Journal of Visualized Experiments

In Vivo Infection with Leishmania amazonensis to Evaluate Parasite Virulence in Mice

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60617R1
Full Title:	In Vivo Infection with Leishmania amazonensis to Evaluate Parasite Virulence in Mice
Section/Category:	JoVE Immunology and Infection
Keywords:	C57BL/6 mice, inflammation response, parasite load, lesion development, cutaneous infection, axenic amastigotes
Corresponding Author:	Maria Fernanda Laranjeira-Silva Universidade de Sao Paulo Sao Paulo, BRAZIL
Corresponding Author's Institution:	Universidade de Sao Paulo
Corresponding Author E-Mail:	mfernandals@usp.br
Order of Authors:	Juliana Ide Aoki
	Ahyun Hong
	Ricardo Andrade Zampieri
	Lucile Maria Floeter-Winter
	Maria Fernanda Laranjeira-Silva
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Sao Paulo, SP, Brazil

UNIVERSITY OF SÃO PAULO
BRAZIL
INSTITUTE OF BIOSCIENCES
Department of Physiology

Maria Fernanda Laranjeira-Silva, Ph.D.
Email: mfernandals@usp.br

Journal of Visualized Experiments
Review Editor

October 2nd, 2019

Re: JoVE60617

Dear Dr. Vineeta Bajaj,

We are submitting a revised version of our manuscript “Murine *in vivo* infection with *Leishmania amazonensis* is a reliable method for a systemic evaluation of the host-parasite interaction” by Aoki et al. As requested, we made the title more concise: “Murine *in vivo* infection with *Leishmania amazonensis* is a reliable method to evaluate the parasite virulence within the host”.

We thank you for the thoughtful handling of this review, which we believe has significantly improved our paper. In this revision we address all the issues raised by the four reviewers, with particular attention to your comments as well.

We look forward to hearing from you.

Sincerely,

Dr. Maria Fernanda Laranjeira-Silva

TITLE:

In Vivo Infection with *Leishmania amazonensis* to Evaluate Parasite Virulence in Mice

AUTHORS AND AFFILIATIONS:

Juliana Ide Aoki[1], Ahyun Hong[1], Ricardo Andrade Zampieri[1], Lucile Maria Floeter-Winter[1], Maria Fernanda Laranjeira-Silva[1]

Department of Physiology, Institute of Bioscience, University of Sao Paulo, Sao Paulo, Brazil

Corresponding Author:

Maria Fernanda Laranjeira-Silva (mfernandals@usp.br)

E-mail Addresses of Co-authors:

Juliana Ide Aoki (juaoki@usp.br)
Ahyun Hong (avery.ahyun.hong@ib.usp.br)
Ricardo Andrade Zampieri (ricardoz@usp.br)
Lucile Maria Floeter-Winter (lucile@ib.usp.br)
Maria Fernanda Laranjeira-Silva (mfernandals@usp.br)

KEYWORDS:

C57BL/6 mice, inflammation response, parasite load, lesion development, cutaneous infection, axenic amastigotes

SUMMARY:

Here, we present a compiled protocol to evaluate the cutaneous infection of mice with *Leishmania amazonensis*. This is a reliable method for studying parasite virulence, allowing a systemic view of the vertebrate host response to the infection.

ABSTRACT:

Leishmania spp. are protozoan parasites that cause leishmaniases, diseases that present a wide spectrum of clinical manifestations from cutaneous to visceral lesions. Currently, 12 million people are estimated to be infected with *Leishmania* worldwide and over 1 billion people live at the risk of infection. *Leishmania amazonensis* is endemic in Central and South America and usually leads to the cutaneous form of the disease, which can be directly visualized in an animal model. Therefore, *L. amazonensis* strains are good models for cutaneous leishmaniasis studies because they are also easily cultivated in vitro. C57BL/6 mice mimic the *L. amazonensis*-driven disease progression observed in humans and are considered one of the best mice strains model for cutaneous leishmaniasis. In the vertebrate host, these parasites inhabit macrophages despite the defense mechanisms of these cells. Several studies use in vitro macrophage infection assays to evaluate the parasite infectivity under different conditions. However, the in vitro approach is limited to an isolated cell system that disregards the organism's response. Here, we compile an in vivo murine infection method that provides a systemic physiological overview of the host-parasite interaction. The detailed protocol for the in vivo infection of C57BL/6 mice with *L. amazonensis* comprises parasite differentiation into infective amastigotes, mice footpad

cutaneous inoculation, lesion development, and parasite load determination. We propose this well-established method as the most adequate method for physiological studies of the host immune and metabolic responses to cutaneous leishmaniasis.

INTRODUCTION:

Leishmaniases are worldwide prevalent parasitic infectious diseases representing important challenges in developing countries and are recognized as one of the most important neglected tropical diseases by the World Health Organization[1,2]. The leishmaniases are characterized by cutaneous, mucosal, and/or visceral manifestations. Cutaneous leishmaniasis is usually caused by *L. amazonensis*, *L. mexicana*, *L. braziliensis*, *L. guyanensis*, *L. major*, *L. tropica* and *L. aethiopica*[3]. This form of the disease is often self-healing in humans due to the induction of protective cellular immune response. However, the cellular immune response may fail, and the disease can progress to disseminated cutaneous leishmaniasis[4,5]. There is no available vaccine due to the diversity among *Leishmania* species and host genetic backgrounds[6,7]. Treatment options are also limited as most of the currently available drugs are either expensive, toxic, and/or may require long-term treatment[8,9]. Besides, there have been reports of drug resistance against the available treatments[10,11].

The causative agent of leishmaniases is the protozoan parasite *Leishmania*. The parasite presents two distinct morphological forms in its life cycle: promastigotes, the flagellated form found in sandflies; and amastigotes, the intracellular form found in the parasitophorous vacuoles of the mammalian host macrophages[12,13]. Amastigotes' ability to invade, survive, and replicate despite the defense mechanisms of the vertebrate host's macrophages are subject to many studies[14–17]. Consequently, several research groups have been describing in vitro macrophage infection assays to evaluate the impact of specific environmental factors, as well as parasite and host genes on parasite infectivity. This assay presents several advantages, such as the ability to adapt studies to a high throughput format, relatively shorter time period to obtain results, and reduced number of laboratory animals sacrificed[18]. However, the findings of in vitro assays are limited because they do not always replicate in vivo studies[14,19–21]. In vivo assays provide a systemic physiological overview of the host-parasite interaction, which cannot be fully mimicked by in vitro assays. For instance, immunological studies can be performed through immunohistochemical assays from collected footpad tissue sections or even from popliteal lymph nodes for analysis of the recovered immune cells[22].

Animals are often used as a model for human diseases in biological and biomedical research to better understand the underlying physiological mechanisms of the diseases[23]. In the case of leishmaniasis, the route, site, or dose of inoculation influence the disease outcome[24–27]. Furthermore, susceptibility and resistance to the infection in humans and mice are highly regulated by the genetic backgrounds of the host and parasite[4,5,22,28–31]. BALB/c mice are highly susceptible to *L. amazonensis* cutaneous infection, showing a rapid disease progression with the parasites' dissemination to the lymph nodes, spleen, and liver[32]. As the disease may progress to cutaneous metastases, the infection can be fatal. In contrast, C57BL/6 mice often develop chronic lesions with persistent parasite loads in *L. amazonensis* infection assays[33]. Thereby, *L. amazonensis* infection with this particular mouse species has been considered an excellent model

to study chronic forms of cutaneous leishmaniasis in humans, because it mimics the disease progression better than the BALB/c mice infection model[5,34].

Hence, we propose that the murine in vivo infection is a useful method for *Leishmania* virulence physiological studies applicable to human disease, allowing a systemic view of the host-parasite interaction. Revisiting well-established assays[22], we present here a compiled step-by-step protocol of the in vivo infection of C57BL/6 mice with *L. amazonensis* that comprises the parasite differentiation into axenic amastigotes, mice footpad cutaneous inoculation, lesion development, and parasite load determination. This protocol can be adapted to other mice strains and *Leishmania* species that cause cutaneous leishmaniases. In conclusion, the method presented here is crucial in identifying new anti-*Leishmania* drug targets and vaccines, as well as in physiological studies of the host immune and metabolic responses to *Leishmania* infection.

PROTOCOL:

All experimental procedures were approved by the Animal Care and Use Committee at the Institute of Bioscience of the University of São Paulo (CEUA 342/2019), and were conducted in accordance with the recommendations and the policies for the Care and Use of Laboratory Animals of São Paulo State (Lei Estadual 11.977, de 25/08/2005) and the Brazilian government (Lei Federal 11.794, de 08/10/2008). All steps described in sections 1–5 should be carried out aseptically inside laminar flow cabinets. Personal protective equipment should be utilized while handling live *Leishmania* parasites.

1. In vitro differentiation of *L. amazonensis* promastigotes into axenic amastigotes[15,20,35–38]

NOTE: *L. amazonensis* (MHOM/BR/1973/M2269) (*La*) parasite was used in this assay. Depending on the study purpose, the infective parasite form can be obtained either by purification of the metacyclic form using a density gradient, as previously described[14], or by differentiation of promastigotes into axenic amastigotes, according to the following protocol.

1.1. Grow *La* promastigotes in a 25 cm^2 cell culture flask containing 10 mL of medium for promastigotes (pro-medium) (pH = 7.0).

1.2. Incubate at 25 ˚C for 3 days.

NOTE: Use promastigote cultures that were passaged in vitro less than 10x to avoid the loss of virulence and aneuploidy changes of parasites[39–44].

1.3. Pipette 5 mL of logarithmic growth phase promastigote culture into a new 25 cm^2 flask.

1.4. Add 5 mL of medium for axenic amastigotes (ama-medium) (pH = 5.2).

1.5. Incubate at 34 ˚C for 3–4 days.

1.6. Split the culture by diluting with ama-medium at a ratio of 1:3 into a new 25 cm^2 flask.

1.7. Incubate at 34 ˚C for 3–5 days.

NOTE: Incubate the axenic amastigotes for up to 5 days, as it represents the end of maturation[37].

2. C57BL/6 footpad infection with *L. amazonensis*

NOTE: Female C57BL/6 mice (6–8 weeks old) were obtained and maintained at the Animal Center of the Biomedical Sciences Institute of the University of São Paulo. Animals received food and water *ad libitum*.

2.1. Count the culture of axenic amastigotes by transferring an aliquot of the parasite suspension diluted in PBS to a Neubauer chamber (i.e., hemocytometer). Count the non-flagellated parasites, which represent amastigotes forms.

NOTE: Alternatively, Trypan blue (1:1) can be used for counting the number of viable amastigotes (i.e., those not stained with Trypan blue).

2.2. Dilute the axenic amastigotes in PBS according to the desired inoculum dose and the number of inoculums intended (1 x 10^6 axenic amastigotes in 50 µL of PBS is recommended).

2.3. Load a tuberculin syringe with a 27 G needle with the prepared parasite suspension.

2.4. Anesthetize a C57BL/6 mouse using 3–5% isoflurane, as recommended by Johns Hopkins University Animal Care and Use Committee[45]. Assess the anesthetic depth by testing the mouse's response to a toe pinch.

2.5. Inoculate 50 µL of the homogenized parasite suspension (1 x 10^6 axenic amastigotes or the desired inoculum dose) in the subplantar tissue of the left hind footpad, using the previously loaded tuberculin syringe (step 2.3).

3. Mouse footpad lesion development

3.1. Measure the progression of the lesion once a week by measuring the thickness of the left (infected) and the right (noninfected) footpads using a caliper.

3.2. Calculate the difference of the thickness between the left and right hind footpads weekly to evaluate lesion progression.

3.3. Plot the calculated differences on the Y-axis and time of infection on the X-axis and calculate the statistical significance.

NOTE: In accordance with the Animal Care and Use Committees' recommendations, animals must be sacrificed before the infected lesion becomes ulcerated, because skin ulcers can lead to secondary infection. As shown in **Supplementary Figure 1**, it takes approximately 10 weeks for the lesions to present signs of ulceration with the parasite dose (10^6 axenic amastigotes) and host strain (C57BL/6) used in this protocol. The mice should be sacrificed for lesion extraction before the signs of ulceration are observed.

4. Mice footpad lesion extraction and parasite limiting dilution

4.1. Prepare a 96 well plate (flat bottom) for the limiting dilution assay[46,47] by adding 180 µL of pro-medium to all wells.

NOTE: Use four plate lanes (half of the plate) for each animal, representing quadruplicate assays (i.e., animal 1 = lanes A-D; animal 2 = lanes E-H).

4.2. Add 1 mL of pro-medium in a glass tissue grinder tube per lesion and weigh the tube.

NOTE: The tube must be kept sterile, so use a sterile lid and weigh the tube with the closed lid, if weighing outside the laminar flow cabinet.

4.3. Sacrifice the animal in a CO_2 chamber, following the Animal Care and Use Committees' recommendations. Disinfect the animal by spraying with 70% ethanol.

4.4. Excise the animal's foot at it heels to extract the infected footpad and spray 70% ethanol on the footpad for disinfection. Ensure the sterilization of scissors and forceps by keeping them soaked in 70% ethanol.

4.5. Place the infected footpad in a sterile Petri dish and dissect using sterile forceps and a scalpel to collect all soft tissues. Discard the bones.

NOTE: Uniformity in the dissection step is recommended to avoid biased tissue recovery.

4.6. Transfer the collected tissues to the glass tissue grinder tube with pro-medium, then weigh the tube again to determine the lesion weight.

NOTE: Avoid placing the forceps and scalpel directly into the medium, as small volumes may be removed, resulting in an underestimated tissue weight. The weight of the lesion is calculated by subtracting the weight of the tube containing pro-medium with the collected tissue from the weight of tube containing only pro-medium.

4.7. Homogenize the tissue 10x using the grinder for complete tissue disruption.

4.8. Allow the mixture to sediment for 10 min, then collect 20 µL of the supernatant.

4.9. Load 20 µL of the supernatant in the 1st column of the 1st lane of the 96 well plate prepared in step 4.1. Repeat this for the next three lanes to have quadruplicates for each animal (i.e., for animal 1 add 20 µL to each well and label A1, B1, C1, and D1).

4.10. Homogenize each well in the 1st column 10x using a multichannel pipette. Then, transfer 20 µL of the diluted samples from the 1st column to the 2nd column.

NOTE: It is important to homogenize each well 10x from one column to the next.

4.11. Repeat step 4.10 to all remaining columns until the last column (12th) and discard the final 20 µL of diluted sample.

NOTE: Change the tips after each dilution to avoid cross-contamination.

4.12. Seal the plates with a film and incubate at 25 ˚C for 7 days in a humid chamber.

5. Lesion parasite load determination

5.1. Analyze the plates after 7 days of incubation using an inverted microscope to determine the last parasite-containing well for each lane.

NOTE: It is also possible to have an indication of the growth of the parasites, or cell density, by visual analysis of the color changes and turbidity of the medium.

5.2. Calculate the parasite tissue load for each lane by dividing the dilution factor per lesion weight.

NOTE: In a specific lane, if parasites are present only in columns 1–5 (i.e., wells A1–A5 are positive for parasite growth), this means that column 5 contained only 1 parasite, column 4 contained 10 parasites, and so on, in multiples of ten. Column 1 would contain 10^4 parasites, which represents the number of parasites in the initial 20 µL of supernatant (the non-diluted sample in step 4.7). Because this 20 µL was diluted with 180 µL of pro-medium, the initial tube contained 5 x 10^5 parasites: (1 x 10^4) / (0.02 mL) = 5 x 10^5 parasites/1 mL. Then, the parasite load in that lesion can be calculated by dividing the initial concentration of the parasite by the lesion weight: (5 x 10^5) / lesion weight (see step 4.6).

5.3. Plot the average of the quadruplicated result for each animal with a log Y-scale.

6. Statistical analysis

6.1. Represent the data as average ± standard deviation using at least five animals per experimental group as replicates.

6.2. Perform statistical analysis using unpaired two-tailed test, considering a p value < 0.05 as significant.

REPRESENTATIVE RESULTS:

Leishmania protozoan parasites exist in two developmental forms during their life cycle in invertebrate and vertebrate hosts: promastigotes, the proliferative forms found in the lumen of the female sandfly; and amastigotes, the proliferative forms found in the parasitophorous vacuoles of the mammalian host cells. Promastigotes have an elongated body of approximately 1.5 µm wide and 20 µm long, with a flagellum typically emerging from the anterior extremity. Amastigotes have a rounded or ovoid body ranging in size from 2–6 µm in length and 1.5–3 µm in width, and possess an inapparent flagellum[12,13] (**Figure 1A**). During the blood meal the invertebrate host, a hematophagous insect of the family Psychodidae, acquires macrophages infected with *Leishmania* amastigotes. Once these cells reach the sandfly digestive tube, amastigotes are released and differentiate to procyclic promastigotes (**Figure 1A**). These forms are noninfective and multiply intensively by binary division and colonize the digestive tube of the insect vector. The procyclic forms then differentiate to metacyclic forms, an infective and fast-moving form, presenting a thinner body and elongated flagellum (**Figure 1A**). The metacyclic forms invade the anterior portions of the esophagus and proventriculus of the sandfly, so that during its next blood meal, regurgitation ensures the inoculation of these infecting forms into a new vertebrate host. In the tegument of the vertebrate host, the parasites are phagocytosed by the macrophages and differentiate into amastigotes inside the parasitophorous vacuoles, where the amastigotes multiply by binary division and complete the life cycle of *Leishmania*[12,13].

Axenic conditions can simulate different host environments in vitro, maintaining the parasite morphology and viability. Axenic conditions for amastigotes were previously described simulating a macrophage's parasitophorous vacuole environment and triggering promastigote in vitro differentiation into the amastigote form[37]. These conditions mimic the acidic environment (pH = 5.5) and the increased temperature of the vertebrate hosts (34 ˚C). **Figure 1B** illustrates promastigotes differentiated to amastigotes by changing these conditions in culture. The viability of these axenic amastigotes can be analyzed by Trypan blue staining, a method based on the principle that live cells possess intact membranes that exclude certain dyes, whereas dead cells do not[48]. Alternatively, we analyzed the viability of axenic amastigotes verifying their ability to transform back to promastigotes when transferred to neutral pH and incubated at 25 ˚C (**Figure 1B**).

Here we propose an in vivo infection method to evaluate the virulence of different *Leishmania* strains. **Figure 2A** represents an in vivo infection assay showing the cutaneous lesion development of C57BL/6 mice footpads that were infected with wild type (*La*-WT) and *Leishmania* Iron Regulator 1 knockout (*La*-LIR1$^{-/-}$) *L. amazonensis* purified metacyclics. LIR1 regulates intracellular iron levels in *Leishmania* mediating iron export and preventing its intracellular accumulation to toxic levels[14]. Observing the progression of the thickness differences of the infected vs. noninfected footpads, we were able to demonstrate that the *La*-LIR1$^{-/-}$ infected mice presented smaller lesions than *La*-WT infected mice (**Figure 2A**). Those findings revealed that LIR1 is essential for *L. amazonensis* in vivo virulence. This demonstrates

the importance and efficacy of this method in assessing the differences of *Leishmania*-driven cutaneous diseases. **Figure 2B** illustrates the noninfected (right) and infected (left) footpads and lesion development 73 days postinfection, showing the differences in swelling and lesion progression of *La*-WT and *La*-LIR1$^{-/-}$ infected mice.

The progression of the *Leishmania* infection consists not only of the lesion development, which represents the inflammatory response, but also the parasite's intracellular replication. To evaluate parasite replication, the parasite load of the lesions was determined by extracting the infected lesion, followed by a limiting dilution assay in a 96 well plate (**Figure 3A**). **Figure 3B** shows the parasite load analysis of the footpad lesions from *La*-WT and *La*-LIR1$^{-/-}$ infected mice after 73 days of infection. From the limiting dilution assay, we detected 10^6-fold fewer parasites in the lesion of the *La*-LIR1$^{-/-}$ infected mice in comparison to *La*-WT, revealing that absence of LIR1 prevents intracellular replication of the amastigotes[14].

One of the advantages of evaluating both lesion development and parasite load is to detect possible differences of parasite intracellular replication and the host inflammatory response. We observed differences between these two phenotypes using the add-back LIR1 (*La*-LIR1^{AB}), which is the *La*-LIR1$^{-/-}$ with the LIR1 ORF integrated back into the ribosomal locus[14]. When *La*-LIR1^{AB} was injected into a mouse's footpad, we observed intermediate-sized lesions compared to *La*-LIR1$^{-/-}$ and *La*-WT infections but a remarkable full parasite load rescue of the *La*-WT phenotype (**Supplementary Figure 2**). These results indicate that *La*-LIR1^{AB} parasites were able to replicate like *La*-WT parasites in a long-term in vivo infection. However, the mouse inflammatory response was not as exacerbated as *La*-WT infections because the lesions were significantly smaller.

Thereby, the method described here was shown to be essential for the identification and characterization of a *L. amazonensis* virulence factor required for the successful amastigote intracellular replication and cutaneous lesion development in the mammalian hosts.

FIGURE AND TABLE LEGENDS:

Figure 1: Morphology of *L. amazonensis* promastigotes and amastigotes. (**A**) Illustrations of the different morphological forms of *Leishmania*: procyclic promastigote, metacyclic promastigote, and amastigote. The scale bar represents 2 μm. (**B**) Pictures of in vitro *L. amazonensis* cultures. In vitro differentiation of promastigotes into axenic amastigotes, and of axenic amastigotes back to promastigotes by changing the pH and temperature conditions, as described in the step-by-step protocol. The pictures were taken using an inverted microscope. The scale bar represents 50 μm.

Figure 2: LIR1 knockout markedly reduces *L. amazonensis* in vivo lesion development. C57BL/6 mice were inoculated in the left hind footpad with 10^6 purified metacyclics of *L. amazonensis* wild type (*La*-WT) and *L. amazonensis* LIR1 knockout (*La*-LIR1$^{-/-}$). (**A**) Footpad cutaneous lesion progression of *La*-WT and *La*-LIR1$^{-/-}$ infected mice analyzed weekly. The data represent the average ± SEM of the infected footpad subtracted by noninfected footpad thickness from five different mice in each group (adapted from Laranjeira-Silva et al.[14]). (**B**) Pictures of the

noninfected and infected footpads of *La*-WT and *La*-LIR1$^{-/-}$ infected mice showing the differences in swelling 73 days postinfection (adapted from Laranjeira-Silva et al.[14]).

Figure 3: LIR1 knockout markedly reduces *L. amazonensis* in vivo intracellular replication. (**A**) An illustration of a 96 well plate representing the 10x serial dilutions of the recovered footpad tissues infected with *L. amazonensis* wild type (*La*-WT) and LIR1 knockout (*La*-LIR1$^{-/-}$). Rows A-D represent the quadruplicate of the serial dilutions of the *La*-WT-footpad lesion sample. Rows E-H represent the quadruplicate of the serial dilutions of the *La*-LIR1$^{-/-}$-footpad lesion sample. The different shades of gray represent the observed cell densities per well (i.e., lighter color means fewer parasites). The wells marked in red represent the last wells that contained parasites per replicate. (**B**) Parasite load in recovered footpad tissues of C57BL/6 mice infected with 10^6 purified metacyclics of *L. amazonensis* wild type (*La*-WT) and *L. amazonensis* LIR1 knockout (*La*-LIR1$^{-/-}$) determined 73 days postinfection. The data represent the average of the parasite load per mg of tissue from five different mice in each group (adapted from Laranjeira-Silva et al.[14]).

Supplementary Figure 1: Skin ulcer as indicative of a secondary infection. Representative pictures of C57BL/6 mice footpads infected with *L. amazonensis* wild type (*La*-WT) taken 80 days postinfection. Red arrows point to signs of ulceration, indicating that the experiment should be terminated.

Supplementary Figure 2: The role of LIR1 on in vivo lesion development and intracellular parasite replication. C57BL/6 mice were inoculated in the left hind footpad with 10^6 purified metacyclics of *L. amazonensis* wild type (*La*-WT), *L. amazonensis* LIR1 knockout (*La*-LIR1$^{-/-}$), and *L. amazonensis* LIR1 add-back (*La*-LIR1^{AB}). (**A**) Footpad cutaneous lesion progression of *La*-WT, *La*-LIR1$^{-/-}$, and *La*-LIR1^{AB} infected mice analyzed weekly. The data represent the average ± SEM of the infected footpad subtracted by noninfected footpad thickness from five different mice in each group (adapted from Laranjeira-Silva et al.[14]). (**B**) Parasite load in recovered footpad tissues from *La*-WT, *La*-LIR1$^{-/-}$, or *La*-LIR1^{AB} infected mice determined 73 days postinfection. The data represent the average of the parasite load per mg of tissue from five different mice in each group (adapted from Laranjeira-Silva et al.[14]).

DISCUSSION:

The in vivo infection assay described in this protocol allows any researcher to evaluate in vivo cutaneous leishmaniasis considering the host-parasite interaction in a systemic scenario. These assays have been used by many groups[22,24,27,29,31,32,34,49] and here we compiled a step-by-step protocol to standardize this method while considering the infrastructure limitations that some groups may have. This protocol can also be used to evaluate virulence of transgenic *Leishmania* parasites by in vivo bioimaging[50–52]. As any other experimental procedure, this assay has limitations and critical steps to execute, such as requiring trained personnel that are comfortable working with mice and have experience in performing subplantar injections to avoid accidental infections. Standardizing protocols is extremely important to avoid biased results and to produce comparable results among different research groups.

The main advantage of using *L. amazonensis* as a model for cutaneous leishmaniases is because the footpad lesion caused by this species can be easily assessed in mice. The swelling of the footpad determined by the method described here represents the sum of two infection phenotypes: host inflammatory response and parasite replication. Both phenotypes can be evaluated separately by associating the parasite tissue load method that reflects parasite intracellular replication with the determination of the lesion thickness progression. Another advantage is that *L. amazonensis*' promastigotes are easily cultivated in vitro. Considering these, any research group can manipulate this *Leishmania* species according to their needs. The findings from *L. amazonensis*' studies may be then compared with other *Leishmania* species to determine whether a specific pathway is evolutionarily conserved or divergent[21,53,54].

In the natural cycle, *Leishmania* transmission to the vertebrate host occurs by the bite of an infected sand fly during the blood meal. The sand fly usually inoculates a few hundred *Leishmania* metacyclic promastigote forms in the insect's saliva. In experimental in vivo infections, the most frequent site of inoculation is the animal's footpad[22]. Intradermal injection into the ear or intraperitoneal injection are alternative sites of inoculation depending on the study purpose because each site presents different phagocytic cell types[24,56]. Therefore, some research groups use laboratory-infected sand flies to infect the animal's ear dermis to mimic the natural transmission[56–59]. However, this protocol presents some restrictions, such as the maintenance of sand fly colonies, which requires facilities not available for most research groups.

The original work describing in vivo C57BL/6 infection with *La*-LIR1$^{-/-}$ has used purified metacyclic promastigotes forms[14]. However, *Leishmania* genetic manipulation can impair either the promastigotes' differentiation into axenic amastigotes[14] or into metacyclic infective forms[20]. Hence, depending on the *Leishmania* strain, the researcher should determine the most adequate method to obtain viable infective parasite forms for their study. The protocol of axenic amastigotes differentiated from promastigote cultures described here can be an easier alternative, producing comparable results in many cases[19,20,35,37,38,64]. This approach avoids the use of other methods that typically result in lower yields of infective parasites, such as incubating promastigotes with specific but not widely available antibodies[65] for metacyclic promastigotes purification, or by density gradient dependent of metacyclics' LPG expression[18,66]. The efficiency of the differentiation protocol can be evaluated by determining the expression levels of amastin-family genes[64,67]. Amastins are members of a conserved gene family that are differentially modulated during the *Leishmania* life cycle[68] and are associated with parasite virulence and pathogenesis[67,69,70]. Other markers can also be used to distinguish amastigote from promastigote forms. For example, gp63 is downregulated in amastigotes, because its role is to protect the promastigotes from the insect's digestive enzymes[71].

The choice of the mouse strain is another critical step to be considered when developing a standardized in vivo infection protocol. Susceptibility and resistance to *Leishmania* infection in mice are mainly regulated by genetic background[29,30,55]. In this protocol, the C57BL/6 strain was chosen because its immune response to *L. amazonensis* is closely related to the mixed Th1-Th2 response in humans[72,73]. Experimental murine infections with *L. amazonensis* have been described to cause moderate lesions in C57BL/6 mice in comparison to other mice strains[28,34,74].

However, depending on the parasite strain, differences in the lesion size are only detectable in susceptible mice strains, like BALB/c[36]. The time course of infection also needs to be considered and correlates with the chosen mice strain[29,26,60–63,75]. Ulcerated footpads should always be avoided as it may represent secondary infections and are often observed at long periods of infection, especially in experiments with susceptible mice strains. Designating the time of the day to start the infection is another step to be considered. As demonstrated in previous studies with *L. amazonensis*, the time of the day of parasite inoculum affects lesion development because the host-parasite interaction is affected in a circadian manner by the pineal-released melatonin during the dark time of day[75].

The major disadvantage of the in vivo infection method is that the experiment requires the use of a substantial number of laboratory animals and takes longer time to obtain final results compared to the in vitro infection method[18]. However, this latter aspect can be also considered as an advantage since the in vivo results reflect the natural time course of disease progression more accurately than the results obtained from in vitro infection. More importantly, the findings from *L. amazonensis* in vivo infection cannot only reflect the transient changes in parasite virulence but also acknowledges the systemic status of the host and all its players. Therefore, considering the several factors mentioned above, the method described in this protocol can be adapted to meet specific experimental needs for characterization of other targets and treatments related to virulence allowing new insights for cutaneous leishmaniasis control.

ACKNOWLEDGMENTS:
We would like to thank Prof. Dr. Niels Olsen Saraiva Câmara from the Animal Center of the Biomedical Sciences Institute of the University of São Paulo for the support and Prof. Dr. Silvia Reni Uliana for providing the glass tissue grinder. This work was supported by Sao Paulo Research Foundation (FAPESP – MFLS' grant 2017/23933-3).

DISCLOSURES:
The authors declare they have no competing financial interests.

REFERENCES:
1. Alvar, J. et al. Leishmaniasis worldwide and global estimates of its incidence. *PloS One.* **7** (5), e35671 (2012).
2. Ashford, R. W. The leishmaniases as emerging and reemerging zoonoses. *International Journal for Parasitololy.* **30** (12–13), 1269–1281 (2000).
3. Burza, S., Croft, S. L., Boelaert, M. Leishmaniasis. *Lancet.* **392** (10151), 951–970 (2018).
4. Scorza, B. M., Carvalho, E. M., Wilson, M. E. Cutaneous Manifestations of Human and Murine Leishmaniasis. *International Journal of Molecular Sciences.* **18** (6), e1296 (2017).
5. Afonso, L. C., Scott, P. Immune responses associated with susceptibility of C57BL/10 mice to Leishmania amazonensis. *Infection and Immunity.* **61** (7), 2952–2959 (1993).
6. Khamesipour, A., Rafati, S., Davoudi, N., Maboudi, F., Modabber, F. Leishmaniasis vaccine candidates for development: a global overview. *Indian Journal of Medical Research.* **123** (3), 423–438 (2006).
7. Kumar, R., Engwerda, C. Vaccines to prevent leishmaniasis. *Clinical & Translational*

Immunology. **3** (3), e13 (2014).
8. Murray, H. W., Berman, J. D., Davies, C. R., Saravia, N. G. Advances in leishmaniasis. *Lancet.* **366** (9496), 1561–1577 (2005).
9. Hotez, P. J., Bottazzi, M. E., Franco-Paredes, C., Ault, S. K., Periago, M. R. The neglected tropical diseases of Latin America and the Caribbean: a review of disease burden and distribution and a roadmap for control and elimination. *PLoS Neglected Tropical Diseases.* **2** (9), e300 (2008).
10. Croft, S. L., Sundar, S., Fairlamb, A. H. Drug resistance in leishmaniasis. *Clinical Microbiology Reviews.* **19** (1), 111–126 (2006).
11. Ponte-Sucre, A. et al. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLoS Neglected Tropical Diseases.* **11** (12), e0006052 (2017).
12. Teixeira, D. E. et al. The cell biology of Leishmania: how to teach using animations. *PLoS Pathogens.* **9** (10), e1003594 (2013).
13. Sunter, J., Gull, K. Shape, form, function and Leishmania pathogenicity: from textbook descriptions to biological understanding. *Open Biology Journal.* **7** (9), 170165 (2017).
14. Laranjeira-Silva, M. F. et al. A MFS-like plasma membrane transporter required for Leishmania virulence protects the parasites from iron toxicity. *PLoS Pathogens.* **14** (6), e1007140 (2018).
15. Aoki, J. I. et al. L-arginine availability and arginase activity: Characterization of amino acid permease 3 in Leishmania amazonensis. *PLoS Neglected Tropical Diseases.* **11** (10), e0006025 (2017).
16. Probst, C. M. et al. A comparison of two distinct murine macrophage gene expression profiles in response to Leishmania amazonensis infection. *BMC Microbiology.* **12**, 22 (2012).
17. Dillon, L. A. et al. Simultaneous transcriptional profiling of Leishmania major and its murine macrophage host cell reveals insights into host-pathogen interactions. *BMC Genomics.* **16**, 1108 (2015).
18. Sarkar, A., Khan, Y. A., Laranjeira-Silva, M. F., Andrews, N. W., Mittra, B. Quantification of Intracellular Growth Inside Macrophages is a Fast and Reliable Method for Assessing the Virulence of Leishmania Parasites. *Journal of Visualized Experiments.* (133), e57486 (2018).
19. Mittra, B., Laranjeira-Silva, M. F., Miguel, D. C., Perrone Bezerra de Menezes, J., Andrews, N. W. The iron-dependent mitochondrial superoxide dismutase SODA promotes. *The Journal of Biological Chemistry.* **292** (29), 12324–12338 (2017).
20. Flannery, A. R., Huynh, C., Mittra, B., Mortara, R. A., Andrews, N. W. LFR1 ferric iron reductase of Leishmania amazonensis is essential for the generation of infective parasite forms. *The Journal of Biological Chemistry.* **286** (26), 23266–23279 (2011).
21. Laranjeira-Silva, M. F., Zampieri, R. A., Muxel, S. M., Beverley, S. M., Floeter-Winter, L. M. Leishmania amazonensis arginase compartmentalization in the glycosome is important for parasite infectivity. *PloS One.* **7** (3), e34022 (2012).
22. Sacks, D. L., Melby, P. C. Animal models for the analysis of immune responses to leishmaniasis. *Current Protocols in Immunology.* (1998).
23. Andersen, M. L., Winter, L. M. F. Animal models in biological and biomedical research - experimental and ethical concerns. *Anais da Academia Brasileira de Ciências.* **91**, e20170238 (2019).
24. Ribeiro-Gomes, F. L. et al. Site-dependent recruitment of inflammatory cells determines the effective dose of Leishmania major. *Infection and Immunity.* **82** (7), 2713–2727 (2014).

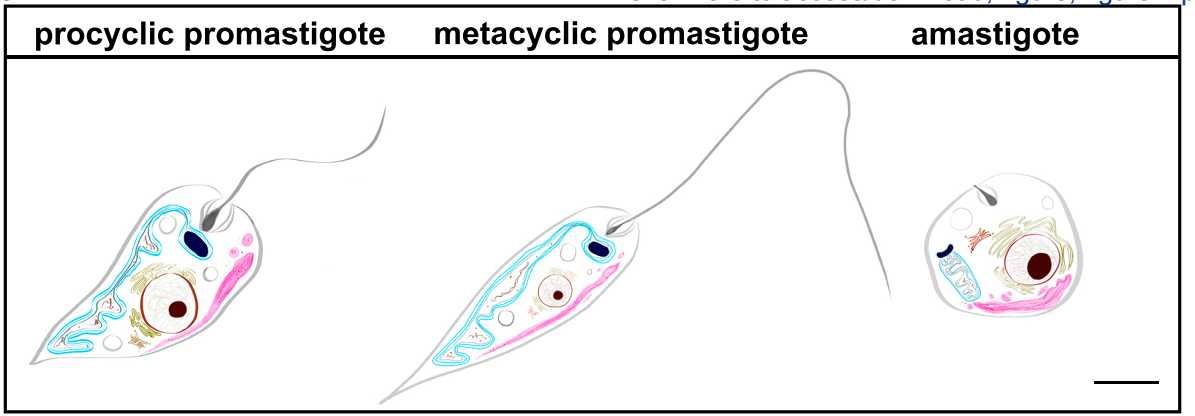
25. Mahmoudzadeh-Niknam, H., Khalili, G., Abrishami, F., Najafy, A., Khaze, V. The route of Leishmania tropica infection determines disease outcome and protection against Leishmania major in BALB/c mice. *The Korean Journal of Parasitology.* **51** (1), 69–74 (2013).
26. Oliveira, D. M. et al. Evaluation of parasitological and immunological parameters of Leishmania chagasi infection in BALB/c mice using different doses and routes of inoculation of parasites. *Parasitology Research.* **110** (3), 1277–1285 (2012).
27. Côrtes, D. F. et al. Low and high-dose intradermal infection with Leishmania major and Leishmania amazonensis in C57BL/6 mice. *Memorias do Instituto Oswaldo Cruz.* **105** (6), 736–745 (2010).
28. Blackwell, J. M. et al. Genetics and visceral leishmaniasis: of mice and man. *Parasite Immunology.* **31** (5), 254–266 (2009).
29. Loeuillet, C., Bañuls, A. L., Hide, M. Study of Leishmania pathogenesis in mice: experimental considerations. *Parasites & Vectors.* **9**, 144 (2016).
30. Alexander, J., Brombacher, F. T Helper1/T Helper2 Cells and Resistance/Susceptibility to Leishmania Infection: Is This Paradigm Still Relevant? *Frontiers in Immunology.* **3**, 80 (2012).
31. Sacks, D., Noben-Trauth, N. The immunology of susceptibility and resistance to Leishmania major in mice. *Nature Reviews Immunology.* **2** (11), 845–858 (2002).
32. Bogdan, C. et al. Experimental Cutaneous Leishmaniasis: Mouse Models for Resolution of Inflammation Versus Chronicity of Disease. *Methods in Molecular Biology.* **1971**, 315–349 (2019).
33. Jones, D. E., Ackermann, M. R., Wille, U., Hunter, C. A., Scott, P. Early enhanced Th1 response after Leishmania amazonensis infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. *Infection and Immunity.* **70** (4), 2151–2158 (2002).
34. Velasquez, L. G. et al. Distinct courses of infection with Leishmania (L.) amazonensis are observed in BALB/c, BALB/c nude and C57BL/6 mice. *Parasitology.* **143** (6), 692–703 (2016).
35. de Menezes, J. P. et al. Leishmania infection inhibits macrophage motility by altering F-actin dynamics and the expression of adhesion complex proteins. *Cellular Microbiology.* **19** (3), 1266 (2017).
36. Mittra, B. et al. A Trypanosomatid Iron Transporter that Regulates Mitochondrial Function Is Required for Leishmania amazonensis Virulence. *PLoS Pathogens.* **12** (1), e1005340 (2016).
37. Zilberstein, D., Nitzan Koren, R. Host-Free Systems for Differentiation of Axenic Leishmania. *Methods in Molecular Biology.* **1971**, 1–8 (2019).
38. Zilberstein, D., Shapira, M. The role of pH and temperature in the development of Leishmania parasites. *Annual Review of Microbiology.* **48**, 449–470 (1994).
Dumetz, F. et al. Modulation of Aneuploidy in *Leishmania donovani* during adaptation to different *in vitro* and *in vivo* environments and its impact on gene expression. *MBio.* **8** (3), e00599–00517 (2017).
39. Sinha, R. et al. Genome Plasticity in Cultured *Leishmania donovani*: Comparison of Early and Late Passages. *Frontiers in Microbiology.* **9**, 1279 (2018).
40. Magalhães, R. D. et al. Identification of differentially expressed proteins from Leishmania amazonensis associated with the loss of virulence of the parasites. *PLoS Neglected Tropical Diseases.* **8** (4), e2764 (2014).
41. Lei, S. M., Romine, N. M., Beetham, J. K. Population changes in Leishmania chagasi promastigote developmental stages due to serial passage. *Journal of Parasitology.* **96** (6), 1134–1138 (2010).

42. Ali, K. S., Rees, R. C., Terrell-Nield, C., Ali, S. A. Virulence loss and amastigote transformation failure determine host cell responses to Leishmania mexicana. *Parasite Immunology.* **35** (12), 441–456 (2013).
43. Rebello, K. M. et al. Leishmania (Viannia) braziliensis: influence of successive in vitro cultivation on the expression of promastigote proteinases. *Experimental Parasitology.* **126** (4), 570–576 (2010).
44. The Johns Hopkins University. Animal Care and Use Committee. Retrieved from http://web.jhu.edu/animalcare/index.html (2019).
45. Titus, R. G., Marchand, M., Boon, T., Louis, J. A. A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. *Parasite Immunology.* **7** (5), 545–555 (1985).
46. Lima, H. C., Bleyenberg, J. A., Titus, R. G. A simple method for quantifying Leishmania in tissues of infected animals. *Parasitology Today.* **13** (2), 80–82 (1997).
47. Strober, W. Trypan Blue Exclusion Test of Cell Viability. *Current Protocols in Immunology.* 10.1002/0471142735.ima03bs21 (1997).
48. Sacks, D., Kamhawi, S. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annual Review of Microbiology.* **55**, 453–483 (2001).
49. Reimão, J. Q. et al. Parasite burden in Leishmania (Leishmania) amazonensis-infected mice: validation of luciferase as a quantitative tool. *Journal of Microbiological Methods.* **93** (2), 95–101 (2013).
50. Buckley, S. M. et al. In vivo bioimaging with tissue-specific transcription factor activated luciferase reporters. *Scientific Reports.* **5**, 11842 (2015).
51. Thalhofer, C. J. et al. In vivo imaging of transgenic Leishmania parasites in a live host. *Journal of Visualized Experiments.* (41), e1980 (2010).
52. Roberts, S. C. et al. Arginase plays a pivotal role in polyamine precursor metabolism in Leishmania. Characterization of gene deletion mutants. *The Journal of Biological Chemistry.* **279** (22), 23668–23678 (2004).
53. Boitz, J. M. et al. Arginase Is Essential for Survival of Leishmania donovani Promastigotes but Not Intracellular Amastigotes. *Infection and Immunity.* **85** (1), e00554–00516 (2017).
54. Rosas, L. E. et al. Genetic background influences immune responses and disease outcome of cutaneous L. mexicana infection in mice. *International Immunology.* **17** (10), 1347–1357 (2005).
55. Belkaid, Y. et al. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis. *Journal of Experimental Medicine.* **188** (10), 1941–1953 (1998).
56. Titus, R. G., Ribeiro, J. M. Salivary gland lysates from the sand fly Lutzomyia longipalpis enhance Leishmania infectivity. *Science.* **239** (4845), 1306–1308 (1988).
57. Lima, H. C., Titus, R. G. Effects of sand fly vector saliva on development of cutaneous lesions and the immune response to Leishmania braziliensis in BALB/c mice. *Infection and Immunity.* **64** (12), 5442–5445 (1996).
58. Theodos, C. M., Ribeiro, J. M., Titus, R. G. Analysis of enhancing effect of sand fly saliva on Leishmania infection in mice. *Infection and Immunity.* **59** (5), 1592–1598 (1991).
59. Kaur, S. et al. Effect of dose and route of inoculation on the generation of CD4+ Th1/Th2 type of immune response in murine visceral leishmaniasis. *Parasitology Research.* **103** (6), 1413–1419 (2008).

60. Rolão, N., Melo, C., Campino, L. Influence of the inoculation route in BALB/c mice infected by Leishmania infantum. *Acta Tropica.* **90** (1), 123–126 (2004).
61. Kébaïer, C., Louzir, H., Chenik, M., Ben Salah, A., Dellagi, K. Heterogeneity of wild Leishmania major isolates in experimental murine pathogenicity and specific immune response. *Infection and Immunity.* **69** (8), 4906–4915 (2001).
62. Baldwin, T. M., Elso, C., Curtis, J., Buckingham, L., Handman, E. The site of Leishmania major infection determines disease severity and immune responses. *Infection and Immunity.* **71** (12), 6830–6834 (2003).
63. Aoki, J. I. et al. RNA-seq transcriptional profiling of Leishmania amazonensis reveals an arginase-dependent gene expression regulation. *PLoS Neglected Tropical Diseases.* **11** (10), e0006026 (2017).
64. Pinto-da-Silva, L. H. et al. The 3A1-La monoclonal antibody reveals key features of Leishmania (L) amazonensis metacyclic promastigotes and inhibits procyclics attachment to the sand fly midgut. *International Journal for Parasitology.* **35** (7), 757–764 (2005).
65. Spath, G. F., Beverley, S. M. A lipophosphoglycan-independent method for isolation of infective Leishmania metacyclic promastigotes by density gradient centrifugation. *Experimental Parasitology.* **99** (2), 97–103 (2001).
66. Aoki, J. I., Laranjeira-Silva, M. F., Muxel, S. M., Floeter-Winter, L. M. The impact of arginase activity on virulence factors of Leishmania amazonensis. *Current Opinion in Microbiology.* **52**, 110–115 (2019).
67. Jackson, A. P. The evolution of amastin surface glycoproteins in trypanosomatid parasites. *Molecular Biology and Evolution.* **27** (1), 33–45 (2010).
68. Rochette, A. et al. Characterization and developmental gene regulation of a large gene family encoding amastin surface proteins in Leishmania spp. *Molecular and Biochemical Parasitology.* **140** (2), 205–220 (2005).
69. Rochette, A., Raymond, F., Corbeil, J., Ouellette, M., Papadopoulou, B. Whole-genome comparative RNA expression profiling of axenic and intracellular amastigote forms of Leishmania infantum. *Molecular and Biochemical Parasitology.* **165** (1), 32–47 (2009).
70. Schneider, P., Rosat, J. P., Bouvier, J., Louis, J., Bordier, C. Leishmania major: differential regulation of the surface metalloprotease in amastigote and promastigote stages. *Experimental Parasitology.* **75** (2), 196–206 (1992).
71. Ji, J., Sun, J., Qi, H., Soong, L. Analysis of T helper cell responses during infection with Leishmania amazonensis. *The American Journal of Tropical Medicine and Hygiene.* **66** (4), 338–345 (2002).
72. Ji, J., Sun, J., Soong, L. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with Leishmania amazonensis. *Infection and Immunity.* **71** (8), 4278–4288 (2003).
73. Felizardo, T. C., Toma, L. S., Borges, N. B., Lima, G. M., Abrahamsohn, I. A. Leishmania (Leishmania) amazonensis infection and dissemination in mice inoculated with stationary-phase or with purified metacyclic promastigotes. *Parasitology.* **134** (12), 1699–1707 (2007).
74. Laranjeira-Silva, M. F., Zampieri, R. A., Muxel, S. M., Floeter-Winter, L. M., Markus, R. P. Melatonin attenuates Leishmania (L.) amazonensis infection by modulating arginine metabolism. *Journal of Pineal Research.* **59** (4), 478–487 (2015).

Figure1

A



B

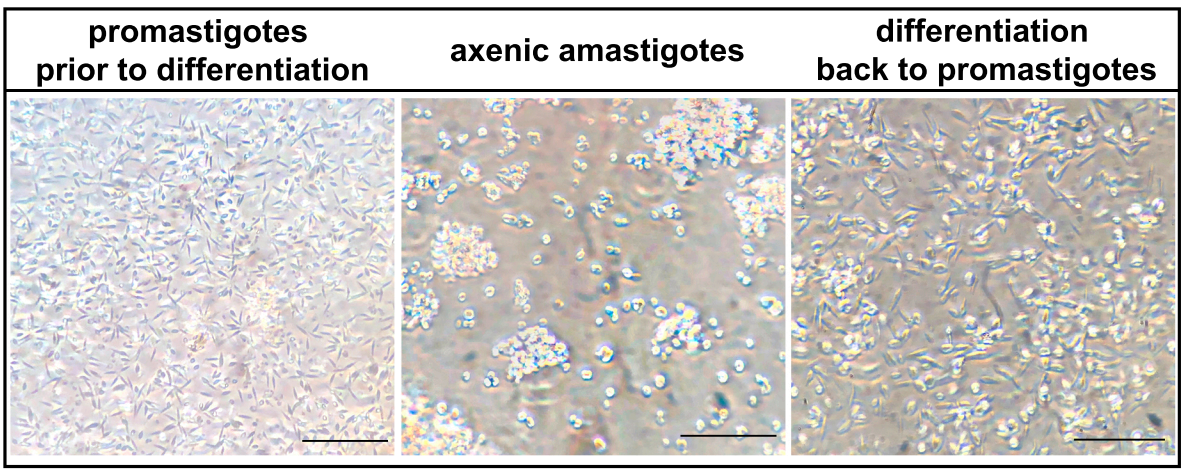


Figure2

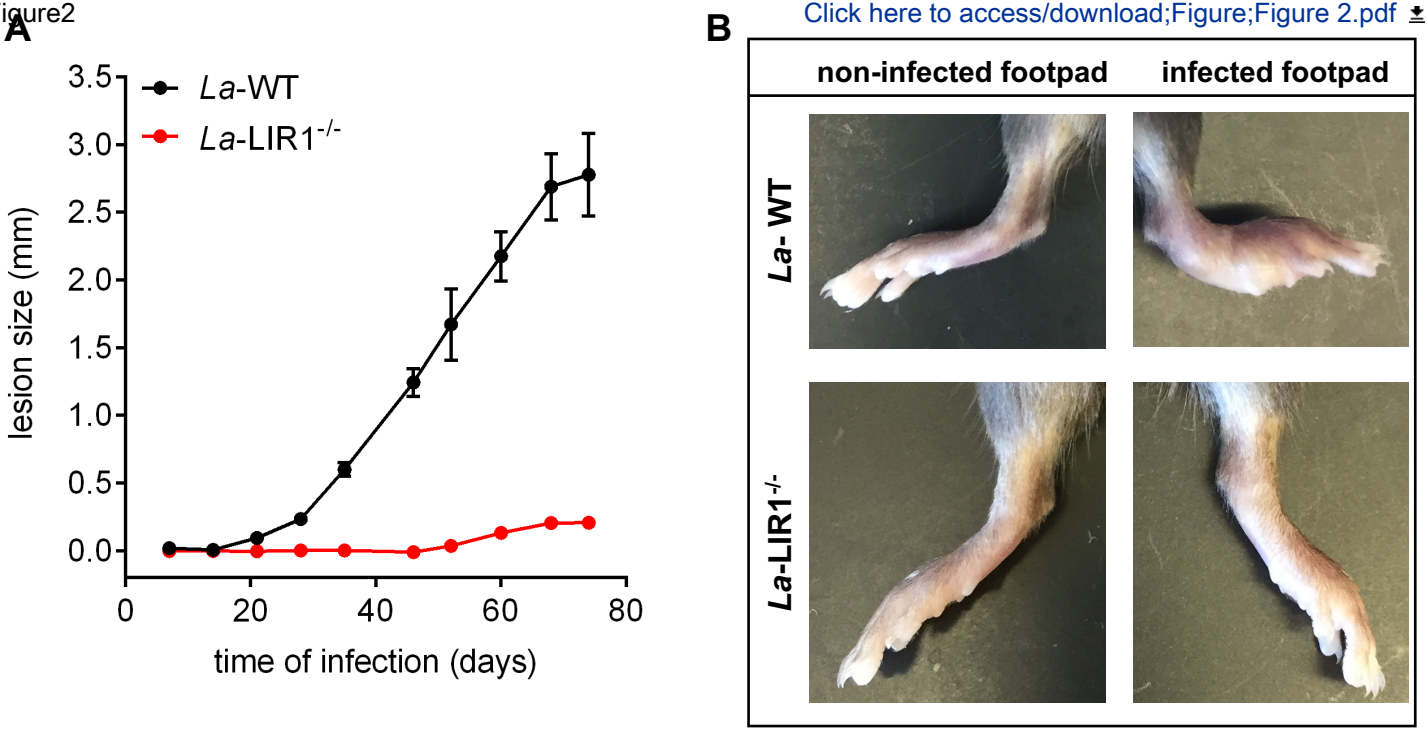
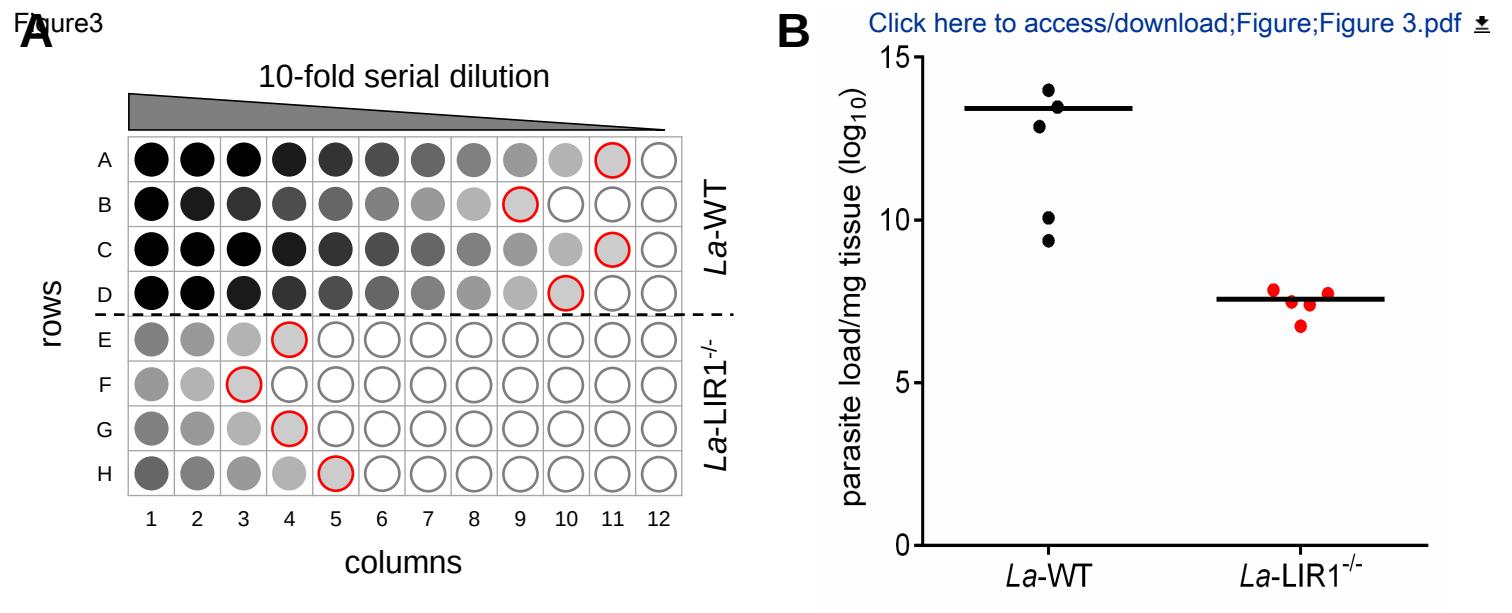


Figure3


A
10-fold serial dilution
rows
A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
8
9
10
11
12
columns
La-WT
La-LIR1-/-
B
Click here to access/download;Figure;Figure 3.pdf
parasite load/mg tissue (log10)
15
10
5
0
La-WT
La-LIR1-/-

Name of Material/Equipment	Company	Catalog Number
96-well plate	Greiner bio-ne	655180
adenine	Sigma	A8626
caliper	Mitutoyo	700-118-20
cell culture flask	Corning	353014
centrifuge	Eppendorf	5804R
CO_2 incubator 34°C	Thermo Scientific	3110
ethanol	Merck	K50237083820
fetal bovine serum	Gibco	12657-029
glass tissue grinder tube	Thomas Scientific	3431 E04
glucose	Synth	G1008.01.AH
GraphPad Prism Software	GraphPad	
hemin	Sigma	H-2250
HEPES	Promega	H5303
incubator 25°C	Fanem	347CD
inverted microscope	Nikon	TMS
isoflurane		
laminar flow cabinet	Veco	VLFS-09
M199 cell culture media	Gibco	31100-035
microcentrifuge tube	Axygen	MCT150C
multichanel pipette	Labsystems	F61978
$NaHCO_3$	Merck	6329
NaOH	Sigma	S8045
Neubauer chamber	HBG	2266
optical microscope	Nikon	E200
parafilm	Bemis	349
penicillin/streptomycin	Gibco	15140122
Petri dishes	TPP	93100
pipetman kit	Gilson	F167360
scale	Quimis	BG2000
scalpel	Solidor	10237580026

serological pipette 10 mL	Nest	327001
tips	Axygen	
Trypan blue	Gibco	15250-061
trypticase peptone	Merck	
tuberculin syringe	BD	305945

Comments/Description
A flat-bottom plate for limiting dilution assay
Supplement added to M199 cell culture media
A caliper to measure the thickness of footpad
A 25 cm^2 volume cell culture flask to cultivate *Leishmania parasite*
An equipament used for separating samples based on its density
An incubator for amastigotes differentiation
A disinfectant for general items
Supplement added to M199 cell culture media
A tube to collect and disrupt infected footpad tissue
Supplement added to M199 cell culture media
A software used to plot the data and calculate statistical significance
Supplement added to M199 cell culture media
Supplement added to M199 cell culture media
An incubator for promastigotes cultivation
An equipament used to visual analyze the promastigote and amastigote cultures
An inhalant anesthetics for mice (3-5%)
A biosafety cabinet used for aseptical work area
A cell culture media for *Leishmania* cultivation
A microtube used for sample collection, processing and storage
A multichannel pipette used for limiting dilution assay
Supplement added to M199 cell culture media
Supplement added to M199 cell culture media
A hemocytometer to count the parasite suspension
An optical equipament used to count parasite
A flexible and resistant plastic to seal the plate
Supplement added to M199 cell culture media
A sterile dish to dissect the footpad tissue
A micropipette kit containing four pipettors (P2 P20 P200 P1000)
An equipament used to weigh collected footpad lesions
A scalpel to cut and collect footpad tissue

A sterile pipette used for transfering mililiter volumes
A pipette tip used for transfering microliter volumes
A dye used to count viable parasites
Supplement added to M199 cell culture media
A syringe with 27G needle to inoculate the parasite suspension

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Murine in vivo infection with Leishmania amazonensis is a reliable method to evaluate the parasite virulence and provide a systemic view of the host-parasite interaction
Author(s):	Juliana Ide Aoki, Ahyun Hong, Ricardo Andrade Zampieri, Lucile Maria Floeter-Winter and Maria Fernanda Laranjeira-Silva

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Maria Fernanda Laranjeira da Silva
Department:	Department of Physiology
Institution:	Institute of Bioscience, University of Sao Paulo, Sao Paulo, Brazil
Title:	Murine in vivo infection with Leishmania amazonensis is a reliable method to evaluate the parasite virulence and provide a systemic view of the host-parasite interaction
Signature:	Maria Fernanda Laranjeira
Date:	07/29/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We appreciate all comments made by the editor. We revised spelling and grammar issues in this revised version of the manuscript.

2. Please make the title concise to reflect the protocol being presented.

Murine *in vivo* infection with *Leishmania amazonensis* is a reliable method to evaluate the parasite virulence and provide a systemic view of the host-parasite interaction

As requested, we changed the title to "Murine *in vivo* infection with *Leishmania amazonensis* is a reliable method to evaluate the parasite virulence within the host".

3. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

As requested, we modified the summary (lines 25-27 of the revised marked version).

4. Please include a single line space between each step, substep and notes in the protocol section.

As requested, we included a single line space between each step, sub-step and notes in the protocol section.

5. 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 and Reagents.
For example: Ficoll, Corning, Durham, USA, Mitutoyo, Japan, GraphPrism Software, M199-pro, Thomas Scientific, Parafilm (Bemis, Neenah, WI, USA), Gibco, Grand Island NY, USA, etc.

As requested, we removed all commercial language from the manuscript. All commercial products were added in the revised Table of Materials and Reagents.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

As requested, we revised the manuscript and all text in the protocol section is written in the imperative tense in the revised manuscript.

7. The Protocol should contain only action items that direct the reader to do something.

As requested, we revised the manuscript and the protocol section contain only action items.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that the Protocol contains only 2-3 actions per step and a maximum of 4 sentences per step.

As requested, we revised the protocol section ensuring only 2-3 actions per step.

9. Please ensure you answer the “how” question, i.e., how is the step performed?

As requested, we revised the manuscript ensuring we answer the “how” question in each step.

10. 1.2: Do you check the O.D in this case? How do you ensure the logarithmic growth phase?

The logarithmic growth phase of the promastigotes´ culture is determined counting the culture daily. This is a well-established practice in any lab working with *Leishmania* cultures. The references regarding this were added in the protocol section 1.

11. In case of 1.1 the culture is grown at 25 degrees, in 1.2 and1.3 it is grown at 34 degrees? Please include the significance of the same.

The temperatures for cultivation of the 2 different forms of *Leishmania amazonensis* are well established, and these temperatures mimic the temperatures found by the parasite in the vertebrate (34ºC) and invertebrate (25ºC) hosts. So, step 1.1 (1.2 of the new version) refers to the promastigotes´ culture, which is kept at 25°C, and steps 1.2 and 1.3 (1.5-1.7 of the new version) refers to the amastigotes´ culture, which is kept at 34°C. References 37 and 38 (line 120 of the revised manuscript) substantiates this matter.

12. 2.3: Do you check for the depth of anesthesia?

Yes, we check the depth of anesthesia by testing the mice´s response to a toe pinch. We added this information as a NOTE (line 174 of the revised manuscript).

13. 4.3, 4.4: what method of sacrificing is used in your experiment? How do you excise the footpad?

The sacrifice was performed using a CO_2 chamber following the Animal Care and Use Committees’ recommendations (step 4.3). The infected footpad was excised using sterile scissors and forceps (step 4.4). We added these information in the protocol section 4 of the revised manuscript.

14. 4.10: Do you plate this on the plates?

Yes, the limiting dilution is done in 96-well plates. We revised the manuscript to improve the description of this step (lines 239-241 of the revised manuscript).

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Steps to be filmed: 2 (2.3-2.5), 3 (3.1) and 4 (4.4-4.11).

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The figures adapted from a previous manuscript were published by PLoS Pathogens (https://doi.org/10.1371/journal.ppat.1007140), which applies the Creative Commons Attribution (CC BY) license policy. Under the CC BY license, authors retain ownership of the copyright for their article, but authors allow anyone to download, reuse, reprint, modify, distribute, and/or copy articles in PLoS journals, so long as the original authors and source are cited. No permission is required from the authors or the publishers, as cab be checked at the website http://journals.plos.org/plospathogens/s/licenses-and-copyright.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

As requested, the discussion section was extensively revised and improved to cover the referred items.

18. Please do not abbreviate the journal titles in the reference section.

As requested, the reference section was modified accordingly.

19. Please ensure that the table of the essential supplies, reagents, and equipment include the name, company, and catalog number of all relevant materials in separate columns.

As requested, we revised the Table of Materials and Reagents.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:

This manuscript provide a detailed protocol on how to grow Leishmania in murine macrophage model. In my opinion this is the best model available. However, a step by step protocol with hint, pitfalls and practical advice is missing. This protocol will be useful to my research group as well as many of my colleagues will use this protocol. Our field of Leishmania research is missing a common protocol for in vivo analyses of parasite development. This manuscript and method is timely and provide a method that we all really need.

Major Concerns:
No comment

We thank are Reviewer #1 for the revision. We are really pleased that Reviewer #1 appreciates the importance and usefulness of this detailed protocol.

Reviewer #2:
So, infections of C57BL/6 mice with L. amazonensis as an experimental model of cutaneous leishmaniasis is a well-established model of cutaneous leishmaniasis. I wouldn't be surprised if many thousand of publications using this model have been published. There is no harm in revisiting established protocols and breathing new life into them. However, when updating old protocols, care should be taken to acknowledge those protocols and then include recent technical additions. The authors are presenting here a protocol for establishing L. amazonensis cutaneous leishmania infections in C57BL/6 mice. If done well, a publication like theirs could introduce new entrants into the field to well established methods.
That said there are several issues with the manuscript that should be addressed. Authors should revise the manuscript to reflect the fact that the protocols for experimental infections are already well established and that those protocols have led to numerous insightful studies on Leishmania pathogenesis. The authors should not suggest that the method that is presented is novel. Both the abstract and the Introduction should be revised accordingly to reflect the fact that this manuscript is not presenting a new protocol but rather a re-visitation of established protocols. The Current Protocols in Immunology originally published in 1991 has protocols for cutaneous Leishmania infections. A Reference such as that should be included in the revised manuscript.

We thank Reviewer #2 for the detailed revision and for acknowledging the importance of revisiting these established protocols that can assist in the introduction of new groups into the field.

We agree the protocol we describe in the present manuscript is a compilation of previous works and our goal is to revisit all these in a standardized detailed protocol. A step by step protocol with practical advices can be useful to several research groups that want to implement this method comparing their results with the ones obtained by other groups. So, as requested, we revised the abstract and introduction to reflect that we are not presenting a new protocol but revisiting well established protocols (lines 43, 47, 97 of the revised manuscript).

Additionally, we would like to emphasize that the Current Protocols in Immunology´s reference from 1998 was included as reference 22 and we added more citations of this specific reference, among other new references, along the manuscript.

They should also consider discussing the possibility of using ear infections as another cutaneous site for establishing cutaneous infections.

We agree with the reviewer and we included the intradermal ear infection as an alternative site for cutaneous infection (lines 435-439 of the revised manuscript).

In the protocols sect 1, they discuss media for cultivation of promastigote forms and the protocol and media for conversion of promastigotes to amastigotes. Although the parasites may change from the long-flagellated forms to the rounded forms, I am sure the authors are aware that rounded form are not necessarily amastigote forms. In this re-visitation of established protocols, it would be useful to use molecular indicators to confirm that a transformation has occurred successfully. For example, the abundant expression of gp63 in promastigote forms contrasts with limited if any expression in amastigotes. It is advised that the protocol includes molecular assays to confirm that transformation has been successful.

We agree it is important to acknowledge that not all rounded forms are necessarily amastigote forms. Previous studies standardized the axenic amastigote differentiation protocol with pH and temperature changes as we used here (Zilberstein et al. 1994 and 2019). Gene expression analyses of specific molecular markers can be performed to confirm the parasite transformation. Among these markers, amastin and gp63 that can be used to distinguish amastigote and promastigote forms as we highlighted in the revised manuscript (lines 463-469).

In the establishment on infections, they suggest to infect in a volume of 50ul. This is most likely much too much volume of liquid to inject in the hind foot of a mouse. 10ul may be more appropriate. Certainly, others have reported on this.

We agree smaller volumes could be easier to inject in the mice footpad, however not all labs have Hamilton glass syringes available for 10uL injections with appropriate accuracy. Besides, most guidelines for use of laboratory animals recommend the use of disposable syringes.

It is not clear when the weight of the infected tissue should be obtained. Should it be after dissection to free the tissue from bone or should it be after sectioning off the intact foot. In the former, the skill of the investigator in dislodging the tissue may influence the amount of tissue recovered, which will result in high parasite/weight values.

The weight of the lesion is calculated by subtracting the weight of the tube containing M199-pro-medium with the collected tissue from the weight of tube containing only M199-pro-medium, so after removing and discarding the bones (steps 4.5, 4.6). We added a NOTE in the revised manuscript recommending uniformity in the dissection step to avoid biased tissue recovery (line 224).

The limiting dilution method is a well-established method for estimating parasite burdens. Titus et al 1995, 1997 are two detailed publications that describe this method along with the statistical considerations. As presented in this manuscript, the method of analysis is flawed. The publications by Titus el al (1995) in Parasite Immunology on evaluation of the progress of Leishmania infections by limiting dilution assays are difficult to obtain. I assume that the difficulties with obtaining that publication may

explain in part why the authors of this manuscript didn't include that reference. This is unfortunate as they would have been made aware of the statistical considerations that need to be addressed when scoring limiting dilution assays.

We apologize for forgetting to include the original Titus' references in the first version of the manuscript. We have these publications and included the references for Titus 1985 and Titus 1997 in the revised manuscript (references 46 and 47).

Reviewer #3:

Manuscript Summary:

The manuscript "Murine in vivo infection with Leishmania amazonensis is a reliable method for a systemic evaluation of the host-parasite interaction" by Aoki et al, describes the protocol to obtain axenic amastigotes (or amastigotes-like) of Leishmania amazonensis, and describes how to perform C57BL/6 footpad infection, monitor the progression of the lesion, obtain amastigotes from the lesion and perform the limiting dilution. These protocols are extremely useful for those working with Leishmania parasites.

Major Concerns:

The main concern is the discussion, which should be improved. I think readers would like to be sure that the axenic amastigotes generated by this protocol are in fact amastigotes and no transitional forms. Do they meet the morphological criteria (non-motile, ovoid shape, and appropriate size), capacity of long-term propagation and infectivity in vitro and in vivo, molecular and biochemical differences when compared with cultured promastigotes?

We thank Reviewer #3 for the careful revision. We are pleased that the Reviewer #3 also appreciates the importance and usefulness of this detailed protocol for the *Leishmania* field.

The discussion section was extensively revised. We included information about gene expression analyses of specific molecular markers that can be performed to ensure the parasite differentiation. Among these markers, we highlighted amastin and gp63 (lines 463-469 of the revised manuscript).

Some Leishmania strains differentiate poorly into metacyclic promastigotes in vitro and the purification of metacyclic forms is expensive and time consuming. In these cases amastigotes harvested from infected tissue (or axenic amastigotes) may be used as inoculum. However, don't you think this is an unnatural way to initiate the infection in the mammalian host? I Think this should be discussed.

We appreciate the reviewer concern and included more references to substantiate the protocol for the use of axenic amastigotes. The promastigotes used by most research groups are also axenic since they are not purified from sandflies. We agree that the use of axenic parasites may be not ideal, however the use of axenic amastigotes has been broadly accepted considering several issues on obtaining purified metacyclics. Besides the reviewer observation that some *Leishmania* strains differentiate poorly into metacyclics, the use of axenic amastigotes also avoids the use of other methods that typically result in lower yields of infective parasites, such as: incubating promastigotes with specific but not-widely available antibodies for metacyclic promastigotes

purification, or by density gradient dependent of metacyclics' LPG expression, as we discussed in the revised manuscript (lines 460-463). So, as requested we revised the discussion section highlighting the most natural way to initiate the infection and the alternatives that can be used depending on the study purpose (lines 432-447).

Line 351- The sentence "Employing the protocol of axenic amastigotes differentiated from promastigote cultures can also be applied and produce comparable results." needs a reference.
We included references to support this sentence (line 460 of the revised manuscript).

Minor Concerns:
Line 112- I recommend using the term amastigote-like or axenic amastigote whenever the authors refer to amastigotes generated in vitro.

As requested, we revised the manuscript to refer to amastigotes generated in vitro as axenic amastigotes.

Line 117- The references Sinha et al, 2018 and Dumetz et al, 2017 are inadequate to justify the sentence "Use promastigote cultures that were passed in vitro less than 10 times". Can you provide a valid reference?

We included more references and modified the sentence to emphasize the virulence attenuation and genomic rearranges observed after *in vitro* passages (lines 135-136).

Line 119- It is important to provide references for "In vitro differentiation of L. amazonensis promastigotes into axenic amastigotes". For example: Flannery et al 2011 or your own nice references, Menezes et al, 2017; Mittra et al, 2017…

We included more references as requested (line 120 of the revised manuscript).

Line 125- The reference Zilberstein and Nitzan refers to the differentiation of L. donovani promastigotes into amastigotes. Does the same apply to L. amazonensis?

Yes, the same was observed for *L. amazonensis*. We included more references to support this (see lines 120 of the revised manuscript).

Line 131- What is the percentage of transformation from promastigotes into amastigotes with the proposed protocol? The sentence "NOTE: Count only non-flagellated parasites (amastigotes forms).", supposes that the transformation is not total, as already demonstrated by other authors. How do you suggest eliminating flagellated forms?

We modified the sentence to "count the non-flagellated parasites" since it is not required to eliminate the flagellated forms, which are significantly underrepresented after the axenic differentiation. In fact, it's rare to find flagellated parasites using this protocol.

Line 138- The parasites are injected subcutaneously or intradermally?

The parasites are injected in the subplantar tissue of the left hind footpad that is the most frequent site of inoculation in experimental *in vivo* infection studies. Intradermal injection into the ear is an alternative site of inoculation depending on the study purpose. We included more about this on the revised discussion section with more references (lines 434-439 of the revised manuscript).

Line 163- When obtaining amastigotes from mouse footpad lesions, it is recommended that the footpad surface be disinfected prior to excisement of the infected tissue to ovoid contamination.

We apologize for forgetting to include the disinfection step. The revised protocol includes ethanol 70% spray for disinfection (lines 215-219 of the revised manuscript).

Line 169- Another important NOTE: avoid placing the forceps and a scalpel directly into the medium as small volumes may be removed, resulting in an underestimate of tissue weight.

We appreciate this suggestion and we included a note about this (lines 230-231 of the revised manuscript).

Reviewer #4:

Major Concerns:

The authors have not referred to or entertained the possibility that axenic amastigotes may not be genetically or phenotypically similar to ex-vivo amastigotes. They are certainly not promastigotes but are the simply another in-vitro entity. Some explanation and substantiation in this regard is required.

Why would you want to initiate a Leishmania infection in-vivo with amastigotes? This has no physiological relevance. Leishmania infections are initiated by promastigotes, and more precisely metacyclic promastigotes.

We thank Reviewer #4 for the careful revision. We appreciate the reviewer concern and included more references to substantiate the protocol for the use of axenic amastigotes. The promastigotes used by most research groups are also axenic since they are not purified from sandflies. We agree that the use of axenic parasites may be not ideal, however the use of axenic amastigotes has been broadly accepted considering several issues on obtaining purified metacyclics. It is important to highlight that some *Leishmania* strains differentiate poorly into metacyclics. Besides this, the use of axenic amastigotes avoids the use of other methods that typically result in lower yields of infective parasites, such as: incubating promastigotes with specific but not-widely available antibodies for metacyclic promastigotes purification, or by density gradient dependent of metacyclics' LPG expression, as we discussed in the revised manuscript (lines 458-463). So, as requested we revised the discussion section to highlight the infection method alternative that better mimics the natural transmission of the parasite by using laboratory-infected sand flies to infect the animal´s ear dermis. However, this protocol presents some restrictions, such as the maintenance of sand fly colonies, which requires facilities not available for most research groups (lines 432-447).

High-dose infections have largely been abandoned in the field due to their questionable relevance to the early stages of physiological disease that is initiated with hundreds, not millions of parasites.

We also modified the introduction adding that the route, site or dose of inoculation influence the disease outcome (lines 84-85 of the revised manuscript). We discuss the sand fly usually inoculates a few hundreds of Leishmania metacyclic promastigote forms with the insect´s saliva (lines 433-434). Since, the goal of this manuscript is to revisit well-stablished protocols to assist in the introduction of new groups into the field, we keep the high-dose infection protocol that most groups are still using. Also, the lower doses are mostly used by groups working with sandfly transmission or saliva, an alternative not available for several groups.

Sub-cutaneous footpad inoculation is not a physiological site of infection, which is intra-dermal.
Sub-cutaneous footpad inoculation is associated with a non-physiological inflammatory response.
In the present manuscript, the site of inoculation is the subplantar tissue of the left hind footpad, which is reported as the most frequent site for experimental *in vivo* cutaneous infections. Intradermal injection into the ear is an alternative site of inoculation depending on the study purpose, however this protocol presents some limitations, as discussed in the revised version (lines 434-447).

The authors have not incorporated the role of dose or site of infection. The authors may not be aware of Cortes et. al. 2010 Mem Inst Oswaldo Cruz Vol.105(6) 736-745, showing dramatically different courses of infection following i.d. infection.

We agree that dose and site of infection are important factors to be considered We included the suggested reference (reference 27).

The authors have not mentioned the complications of immune cell recovery from the footpad, which is complicated by the bones, fat, and muscle associated with the footpad.

We included in the introduction section of the revised manuscript that for immunological studies, immunohistochemical assays from footpad tissue sections or even analysis of recovered immune cells from popliteal lymph nodes can be performed (lines 79-81).

Editorial comments:

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We appreciate all comments made by the editor. We revised spelling and grammar issues in this revised version of the manuscript.

2. Please make the title concise to reflect the protocol being presented.

Murine *in vivo* infection with *Leishmania amazonensis* is a reliable method to evaluate the parasite virulence and provide a systemic view of the host-parasite interaction

As requested, we changed the title to "Murine *in vivo* infection with *Leishmania amazonensis* is a reliable method to evaluate the parasite virulence within the host".

3. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

As requested, we modified the summary (lines 25-27 of the revised marked version).

4. Please include a single line space between each step, substep and notes in the protocol section.

As requested, we included a single line space between each step, sub-step and notes in the protocol section.

5. 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 and Reagents.
For example: Ficoll, Corning, Durham, USA, Mitutoyo, Japan, GraphPrism Software, M199-pro, Thomas Scientific, Parafilm (Bemis, Neenah, WI, USA), Gibco, Grand Island NY, USA, etc.

As requested, we removed all commercial language from the manuscript. All commercial products were added in the revised Table of Materials and Reagents.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

As requested, we revised the manuscript and all text in the protocol section is written in the imperative tense in the revised manuscript.

7. The Protocol should contain only action items that direct the reader to do something.

As requested, we revised the manuscript and the protocol section contain only action items.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that the Protocol contains only 2-3 actions per step and a maximum of 4 sentences per step.

As requested, we revised the protocol section ensuring only 2-3 actions per step.

9. Please ensure you answer the “how” question, i.e., how is the step performed?

As requested, we revised the manuscript ensuring we answer the “how” question in each step.

10. 1.2: Do you check the O.D in this case? How do you ensure the logarithmic growth phase?

The logarithmic growth phase of the promastigotes´ culture is determined counting the culture daily. This is a well-established practice in any lab working with *Leishmania* cultures. The references regarding this were added in the protocol section 1.

11. In case of 1.1 the culture is grown at 25 degrees, in 1.2 and1.3 it is grown at 34 degrees? Please include the significance of the same.

The temperatures for cultivation of the 2 different forms of *Leishmania amazonensis* are well established, and these temperatures mimic the temperatures found by the parasite in the vertebrate (34ºC) and invertebrate (25ºC) hosts. So, step 1.1 (1.2 of the new version) refers to the promastigotes´ culture, which is kept at 25°C, and steps 1.2 and 1.3 (1.5-1.7 of the new version) refers to the amastigotes´ culture, which is kept at 34°C. References 37 and 38 (line 120 of the revised manuscript) substantiates this matter.

12. 2.3: Do you check for the depth of anesthesia?

Yes, we check the depth of anesthesia by testing the mice´s response to a toe pinch. We added this information as a NOTE (line 174 of the revised manuscript).

13. 4.3, 4.4: what method of sacrificing is used in your experiment? How do you excise the footpad?

The sacrifice was performed using a CO_2 chamber following the Animal Care and Use Committees’ recommendations (step 4.3). The infected footpad was excised using sterile scissors and forceps (step 4.4). We added these information in the protocol section 4 of the revised manuscript.

14. 4.10: Do you plate this on the plates?

Yes, the limiting dilution is done in 96-well plates. We revised the manuscript to improve the description of this step (lines 239-241 of the revised manuscript).

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Steps to be filmed: 2 (2.3-2.5), 3 (3.1) and 4 (4.4-4.11).

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The figures adapted from a previous manuscript were published by PLoS Pathogens (https://doi.org/10.1371/journal.ppat.1007140), which applies the Creative Commons Attribution (CC BY) license policy. Under the CC BY license, authors retain ownership of the copyright for their article, but authors allow anyone to download, reuse, reprint, modify, distribute, and/or copy articles in PLoS journals, so long as the original authors and source are cited. No permission is required from the authors or the publishers, as cab be checked at the website http://journals.plos.org/plospathogens/s/licenses-and-copyright.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

As requested, the discussion section was extensively revised and improved to cover the referred items.

18. Please do not abbreviate the journal titles in the reference section.

As requested, the reference section was modified accordingly.

19. Please ensure that the table of the essential supplies, reagents, and equipment include the name, company, and catalog number of all relevant materials in separate columns.

As requested, we revised the Table of Materials and Reagents.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:

This manuscript provide a detailed protocol on how to grow Leishmania in murine macrophage model. In my opinion this is the best model available. However, a step by step protocol with hint, pitfalls and practical advice is missing. This protocol will be useful to my research group as well as many of my colleagues will use this protocol. Our field of Leishmania research is missing a common protocol for in vivo analyses of parasite development. This manuscript and method is timely and provide a method that we all really need.

Major Concerns:
No comment

We thank are Reviewer #1 for the revision. We are really pleased that Reviewer #1 appreciates the importance and usefulness of this detailed protocol.

Reviewer #2:
So, infections of C57BL/6 mice with L. amazonensis as an experimental model of cutaneous leishmaniasis is a well-established model of cutaneous leishmaniasis. I wouldn't be surprised if many thousand of publications using this model have been published. There is no harm in revisiting established protocols and breathing new life into them. However, when updating old protocols, care should be taken to acknowledge those protocols and then include recent technical additions. The authors are presenting here a protocol for establishing L. amazonensis cutaneous leishmania infections in C57BL/6 mice. If done well, a publication like theirs could introduce new entrants into the field to well established methods.
That said there are several issues with the manuscript that should be addressed. Authors should revise the manuscript to reflect the fact that the protocols for experimental infections are already well established and that those protocols have led to numerous insightful studies on Leishmania pathogenesis. The authors should not suggest that the method that is presented is novel. Both the abstract and the Introduction should be revised accordingly to reflect the fact that this manuscript is not presenting a new protocol but rather a re-visitation of established protocols. The Current Protocols in Immunology originally published in 1991 has protocols for cutaneous Leishmania infections. A Reference such as that should be included in the revised manuscript.

We thank Reviewer #2 for the detailed revision and for acknowledging the importance of revisiting these established protocols that can assist in the introduction of new groups into the field.

We agree the protocol we describe in the present manuscript is a compilation of previous works and our goal is to revisit all these in a standardized detailed protocol. A step by step protocol with practical advices can be useful to several research groups that want to implement this method comparing their results with the ones obtained by other groups. So, as requested, we revised the abstract and introduction to reflect that we are not presenting a new protocol but revisiting well established protocols (lines 43, 47, 97 of the revised manuscript).

Additionally, we would like to emphasize that the Current Protocols in Immunology´s reference from 1998 was included as reference 22 and we added more citations of this specific reference, among other new references, along the manuscript.

They should also consider discussing the possibility of using ear infections as another cutaneous site for establishing cutaneous infections.

We agree with the reviewer and we included the intradermal ear infection as an alternative site for cutaneous infection (lines 435-439 of the revised manuscript).

In the protocols sect 1, they discuss media for cultivation of promastigote forms and the protocol and media for conversion of promastigotes to amastigotes. Although the parasites may change from the long-flagellated forms to the rounded forms, I am sure the authors are aware that rounded form are not necessarily amastigote forms. In this re-visitation of established protocols, it would be useful to use molecular indicators to confirm that a transformation has occurred successfully. For example, the abundant expression of gp63 in promastigote forms contrasts with limited if any expression in amastigotes. It is advised that the protocol includes molecular assays to confirm that transformation has been successful.

We agree it is important to acknowledge that not all rounded forms are necessarily amastigote forms. Previous studies standardized the axenic amastigote differentiation protocol with pH and temperature changes as we used here (Zilberstein et al. 1994 and 2019). Gene expression analyses of specific molecular markers can be performed to confirm the parasite transformation. Among these markers, amastin and gp63 that can be used to distinguish amastigote and promastigote forms as we highlighted in the revised manuscript (lines 463-469).

In the establishment on infections, they suggest to infect in a volume of 50ul. This is most likely much too much volume of liquid to inject in the hind foot of a mouse. 10ul may be more appropriate. Certainly, others have reported on this.

We agree smaller volumes could be easier to inject in the mice footpad, however not all labs have Hamilton glass syringes available for 10uL injections with appropriate accuracy. Besides, most guidelines for use of laboratory animals recommend the use of disposable syringes.

It is not clear when the weight of the infected tissue should be obtained. Should it be after dissection to free the tissue from bone or should it be after sectioning off the intact foot. In the former, the skill of the investigator in dislodging the tissue may influence the amount of tissue recovered, which will result in high parasite/weight values.

The weight of the lesion is calculated by subtracting the weight of the tube containing M199-pro-medium with the collected tissue from the weight of tube containing only M199-pro-medium, so after removing and discarding the bones (steps 4.5, 4.6). We added a NOTE in the revised manuscript recommending uniformity in the dissection step to avoid biased tissue recovery (line 224).

The limiting dilution method is a well-established method for estimating parasite burdens. Titus et al 1995, 1997 are two detailed publications that describe this method along with the statistical considerations. As presented in this manuscript, the method of analysis is flawed. The publications by Titus el al (1995) in Parasite Immunology on evaluation of the progress of Leishmania infections by limiting dilution assays are difficult to obtain. I assume that the difficulties with obtaining that publication may

explain in part why the authors of this manuscript didn't include that reference. This is unfortunate as they would have been made aware of the statistical considerations that need to be addressed when scoring limiting dilution assays.

We apologize for forgetting to include the original Titus' references in the first version of the manuscript. We have these publications and included the references for Titus 1985 and Titus 1997 in the revised manuscript (references 46 and 47).

Reviewer #3:

Manuscript Summary:

The manuscript "Murine in vivo infection with Leishmania amazonensis is a reliable method for a systemic evaluation of the host-parasite interaction" by Aoki et al, describes the protocol to obtain axenic amastigotes (or amastigotes-like) of Leishmania amazonensis, and describes how to perform C57BL/6 footpad infection, monitor the progression of the lesion, obtain amastigotes from the lesion and perform the limiting dilution. These protocols are extremely useful for those working with Leishmania parasites.

Major Concerns:

The main concern is the discussion, which should be improved. I think readers would like to be sure that the axenic amastigotes generated by this protocol are in fact amastigotes and no transitional forms. Do they meet the morphological criteria (non-motile, ovoid shape, and appropriate size), capacity of long-term propagation and infectivity in vitro and in vivo, molecular and biochemical differences when compared with cultured promastigotes?

We thank Reviewer #3 for the careful revision. We are pleased that the Reviewer #3 also appreciates the importance and usefulness of this detailed protocol for the *Leishmania* field.

The discussion section was extensively revised. We included information about gene expression analyses of specific molecular markers that can be performed to ensure the parasite differentiation. Among these markers, we highlighted amastin and gp63 (lines 463-469 of the revised manuscript).

Some Leishmania strains differentiate poorly into metacyclic promastigotes in vitro and the purification of metacyclic forms is expensive and time consuming. In these cases amastigotes harvested from infected tissue (or axenic amastigotes) may be used as inoculum. However, don't you think this is an unnatural way to initiate the infection in the mammalian host? I Think this should be discussed.

We appreciate the reviewer concern and included more references to substantiate the protocol for the use of axenic amastigotes. The promastigotes used by most research groups are also axenic since they are not purified from sandflies. We agree that the use of axenic parasites may be not ideal, however the use of axenic amastigotes has been broadly accepted considering several issues on obtaining purified metacyclics. Besides the reviewer observation that some *Leishmania* strains differentiate poorly into metacyclics, the use of axenic amastigotes also avoids the use of other methods that typically result in lower yields of infective parasites, such as: incubating promastigotes with specific but not-widely available antibodies for metacyclic promastigotes

purification, or by density gradient dependent of metacyclics' LPG expression, as we discussed in the revised manuscript (lines 460-463). So, as requested we revised the discussion section highlighting the most natural way to initiate the infection and the alternatives that can be used depending on the study purpose (lines 432-447).

Line 351- The sentence "Employing the protocol of axenic amastigotes differentiated from promastigote cultures can also be applied and produce comparable results." needs a reference.
We included references to support this sentence (line 460 of the revised manuscript).

Minor Concerns:
Line 112- I recommend using the term amastigote-like or axenic amastigote whenever the authors refer to amastigotes generated in vitro.

As requested, we revised the manuscript to refer to amastigotes generated in vitro as axenic amastigotes.

Line 117- The references Sinha et al, 2018 and Dumetz et al, 2017 are inadequate to justify the sentence "Use promastigote cultures that were passed in vitro less than 10 times". Can you provide a valid reference?

We included more references and modified the sentence to emphasize the virulence attenuation and genomic rearranges observed after *in vitro* passages (lines 135-136).

Line 119- It is important to provide references for "In vitro differentiation of L. amazonensis promastigotes into axenic amastigotes". For example: Flannery et al 2011 or your own nice references, Menezes et al, 2017; Mittra et al, 2017…

We included more references as requested (line 120 of the revised manuscript).

Line 125- The reference Zilberstein and Nitzan refers to the differentiation of L. donovani promastigotes into amastigotes. Does the same apply to L. amazonensis?

Yes, the same was observed for *L. amazonensis*. We included more references to support this (see lines 120 of the revised manuscript).

Line 131- What is the percentage of transformation from promastigotes into amastigotes with the proposed protocol? The sentence "NOTE: Count only non-flagellated parasites (amastigotes forms).", supposes that the transformation is not total, as already demonstrated by other authors. How do you suggest eliminating flagellated forms?

We modified the sentence to "count the non-flagellated parasites" since it is not required to eliminate the flagellated forms, which are significantly underrepresented after the axenic differentiation. In fact, it's rare to find flagellated parasites using this protocol.

Line 138- The parasites are injected subcutaneously or intradermally?

The parasites are injected in the subplantar tissue of the left hind footpad that is the most frequent site of inoculation in experimental *in vivo* infection studies. Intradermal injection into the ear is an alternative site of inoculation depending on the study purpose. We included more about this on the revised discussion section with more references (lines 434-439 of the revised manuscript).

Line 163- When obtaining amastigotes from mouse footpad lesions, it is recommended that the footpad surface be disinfected prior to excisement of the infected tissue to ovoid contamination.

We apologize for forgetting to include the disinfection step. The revised protocol includes ethanol 70% spray for disinfection (lines 215-219 of the revised manuscript).

Line 169- Another important NOTE: avoid placing the forceps and a scalpel directly into the medium as small volumes may be removed, resulting in an underestimate of tissue weight.

We appreciate this suggestion and we included a note about this (lines 230-231 of the revised manuscript).

Reviewer #4:
Major Concerns:
The authors have not referred to or entertained the possibility that axenic amastigotes may not be genetically or phenotypically similar to ex-vivo amastigotes. They are certainly not promastigotes but are the simply another in-vitro entity. Some explanation and substantiation in this regard is required.

Why would you want to initiate a Leishmania infection in-vivo with amastigotes? This has no physiological relevance. Leishmania infections are initiated by promastigotes, and more precisely metacyclic promastigotes.

We thank Reviewer #4 for the careful revision. We appreciate the reviewer concern and included more references to substantiate the protocol for the use of axenic amastigotes. The promastigotes used by most research groups are also axenic since they are not purified from sandflies. We agree that the use of axenic parasites may be not ideal, however the use of axenic amastigotes has been broadly accepted considering several issues on obtaining purified metacyclics. It is important to highlight that some *Leishmania* strains differentiate poorly into metacyclics. Besides this, the use of axenic amastigotes avoids the use of other methods that typically result in lower yields of infective parasites, such as: incubating promastigotes with specific but not-widely available antibodies for metacyclic promastigotes purification, or by density gradient dependent of metacyclics' LPG expression, as we discussed in the revised manuscript (lines 458-463). So, as requested we revised the discussion section to highlight the infection method alternative that better mimics the natural transmission of the parasite by using laboratory-infected sand flies to infect the animal´s ear dermis. However, this protocol presents some restrictions, such as the maintenance of sand fly colonies, which requires facilities not available for most research groups (lines 432-447).

High-dose infections have largely been abandoned in the field due to their questionable relevance to the early stages of physiological disease that is initiated with hundreds, not millions of parasites.

We also modified the introduction adding that the route, site or dose of inoculation influence the disease outcome (lines 84-85 of the revised manuscript). We discuss the sand fly usually inoculates a few hundreds of Leishmania metacyclic promastigote forms with the insect´s saliva (lines 433-434). Since, the goal of this manuscript is to revisit well-stablished protocols to assist in the introduction of new groups into the field, we keep the high-dose infection protocol that most groups are still using. Also, the lower doses are mostly used by groups working with sandfly transmission or saliva, an alternative not available for several groups.

Sub-cutaneous footpad inoculation is not a physiological site of infection, which is intra-dermal.
Sub-cutaneous footpad inoculation is associated with a non-physiological inflammatory response.
In the present manuscript, the site of inoculation is the subplantar tissue of the left hind footpad, which is reported as the most frequent site for experimental *in vivo* cutaneous infections. Intradermal injection into the ear is an alternative site of inoculation depending on the study purpose, however this protocol presents some limitations, as discussed in the revised version (lines 434-447).

The authors have not incorporated the role of dose or site of infection. The authors may not be aware of Cortes et. al. 2010 Mem Inst Oswaldo Cruz Vol.105(6) 736-745, showing dramatically different courses of infection following i.d. infection.

We agree that dose and site of infection are important factors to be considered We included the suggested reference (reference 27).

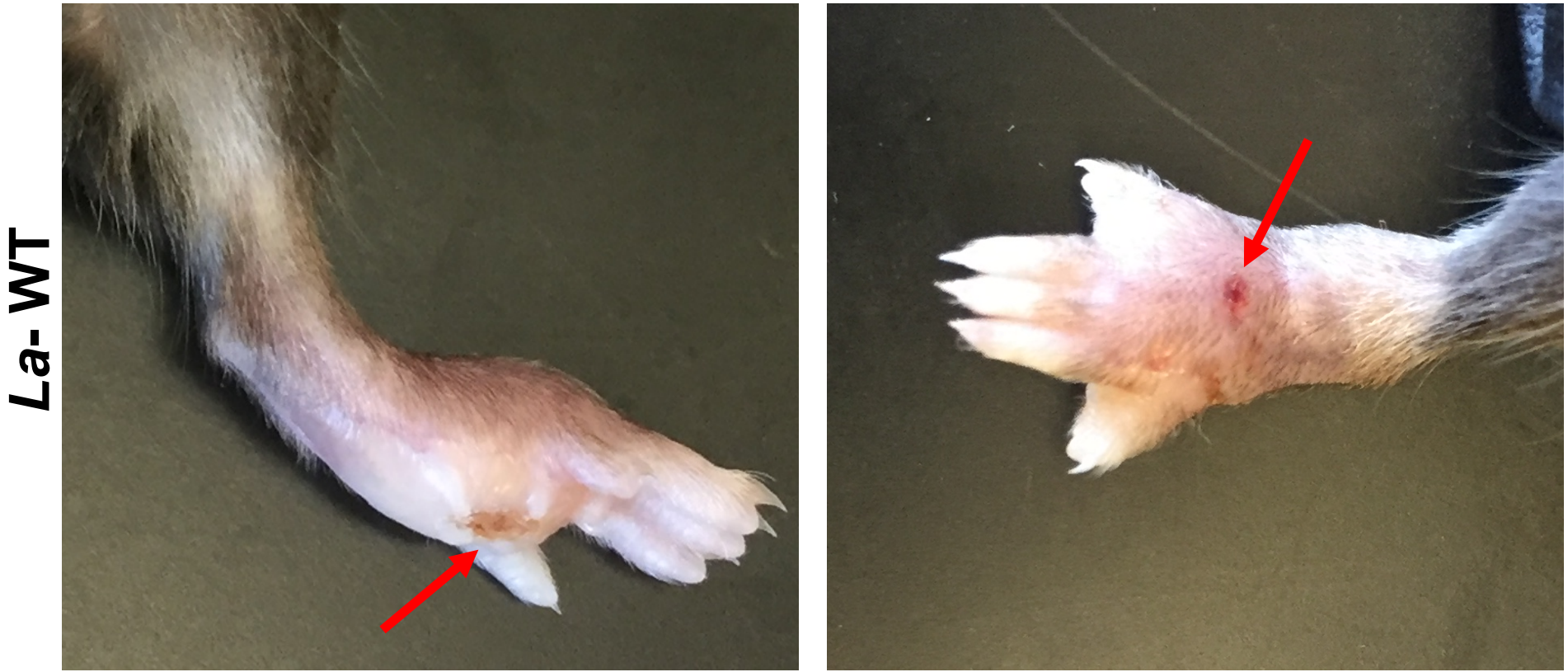
The authors have not mentioned the complications of immune cell recovery from the footpad, which is complicated by the bones, fat, and muscle associated with the footpad.

We included in the introduction section of the revised manuscript that for immunological studies, immunohistochemical assays from footpad tissue sections or even analysis of recovered immune cells from popliteal lymph nodes can be performed (lines 79-81).

Supplemental Figure 1

ulcerated skin in the infected footpad

Click here to access/download;Supplemental



Supplemental Figure 2