Chapter 35

Evaluating the Role of Host AMPK in *Leishmania* Burden

Diana Moreira, Jérôme Estaquier, Anabela Cordeiro-da-Silva, and Ricardo Silvestre

Abstract

The study of host AMP-activated protein kinase (AMPK) activation during *Leishmania* infection imposes distinct types of techniques to measure protein expression and activation, as well as to quantify, at transcription and translational levels, its downstream targets. The investigation of host AMPK protein modulation during *Leishmania* infection should primarily be assessed during in vitro infections using as a host murine bone marrow-derived macrophages (BMMos). The infection outcome is assessed measuring the percentage of infected cells in the context of BMMos. To evaluate AMPK activity during infection, the expression of AMPK-phosphorylated at Thr172 as well as the transcription and translational levels of its downstream targets are evaluated by quantitative PCR and immunoblotting. The modulation of AMPK activity in vivo is determined specifically in sorted splenic macrophages harboring *Leishmania* parasites recovered from infected mice using fluorescent-labeled parasites in the infectious inocolum. The modulation of AMPK activity was assessed by AMPK activators and inhibitors and also using AMPK, SIRT1, or LKB1-KO mice models. The infection outcome in BMMos and in vivo was further determined using these two different approaches. To finally understand the metabolic impact of AMPK during infection, in vitro metabolic assays in infected BMMos were measured in the bioenergetic profile using an extracellular flux analyzer.

Key words Leishmania, AMPK, Bioenergetic profile, Extracellular flux analyzer, AMPK activators and inhibitors, SIRT1, Mitochondria, Cell metabolism, Macrophages

1 Introduction

Leishmania spp. is the causative agent of leishmaniasis, a neglected tropical disease transmitted by the bite of an infected female sand fly [1]. These parasites are mainly phagocyted by macrophages being able to subvert their intracellular signaling pathways and compete for similar resources [2–4]. A key question in the context of host-pathogen interactions is how pathogens survive in a hostile environment and how they hijack host machinery for their own benefit.

To address AMPK signaling during *Leishmania* infection, bone marrow precursors are differentiated in vitro with macrophage colony-stimulating factor (M-CSF) and used as a target cell for

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infection (Subheading 3.1). This differentiation procedure leads to a higher yield and reproducibility, being these bone marrow-derived macrophages (BMMos) commonly considered as a model for the role of resident macrophages [5]. The infection is performed at early (6 h post-infection) and at later time points of infection (18 h post-infection) to acquire a dynamic profile of the infection process. The level of infected cells was obtained through the infection of BMMos with CFSE-labeled parasites being the percentage of $CFSE^+$ or $eFluor670^+$ cells, obtained by FACS analysis, a direct measurement of parasites internalization [6, 7] (Subheading 3.1). A direct analysis of AMPK activation in a context of infection is addressed through immunoblotting of total and phosphorylated Thr172 both in infected BMMos (Subheading 3.1) and in sorted infected splenic macrophages (Subheading 3.2). The modulation of AMPK activity during infection can be obtained establishing a pharmacological approach where an AMPK activator (AICAR) and/or inhibitor (compound C) can be used isolated or in combination, as was described by us and by other authors in different contexts of infection [7–9]. The establishment on an in vivo infection (Subheading 3.2) using myeloid-restricted (Mac)-AMPK, SIRT1, or LKB1 KO mice (Subheading 3.3) is imperative to evaluate the impact of AMPK for the infection outcome in *Leishmania*-parasitized organs. SIRT1 has been investigated in different contexts as a potential modulator of AMPK activation. On one hand SIRT1 protein has been described as an upstream activator of AMPK through LKB1 deacetylation and on the other hand has been considered a downstream target of AMPK, becoming activated by the increase levels of NAD^+ [10–13]. The parasite load in *Leishmania*-parasitized organs is determined ultimately by real-time quantitative PCR (qRT-PCR) (Subheading 3.2) [7, 14]. The metabolic impact of AMPK during *Leishmania* infection can be finally addressed using the extracellular flux analyzer in infected BMMos. Host bioenergetic profile at real time is traced at basal conditions and in response to distinct pharmacological agents, which allow the quantification of metabolic parameters as extracellular acidification rate (ECAR), oxygen consumption rate (OCR), spare respiratory capacity (SRC), and glycolytic capacity (Subheading 3.3). Overall, with these techniques we could trace the activation of host AMPK network during *Leishmania* infection and the impact on parasite survival.

2 Materials

2.1 Animals and Parasites

1. Myeloid cell-specific (Mac)-Sirt1 KO mice, Mac-AMPKα1 KO, Mac-LKB1 KO on C57BL/6 genetic background, and the respective littermate lox control (Lysozyme M-$Cre^{+/+}$

Sirt1$^{flox/flox}$) mice. All animals used in experiments are aged from 6 to 12 weeks.

2. *L. infantum* (MHOM/MA/67/ITMAP-263) promastigotes.

2.2 Culture Reagents

1. Macrophage medium: DMEM, 4.5 g/L glucose, 20 mM HEPES, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin.
2. *Leishmania* medium: RPMI 1640, 20 mM HEPES, 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin.
3. Macrophage growth factor: 100 μg/ml M-CSF.
4. XF medium: unbuffered DMEM, 4.5 g/L glucose, 2% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin.
5. Carboxyfluorescein succinimidyl ester (CFSE, FITC) and eFluor670 (APC): 5 mM stock solution.
6. AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide).
7. Compound C (also known as Dorsomorphin).
8. SRT1720.

2.3 FACS Staining

1. DMEM-EDTA: DMEM, 50 mM EDTA.
2. Phosphate buffer saline (PBS): 145 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.
3. PBS-FBS: PBS, 2% (v/v) FBS.
4. Probes: 14.6 mM 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) and 1 mg/ml 7-Aminoactinomycin D (7-AAD).

2.4 Antibodies

1. FACS: anti-mouse monoclonal antibodies—anti-CD11b-PE (M1/70), anti-Ly6C-PerCP/Cy5.5 (HK1.4), anti-Ly6G-Pacific Blue (1A8), and anti-F4/80-PerCP-Cy5.5 (BM8).
2. Cell magnetic separation: CD3ε and CD19 microbeads.
3. PBS-EDTA buffer: PBS, 0.5% (w/v) BSA (bovine serum albumin), 2 mM EDTA, pH 7.2.
4. Immunoblot: total AMPKα (23A3), AMPKα phosphorylated at Thr172, total SIRT1 (H-300), total PGC1β (E-9), β-actin (C4), total PGC1α (4C1.3), and horseradish peroxidase-coupled secondary reagents.

2.5 Quantitative PCR Analysis

1. TRIzol reagent.
2. Chloroform.
3. Isopropanol.

t.1 **Table 1**
List of primers used for real-time quantitative PCR (qRT-PCR)

t.2 Genes	Forward sequence	Reverse sequence	Accession number
t.3 *Ppargc1a*	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG	NM_008904
t.4 *Slc2a4*	ACATACCTGACAGGGCAAGG	CGCCCTTAGTTGGTCAGAAG	NM_009204
t.5 *RPS29*	CACCCAGCAGACAGACAAACTG	GCACTCATCGTAGCGTTCCA	NM_009093

4. Ethanol 75% (c/v) in H_2O.
5. H_2O RNase free.
6. RNeasy micro kit.
7. QIAmp DNA micro kit.
8. cDNA synthesis Kit.
9. SYBR Green Supermix.
10. Primers: R221 and R332 primers parasite load quantification and primers for real-time quantitative PCR listed in Table 1.
11. DNazol.

2.6 Immunoblot

1. Lysis buffer: 50 mM Tris-HCL, pH 7.4, 1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 25 mM sodium-β-glycerophosphate, 1 mM DTT, 0.5 mM PMSF, protease and phosphatase inhibitor cocktails.
2. Dc protein assay with reagent S (Bio-Rad).
3. SDS polyacrylamide gel 10% resolving gel buffer (4.5 mL): 1.9 mL H_2O, 1.7 mL 30% acrylamide, 1.3 mL Tris-HCL pH 8.8, 50 µL of 10% SDS, 50 µL of 10% APS, 2 µL of TEMED. Stacking gel (3 mL): 2.1 mL of H_2O, 500 µL of 30% acrylamide, 380 µL of 0.5 M Tris-HCL pH 6.8, 30 µL of 10% SDS, 30 µL of 10% APS, 3 µL of TEMED.
4. Transfer pack with nitrocellulose membrane.
5. Western blot transfer system.
6. Ponceau S.
7. Block/diluent solutions: 10% (w/v) BSA, 0.05% (v/v) Tween 20.
8. SuperSignal West Pico or West Dura chemiluminescent substrate.
9. Striping solution I: 0.2 M glycine, 0.5 M NaCl, pH 2.8.
10. Striping solution II: 0.5 M Acetic acid, 0.5 M NaCl, pH 2.5.

2.7 Equipment

1. Gamma irradiator.
2. Flow cytometer.
3. Western blot transfer system.
4. Microplate reader.
5. Western blot detection system.
6. Protein expression quantification software.
7. Spectrophotometer.
8. Real-time PCR thermal cycler.
9. Magnetic cell separator system.
10. Extracellular flux analyzer.

3 Methods

3.1 Infection of Bone Marrow-Derived Macrophages with L. infantum

3.1.1 Isolation and Culture of Mouse Bone Marrow-Derived Macrophages

1. Anesthetize mice through isoflurane inhalation and euthanize by cervical dislocation.
2. Isolate the femurs and tibias of each mouse with a sterile scalp and scissor from the hind legs (*see* **Note 1**).
3. Holding the femurs and the tibias with the help of a scissor, cut off with a scalpel each tip of the bones. Recover bone marrow precursors by flushing the bone marrow with 3–5 mL of ice-cold complete macrophage medium (per femur or tibia) with the help of a syringe and a 26 G needle.
4. Centrifuge bone marrow cells at 300 × *g* for 10 min at 4 °C and resuspend in complete macrophage medium.
5. Plate the bone marrow precursor cells without counting in a 75 cm^2 culture flask in a volume of 15 mL of complete macrophage medium. Incubate at 37 °C with 5% CO_2 for 4–6 h.
6. Discard the adherent cells (differentiated macrophages from the stroma), and recover the bone marrow precursors present in the supernatant. Count with trypan blue and seed the bone marrow precursors in macrophage medium with 20 ng/ml of M-CSF at a proportion of 1×10^5 cells in 200 µL of medium in 96-well plates, 2×10^5 cells in 400 µL of medium in 24-well plates, and 1×10^6 cells in 2 mL of medium in 6-well plates.
7. Renew M-CSF growth factor at day 4 of culture in each well by adding 20 ng/ml of M-CSF. Macrophages acquired a definitive differentiation status at day 7 of culture, being defined as bone marrow-derived macrophages (BMMos) (*see* **Note 2**).

3.1.2 Labeling of Promastigotes and Infection Assay

1. Start a 5 mL culture of *L. infantum* promastigotes at a concentration of 1×10^6 promastigotes/ml of *Leishmania* medium (*see* **Note 3**).

2. At day 5 of growth, wash *L. infantum* promastigotes twice with 5 mL of PBS and resuspend the parasites in 1 mL of PBS. Count the parasites by diluting 1/10 in 2% (v/v) glutaraldehyde that fix the parasites. Dilute the *Leishmania* culture to 6×10^7 promastigotes in 1 mL of PBS.
3. Label with 5 μM CFSE for 10 min or 1 μM eFluor670 for 5 min at 37 °C followed by 5 min incubation at 4 °C to stop the reaction (*see* **Note 4**).
4. Wash the parasites twice with 5 mL of PBS, and resuspend in 1 mL of *Leishmania* medium before proceeding to infections.
5. Count the parasites by diluting 1/10 in 2% (v/v) glutaraldehyde and infect 7-day differentiated BMMos with labeled and unlabeled *L. infantum* promastigotes at a 1:10 ratio.
6. Irradiate 6×10^7 promastigotes suspended in 1 mL of *Leishmania* culture medium at 3000 Gy. Perform control experiments with those irradiated parasites using the previous co-culture ratio.
7. After 4 h of incubation, remove the medium of each well. Wash the cells, at least twice, with macrophage culture medium pipetting up and down several times.
8. For each round of washing, observe under the microscope if the parasites are still present in the supernatant. Repeat the washing procedure until the complete removal of non-internalized parasites.
9. Detach BMMos after 6 and 18 h post-infection. Remove the macrophage medium and add an equivalent volume of DMEM-EDTA solution. Wait 5 min at room temperature, and recover the BMMos by pipetting up and down several times.
10. Centrifuge at $300 \times g$ for 10 min at room temperature.
11. Resuspend BMMos in 200 μL of PBS-2%FBS solution, and incubate for 15 min at room temperature with 7-AAD at 1 μg/ml.
12. Transfer the cells to the cytometer tubes. Acquire the samples in a flow cytometer.
13. In the cytometer adopt the following gate strategy: exclude the death cells that are stained positively to 7-AAD. In the viable population (7-AAD^-), gate the cells that stained positively to eFluor670 (eFluor670^+) or CFSE (CFSE^+).
14. Obtain the percentage of infected cells by the % of CFSE^+ or eFluor670^+ cells. Determine the cell viability by the percentage of negative 7-AAD stained cells (*see* **Note 5**).

3.2 Infection of Mice with *L. infantum* and Sorting of Infected Splenic Macrophages

3.2.1 In Vivo Infection

1. Infect mice intraperitoneally, using a 26 G needle, with 1×10^8 CFSE-labeled *L. infantum* promastigotes resuspended in 200 µL of sterile PBS.

3.2.2 Parasite Load Quantification

1. At 10 days post-infection, anesthetize mice through isoflurane inhalation and euthanize by cervical dislocation. Remove and weigh spleen and liver. Transfer both organs to a 70 µm mesh cell strainer, within a small petri dish, and use the syringe plunger to process the organs to generate a single cell suspension. Centrifuge at 300 × *g* for 10 min at 4 °C, and resuspend in 5 mL of complete macrophage medium.
2. Extract DNA from 10 mg of spleen and liver (single cell suspensions) or 3×10^6 bone marrow cells by DNazol, according to the manufacturer instructions. Dissolve DNA in 100 µL of nuclease-free water. Quantify the total DNA in a NanoDrop spectrophotometer and prepare a twofold serial concentrations dilution adjusted for each tissue.
3. Quantify *Leishmania infantum* DNA by qPCR using 1000 nM of R223 and 500 nM of R333 primers for the small subunit rRNA (SSUrRNA) [15]. As a template use 400 ng of total DNA in 20 µL of reaction with SYBR Green Supermix, according to the manufacturer's instructions.
4. Perform a touchdown qPCR in Bio-Rad My Cycler iQ5, with a final annealing temperature of 65 °C [16]. The denaturation temperature is at 94 °C (5 s) and synthesis at 72 °C (10 s) with 30 cycles (*see* **Note 6**).
5. Extrapolate CTs from a standard curve constructed previously with a serial dilutions of *L. infantum* DNA (strain MHOM/MA/67/ITMAP-263) diluted in host DNA (from spleen of naïve mice). Calculate then *Leishmania* content expressed by parasites/µg of DNA (*see* **Note 7**).

3.2.3 Sorting of Infected Splenic Macrophages

1. Euthanize naïve and CFSE-*L. infantum* promastigote infected mice at 18 or 48 h post-infection. Collect the spleens into a falcon with 5 mL of macrophage culture medium.
2. Process the organs as **step 2** in Subheading 3.2.1. Determine the cell number.
3. Wash the cells twice with 5 mL PBS. Pipette off supernatant completely and resuspend cell pellet in 80 µL of PBS-EDTA buffer per 10^7 cells.
4. Deplete splenic T and B lymphocytes using CD3ε and CD19 microbeads coupled with depletion columns using a magnetic cell isolation separator.

5. Label the remaining cell suspension with 2 μg/ml anti-CD11b-PE, anti-Ly6C-PerCP/Cy5.5 and anti-Ly6G-Pacific Blue diluted in 25 μL of PBS-FBS solution for 30 min at 4 °C in the dark.
6. Wash the cells twice with 200 μL of PBS-FBS solution, and resuspend the cell pellet in 200 μL of PBS-FBS solution. Transfer the cell suspension to the cytometer tubes.
7. Sort the cells according to the surface expression of $CD11b^{+}Ly6C^{int/high}Ly6G^{low}$ and CFSE expression gated on infected ($CFSE^{+}CD11b^{+}Ly6C^{int/high}Ly6G^{low}$) or bystander ($CFSE^{-}CD11b^{+}Ly6C^{int/high}Ly6G^{low}$) splenic macrophages. As a control, sort $CD11b^{+}Ly6C^{int/high}Ly6G^{low}$ cells from the spleen of non-infected mice. The purity of the separation should be higher than 90%.
8. Count the cells obtained after the sorting assay using trypan blue to exclude dead cells.
9. Sorted splenic macrophages are rinse with PBS by centrifugation at 300 × *g* for 10 min at 4 °C.
10. Resuspend 1×10^5 sorted macrophages in 350 μL of RLT buffer with 7 μL of 2-mercaptoethanol, and isolate RNA according to RNeasy micro kit manufacturer's instructions.
11. Analyze the transcription levels of Ppargc1a (PGC-1α) and Slc2a4 (GLUT4) by qPCR using RPS29 as housekeeping gene.
12. Resuspend 1×10^6 sorted macrophages in ice-cold lysis buffer for 30 min at 4 °C with shaking. The supernatant is recovered after centrifugation at 17,000 × *g* during 20 min at 4 °C.
13. Analyze AMPK activation by immunoblot using the experimental procedure described in Chapter 27 (*see* **Note 8**).

3.3 Modulation of AMPK in a Context of Host-*L. infantum* interaction

1. Infect KO mouse models (Mac-Sirt1 KO mice, Mac-AMPKα1 KO, Mac-LKB1) and the respective littermate lox controls, as described in Subheading 3.2.
2. Evaluate the parasite load by qPCR as described in Subheading 3.2.

3.3.1 Modulation of AMPK Activity During In Vivo *L. infantum* Infection

3.3.2 Modulation of AMPK Activity in In Vitro *L. infantum*-Infected BMMos

1. Treat BMMos, from WT, Mac-SIRT1 KO, Mac-AMPK KO, and Mac-LKB1 KO mice, previously infected with CFSE or eFluor670-*L. infantum* promastigotes, at 6 h post-infection with 440 μM AICAR, 440 μM AICAR, and 5 μM compound C, 1 μM SRT1720 or left untreated (*see* **Note 9**).

2. After 24 h of infection, detach BMMos by using the DMEM-EDTA solution and centrifuge at 300 × *g* for 10 min at room temperature.
3. Resuspend cells in 200 μL of PBS-2%FBS solution and incubate for 15 min at room temperature with 7-AAD at 1 μg/ml.
4. Evaluate the infection rate as described in the Subheading 3.1.

3.3.3 *In Vitro Metabolic Assays of Infected Bone Marrow Macrophages*

1. Collect bone marrow precursors as described in the Subheading 3.1. Perform the 7-day differentiation process in 75 cm^2 culture flasks.
2. After 7 days in culture, scrap the cells and seed BMMo at 2×10^5 cells/well in 400 μL of complete macrophage medium in XF-24 cell culture plates. Let the cells to adhere during an overnight period (*see* **Note 10**).
3. This procedure warrants a homogeneous distribution of the cells under the XF-24 plate surface, decreasing the variability among wells.
4. After an overnight period, infect the cells with irradiated or not *L. infantum* promastigotes at a 1:10 ratio, during 6 and 18 h.
5. One hour before the defined times of infection, wash the cells with pre-warmed XF medium. In the final wash, add 200 μL of XF medium to each well, and incubate for an hour at 37 °C without CO_2.
6. Determine the real-time measurement of bioenergetic profile (eight wells per condition), oxygen consumption rate (OCR), and extracellular acidification rate (ECAR), under basal conditions and in response to oligomycin (1 μM), fluoro-carbonyl cyanide phenylhydrazone (FCCP—1 μM), rotenone (1 μM), and antimycin A (1 μM), using an extracellular flux analyzer, at 6 and 18 h post-infection (Fig. 1).

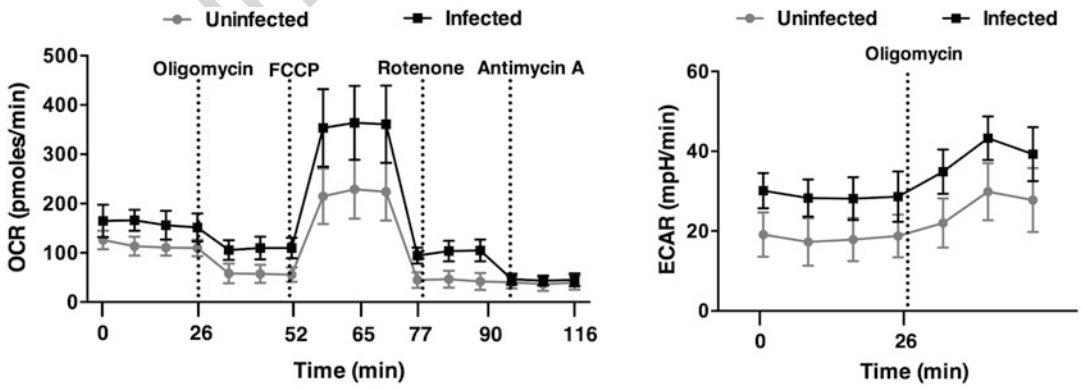

Fig. 1 A representative image of the real-time measurement of oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in uninfected and infected BMMo. The different bioenergetic profiles are obtained under basal conditions and in response to oligomycin (1 μM), fluoro-carbonyl cyanide phenylhydrazone (FCCP—1 μM), rotenone (1 μM), and antimycin A (1 μM), using an XF-24 Extracellular Flux Analyzer

7. Obtain the non-mitochondrial respiration by subtracting the rotenone/antimycin A values. Calculate the spare respiratory capacity (SRC) by subtracting FCCP from basal OCR values, and define the glycolytic capacity as the variation between oligomycin and basal ECAR values. Normalize the values for all the conditions in relation to the protein content.

4 Notes

1. To maintain aseptic conditions and to easily remove the surrounding tissue, collect the femurs and tibias into alcohol solution for few seconds transferring afterward to DMEM medium.
2. Determine the purity of the macrophage culture by quantifying by flow cytometry the percentage of the cells expressing simultaneously the two surface markers CD11b and F4/80, which should be superior to 90%.
3. Maintain a cloned line of virulent *L. infantum* (MHOM/MA/67/ITMAP-263) by weekly subpassages at 26 °C in *Leishmania* medium. Use *L. infantum* promastigotes under four to ten passages in all the experiments.
4. eFluor 670 has a peak emission at 670 nm being detected with a 660/20 band-pass filter (equivalent to APC, Alexa Fluor™ 647, or eFluor™ 660) while CFSE peak emission of 521 nm being detected with a 530/30 nm band-pass emission filter (equivalent to GFP or FITC). Given that both dyes display similar results, the choice of labeling dye relates to the disponibility of the flow cytometry detectors and possibility to multicolor flow cytometry.
5. Giemsa staining is an alternative procedure to determine the percentage of infected cells. Upon **step 8**, Subheading 3.1.2, remove the macrophage medium and wash BMMos with 1 mL of PBS. Add 1 mL of 1% (v/v) of paraformaldehyde for 20 min. Rinse twice with 1 mL of PBS and add 1 mL of Giemsa stain previously diluted 1:20 in deionized water. Incubate for 30 min and rinse with deionized water. Air dry and count the infected cells and the number of intracellular parasites in a microscope at a 400× magnification. Cell nucleus and the parasites acquire a purple coloration. The volumes given consider an infection protocol for 2×10^5 BMMos in 400 μL of medium in 24-well plates. Up or downscale accordingly to the chosen infection protocol.
6. Whenever the qPCR gave a positive (with the expected melting curve) but unquantifiable value or a doubtable specific product (aberrant melting curve), perform a nested PCR [17] that has a

higher sensitivity (0.01 parasites) than the qPCR (0.6 parasites) to confirm the positivity of the quantitative result. For the first amplification reaction, use 300 nM of R221 and R332 primers [17]. For the second reaction, use 10 μL of the first PCR product diluted 1:40, which will serve as a template with the same R223 and R333 primers (300 nM and 150 nM, respectively) used for the qPCR.

7. As alternatives, the detection and quantification of *L. infantum* kinetoplastid DNA can be performed by TaqMan-based qPCR assay [18] or the parasite burden determined by limiting dilution assay [19]. For the former, reaction mixtures are composed of ABI TaqMan PCR 2× (Applied Biosystems), 375 nM of direct primer (CTTTTCTGGTCCTCCGGGTAGG), 375 nM of reverse primer (CCACCCGGCCCTATTTTACACCAA), 250 nM of hydrolysis probe (5′FAM-TTTTCGCAGAACGCCCCTACCCGC-3′TAMRA), and 100 ng of sample DNA. Thermocycling settings consist of one hold of 10 min at 95 °C followed by a two-step temperature (95 °C for 15 s and 60 °C for 60 s) over 40 cycles. A standard curve is established corresponding to a range of 50,000–0.01 parasites. Sample normalization is performed by quantifying a host gene (murine albumin), in 10 μL parallel reactions consisting of SYBR Green ROX Mix 2×, 100 nM of forward primer (CCATTGGTGAGACCAGAGGT), 100 nM of reverse primer (GAGGCAGGCAGCTTTATCAG), 100 ng of DNA, and the same thermal profile used for parasite quantification. A calibration curve ranging from 10,000 to 0.1 cells is established and parasite load expressed as the number of parasites per million of host cells. For the parasite burden, remove the organs from the mice (spleen and liver), weight and homogenize in RPMI medium. After cell counting, perform a subsequent twofold dilutions, in quadruplicate, in 96-well plates, and then incubate at 26 °C for 15 days. Record the presence or absence of motile promastigotes in each well. Calculate the number of parasites per gram of organ (parasite burden) as follows: parasite burden = [(geometric mean of reciprocal titer from each quadruplicate cell culture/weight of homogenized organ) reciprocal fraction of the homogenized organ inoculated into the first well].

8. AICAR is an AMPK activator, compound c is an AMPK inhibitor, and SRT1720 is an activator of SIRT1. BMMos from Mac-SIRT1 KO mice are treated with AICAR and AICAR + compound c, and BMMos from Mac-AMPK KO and Mac-LKB1 KO mice are treated with SRT1720, respectively.

9. In the immunoblot assays, use as a readout the expression of total AMPK, AMPK-PThr172, and PGC-1α.

10. The procedure warrants a homogeneous distribution of the cells under the XF-24 plate surface, decreasing the variability among wells.

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References

1. Murray HW, Berman JD, Davies CR, Saravia NG (2005) Advances in leishmaniasis. Lancet 366:1561–1577
2. Estaquier J, Vallette F, Vayssiere JL, Mignotte B (2012) The mitochondrial pathways of apoptosis. Adv Exp Med Biol 942:157–183
3. Rodrigues V, Cordeiro-da-Silva A, Laforge M, Ouaissi A, Silvestre R, Estaquier J (2012) Modulation of mammalian apoptotic pathways by intracellular protozoan parasites. Cell Microbiol 14:325–333
4. Rodrigues V, Cordeiro-da-Silva A, Laforge M, Ouaissi A, Akharid K, Silvestre R, Estaquier J (2014) Impairment of T cell function in parasitic infections. PLoS Negl Trop Dis 8:e2567
5. Manzanero S (2012) Generation of mouse bone marrow-derived macrophages. Methods Mol Biol 844:177–181
6. Resende M, Moreira D, Augusto J, Cunha J, Neves B, Cruz MT, Estaquier J, Cordeiro-da-Silva A, Silvestre R (2013) *Leishmania*-infected MHC class II high dendritic cells polarize CD4+ T cells toward a nonprotective T-bet+ IFN-gamma+ IL-10+ phenotype. J Immunol 191:262–273
7. Moreira D, Rodrigues V, Abengozar M, Rivas L, Rial E, Laforge M, Li X, Foretz M, Viollet B, Estaquier J, Cordeiro da Silva A, Silvestre R (2015) *Leishmania infantum* modulates host macrophage mitochondrial metabolism by hijacking the SIRT1-AMPK axis. PLoS Pathog 11:e1004684
8. Bulusu V, Thakur SS, Venkatachala R, Balaram H (2011) Mechanism of growth inhibition of intraerythrocytic stages of *Plasmodium falciparum* by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Mol Biochem Parasitol 177:1–11
9. Kondratowicz AS, Hunt CL, Davey RA, Cherry S, Maury WJ (2013) AMP-activated protein kinase is required for the macropinocytic internalization of ebolavirus. J Virol 87:746–755
10. Fulco M, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve AA, Sartorelli V (2008) Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. Dev Cell 14:661–673
11. Hou X, Xu S, Maitland-Toolan KA, Sato K, Jiang B, Ido Y, Lan F, Walsh K, Wierzbicki M,

Verbeuren TJ, Cohen RA, Zang M (2008) SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. J Biol Chem 283:20015–20026

12. Lan F, Cacicedo JM, Ruderman N, Ido Y (2008) SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1. Possible role in AMP-activated protein kinase activation. J Biol Chem 283:27628–27635
13. Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J (2009) AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 458:1056–1060
14. Cunha J, Carrillo E, Sanchez C, Cruz I, Moreno J, Cordeiro-da-Silva A (2013) Characterization of the biology and infectivity of Leishmania infantum viscerotropic and dermotropic strains isolated from HIV+ and HIV- patients in the murine model of visceral leishmaniasis. Parasit Vectors 6:122
15. van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB (1992) Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol 51:133–142
16. Miro G, Oliva G, Cruz I, Canavate C, Mortarino M, Vischer C, Bianciardi P (2009) Multicentric, controlled clinical study to evaluate effectiveness and safety of miltefosine and allopurinol for canine leishmaniosis. Vet Dermatol 20:397–404
17. Cruz I, Chicharro C, Nieto J, Bailo B, Canavate C, Figueras MC, Alvar J (2006) Comparison of new diagnostic tools for management of pediatric Mediterranean visceral leishmaniasis. J Clin Microbiol 44:2343–2347
18. Rodrigues V, Laforge M, Campillo-Gimenez L, Soundaramourty C, Correia-de-Oliveira A, Dinis-Oliveira RJ, Ouaissi A, Cordeiro-da-Silva A, Silvestre R, Estaquier J (2014) Abortive T follicular helper development is associated with a defective humoral response in *Leishmania infantum*-infected macaques. PLoS Pathog 10:e1004096
19. Silvestre R, Cordeiro-Da-Silva A, Santarem N, Vergnes B, Sereno D, Ouaissi A (2007) SIR2-deficient *Leishmania infantum* induces a defined IFN-gamma/IL-10 pattern that correlates with protection. J Immunol 179:3161–3170