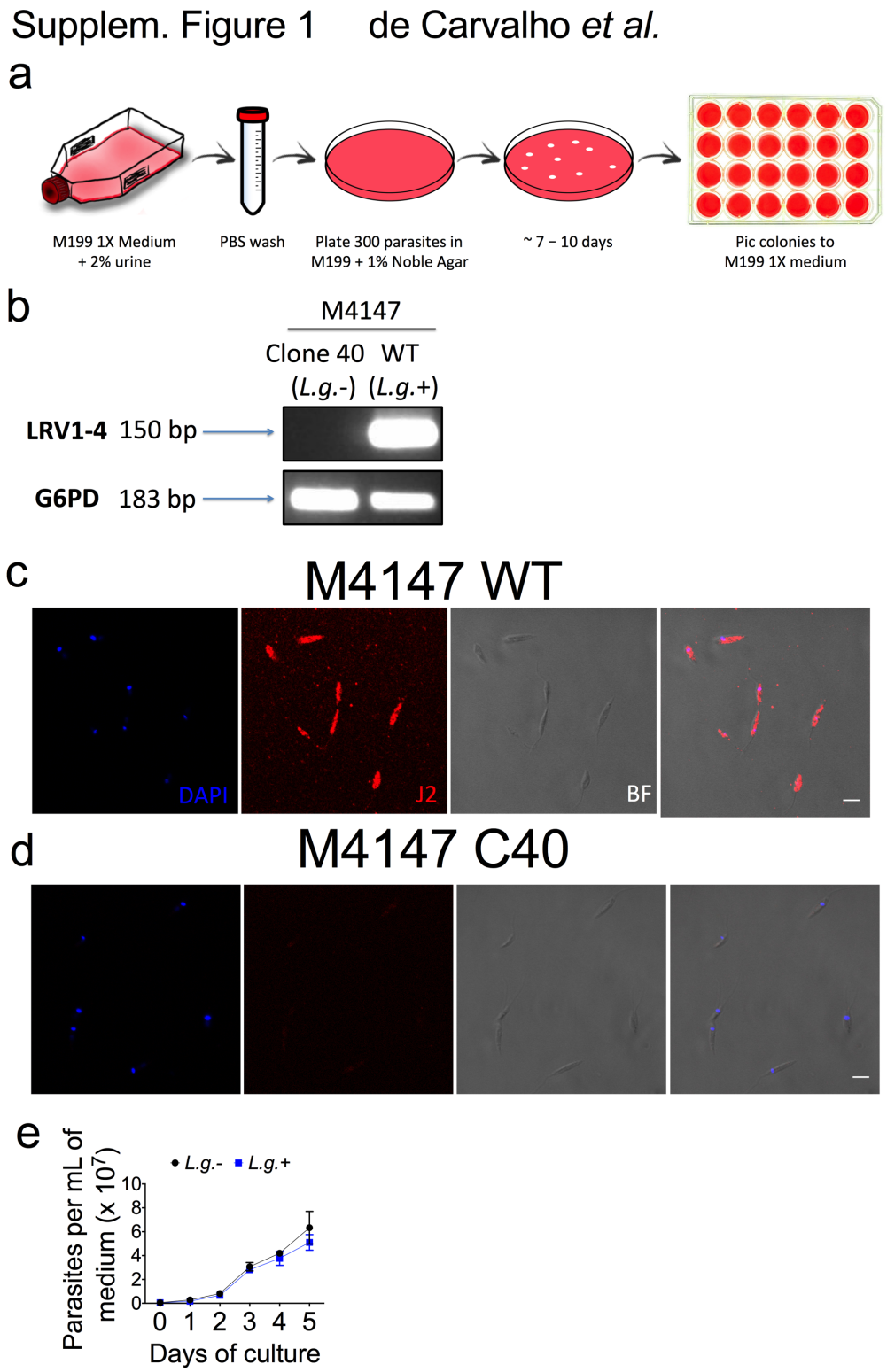
# *Leishmania* RNA virus exacerbates Leishmaniasis by subverting innate immunity via TLR3-mediated NLRP3 inflammasome inhibition

de Carvalho *et al*.

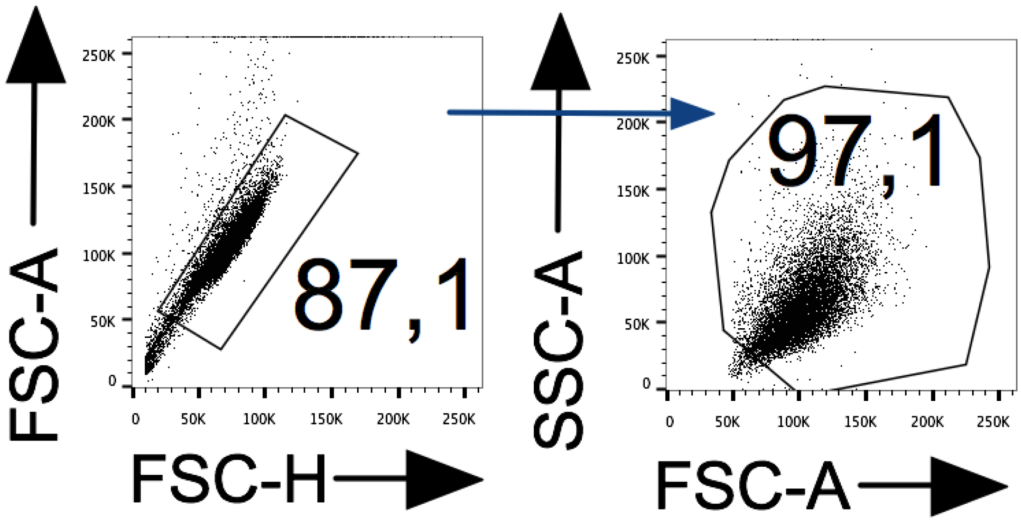
Supplementary Material (Figures and Tables)



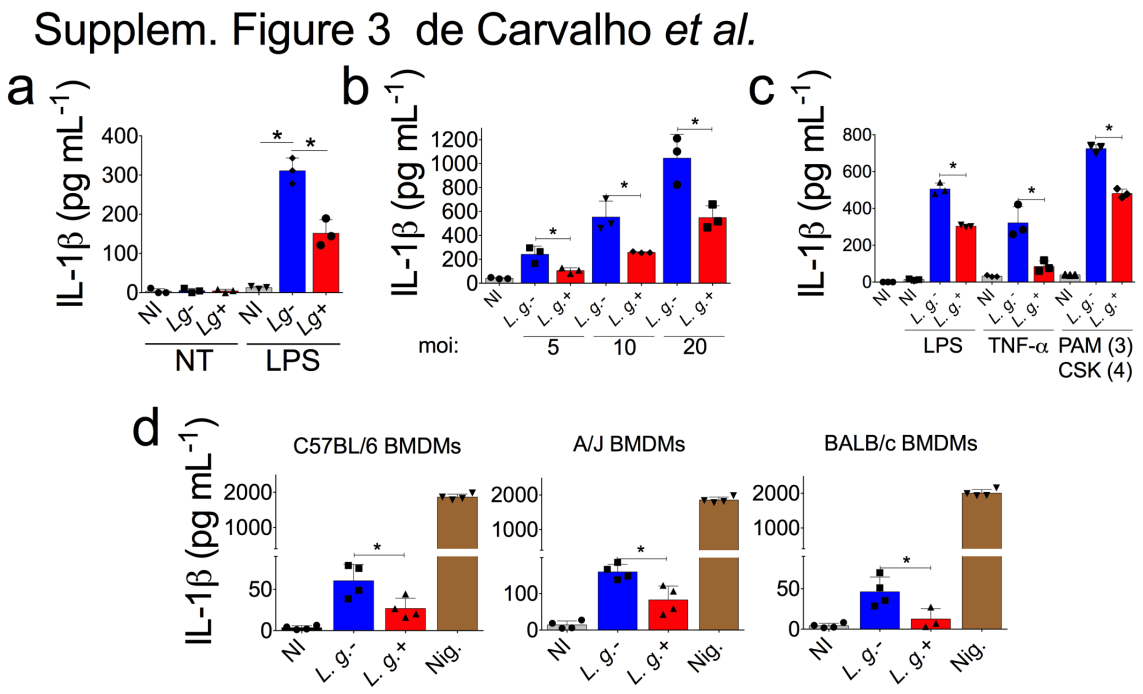

**Supplementary Figure 1. Generation of the clone 40 (*L.g.*-).** (**a**) Schematics of clone 40 generation (*L.g.*-). (**b**) PCR for LRV product (150 bp) and *Leishmania* G6PD (183 bp) in both clones (*L.g.*- and *L.g.*+). (**c,d**) Confocal microscopy in $8x10^6$ fixed and permeabilized promastigotes from the WT strain and its LRV

negative clone 40. Parasites were stained for nuclei (DAPI) and dsRNA (J2 antibody). Scale bar: 5μM. BF: Bright field. (**e**) The growth of both clones was followed in Schneider's insect medium and parasite concentration was determined daily by manual counting in Neubauer's chamber. The results are shown as mean ± SD.

Supplem. Figure 2  de Carvalho *et al.*

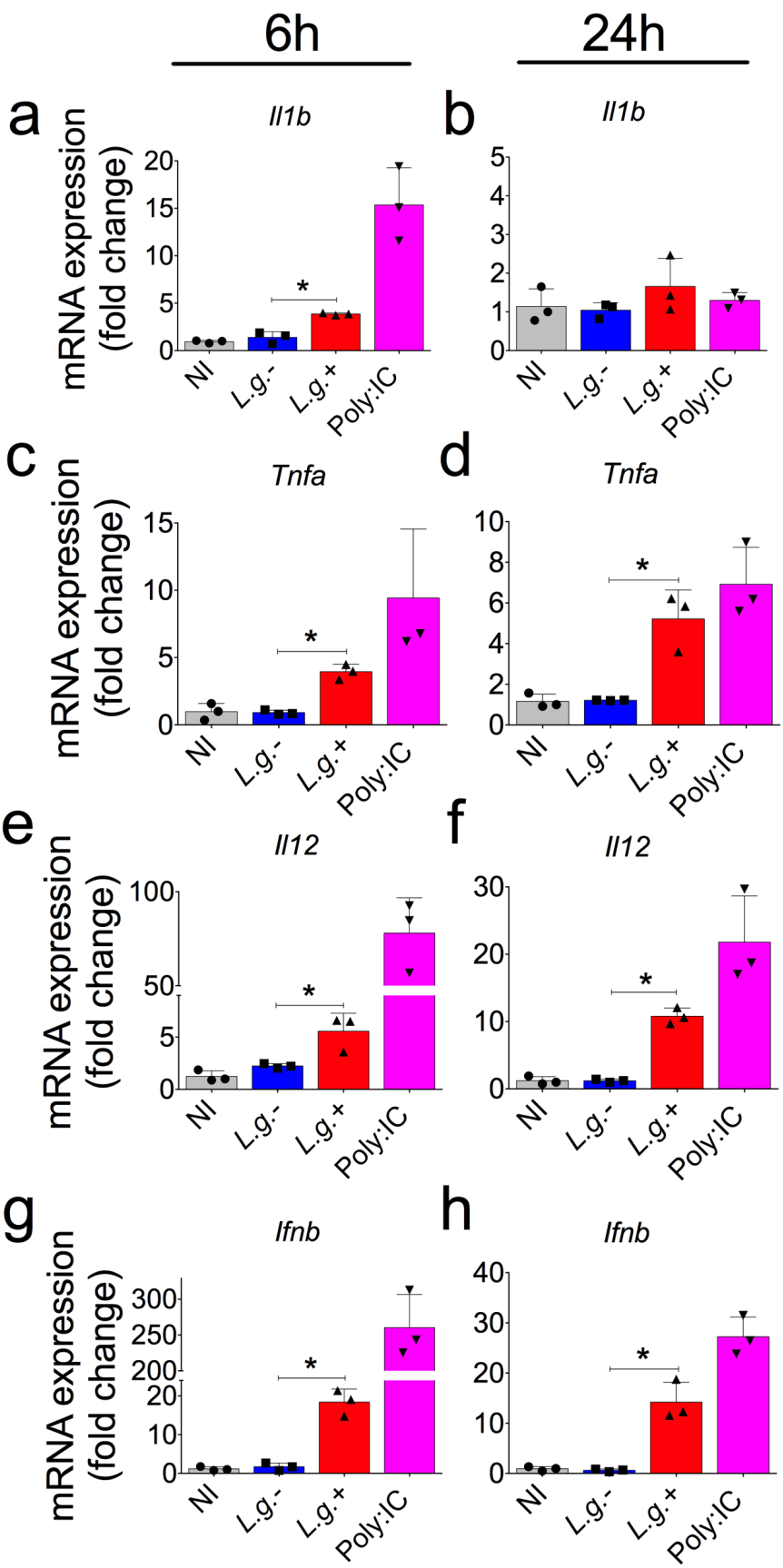



**Supplementary Figure 2. Gating strategy in flow-cytometric analysis.** Schematics of gating strategy demonstrating how true BMDMs were gated for subsequent analysis of infected cells, cell death and caspase-1 staining. BMDMs were initially gated to exclude debris and doublets (FSC-A/FSC-H). Then, true BMDMs were gated according to their granularity and size (SSC-A/FSC-A). Next, cells were gated according to the objective/staining in each experiment of the current study.



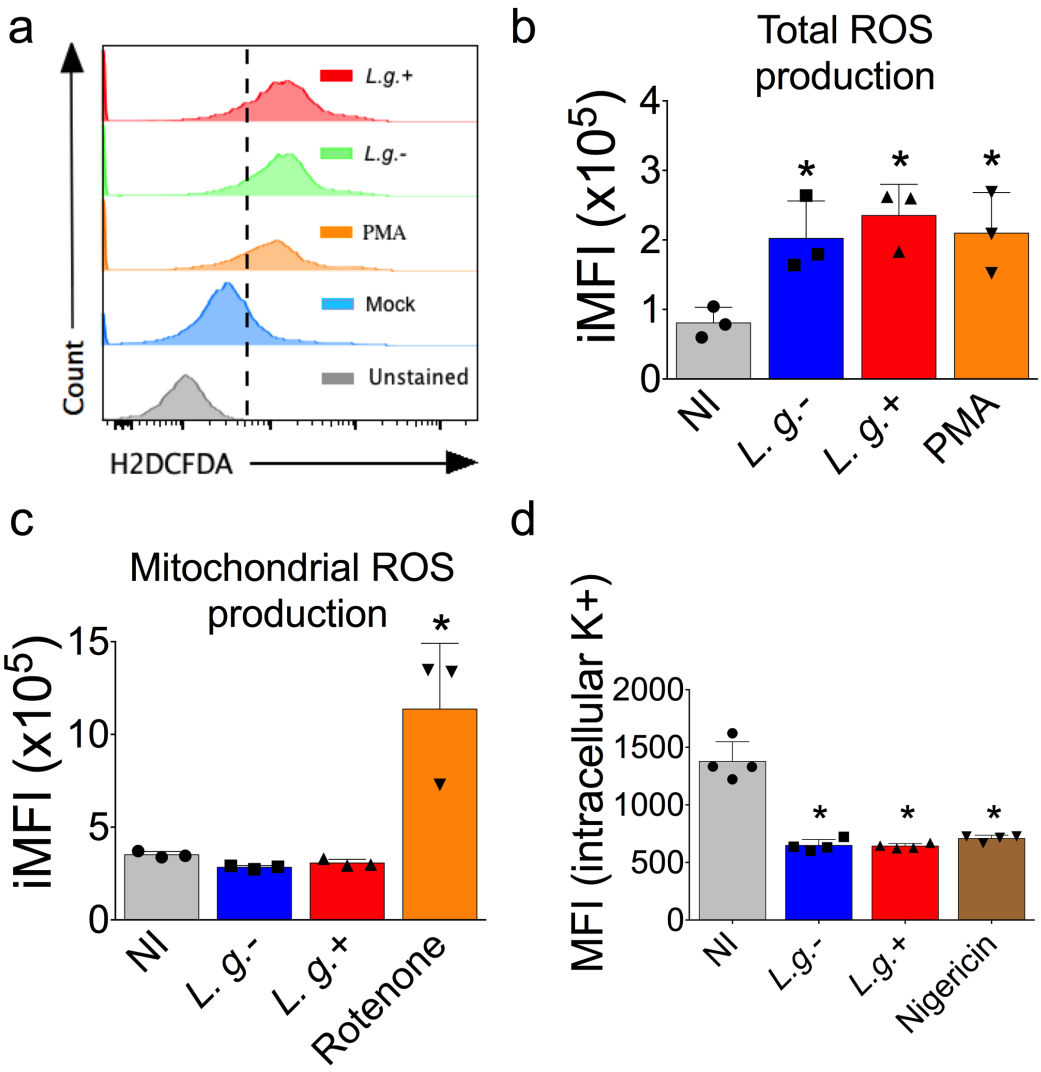

**Supplementary Figure 3. LRV attenuates inflammasome activation by *L.g.*** (**a**) C57BL/6 BMDMs were left unprimed or primed with LPS (500 ng/mL) for 4 hours, and then infected with SP Promastigotes of *L.g.* clones at MOI 10. (**b**) LPS-primed (500 ng/mL) BMDMs were infected with three different MOI of SP Promastigotes of *L.g.*- or *L.g.*+. (**c**) Cells were primed for 4h with either LPS (500 ng/mL), PAM(3)CSK(4) (300 ng/mL) or TNF-α (10 ng/mL), and then infected for 24h with *L.g.- or L.g.*+. (**d**) BMDMs from different mouse strains were primed with LPS and infected for 24h with *L.g.* clones, or treated with 20 μM of Nigericin (positive control). After 24 hours, supernatants were collected and ELISA for IL-1β was performed in cell-free supernatants. One representative of at least two independent experiments performed with technical replicates is shown. The results are shown as mean ± SD. Statistical analysis was performed by unpaired Student's *t test*, and $P<0.05$ (*) was considered statistically significant.

Supplem. Figure 4 de Carvalho *et al.*



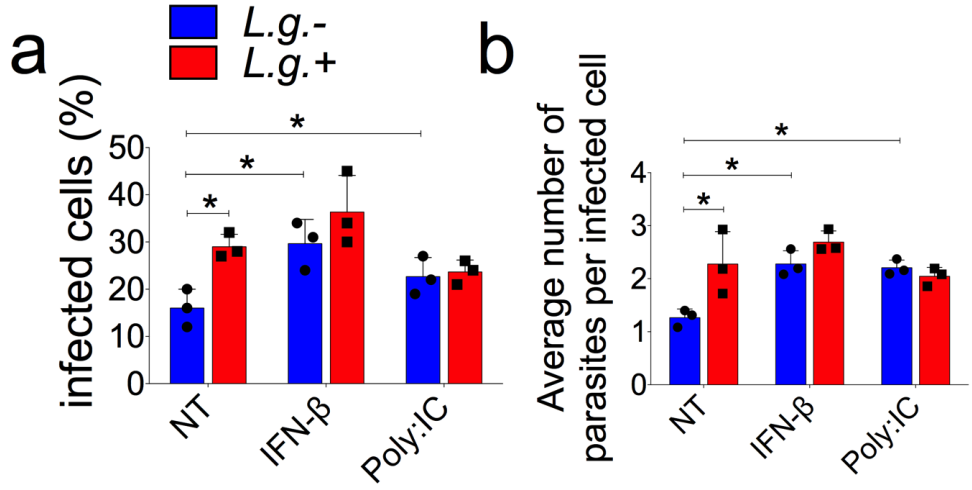
**Supplementary Figure 4. LRV induces transcription of different inflammatory genes.** C57BL/6 BMDMs were infected with *L.g.*- or *L.g.*+ for 6 (**a,c,e,g**) or 24 hours (**b,d,f,h**). qPCR for *Il1b* (**a,b**), *Tnfa* (**c,d**), *Il12* (**e,f**) and *Ifnb* (**g,h**) was performed. Poly:IC (5 μg/mL) was used as a positive control. One representative of two independent experiments performed with technical replicates is shown. The results are shown as mean ± SD. Statistical analysis was performed by unpaired Student's *t test*, and $P<0.05$ (*) was considered statistically significant.

Supplem. Figure 5 de Carvalho *et al.*



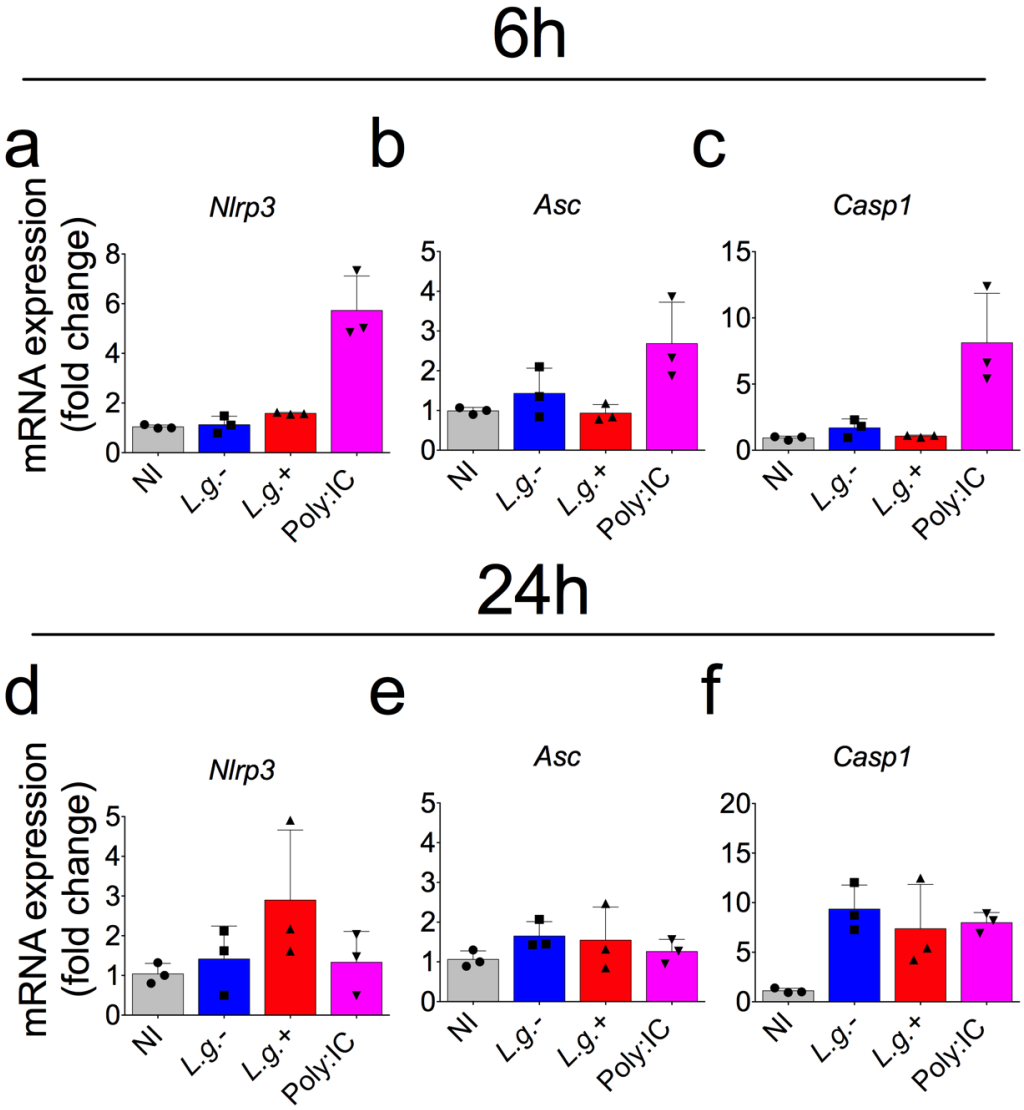

**Supplementary Figure 5. LRV does not interfere with the generation of second signals by *L.g.*** (**a-c**) C57BL/6 BMDMs were infected with either *L.g.*- or *L.g.*+ for 90 minutes. Then, a fluorescent dye staining total (**a,b**) or mitochondrial (**c**) ROS was added for additional 30 minutes. Cells were harvested and analyzed by FACS. (**d**) Cells were incubated with APG-2 dye and 12 hours after infection, levels of intracellular potassium were determined by a High Content Screening Confocal equipment. The results are shown as mean ± SD. Statistical analysis was performed by unpaired Student's *t test*, and $P<0.05$ (*) was considered statistically significant. One representative of two independent experiments performed with technical replicates is shown.

Supplem. Figure 6 de Carvalho *et al.*

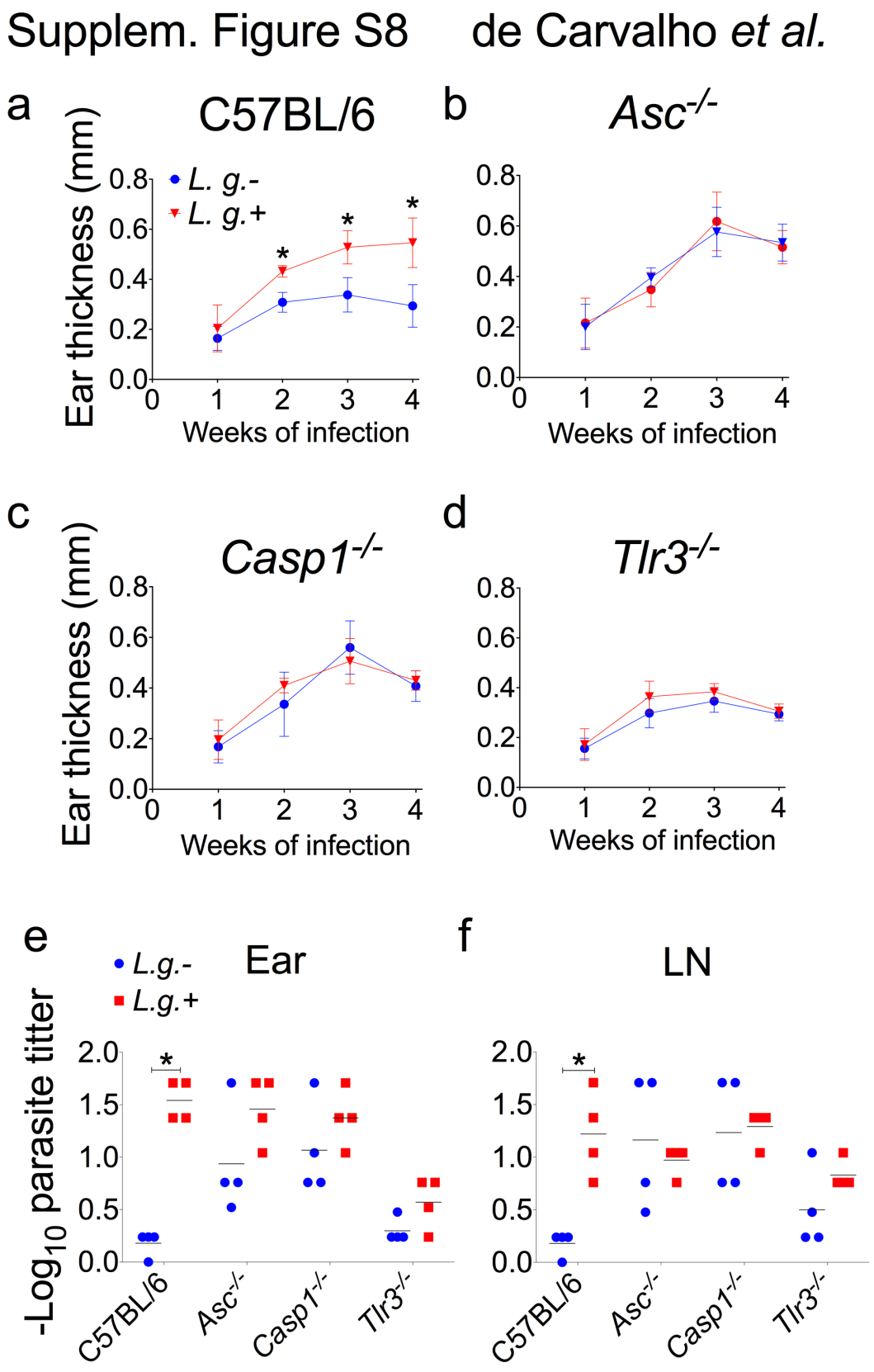



**Supplementary Figure 6. TLR3, TRIF, IFN-β and NLRP3 are required for LRV-mediated parasite survival.** (**a,b**) C57BL/6 BMDMs were infected with metacyclic parasites from either *L.g.*- or *L.g.*+ at a MOI of 1. After 1h of infection, cells were washed and replaced with fresh medium containing either Poly:IC (5 µg/mL) (I) or IFN-β (1000 U/mL), and left for 48 hours in culture. The results are shown as mean ± SD. Statistical analysis was performed by unpaired Student's *t test*, and $P<0.05$ (*) was considered statistically significant. One representative of two independent experiments performed with technical replicates is shown.

Supplem. Figure 7 de Carvalho *et al.*




**Supplementary Figure 7. LRV does not alter the transcription of inflammasome components.** C57BL/6 BMDMs were infected with *L.g.*- or *L.g.*+. After 6 (**a-c**) or 24h of infection (**d-f**), total RNA was extracted. Levels of *Nlrp3* (**a,d**), *Asc* (**b,e**) and *casp1* (**c,f**) transcripts were determined by qPCR analysis. Poly:IC (5 µg/mL) was used as a positive control. The results are shown as mean ± SD. One representative of two independent experiments performed with technical replicates is shown.




**Supplementary Figure 8. LRV exacerbates *L.g.* infection via ASC, CASP1 and TLR3 *in vivo*.** (**a-d**) C57BL/6 (**a**), *Asc*$^{-/-}$ (**b**), *Casp1*$^{-/-}$ (**c**) and *Tlr3*$^{-/-}$ (**d**) mice were injected with $10^6$ Stationary-phase promastigotes of *L.g.*- or *L.g.*+ (n = 4-5

mice per group), and ear thicknesses were followed weekly (**a-d**). 4 weeks after infection, parasite titers were determined in the ear (**e**) and draining lymph node (**f**) from all mice used in this experiment. The results are shown as mean **(e,f)** or mean ± SD **(a,b,c,d)** from data obtained from two independent experiments performed with biological replicates. Statistical analysis was performed by two-way ANOVA with Bonferroni's multiple comparison test. $P<0.05$ (*) was considered statistically significant.

Supplem. Table 1 de Carvalho *et al.*

| Patient number | Infectious specie | Region | Outcome | LRV Status |
|---|---|---|---|---|
| 1 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 2 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 3 | *Leishmania spp.* | Rondônia, Brazil | MCL | NEGATIVE |
| 4 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 5 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 6 | *L. braziliensis* | Rondônia, Brazil | CL | NEGATIVE |
| 7 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 8 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 9 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 10 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 11 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 12 | *L. braziliensis* | Rondônia, Brazil | MCL | POSITIVE |
| 13 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 14 | *L. braziliensis* | Rondônia, Brazil | CL | NEGATIVE |
| 15 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 16 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 17 | *Leishmania spp.* | Rondônia, Brazil | CL | POSITIVE |
| 18 | *L. guyanensis* | Rondônia, Brazil | CL | NEGATIVE |
| 19 | *L. braziliensis* | Rondônia, Brazil | MCL | POSITIVE |
| 20 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 21 | *L. guyanensis* | Rondônia, Brazil | CL | POSITIVE |
| 22 | *L. braziliensis* | Rondônia, Brazil | CL | NEGATIVE |
| 23 | *L. braziliensis* | Rondônia, Brazil | CL | NEGATIVE |
| 24 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 25 | *L. braziliensis* | Rondônia, Brazil | CL | NEGATIVE |
| 26 | *L. braziliensis* | Rondônia, Brazil | MCL | NEGATIVE |
| 27 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 28 | *L. braziliensis* | Rondônia, Brazil | MCL | POSITIVE |
| 29 | *Leishmania spp.* | Rondônia, Brazil | MCL | POSITIVE |
| 30 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 31 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 32 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 33 | *L. guyanensis* | Rondônia, Brazil | MCL | NEGATIVE |
| 34 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 35 | *L. braziliensis* | Rondônia, Brazil | MCL | POSITIVE |
| 36 | *L. braziliensis* | Rondônia, Brazil | MCL | POSITIVE |
| 37 | *L. braziliensis* | Rondônia, Brazil | CL | NEGATIVE |
| 38 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 39 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 40 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |
| 41 | *Leishmania spp.* | Rondônia, Brazil | CL | NEGATIVE |
| 42 | *L. guyanensis* | Rondônia, Brazil | CL | NEGATIVE |
| 43 | *L. braziliensis* | Rondônia, Brazil | CL | POSITIVE |

**Supplementary Table 1. Cervical brushes obtained from patients.** The diagram shows the distribution of the samples tested in figure 1, according to the specie of the infecting parasite, place of Isolation, outcome of the disease (CL= Cutaneous Leishmaniasis; MCL = Mucocutaneous Leishmaniasis), and LRV status.

| Collection Label | Specie | Place of Isolation | LRV Status |
|---|---|---|---|
| IOCL 3569 (299) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3570 (303) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3571 (304) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3622 (476) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3625 (313) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3639 (390) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3626 (314) | *L. braziliensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3642 (412) | *L. braziliensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3710 (666) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3708 (667) | *L. braziliensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3714 (760) | *L. braziliensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3713 (767) | *L. braziliensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3711 (818) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3712 (820) | *L. braziliensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3637 (384) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3540 (268) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3539 (271) | *L. guyanensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3460 (391) | *L. guyanensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3546 (276) | *L. braziliensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3538 (251) | *L. guyanensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3549 (283) | *L. braziliensis* | Rondônia, Brazil | NEGATIVE |
| IOCL 3567 (291) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |
| IOCL 3545 (275) | *L. braziliensis* | Rondônia, Brazil | POSITIVE |

**Supplementary Table 2. Clinical isolates obtained from patients.** The diagram shows the distribution of the *L.b.* and *L.g.* parasites tested in figure 10, according to their International code, FIOCRUZ collection label and LRV status.