Endosymbiotic RNA virus inhibits *Leishmania*-induced caspase-11 activation

**HIGHLIGHTS**
- LRV partially blocks NLRP3 inflammasome activation by *L. g.* via Casp11
- LRV interferes directly with caspase-11 activation by *L. g.*
- Caspase-11 blockage by LRV increases *L. g.* replication and disease pathogenesis
- TLR3 and ATG5 are required for LRV-mediated inhibition of Casp11

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Endosymbiotic RNA virus inhibits Leishmania-induced caspase-11 activation

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SUMMARY

New World species of the intracellular protozoan parasites of the Leishmania genus can cause mucocutaneous leishmaniasis. The presence of an endosymbiotic Leishmania RNA virus (LRV) in Leishmania guyanensis (L.g.) promotes disease exacerbation and the development of mucocutaneous disease. It was previously reported that LRV blocks the NLRP3 inflammasome, but additional mechanisms remain unclear. Here, we investigated whether LRV interferes with the inflammasome via caspase-11, which induces non-canonical NLRP3 activation and was reported to be activated by Leishmania. By using macrophages and mice, we found that LRV inhibits caspase-11 activation and IL-1β release by L.g. in a TLR3- and ATG5-dependent manner. Moreover, LRV exacerbates disease in C57BL/6 mice but not in Casp11−/−, Nlrp3−/−, and 129 mice, a mouse strain that is naturally mutant for caspase-11. These results demonstrate that LRV interferes with caspase-11 activation by Leishmania, expanding our understanding about the mechanisms by which LRV promotes disease exacerbation.

INTRODUCTION

Leishmaniases comprise a large group of inflammatory insect-borne diseases that may range from self-healing cutaneous lesions to mucocutaneous and visceral outcomes, affecting millions of people worldwide and causing thousands of deaths per year (Hartley et al., 2012; Pigott et al., 2014; Scott and Novais, 2016). Although the host immune response against the parasite has extensively been studied, only recently the specific mechanisms of innate immune recognition began to be unraveled (reviewed in de Carvalho and Zamboni, 2020). Recent studies have shown that caspase-11 is efficiently activated by Leishmania parasites to induce non-canonical NLRP3 activation, expanding the importance of caspase-11 to parasitic diseases (de Carvalho et al., 2019a). Furthermore, it was demonstrated that parasites harboring the Leishmania RNA virus (LRV) induce less inflammasome activation by a TLR3- and autophagy-dependent mechanism, suggesting that this pathway could be responsible for the debilitating mucocutaneous form of leishmaniasis observed in patients (de Carvalho et al., 2019b; Hartley et al., 2016; Ives et al., 2011; Rossi et al., 2017). However, whether LRV affects caspase-11 and the non-canonical activation of NLRP3 by Leishmania remains unknown.

Here, we use mouse bone-marrow-derived macrophages (BMDMs) and mouse models of leishmaniasis to investigate the impact of LRV in non-canonical NLRP3 activation by Leishmania parasites. By using the M4147 L. guyanensis strain, which harbors high levels of LRV (L.g.+), and an M4147-derived LRV negative clone (L.g.) that spontaneously lost LRV (de Carvalho et al., 2019b), we found that LRV blocks caspase-11 activation by L.g. We further show that this process is TLR3/ATG5 dependent and leads to inhibition of the non-canonical pathway for NLRP3 activation, thus favoring parasite persistence and increasing disease susceptibility in mice. Collectively, these data advance toward the understanding of the mechanisms by which LRV promotes inhibition of inflammasome activation and disease exacerbation.

RESULTS

Leishmania RNA virus (LRV) interferes with inflammasome activation via caspase-11

NLRP3 activation and overall inflammation is a hallmark of many inflammatory and infectious diseases including leishmaniasis, although the complete mechanisms of inflammasome regulation in response to
infection by \textit{Leishmania} parasites are still under intense investigation (reviewed in Broz and Dixit, 2016; de Carvalho and Zamboni, 2020; Zamboni and Sacks, 2019). In this context, LRV emerged as an important modulator of innate immunity during \textit{Leishmania} infection since it was shown to trigger TLR3 activation and negatively regulate NLRP3 assembly by the parasite (de Carvalho et al., 2019b; Hartley et al., 2016; Ives et al., 2011; Rossi et al., 2017). Importantly, studies from our and other labs recently demonstrated that \textit{Leishmania} is capable of triggering caspase-11 activation (Chaves et al., 2019; de Carvalho et al., 2019a), a process that was previously reported to occur via bacterial lipopolysaccharide (LPS) (Hagar et al., 2013; Kayagaki et al., 2013). Thus, we decided to investigate whether LRV could exert any impact on caspase-11 and non-canonical inflammasome activation by \textit{L. guyanensis}. To address this question, we initially infected wild-type (WT), \textit{Nlrp3}^−/−, and \textit{Casp11}^−/− BMDMs with both \textit{L.g.}− and \textit{L.g.}+ at MOI 10, and measured caspase-1 activation and IL-1β production. Western blotting for cleaved caspase-1 (Figure 1A), flow cytometric analysis of active caspase-1 staining (using FAM-YVAD fluorescent dye) in BMDMs from wild-type \textit{C57BL/6}, \textit{Nlrp3}^−/−, and \textit{Casp11}^−/− mice, 24 hr after infection with SP \textit{L.g.}− and \textit{L.g.}+ at MOI 10. A representative contour plot (B), the percentage of active caspase-1+ cells (FAM-YVAD+) (C), and the integrated mean of fluorescence intensity (iMFI) (D) are shown.

(F) ELISA assay quantifying IL-1β production in cell-free supernatants from LPS-primed wild-type \textit{C57BL/6} and \textit{Casp11}^−/− BMDMs, 24 hr after infection with SP \textit{L.g.}− and \textit{L.g.}+ at MOI 10 or after 20 hr of infection with parasites and 4 hr of infection with \textit{Legionella pneumophila} lacking flagellin (\textit{flaA}).

The results are shown as mean ± SD, and statistical analysis was performed by unpaired Student’s t test (C–F). *, p < 0.05. SD, standard deviation. One representative of at least three independent experiments performed with technical replicates is shown.
CASP11-dependent manner. We also performed the coinfection experiment using *Legionella pneumophila* lacking flagellin (*flaA*), a bacteria that is known to induce caspase-11 activation and non-canonical activation of NLRP3 (Case et al., 2013). Briefly, we infected LPS-primed BMDMs with *L.g.−* or *L.g.+* for 20 hr and then infected these cells with *flaA* for additional 4 hr. After these total 24 hr, cell-free supernatants from infected or non-infected (controls) were collected and IL-1β production was assessed by ELISA. While we found that *L.g.+* decreases IL-1β production in *flaA*−infected C57BL/6 macrophages, this effect is completely abolished in Casp11−/−/C0−/C0 BMDMs (Figure 1F). Collectively, these results suggest that *L. guyanensis* induces NLRP3 and caspase-11 activation via caspase-11, and the presence of LRV is capable of blocking non-canonical activation of the inflammasome.

**LRV inhibits *Leishmania guyanensis*-induced caspase-11 activation**

Next, we addressed the impact of LRV in caspase-11 activation by measuring caspase-11 directly, as previously described (Cunha et al., 2015). For this purpose, we primed macrophages with PAM3CSK4, a TLR2 agonist, and incubated cells with biotin-VAD dye for 15 min prior to infection (more details in the Transparent methods section). This dye binds to all active forms of caspases, enabling its detection via incubation with streptavidin beads and subsequent antibody-mediated detection by Western blotting. By pulling down the active forms of caspase-11 upon infection, we found that different than *L.g.−*, *L.g.+* fails to induce robust caspase-11 activation in PAM3CSK4 (Figure 2A) or TNF-α-primed macrophages (Figure 2B). These data support the important role of LRV in blocking *L.g.*-induced caspase-11 activation and the non-canonical NLRP3 activation by *L.g.*

![Figure 2. LRV interferes with caspase-11 activation in macrophages infected by *L. guyanensis*](image)

**LRV exacerbates *L.g.* pathogenesis in mice and enhances parasite replication in BMDMs via caspase-11**

Our results obtained using BMDMs demonstrate that LRV attenuates non-canonical inflammasome activation, suggesting that caspase-11 might play an important role for *L.g.* replication and pathogenesis in vitro.
In order to address these questions, we initially infected C57BL/6 WT, Nlrp3−/−, and Casp11−/− BMDMs with both parasites (L.g.- and L.g.+ at MOI 3). After 1 hr of infection, cells were washed and left in culture for 1 or 48 hr. Parasite killing was evaluated by Panotico Giemsa. The percentage of infected BMDMs at 1 (A) or 48 hr (C) and the average number of parasites at 1 (B) or 48 hr (D) after infection are shown. The results are shown as mean ± SD, and statistical analysis was performed by unpaired Student’s t test. *p < 0.05. SD, standard deviation. One representative of three independent experiments performed with technical replicates is shown.

These results encouraged us to investigate the participation of caspase-11 in LRV-mediated disease exacerbation in vivo. To tackle this question, we infected C57BL/6 WT, Nlrp3−/−, and Casp11−/− BMDMs with both parasites (L.g.- and L.g.+) and assessed parasite killing by Giemsa staining. At 1 hr after infection, no differences were observed in parasite internalization, as demonstrated by the percentage of infected cells (Figure 3A) and average number of parasites per infected cell (Figure 3B). By contrast, L.g.+ killing was significantly hampered compared to L.g.- after 48 hr of infection in WT BMDMs (Figures 3C and 3D). Interestingly, this phenomenon was not observed in NLRP3- and CASP11-deficient macrophages (and also not in Tlr3−/− BMDMs), suggesting that LRV modulates parasite killing in vitro by interfering with the activation of the non-canonical inflammasome (Figures 3C and 3D).

LRV does not interfere with inflammasome and disease pathogenesis in naturally caspase-11-deficient mice

The 129 mouse lineage has been used as a model to study infectious diseases, but differently from C57BL/6, this mouse strain carries polymorphisms that lead to a premature stop and inactive casp11 gene (Kayagaki et al., 2011; Kenneth et al., 2012). Thus, we decided to investigate whether LRV-mediated inflammasome inhibition and disease progression would also be observed in this mouse strain. We initially infected C57BL/6 or 129 derived BMDMs with L.g.- and L.g.+ parasites and assessed IL-1β production by

![Figure 3. LRV increases L. guyanensis replication in macrophages via caspase-11 and NLRP3](image-url)
these cells. As expected, L.g.+ induced less cytokine secretion compared to L.g.- in C57BL/6-derived WT BMDMs, a process that was completely dependent on NLRP3, ASC, and caspase-1 (Figure 5A). However, in 129 derived WT BMDMs, L.g.+ and L.g.- induced similar levels of IL-1β (Figure 5B), differently from what were observed in C57BL/6-derived WT BMDMs. Remarkably, we observed a significant increase in the amount of IL-1β generated by 129 derived BMDMs upon infection with L.g.-, when compared to C57BL/6-derived macrophages, which we believe to be a direct result of genetic differences between these strains. For instance, the 129 mouse lineage has a NRAMP1 iron transporter that is functional and plays a role in antimicrobial immunity against intracellular pathogens, while C57BL/6 does not (Skamene et al., 1998). In addition to this, 129 and C57BL/6 express distinct functional alleles of the Nlrp1 gene, which controls inflammasome activation and IL-1β production (Chavarria-Smith and Vance, 2015). Thus, these and possibly other genetic variations may explain different levels of IL-1β induced in response to Leishmania during infection in these two lineages of BMDMs. These data obtained in vitro further prompted us to evaluate the potential effects of LRV in L.g.-infected 129 mice. We found that in vivo infection of 129 mice with L.g.+ did not result in increased ear thickness (Figure 6A) compared to L.g.-, differently from what is observed in C57BL/6 mice (Figure 6B). Corroborating these data, we observed higher parasites titers in the ear (Figure 6C) and draining lymph nodes (Figure 6D) of L.g.+-infected C57BL/6 mice compared to L.g.-, but these differences were not observed in L.g.-infected 129 mice. Taken together, these results obtained using a mouse strain that is naturally deficient for caspase-11 provide additional support to our assertion that caspase-11 is required for LRV-induced inflammasome modulation.

LRV blocks L.g.-induced caspase-11 activation via TLR3 and ATG5

The results presented so far suggest that LRV interferes with the activation of caspase-11 and consequently with the non-canonical activation of the NLRP3 inflammasome in macrophages, a process that increases L.g. replication in vitro and disease pathogenesis in vivo. We previously demonstrated that viral dsRNA induces activation of TLR3, triggering autophagy and therefore blocking NLRP3 activation (de Carvalho et al., 2019b). We then decided to address the possible mechanisms by which LRV could potentially interfere with caspase-11 activation. For this purpose, we performed the pull-down experiment previously described

Figure 4. LRV exacerbates L. guyanensis infection in vivo via caspase-11 and NLRP3

Wild-type C57BL/6 (A), Nlrp3−/− (B), and Casp11−/− mice (C) were infected with 10⁵ metacyclic promastigotes of L. guyanensis without the virus (L.g.-) or with the virus (L.g.+ ) and ear thicknesses were followed weekly, during 4 weeks. After that period, ears were removed and processed, and parasite titers were determined in the ear (D) and draining lymph nodes (E). The results are shown as mean ± SD, and statistical analysis was performed by unpaired Student’s t test (D,E) or two-way ANOVA with Bonferroni’s multiple comparison test (A-C). *, p < 0.05. SD, standard deviation. One representative of three independent experiments performed with biological replicates (n = 4–6 mice per group) is shown.
utilizing Tlr3\textsuperscript{-/-} and LysM\textsuperscript{Cre/+}\textsuperscript{Atg5Fl/Fl} BMDMs, which lack TLR3 and ATG5 (a critical gene involved with autophagy induction), respectively. Interestingly, we found that caspase-11 activation was restored upon L.g.+ infection in BMDMs lacking either one of these molecules (Figures 7A and 7B), providing insights toward the mechanisms by which LRV modulates caspase-11. Although toll-like receptors (TLRs) are known to serve as a priming and therefore facilitate inflammasome activation (Swanson et al., 2019), these results corroborate previous findings suggesting that, during Leishmania infection, LRV-mediated TLR3 activation acts as a negative regulator of NLRP3, accounting to consolidate the concept that Leishmania orchestrates a differential innate immune signaling in macrophages. Although more studies are required to rule out the exact mechanisms by which LRV-induced TLR3 and ATG5 activation interferes with caspase-11 activation, this study advances in the understanding of NLRP3 modulation by LRV and reinforces the important participation of caspase-11 and the non-canonical inflammasome in the pathogenesis of leishmaniasis.

DISCUSSION

Activation of the NLRP3 inflammasome is a hallmark of leishmaniasis, and this platform is very important for the pathogenesis and the outcome of the disease (Zamboni and Sacks, 2019). We have previously demonstrated that the NLRP3 inflammasome is important to explain the previously reported effects of the LRV in disease exacerbation and induction of mucocutaneous leishmaniasis (de Carvalho et al., 2019b; Ives et al., 2011; Olivier and Zamboni, 2020). Here, we expand these findings indicating that caspase-11 is a target of LRV and required for the LRV-mediated inflammasome inhibition. Our data also highlight an important signaling axis involving caspase-11 activation to Leishmania pathogenesis and disease progression in infections caused by New World Leishmania species that harbors LRV. Although we show that this phenomenon is driven by TLR3 and ATG5, the precise mechanisms by which this pathway regulates caspase-11 activation were not evaluated in this study. Intriguingly, TLRs have been described as important receptors involved with the generation of first signal for inflammasome activation, often contributing to NLRP3-dependent cytokine production (Swanson et al., 2019). However, in the context of Leishmania infection, we show that LRV-driven TLR3 activation plays an opposing role in caspase-11 and NLRP3, contributing to inhibition, rather than assembly of the non-canonical inflammasome. We believe that this differential mechanism relies as a direct result of the parasite’s manipulation of intracellular signaling pathways, such as NF-kB-dependent inflammatory gene’s transcription (Olivier and Zamboni, 2020; Zamboni and Sacks, 2019). However, the exact factors responsible for this different TLR3-mediated outcome remain to be addressed in future studies.

In this work, we combined a series of genetic tools (in vitro and in vivo) and different inflammasome readouts to identify caspase-11 as the target used by LRV to inhibit the inflammasome and promote...
parasite replication and disease exacerbation. So far, studies addressing the impact of LRV in the course of Leishmania infection employed genetically modified parasites (Castiglioni et al., 2017; Eren et al., 2016; Hartley et al., 2016; Ives et al., 2011) or clinical isolates (Kariyawasam et al., 2017) and they indeed reached important results. In this study, we used a previously obtained spontaneously derived L.g.- clone that lost the virus (de Carvalho et al., 2019b), offering us an excellent model to assess the LRV-mediated effects in the modulation of innate immunity and disease. Noteworthy, the differences observed in inflammasome activation by L.g.+ and L.g.- parasites could be complemented by using extracellular vesicles containing LRV, as previously described (Atayde et al., 2019; de Carvalho et al., 2019b).

Although one could argue that L.g.- does not cause significant pathology (as indicated by an increase in skin swelling in 129 infected mice), L.g.- parasites are found in similar titers in both C57BL/6 and 129 mice, both in the ear and draining lymph node, suggesting that the parasite is resisting killing/multiplying even in the absence of substantial immunopathology in the infected site. Importantly, it is worth mentioning that the similar parasite titers found in 129 mice upon infection with either L.g.- or L.g.+ do not necessarily represent a role for caspase-11 since other factors related to the background of this mouse could be responsible for this phenotype (examples: polymorphisms in genes involved with parasite replication or inflammatory reactions, natural resistance to infections, etc). A nice example of this is the Slc11a11 gene, which codifies the NRAMP1 protein in myeloid cells. The 129 mouse strain produces a mature version of this protein and therefore is able to control intracellular pathogens such as Salmonella enterica, Leishmania donovani, and Mycobacterium tuberculosis. On the other hand, C57BL/6 mouse has a point mutation that prevents formation of a mature version of NRAMP1 and is therefore susceptible to these infections (Cellier et al., 1996; Skamene et al., 1998; Zhang et al., 2018). Nonetheless, our data in C57BL/6 mice

Figure 6. Disease progression upon L.g.+ and L.g.- infection is similar in 129 mice
Wild-type 129 and C57BL/6 mice were infected with 10^5 metacyclic promastigotes of L. guyanensis without the virus (L.g.-) or with the virus (L.g.+), and ear thicknesses were followed weekly, during 4 weeks (A and B). After that period, ears and draining lymph nodes (LNs) were removed and processed, and parasite titers were determined in the ear (C) and LNs (D). The results are shown as mean ± SD, and statistical analysis was performed by unpaired Student’s t test (C and D) or two-way ANOVA with Bonferroni’s multiple comparison test (A, B). *, p < 0.05. SD, standard deviation. One representative of three independent experiments performed with biological replicates (n = 5 mice per group) is shown.
unequivocally demonstrate that LRV utilizes caspase-11 and the inflammasome pathway to exacerbate infection in this mouse model of leishmaniasis.

Taken together, the data presented in this manuscript suggest that caspase-11 is an additional target for LRV, representing a potential molecule involved with increased disease pathology during infection with LRV + Leishmania. Understanding how to overcome the signaling axis triggered by the virus could represent a potential therapeutic target for the development of better therapies in patients suffering from severe and highly debilitating mucocutaneous leishmaniasis.

Limitations of the study
The current study demonstrates that Leishmania RNA virus promotes inhibition of the non-canonical inflammasome pathway induced upon L. guyanensis infection, by interfering with caspase-11 activation. Caspase-11 is known to operate upstream of NLRP3 (Hagar et al., 2013; Kayagaki et al., 2013), and it is activated in response to Leishmania infection (de Carvalho et al., 2019a). Although the findings represent an advance in our understanding of LRV-mediated effects on the NLRP3 inflammasome and intracellular signaling pathways in mammalian cells, we do not show the precise mechanisms by which LRV interferes with caspase-11 activation. This issue remains to be addressed and should be the subject of further studies.

Resource availability

Lead contact
Further information and requests for resources and reagents should be directed to our Lead Contact, Dario S. Zamboni (dszamboni@fmrp.usp.br).

Material availability
This study did not generate new materials or reagents.
Data codes/sets were not generated or analyzed in this study. For questions regarding the raw data from the current study, please contact the corresponding author. All softwares used in this study are commercially available.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.102004.

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AUTHOR CONTRIBUTIONS
R.V.H.C., D.S.L.J., and C.V.O. designed and performed experiments and generated tools for proper work development. R.V.H.C. and D.S.Z. analyzed the data, discussed hypotheses, generated figures, and wrote the article. All authors revised the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Endosymbiotic RNA virus inhibits *Leishmania*-induced caspase-11 activation

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Supplemental information

Transparent Methods

Mice
Six to eight weeks old female wild-type 129S1/SvImJ (JAX number 002448) and C57BL/6, as well as knockout animals in C57BL/6 background Nlrp3−/−, Asc−/−, Tlr3−/−, Casp11−/−, LysMCre/+Atg5Fl/+ and LysMCre/+Atg5Fl/Fl were used. Animals were bred and maintained under specific pathogen-free conditions at the animal facilities of the University of São Paulo, FMRP/USP. Experiments were approved and conducted according to institutional guidelines for animal care (Protocol number 014/2016).

Parasites and infection
The parasite strains used in this study were the wild-type strain of *L. guyanensis* M4147 (MHOM/BR/75/M4147), harboring high levels of LRV1 (*L.g.+*) and its LRV negative-derived clone (*L.g.-*), as previously described (de Carvalho et al., 2019). Parasites were cultured at 25 °C in Schneider’s Drosophila medium (Invitrogen, Carlsbad, CA), pH 7.0, supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL), 2 mM L-glutamine, and 2% biopterin, pH 6.5. For in vivo infections, mice were infected with 10⁵ metacyclic promastigotes (purified using the gradient-density FICOLL method (Spâth and Beverley, 2001) in 10 μL of PBS, through an intradermal injection into the left ear. Ear thicknesses were measured weekly with a dial gauge caliper and compared to the thickness of the uninfected ear of each individual mouse. Parasite burdens were quantified by
limiting dilution assay, which consisted of preparing ear and lymph node's homogenates from infected mice after 4 weeks of infection, plating undiluted samples at the first lane of 96 well plates, and serial diluting each (1:5 for ear, 1:2 for lymph node) sample up to 12x in complete Schneider's medium, therefore allowing parasite's growth. After 7 days in culture, each well in each dilution was checked and parasites were quantified, as previously described (de Carvalho et al., 2019). Coinfection experiments with *Legionella pneumophila* lacking flagellin (*FlaA*) were performed as previously described (de Carvalho et al., 2019).

Briefly, we infected LPS-primed BMDMs with *L.g.-* or *L.g.+* for 20 hours, and then infected these cells with *flaA* for additional 4 hours. After these total 24 hours, cell-free supernatants from infected or non-infected (controls) were collected and IL-1β production was assessed by ELISA (R&D Systems), according to the manufacturer's protocol.

**Bone marrow-derived macrophages (BMDM) and killing experiments**

Femurs and tibias were isolated and flushed with PBS, and total bone marrow cells were cultured in RPMI supplemented with 30% L929 cell-conditioned medium and 20% FBS. After 7 days in culture, mature BMDMs were harvested and infected with stationary phase or metacyclic promastigotes at MOI 3. 1 hour after infection, wells were washed, and fresh media was added to the infected cultures. The Leishmanicidal activity of the cells was determined at 48 hours after infection by counting the Giemsa-stained cytospin preparations under a light microscope with a 40X objective. The infection rate was determined by scoring the infected and uninfected cells (100 BMDMs) and the average number of intracellular parasites per infected BMDM.
**Caspase-1 activation assays**

BMDMs were cultured and infected with stationary phase *L.g.+* and *L.g.-* (MOI 10). 24 hours after infection, macrophages were stained for 1 hour with FAM–YVAD–fluoromethyl ketone (FAM–YVAD–FMK; Immunochemistry Technologies), as recommended by the manufacturer’s instructions. The active CASP1 was then measured by FACS. Both the percentage of active-caspase-1 macrophages and the integrated mean of fluorescence intensity (iMFI, which represents the average of caspase-1 activation per cell) were evaluated. Data were acquired on a FACS ACCURI C6 flow cytometer (BD Biosciences) and analyzed with the FlowJo software (Tree Star).

**Cytokine detection by ELISA**

BMDMs were primed for 4 hours with 500 ng/mL of LPS, a TLR4 agonist, and then infected with *L.g.-* or *L.g.+* at MOI 10. After 24 hours of infection, cell-free supernatants were collected and the levels of IL-1β were measured by ELISA (BD Biosciences), according to the manufacturer’s instructions.

**Pull-down of active caspase-11**

Macrophages were seeded in 6-well plates. Cells were plated in triplicates for each condition, 5x10⁶ BMDMs/well. RPMI 10% FBS was used as media for the entire assay. Cells were primed with either TNF-α (10 ng/mL) or PAM(3)CSK(4) (300 ng/mL) for 4 hours, and 15 minutes prior to infection, medium was renewed with 20 mM biotin-VAD-FMK (Enzo). Infection was performed with an additional 1
mL per well, totalizing 2 mL per well (not discarding the previous medium with b-VAD). 24 hours after infection, medium was removed and 500 microliters of RIPA 4% protease inhibitor (PI) was added to each triplicate (ex: 200 microL in the first replicate, and 150 microL on the other 2). Samples were centrifuged at 14000 G/15 min/4 degrees Celsius. As debris were sedimented, supernatant was transferred to a new 1.5 mL eppendorf. Next, the total amount of proteins in each sample was quantified by Bradford (Cepham Life Sciences). Samples were then equalized to 1 microgram of protein and incubated in a rotator with streptavidin-beads overnight (ON), at 4 degrees Celsius. Unincubated samples were used as loading controls for caspase-11 and actin (total protein in cell lysates). After incubating ON, the active caspases were bound to the streptavidin beads, and 500 microL of RIPA 4% PI to each sample was added. Next, samples were centrifuged 11000G/2 min. We did that for a total of three times. Finally, supernatants were discarded and we added 30 microL of sample buffer to each sample, boiling them at 100 degrees Celsius for 5 minutes, what induces active caspases dissociation from the beads. Supernatants were recovered in a new eppendorf, and these samples countaining active caspases were ready to be runned in SDS-PAGE and analysis by western blotting. Rabbit anti-Casp11 antibody clone Ab180673 (Abcam) (1:1000), mouse anti-β-actin (C4) (Santa cruz, sc-47778) and specific horseradish peroxidase-conjugated secondary antibodies (1:3000; KPL) were diluted in blocking buffer for the incubations. The ECL luminol reagent (GE Healthcare) was used for protein detection.

Western blot for caspase-1

A total of 10^7 BMDMs were seeded per well, primed with ultrapure LPS (500
ng/mL) for 4 h and left uninfected or infected for 48 hours. After this period, the
supernatants were collected and precipitated with 50% trichloroacetic acid (TCA)
and acetone. Cells were lysed in RIPA buffer containing protease inhibitor
cocktail (Roche). The lysates and supernatants were resuspended in Laemmlı
buffer, boiled, resolved by SDS-PAGE and transferred (Semidry Transfer Cell,
Bio-Rad) to a nitrocellulose membrane (GE Healthcare). Rat anti-Casp1 p20
monoclonal antibody clone 4B4 (Genentech) (1:500) and specific anti-rat
horseradish peroxidase-conjugated antibodies (1:3000; KPL) were diluted in
blocking buffer for the incubations. The ECL luminol reagent (GE Healthcare)
was used for the protein detection.

**Statistical analysis**

Two-way analysis of variance (ANOVA) followed by Bonferroni post-test was
used for the comparison of multiple groups. The differences in the values
obtained for two different groups were determined using Student’s t test. The
Prism 5.0 software (GraphPad, San Diego, CA) was used. A difference was
considered statistically significant when P<0.05.

**Supplemental references**

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