages infected by *L. braziliensis* from BA5456 (**A-C**) or BA788 (**D-E**) strains (four hours of infection). (**G**) Macrophage-derived viable *L. braziliensis* after four hours of infection with BA788 strain. (**H**) Representative images of human macrophages infected by *L. braziliensis* (BA788 strain) showing low (left) and high infection rate (right). Arrows = intracelular amastigotes; Scale bar 10 µm; *$p < 0.05$; (**A,B**) n = 3; (**D,E**) n = 6; (**G**) n = 6.

Figure 3. *L. braziliensis*-induced production of inflammatory mediators by human macrophages in four hours after infection. (**A**) LTB_4, (**B**) IL-6 and (**C**) TNF-*α* production in culture supernatant after four hours infection by *L. braziliensis* (BA788 strain) measured by ELISA. **$p < 0.01$; n = 5.

Figure 4. ROS production by human macrophages after *in vitro* infection with *L. braziliensis*. (**A**) Representative images of ROS fluorescent labelling in cultured human macrophages after four hours of infection by *L. braziliensis* (BA788 strain). (**B**) Quantification of ROS production based on the corrected total cell fluorescence (CTCF) using Image J. Green = ROS; Blue = nucleus; Scale bar 10 µm; * $p < 0.05$; n =6.

DISCUSSION

The protocol presented herein for human monocytes differentiation into macrophages followed by the infection with two strains of *L. braziliensis* allows the evaluation of several aspects of parasite-cell interaction. These tools can be crucial to elucidate unanswered questions about CL. With the establishment of this protocol, our group was able to uncover some aspects of the immune response of macrophages obtained from individuals with diabetes and CL[14].

The differentiation process of monocytes into human macrophages is complex and requires attention from the first day of culture. The researcher must monitor the differentiation, checking

the development of the culture by cellular morphology daily. Usually, the culture of monocytes for seven days with M-CSF containing medium is sufficient for complete differentiation. It is important to mention that cell morphology depends on the donor, thus several stages of cellular differentiation can be observed between donors. This can be overcome with an increase in the number of donors, which will allow the identification of outliers. Moreover, the use of M-CSF is crucial for fully differentiation into macrophages[17]; otherwise, it will result in a highly heterogeneous population of dendritic cells-like, macrophage-like cells and monocytes. Other growth factors or a combination of these has been used as tolls to further polarize macrophages into M1 or M2 profile[17, 18]. Several studies have shown that macrophages cultured with M-CSF develop an M2 profile, while cells treated with GM-CSF exhibit an M1 profile[19]. However, macrophages cultured with M-CSF can polarize to the M1 profile after stimulation[20]. Based on the results, we were unable to determine a macrophage profile prior to infection, but hypothesize that this probably trended toward an M2 profile. On the other hand, after 4 hours of infection, macrophages produced high levels of pro-inflammatory cytokines and ROS, which is characteristic of a classic macrophage profile. Another relevant aspect about the infection of macrophages with *Leishmania* is the dispersion observed in the percentage of infected cells (infection rate). This is a marked feature of assays with human macrophages, also due to the responsiveness of each donor. To minimize this effect, the stationary-phase of *Leishmania* cultures should be confirmed and methods to purify metacyclic promastigotes can be considered[21–23]. In addition, our findings show that increased infection periods result in a decrease in the infection rate, since human macrophages seem to be able to control *L. braziliensis*.

The interaction of macrophages and *Leishmania* involves the production of mediators that are a combination of a protective response of the host cell to kill the parasite and escape mechanisms developed by each *Leishmania* species. Thus, to define the profile of mediators produced during the infection is essential to understand the pathogenesis of CL[24]. Based on our protocol, it is possible to measure pro-inflammatory mediators after 4 hours of infection with *L. braziliensis*. With this method, we were also able to show this inflammatory response of macrophages from individuals with diabetes after infection *in vitro* with *L. braziliensis*[14]. The possibility to evaluate different inflammatory mediators is crucial to better understand CL as a chronic inflammatory skin disease[24–26].

The production of mediators involved in parasite killing also play a significant role in the disease development and outcome. It has already been described that ROS production is one of the most efficient mechanism to control *L. braziliensis* infection[13,14]. The protocol presented herein allow the evaluating of ROS production within the macrophages infected by *L. braziliensis* using fluorescence microscope. ROS production seems to be key to define susceptibility to the infection[14,25]. Unlike other methods, the quantification of ROS through fluorescence microscopy has the advantage of locally identifying production inside the cell. Other methods only allow the indirect quantification of ROS production from an entire cell population, without considering that cells can respond differently to the same stimulus[13,25].

In summary, the results show that the protocol described herein enables studies that aim to explore the interaction between macrophages and *L. braziliensis*, assessing aspects such as the infection rate, parasite killing and production of mediators. This allows to extrapolate the findings and to correlate it with naturally occurring mechanisms.

DISCLOSURES

The authors declare they have no competing financial interests.

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Figure 1

Click here to access/download;Figure;Figure_1.tiff

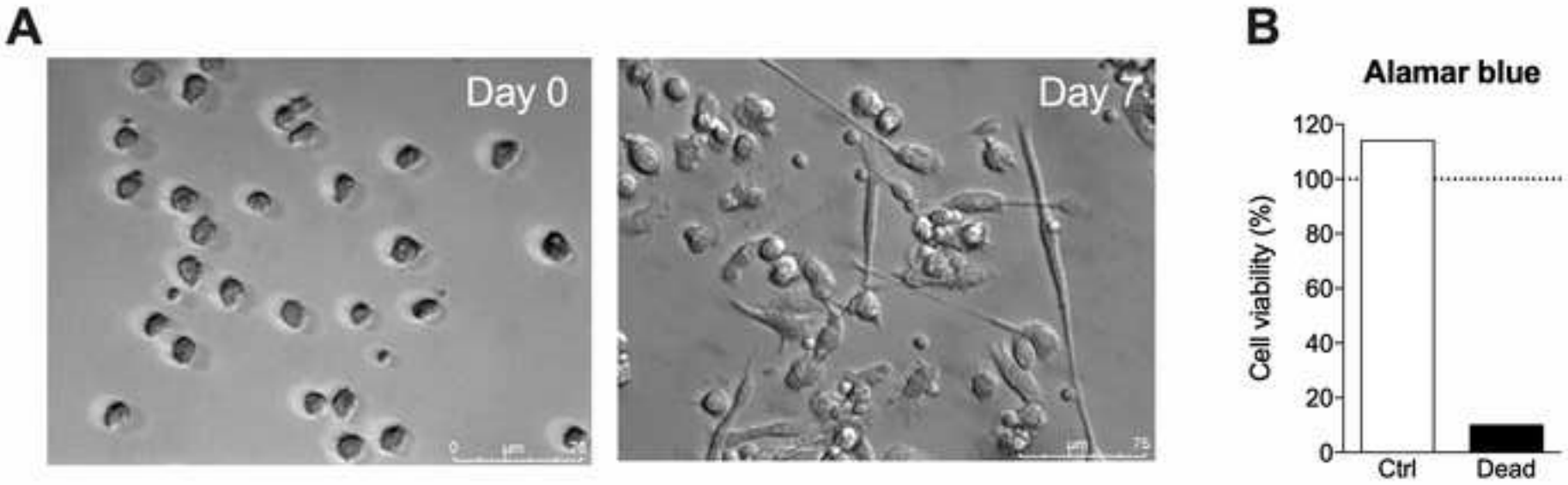


Figure 2

Click here to access/download;Figure;Figure_2.tiff

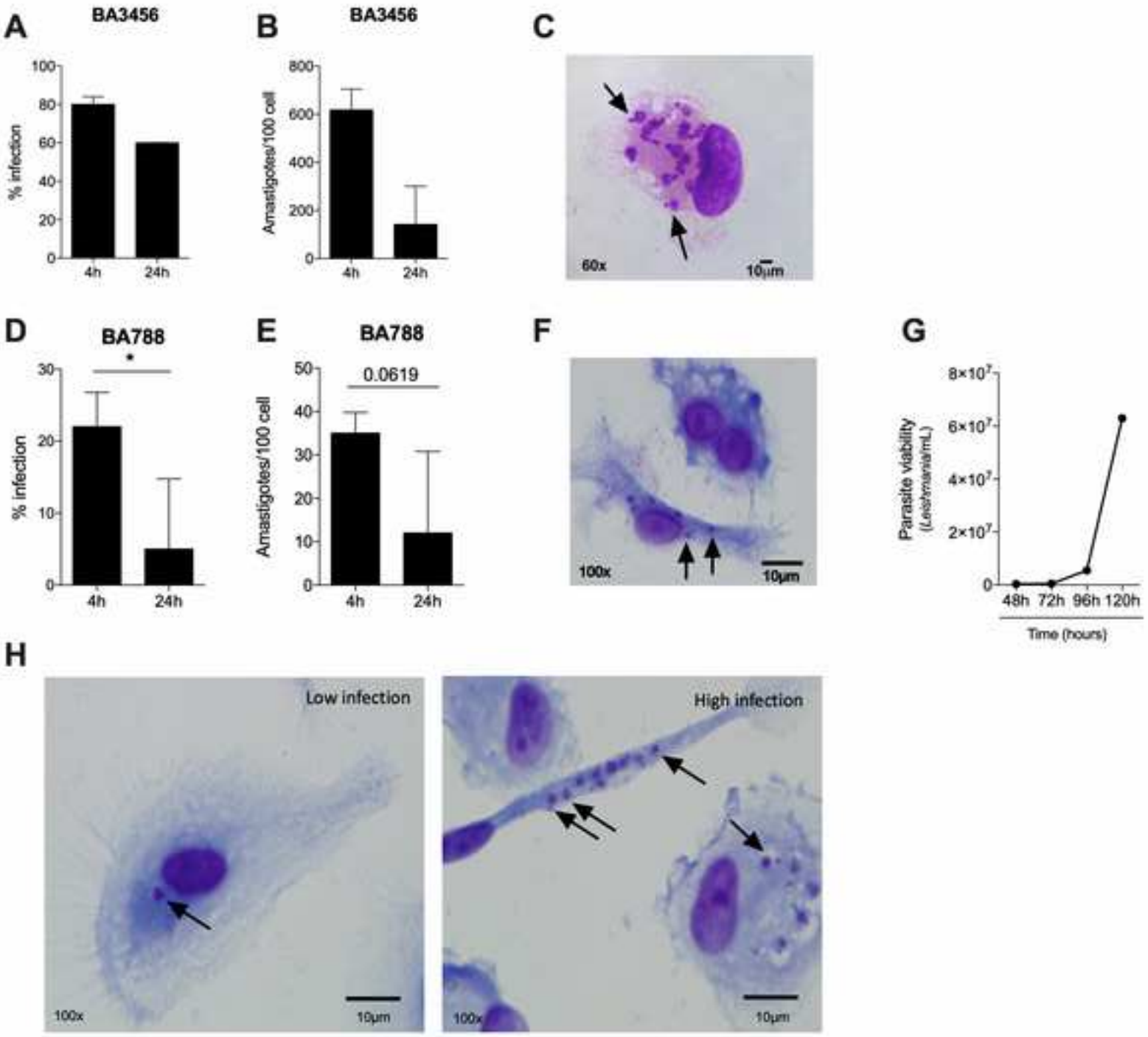


Figure 3

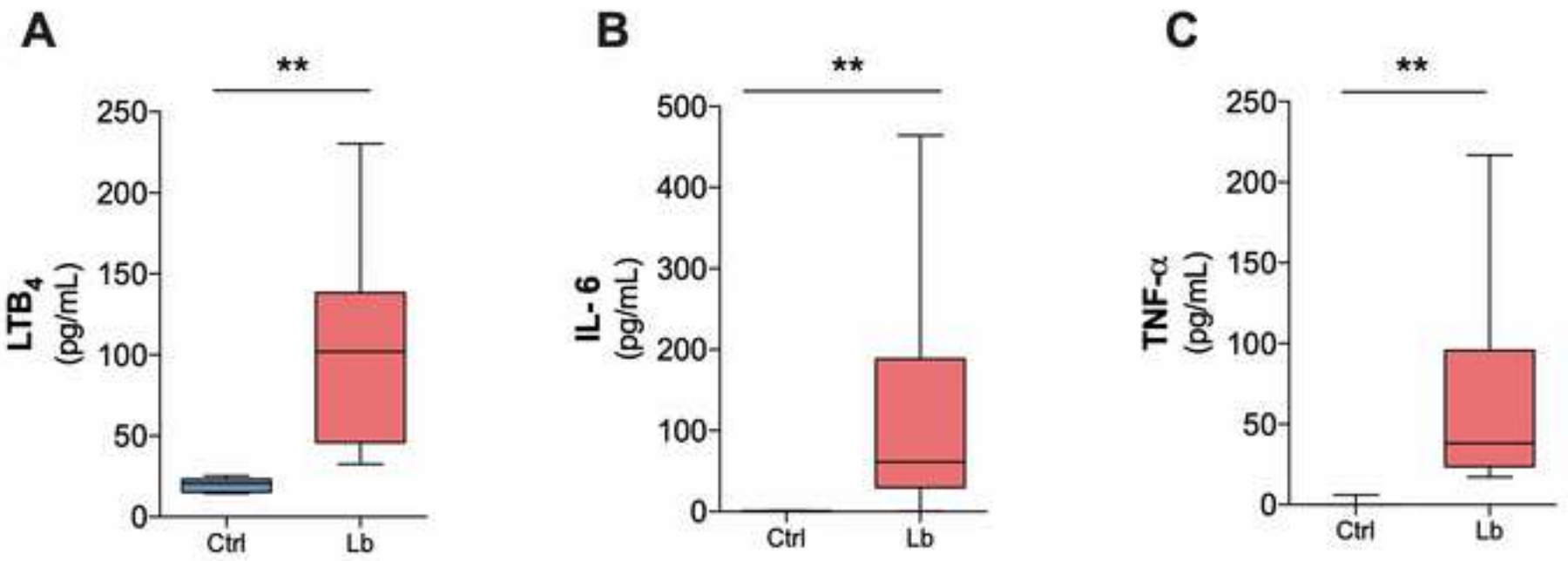
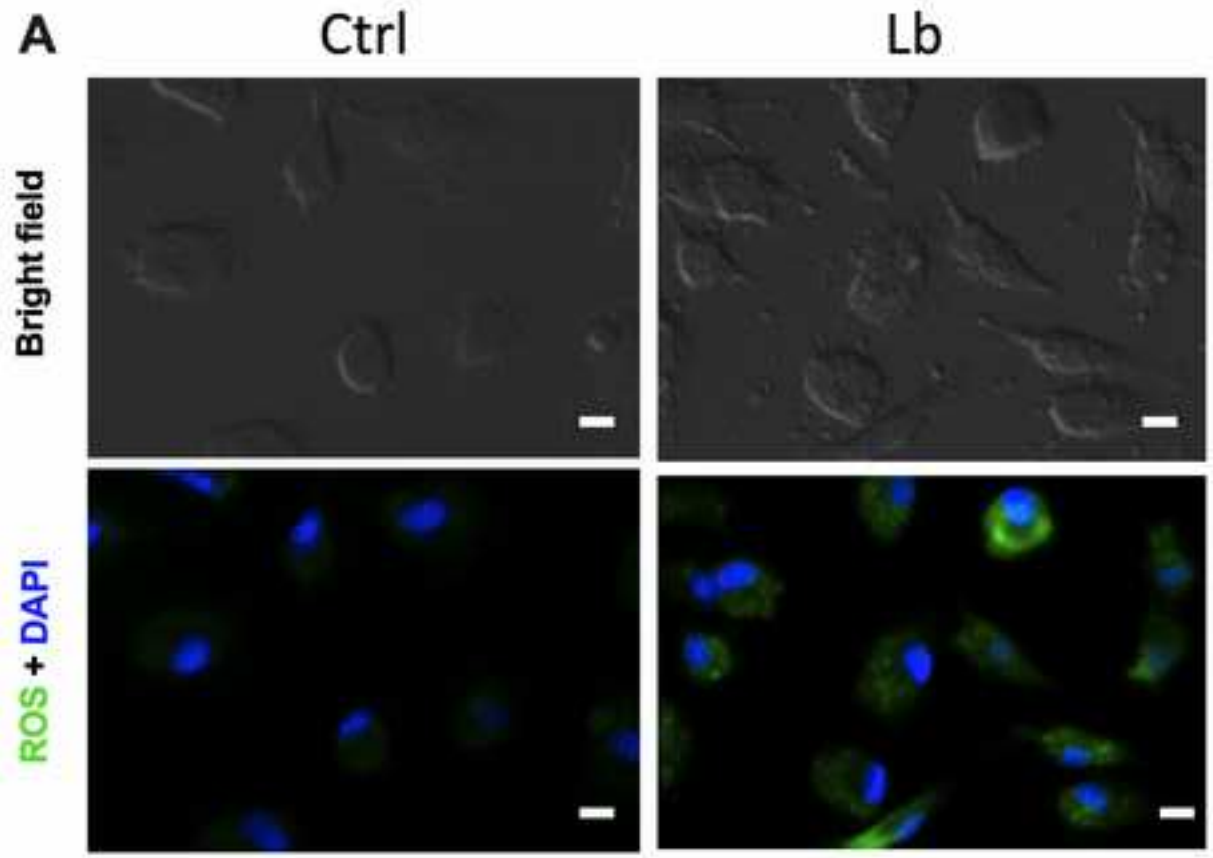
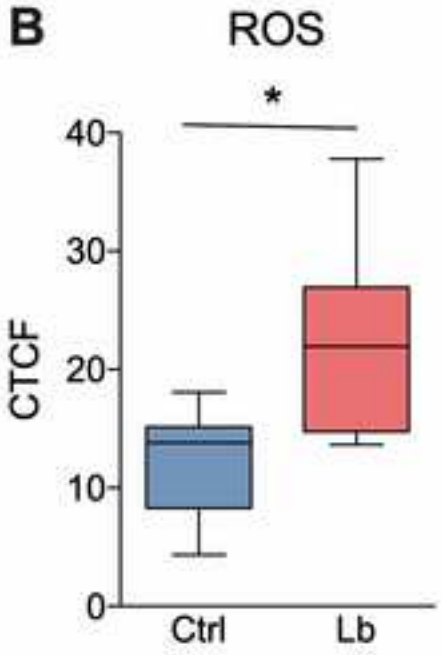


Figure 4

Click here to access/download;Figure;Figure_4.tiff





Name of Material/ Equipment	Company	Catalog Number
AlamarBlue Cell Viability Reagent	Invitrogen	DAL1100
Cell Culture Flask 25 cm²	SPL	70125
CellROX Green Reagent	Invitrogen	C10444
Coverslip circles 13 mm	Perfecta	10210013CE
DAPI (4',6-diamidino-2-phenylindole)	ThermoFisher	D1306
Disposable support for blood collection	BD Vacutainer	364815
Eclipse blood collection needle 21 g x 1.25 in	BD Vacutainer	368607
Entellan	Sigma Aldrich	107961
Falcon Conical Tubes, 15 mL	Sigma Aldrich	CLS430791-500EA
Falcon Conical Tubes, 50 mL	StemCell Technologies	100-0090
Fetal Bovine Serum	Gibco	A4766801
Formaldehyde 3.7%	Merck	252549
Glass slide 25,4x76,2mm	Perfecta	0200
Histopaque	Sigma Aldrich	10771
Human IL-6 ELISA Kit	RD	DY206
Human M-CSF Recombinant Protein	PeproTech	300-25
Human TNF-a ELISA Kit	RD	DY210
Leukotriene B4 ELISA Kit	Cayman	520111
Methanol	Merck	MX0482
Penilicin-Sreptomycin-Glutamine (100x)	ThermoFisher	10378-016
Phosphate Buffered Saline pH 7.2 (10x)	Gibco	70013032
Plasma tube, 158 USP units of sodium heparin (spray coated)	BD Vacutainer	367874
Quick H&E Staining Kit (Hematoxylin and Eosin)	abcam	ab245880
RPMI 1640 Medium	Gibco	11875093
Schneider's Insect Medium	Sigma Aldrich	S0146
Tissue Culture 24-wells Plate	TPP	Z707791-126EA
Trypan Blue	Gibco	15250061

Comments/Description

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Point-by-point reply to reviewer's comments

The authors would like to thank the reviewers for their careful and thorough reading of our manuscript. We also appreciate the comments and suggestions received, which have improved the quality of our work. Please find detailed responses for each point raised by the reviewer's concern below:

Reviewer #1

Major Concerns

Reviewer #1's concern 1:

The clarity and precision of the title of the manuscript should be priorities, I would suggest its reformulation.

*R. We appreciate the suggestion, and the title now reads "**Obtaining macrophages from human monocytes to analyze the infection rate and innate mechanisms against Leishmania braziliensis**".*

Reviewer #1's concern 2:

It would be interesting to include an analysis of the phenotypic markers of macrophages of the cells obtained after the 7 days of culture with GM-CSF, and to compare them with the cells obtained from the culture without GM-CSF.

R. In fact, M-CSF is the growth factor used to induce macrophage differentiation from monocytes. It is known that the absence of a growth factor induces a heterogeneous population, with cells that phenotypically resemble macrophages, dendritic cells and monocytes (1–4)*. Besides, macrophages are adherent cells and their removal from culture plates induces changes in the expression profile that could artificially bias the results. Nevertheless, few studies have phenotypically characterized monocyte-derived macrophages by flow cytometry. It has been shown that M-CSF induces the expression of CD163, a M2 marker, CD80, CLEC10A and CD200R* (5)*. Besides their phenotype, cell morphology of M-CSF cultured monocytes more closely resembles tissue macrophages (elongated) when compared to cells cultured without growth factors (rounded)* (1,2)*.*

Reviewer #1's concern 3:

The manuscript needs a careful grammar English revision.

R. We thank the reviewer and an English native speaker has revised the manuscript.

Minor Concerns

1. Some sentences all through the manuscript need to reformulated or fixed, as line 25 "detecting and phagocyte microorganisms"; Line 31: "From the whole blood, monocytes are obtained by adhesion to the culture plate"; Line 134-135: "Separate the supernatant in a new tube and centrifuge at 1800 xg for 10 min at 4ºC to remove dead parasites".

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R. The sentences have been reformulated as follows:

- *Line 25 "They are specialized in microorganism recognition and phagocytosis";*

- *Line 31 "Peripheral blood mononuclear cells (PBMCs) were isolated by centrifuging with Ficoll reagent. After, PBMCs were plated to allow monocytes adherence to culture plate and non-adherent cells were removed by washing"*
- *Line 134-135 "Monitor parasite growth daily and, at the stationary phase, centrifuge the Leishmania culture at 100xg for 10 min at 4º C to remove dead parasites (precipitated at the bottom of the tube). Transfer the supernatant to a new tube and centrifuge at 1800xg for 10 min at 4ºC to recover viable parasites"*

2. Please, indicate the density of Ficoll-Histopaque used in this protocol.

R. Ficoll-Histopaque density was mentioned at line 83 (1.077 g/ml - Sigma).

3. In place of the word "break" used several times by the authors throughout the manuscript, I imagine they mean "brake. Please change the word accordingly.

R. We apologize for this oversight and have changed the word throughout the text.

4. Indicate where the PBMC layer is supposed to be observed after the centrifugation of the gradient.

R. We have included a detailed description of the PBMC layer at lines 92 -94, as follows: "Remove the plasma above the PBMC ring with a pipette (the buffy coat layer is located between plasma and ficoll layers, bellow that, there are red blood cells/granulocytes pellet)".

5. It is desirable to specify the DAPI used in this protocol

R. DAPI ProLong Gold antifade (Thermo Fisher Scientific) *was mentioned in line* 205.

6. Please, check whether the cytokine measurements were actually performed just 4 hours after infection?

R. Yes, the assays to quantify cytokine production were conducted after 4 hours of parasite infection. This has already been reported by our group (6,7).

7. Figure 1B: it is not clear how the cell viability analysis was carried out.

R. We appreciate the observation. We included the procedure on lines 131 – 135 and 235 -240, as follows: "To analyze cell viability, another culture was done in parallel with the same donors in a 96-well plate (2x105 per well). After differentiation in macrophages (seven days), 20 µL of the AlamarBlue (ThermoFisher) reagent was added and after 7 hours of incubation, plate was read on a spectrophotometer at a wavelength of 570 nm and 600 nm" and "After 7 days of differentiation, macrophages were incubated with the Alamarblue reagent for 24 hours until reading. This method allows to quantify the capacity to reduce resazurin to resorufin, differentiating viable from dead cells. The "ctrl" group refers to macrophages after 7 days of culture with supplemented medium and the "dead" group, to macrophages submitted to osmotic lysis during its differentiation, as a control of the technique".

We also have included the reference for each group in the figure legend. "Ctrl = macrophages in culture with supplemented medium; Dead = macrophages subjected to osmotic lysis".

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Reviewer #2

Minor Concerns

1. Line 115: "3. Remove the supernatants with a pipette and add..." After adhesion for 30 min of monocytes, authors recommend to remove the supernatants and add medium. At this moment, all non-adherent cells are removed without washings with warm medium?

R. We appreciate the observation. This step includes washing the cells with saline at room temperature to remove non-adherent cells in the supernatant. We have included this point in the methodology, as follows: "Remove the supernatants and wash once with 0.9% saline at room temperature to remove non-adherent cells. After washing, remove the saline and add 250 µL of supplemented RPMI medium room temperature (10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 ng/mL M-CSF) to each well" on the lines 123 – 127.

2. Line 121 - end of the differentiation of the macrophages: According to the protocol plating 2 millions of PBMC it will be achieved 2 x 10(5) macrophages. Thus, it seems that it is not necessary to detach the cells, count and replated again, because macrophages were derived on coverslips. Are the authors confident that in each well there is the same amount of macrophages to have homogenous results (especially for measurements of inflammatory mediators in supernatants)?

R. The reviewer is right, but it is important to mention that these values are based on the average of cell count. Considering that the human blood is composed of 10% monocytes, on average, our protocol is based on this efficiency, which we have previously standardized. However, there is the possibility to detach and count the cells. We do not recommend this step due to the stress the cells are submitted, which can alter their phenotype and response. Moreover, the efficacy of these methods to detach and count the cells are controversial and can result in a significant cell loss. We detail the plating performance in the methodology section on lines 113 - 115.

3. Line 127: 100mg/mL streptomycin - change to 100 ug/mL

R. It was altered.

4. Line 140: "8. Remove the supernatant from each well." I think it is good to complete this phrase with ... containing the differentiated macrophages. Then the read is back to the macrophages.

R. The sentence now reads: "Remove the supernatant from each well containing the differentiated macrophages.".

5. Line 146: "11. Remove the supernatant to measure inflammatory mediators.." Without washings to remove excess or non-internalized parasites after 4h of incubation?

R. We appreciate the observation. In fact, we centrifuge to remove non-internalized parasites. We detail this in the methodology on lines 169-172. The sentence now reads: "Centrifuge the collected supernatant 1800xg for 10 min at room temperature. Transfer the supernatant to a new tube. This procedure is performed to remove non-internalized parasites. Supernatants can be frozen and kept at -80°C".

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6. Line 155: "1. Add 300 µL of methanol..." Please, complete the phrase with: after removing all the supernatant.

R. We completed the sentence as suggested.

7. Line 167: (Parasite viability) - Why this time of 4 h for viability of the parasites and no other time points?

R. The differentiation of human macrophages is a cell culture with a relatively long time (7 days), our protocol was thought of as the quickest way to evaluate L.b infection. The macrophage is an innate cell that responds quickly to stimuli, we evaluated shorter times, such as 30 minutes and 3 hours, 4 hours proved to be the best time to evaluate the immune defense mechanisms. Besides, a short infection time, such as 4 hours, allows this protocol to be used for testing drugs by incubating after this period.

8. Figure 1 - The cell viability after 7 days of differentiation presented in Fig.1B, the method was not described or cited, please include it.

R. We apologize for this oversight. The procedure was included on lines 131-135, as following: "To analyze cell viability, another culture was done in parallel with the same donors in a 96-well plate ($2x10^5$ per well). After differentiation in macrophages (seven days), 20 µL of the AlamarBlue (ThermoFisher) reagent was added and after 7 hours of incubation, plate was read on a spectrophotometer at a wavelength of 570 nm and 600 nm."

9. Figure 3. Caption: To refer the time of incubation.

R. We have included 4 hours to indicate the time of incubation.

10. Lines 303-307 - Discussion - Could you conclude if this macrophage is M1 according your results? As other studies show that M-CSF and GM-CSF can lead to different profiles of macrophages, as M1 and M2.

R. Indeed, the combination of growth factors, such as M-CSF and GM-CSF with other mediators, has been used for macrophage polarization. Several studies have shown that macrophages cultured with M-CSF develop a M2 profile, while GM-CSF treatment, a M1 profile (3)*. However, macrophages cultured with M-CSF can polarize to the M1 profile after stimulation* (4)*. Based on our results, we cannot determine macrophage profile before the infection, but it probably trend toward a M2 profile. On the other hand, after 4 hours of infection, these macrophages produce high levels of pro-inflammatory cytokines and ROS, which characterizes a classic macrophage profile. These points were added to the discussion section lines 332-338.*

11. References: there are several names of microorganisms not italicized.

R. These references were updated.

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