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Inflammasome-derived IL-1β production induces nitric oxide–mediated resistance to *Leishmania*

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Parasites of the *Leishmania* genus are the causative agents of leishmaniasis in humans, a disease that affects more than 12 million people worldwide. These parasites replicate intracellularly in macrophages, and the primary mechanisms underlying host resistance involve the production of nitric oxide (NO). In this study we show that the Nlrp3 inflammasome is activated in response to *Leishmania* infection and is important for the restriction of parasite replication both in macrophages and *in vivo* as demonstrated through the infection of inflammasome-deficient mice with *Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania infantum chagasi*. Inflammasome-driven interleukin-1β (IL-1β) production facilitated host resistance to infection, as signaling through IL-1 receptor (IL-1R) and MyD88 was necessary and sufficient to trigger inducible nitric oxide synthase (NOS2)-mediated production of NO. In this manuscript we identify a major signaling platform for host resistance to *Leishmania* spp. infection and describe the molecular mechanisms underlying *Leishmania*-induced NO production.

Leishmaniasis is caused by protozoan parasites of the *Leishmania* genus, which includes several species wildly distributed in South America and Asia[1]. Whereas *L. (L.) amazonensis* and *L. (Viannia) braziliensis* induce the cutaneous and mucocutaneous forms of the disease, *L. (L.) infantum chagasi* causes a life-threatening visceral form of leishmaniasis[1,2].

Leishmania parasites survive and multiply in macrophages, which are able to produce leishmanicidal products that contribute effectively to the restriction of parasite proliferation[3–5]. A potent leishmanicidal factor produced by macrophages is NO produced by the enzyme NOS2, which is tightly regulated in response to infection by pathways that are not completely elucidated[6–8].

Macrophage activation is crucial for the initiation of protective immune responses to different diseases, including leishmaniasis. Activation is achieved when host cell receptors sense microbial components or stress signals. Members of the Nod-like receptor (NLR) family of proteins have emerged as important innate immune sensors of microbes and damage[9]. Certain NLRs regulate the assembly of the inflammasome, a multimeric complex that contains active caspase-1 (reviewed in ref. 10). The Nlrp3 inflammasome requires the adaptor protein Asc (also called Pycard) and has been extensively studied because of its association with important chronic inflammatory diseases, including gout, Alzheimer's disease and type 2 diabetes[11]. The Nlrp3 inflammasome responds to microbial RNA and perturbations in the membranes of innate immune cells; this feature suggests that Nlrp3 is a sensor of damage (reviewed in ref. 10). Through mechanisms that are not yet elucidated, the assembly of the canonical Nlrp3 inflammasome requires the efflux of potassium $(K)^+$ and is impaired by inhibitors of K^+ transporters, lysosomal cathepsins and reactive oxygen species[12–15]. Once activated, caspase-1 induces processing and secretion of IL-1β, which is transcriptionally regulated when microbial components are sensed by pattern recognition receptors[16].

The activation of the inflammasome in response to infection by intracellular pathogens has been extensively investigated. However, only a few studies have addressed the role of the inflammasome in the restriction of infection by these microbes, and the mechanisms that underlie inflammasome-mediated host resistance are largely unknown[17–20]. In this study we addressed the role of the inflammasome in the host response to intracellular protozoan parasites of the *Leishmania* genus. We found that the Nlrp3 inflammasome is engaged in response to *L. amazonensis* infection and that activation of this molecular platform has a crucial role in the restriction of parasite replication both in isolated macrophages and *in vivo*. Notably, we found that IL-1β is important for host resistance to infection, as IL-1β signaling through IL-1R and MyD88 contributes to induce NOS2-mediated production of NO, which is a major host defense mechanism against *Leishmania* spp.

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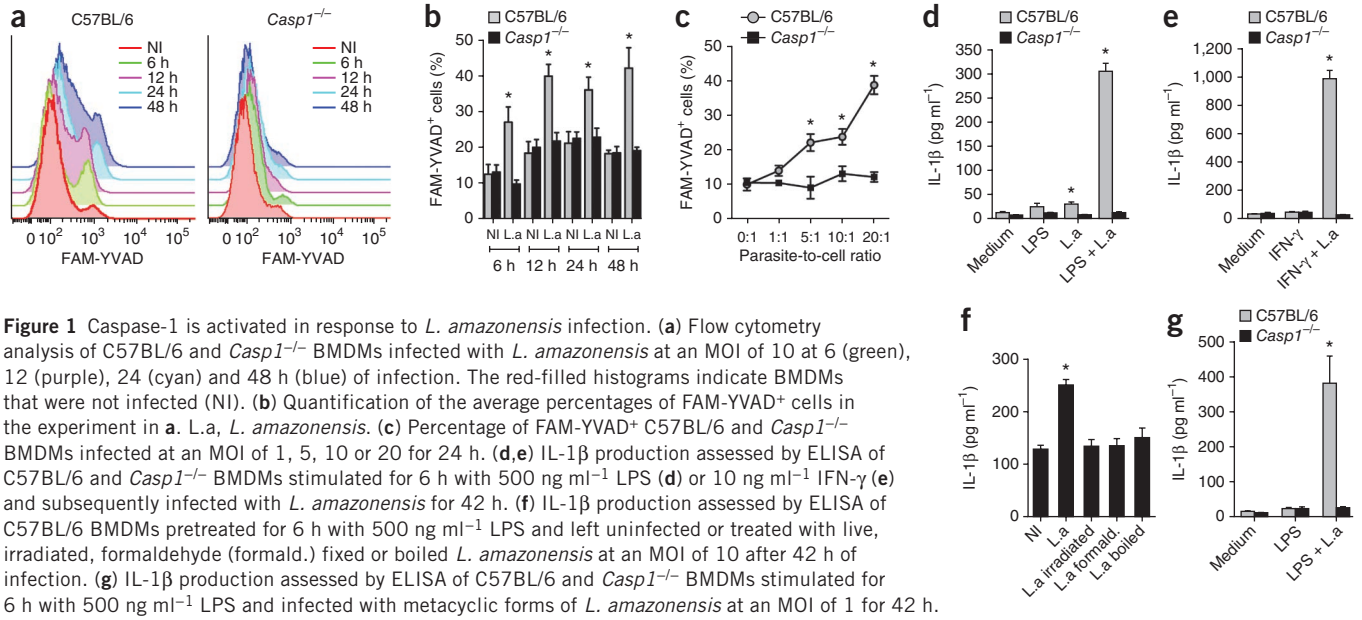

Figure 1 Caspase-1 is activated in response to *L. amazonensis* infection. (**a**) Flow cytometry analysis of C57BL/6 and *Casp1*$^{-/-}$ BMDMs infected with *L. amazonensis* at an MOI of 10 at 6 (green), 12 (purple), 24 (cyan) and 48 h (blue) of infection. The red-filled histograms indicate BMDMs that were not infected (NI). (**b**) Quantification of the average percentages of FAM-YVAD^{+} cells in the experiment in **a**. L.a, *L. amazonensis*. (**c**) Percentage of FAM-YVAD^{+} C57BL/6 and *Casp1*$^{-/-}$ BMDMs infected at an MOI of 1, 5, 10 or 20 for 24 h. (**d**,**e**) IL-1β production assessed by ELISA of C57BL/6 and *Casp1*$^{-/-}$ BMDMs stimulated for 6 h with 500 ng ml^{-1} LPS (**d**) or 10 ng ml^{-1} IFN-γ (**e**) and subsequently infected with *L. amazonensis* for 42 h. (**f**) IL-1β production assessed by ELISA of C57BL/6 BMDMs pretreated for 6 h with 500 ng ml^{-1} LPS and left uninfected or treated with live, irradiated, formaldehyde (formald.) fixed or boiled *L. amazonensis* at an MOI of 10 after 42 h of infection. (**g**) IL-1β production assessed by ELISA of C57BL/6 and *Casp1*$^{-/-}$ BMDMs stimulated for 6 h with 500 ng ml^{-1} LPS and infected with metacyclic forms of *L. amazonensis* at an MOI of 1 for 42 h. One representative of three (**a**–**f**) or two (**g**) independent experiments performed in triplicate is shown. Error bars (**b**–**g**), s.d. $^{*}P < 0.05$ (two-way analysis of variance (ANOVA) with Bonferroni's post test (**b**,**c**) or Student's *t* test (**d**–**g**)) compared to *Casp1*$^{-/-}$ (**b**–**e**,**g**) or NI (**f**).

RESULTS

The inflammasome is activated in response to *L. amazonensis*

Although caspase-1 is activated in macrophages in response to several intracellular and extracellular bacterial pathogens, the ability of parasites from the *Leishmania* genus to trigger caspase-1 activation is unknown. To determine whether macrophages activate caspase-1 in response to *L. amazonensis* infection, we infected bone marrow–derived macrophages (BMDMs) from C57BL/6 mice with stationary-phase forms of *L. amazonensis* and directly measured caspase-1 activation using a fluorescent dye that stains the active form of caspase-1 (FAM-YVAD)[20]. We found that *L. amazonensis* infection triggered caspase-1 activation in C57BL/6 but not in caspase-1–deficient (*Casp1*$^{-/-}$) BMDMs (**Fig. 1a**–**c**). The secretion of IL-1β is dependent on active caspase-1. We observed that BMDMs obtained from C57BL/6 mice, but not those from *Casp1*$^{-/-}$ mice, produced significant amounts of IL-1β in response to *L. amazonensis* infection in cells pretreated with lipopolysaccharide (LPS) or interferon-γ (IFN-γ) (**Fig. 1d**,**e**). To further evaluate whether caspase-1 activation is induced in response to live parasites, we infected BMDMs with live or nonviable (dead) *L. amazonensis* parasites and measured IL-1β secretion. Live *L. amazonensis* effectively induced IL-1β secretion, whereas nonviable parasites (boiled, inactivated with ultraviolet light or formaldehyde fixed) did not induce IL-1β secretion (**Fig. 1f**). We also observed these effects in response to infection with metacyclic forms of the parasite (**Fig. 1g**).

To determine which NLR is responsible for caspase-1 activation in response to *L. amazonensis* infection, we performed experiments using BMDMs deficient in different inflammasome components. The FAM-YVAD assay revealed that BMDMs from *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice induced neither caspase-1 activation (**Fig. 2a**,**b**) nor IL-1β secretion after infection with *L. amazonensis* (**Fig. 2c**). In this experiment we also used a combination of LPS and nigericin, which induces activation of the Nlrp3 inflammasome[16,17]. Moreover, we performed western blot assays with BMDMs treated with nigericin or *L. amazonensis* to further confirm the activation of the Nlrp3 inflammasome in response to *L. amazonensis* infection. We observed that *L. amazonensis* infection induced the processing and secretion of the caspase-1 subunit p20 and the IL-1β subunit p19 in a Nlrp3-dependent, Asc-dependent and caspase-1–dependent manner (**Fig. 2d**). The reduced amount of IL-1β production and caspase-1 cleavage in *L. amazonensis*–infected macrophages as compared to cells treated with LPS and nigericin supports the hypothesis that *L. amazonensis* may modulate and perhaps inhibit inflammasome activation in some instances, a feature that will require further investigation. Nonetheless, the inflammasome is engaged in response to *Leishmania* infection, and to further evaluate inflammasome assembly *in situ*, we transduced BMDMs with a retroviral construct encoding GFP-tagged Asc (Asc-GFP). We infected the transduced BMDMs with *L. amazonensis* and found formation of Asc foci in the infected cells at 2 and 6 h after infection with either stationary-phase or metacyclic forms of *L. amazonensis* (**Fig. 2e**–**h**), thus indicating the assembly of the inflammasome in response to *L. amazonensis* infection.

Potassium efflux is crucial for the activation of the Nlrp3 inflammasome[16,21]. Moreover, CA-074-ME (a compound that inhibits both cathepsin B and cathepsin L) and glibenclamide (a drug that inhibits ATP-sensitive potassium channels) both inhibit activation of the Nlrp3 inflammasome[15,22,23]. To further investigate the role of Nlrp3 in the activation of caspase-1 in response to *L. amazonensis* infection, we determined whether glibenclamide, CA-074-ME and extracellular K^{+} interfere with caspase-1 activation during the macrophage response to *L. amazonensis* infection. FAM-YVAD^{+} staining and IL-1β secretion demonstrated that the addition of glibenclamide, CA-074-ME or potassium chloride (KCl), but not sodium chloride (NaCl), impaired *L. amazonensis*–induced caspase-1 activation (**Supplementary Fig. 1**). Taken together these results indicate that potassium, cathepsins and K^{+} channels are all required for *L. amazonensis*–induced Nlrp3 inflammasome activation, thus supporting a key role for this inflammasome in caspase-1 activation in BMDMs infected with *L. amazonensis*.

Inflammasome facilitates restriction of *Leishmania* infection

To evaluate the role of the inflammasome in the restriction of *L. amazonensis* replication in BMDMs, we measured parasite

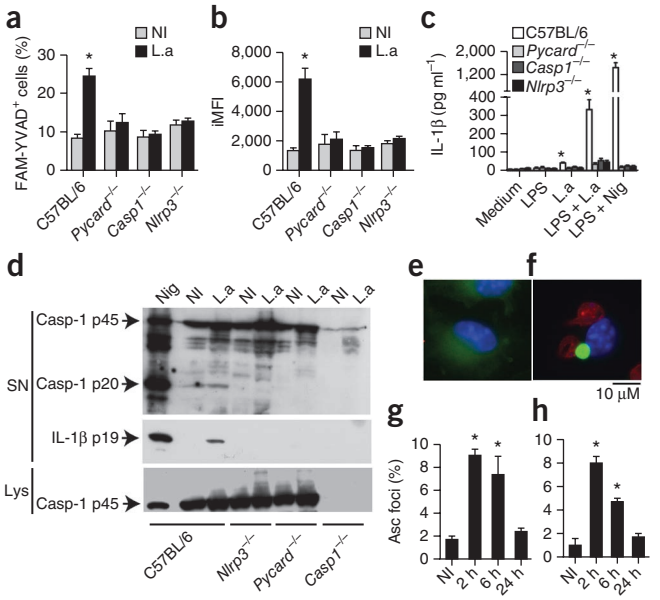

Figure 2 The Nlrp3 inflammasome is required for caspase-1 activation in macrophages infected with *L. amazonensis*. (**a**,**b**) Flow cytometry analysis of FAM-YVAD staining of C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs infected with stationary-phase *L. amazonensis* at an MOI of 10 for 42 h. (**c**) IL-1β production from C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs stimulated for 6 h with LPS (500 ng ml^{-1}) and infected with stationary-phase *L. amazonensis* at an MOI of 10 for 42 h or treated with 20 μM nigericin (Nig) for 1 h. (**d**) Immunoblotting for caspase-1 p20 and IL-1β p19 in cell-culture supernatants (SN) and cell lysates (Lys) of BMDMs stimulated for 6 h with LPS (500 ng ml^{-1}) and infected or not infected with stationary-phase *L. amazonensis* at an MOI of 10 for 42 h. (**e**–**h**) Immunofluorescence and quantification of *Casp1*$^{-/-}$ BMDMs transduced with a retroviral construct encoding Asc-GFP and infected with stationary-phase *L. amazonensis* at an MOI of 5 (**f**,**g**) or with metacyclic promastigotes at an MOI of 3 (**h**). (**e**) Uninfected *Casp1*$^{-/-}$ BMDMs expressing Asc-GFP dispersed in the cytoplasm (green). (**f**) BMDMs infected with *L. amazonensis* (red) for 2 h showing the Asc-GFP foci (green). (**g**,**h**) Quantification of the percentage of Asc foci at 2, 6 and 24 h after infection of the BMDMs. One representative of three experiments performed in triplicate is shown throughout. Error bars (**a**–**c**,**g**,**h**), s.d. *$P < 0.05$ (Student's *t* test (**a**–**c**) or one-way ANOVA with Bonferroni's post test (**g**,**h**)) compared to NI (**a**,**b**,**g**,**h**) or similarly treated *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs (**c**).

replication in BMDMs obtained from C57BL/6 or inflammasome-deficient mice. We also used BMDMs from *Nos2*$^{-/-}$ mice because NOS2-induced NO is essential for the restriction of parasite growth[8,24,25]. Although parasite internalization by the BMDMs was similar after 1 h of infection, the BMDMs from *Pycard*$^{-/-}$, *Casp1*$^{-/-}$, *Nlrp3*$^{-/-}$ and *Nos2*$^{-/-}$ mice had higher percentages of infected cells (**Fig. 3a**) and higher numbers of intracellular amastigotes (**Fig. 3b**,**c**) after 24, 48, 72 and 96 h of infection compared to C57BL/6 BMDMs. We performed similar experiments using metacyclic forms of *L. amazonensis* and found that the parasites replicated more efficiently in cells deficient in the inflammasome or NOS2 (**Fig. 3d**). To further evaluate *L. amazonensis* replication in BMDMs, we used GFP-expressing *L. amazonensis* (La-GFP$^+$) to quantify parasite replication using flow cytometry. To validate the effectiveness of this method, we infected BMDMs with different multiplicities of infection (MOIs) of La-GFP$^+$ for 3 h and determined the percentage of GFP$^+$ BMDMs using flow cytometry. We observed a direct correlation between the MOI used for infection, the percentage of GFP$^+$ BMDMs and the integrated mean fluorescence intensity (iMFI) of the infected BMDMs (**Supplementary Fig. 2**). We then used the La-GFP$^+$ parasites to evaluate the role of the inflammasome in the restriction of intracellular parasite replication using flow cytometry. We observed that the parasite loads in the *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ BMDMs were significantly higher than those in C57BL/6 BMDMs and were similar to those in *Nos2*$^{-/-}$ BMDMs, as demonstrated by the percentage of GFP$^+$ BMDMs at 48 h after infection (**Fig. 3e**) and the MFIs of the GFP$^+$ BMDMs during a 96-h growth kinetics analysis (**Fig. 3f**). We further investigated whether treatment of the BMDMs with the inhibitors described above affects the restriction of parasite replication. We observed that the C57BL/6 BMDMs treated with CA-074-ME, glibenclamide or KCl, but not NaCl, were more susceptible to *L. amazonensis* replication, as demonstrated by the percentage of GFP$^+$ BMDMs and the iMFIs of the infected BMDMs; however, these treatments did not alter parasite replication in *Pycard*$^{-/-}$ or *Casp1*$^{-/-}$ cells (**Fig. 3g**,**h**). Taken together, these results indicate that the activation of the Nlrp3 inflammasome is important for the restriction of *L. amazonensis* replication in BMDMs.

To evaluate the role of the inflammasome in infection of mice, we used an ear model of infection[26]. We observed a significant increase in the size of the lesions and skin necroses in the ears of *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice compared to those in the ears of C57BL/6 mice (**Fig. 4a**,**b**). Consistent with the increased lesion size, *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice also harbored increased parasite loads in the ears, lymph nodes and spleen as measured by a limiting dilution of organ homogenates after 8 weeks of infection (**Fig. 4c**). We obtained similar results in mice infected with metacyclic promastigotes of *L. amazonensis*, the infective forms of the parasite that are injected through insect bites in natural infections (**Fig. 4d**–**f**). We also performed infections in an A/J mouse background, which is resistant to *L. amazonensis*[27], to evaluate whether the observed phenomena were limited to the mouse background chosen for our experimental model. Therefore, we generated caspase-1–deficient mice in an A/J background (A/J-*Casp1*$^{-/-}$) by backcrossing mice carrying the caspase-1–deficient allele with A/J mice for ten generations. The experiments performed with this mouse strain indicated that the caspase-1 deficiency in A/J-*Casp1*$^{-/-}$ mice increased lesion development, skin necrosis and parasite load compared to infected A/J control mice (**Fig. 4g**–**i**). Taken together, these results suggest that regardless of the mouse background and parasite forms used for infection, the inflammasome is important for the efficient restriction of parasite infection in cutaneous leishmaniasis.

To investigate inflammasome activation in infected mice, we quantified the production of IL-1β in cells obtained from *L. amazonensis*–infected mice. We found substantial production of IL-1β in lymph-node and spleen cells obtained from C57BL/6 mice infected for 2 or 5 weeks. The production of IL-1β detected in these *ex vivo* experiments was dependent on the inflammasome, as we detected no IL-1β in the supernatants of cultures of cells obtained from infected *Pycard*$^{-/-}$ or *Casp1*$^{-/-}$ mice (**Supplementary Fig. 3**).

Inflammasome triggers NO-mediated resistance to infection

We assessed the role of IL-1β in host resistance to *L. amazonensis* infection because the processing of this cytokine in BMDMs is dependent strictly on the inflammasome and because polymorphisms within the human *IL1B* gene are associated with severity of disease[28,29]. We quantified parasite replication in C57BL/6 and inflammasome-deficient BMDMs treated with different concentrations of an IL-1R antagonist (IL-1Ra)[30]. We observed that inhibiting endogenous IL-1R signaling resulted in a dose-dependent increase

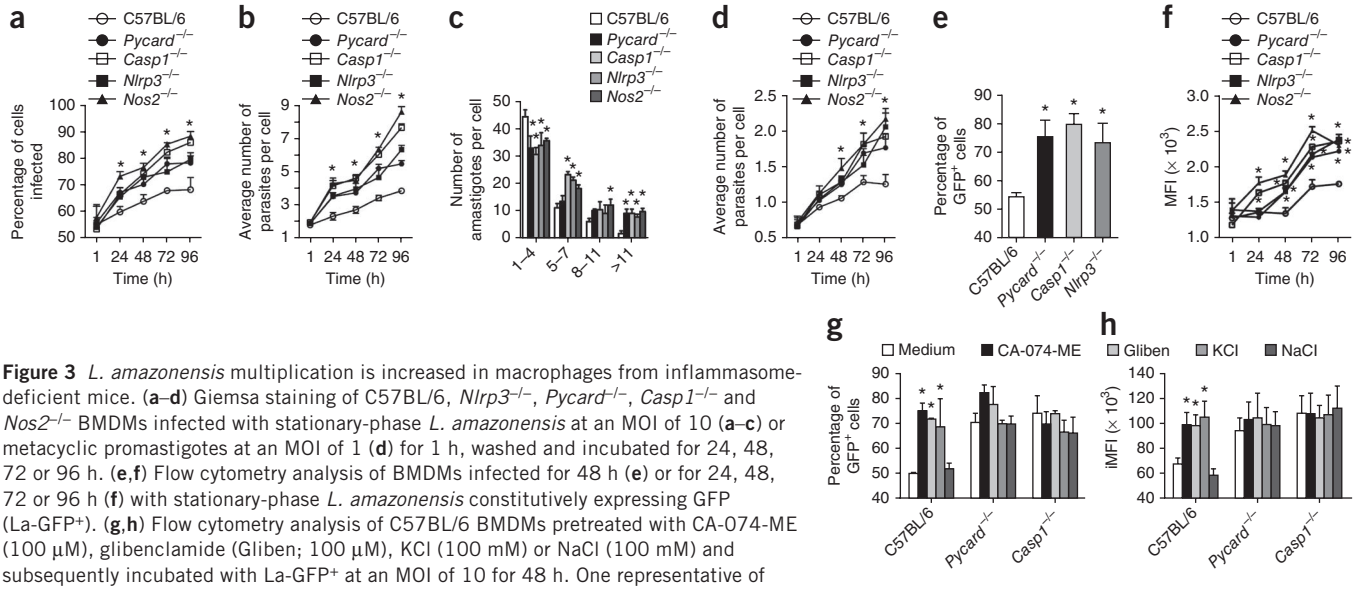

Figure 3 *L. amazonensis* multiplication is increased in macrophages from inflammasome-deficient mice. (**a**–**d**) Giemsa staining of C57BL/6, *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nos2*$^{-/-}$ BMDMs infected with stationary-phase *L. amazonensis* at an MOI of 10 (**a**–**c**) or metacyclic promastigotes at an MOI of 1 (**d**) for 1 h, washed and incubated for 24, 48, 72 or 96 h. (**e**,**f**) Flow cytometry analysis of BMDMs infected for 48 h (**e**) or for 24, 48, 72 or 96 h (**f**) with stationary-phase *L. amazonensis* constitutively expressing GFP (La-GFP^{+}). (**g**,**h**) Flow cytometry analysis of C57BL/6 BMDMs pretreated with CA-074-ME (100 μM), glibenclamide (Gliben; 100 μM), KCl (100 mM) or NaCl (100 mM) and subsequently incubated with La-GFP^{+} at an MOI of 10 for 48 h. One representative of three independent experiments performed in triplicate is shown throughout. Error bars (**a**–**h**), s.d. $^{*}P < 0.05$ (two-way ANOVA with Bonferroni's post test (**a**–**d**,**f**) or Student's *t* test (**e**,**g**,**h**)) for *Pycard*$^{-/-}$, *Casp1*$^{-/-}$, *Nlrp3*$^{-/-}$ and *Nos2*$^{-/-}$ compared to C57BL/6 (**a**–**f**) or medium (**g**,**h**).

in the susceptibility of C57BL/6 BMDMs to infection; in contrast, the susceptibility of *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nos2*$^{-/-}$ BMDMs was not affected by IL-1Ra treatment (**Fig. 5a**). To evaluate the role of IL-1β in the leishmanicidal activity of BMDMs, we treated C57BL/6 BMDMs with recombinant IL-1β or IFN-γ and measured parasite replication. We observed a dose-dependent restriction of parasite replication in BMDMs treated with either IL-1β or IFN-γ (**Fig. 5b**). To address whether the leishmanicidal activity induced through IL-1β required components of the inflammasome, we treated BMDMs from C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nos2*$^{-/-}$ mice with exogenous IL-1β and measured parasite replication. We observed that the addition of exogenous IL-1β triggered pronounced leishmanicidal activity in C57BL/6 BMDMs and, to a lesser extent, in BMDMs obtained from inflammasome-deficient mice (**Fig. 5c**). To determine whether IL-1β contributes to NO production in infected BMDMs, we measured the production of nitrite in the supernatants of *L. amazonensis*–infected C57BL/6 BMDMs treated with IL-1β or IFN-γ. We observed a dose-dependent production of NO_2^- in response to either IFN-γ or IL-1β (**Fig. 5d**). BMDMs from *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice, but not *Nos2*$^{-/-}$ mice, produced NO in response to IL-1β (**Fig. 5e**). Notably, the addition of exogenous IL-1β only partially restored the leishmanicidal activity (and NO production) in inflammasome-deficient BMDMs (**Fig. 5c**,**e**). Thus, we speculate that the inflammasome is involved in additional processes leading to macrophage resistance.

Because IFN-γ is crucial for NO production[31–33], we investigated the requirement of the inflammasome for IFN-γ production in BMDMs infected with *L. amazonensis*. We infected BMDMs from C57BL/6 and *Casp1*$^{-/-}$ mice with *L. amazonensis* and measured the expression of IFN-γ using quantitative RT-PCR. We observed that caspase-1–deficient BMDMs had impaired expression of *Nos2* and

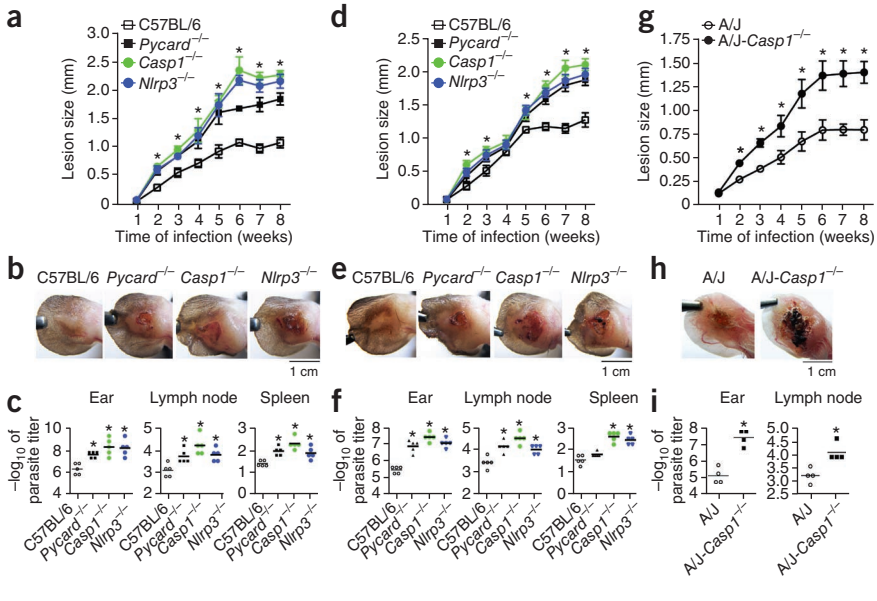

Figure 4 The Nlrp3 inflammasome is important for the *in vivo* restriction of *L. amazonensis* infection. (**a**–**c**) Quantification of lesion development (**a**), representative images of ear lesions after 8 weeks of infection (**b**) and limiting dilution analysis of parasite burden in the infected ear, draining lymph node and spleen at 8 weeks of infection (**c**) in C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice in the C57BL/6 genetic background infected in the ear dermis with 10^6 stationary-phase *L. amazonensis* promastigotes. (**d**–**f**) As in **a**–**c** but using infection with 10^5 metacyclic promastigotes of *L. amazonensis*. (**g**–**i**) As in **a**–**c** but using A/J and A/J-*Casp1*$^{-/-}$ mice infected in the ear dermis with 10^6 stationary-phase *L. amazonensis* promastigotes. One representative of six (**a**–**c**) or three (**d**–**i**) independent experiments using four or five mice per group is shown (with the symbols in **c**, **f** and **i** each representing an individual mouse). Error bars (**a**,**d**,**g**), s.d. $^{*}P < 0.05$ (two-way ANOVA with Bonferroni's post test (**a**,**d**,**g**) or Student's *t* test (**c**,**f**,**i**)) for *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ compared to C57BL/6 (**a**,**c**,**d**,**f**) or A/J-*Casp1*$^{-/-}$ compared to A/J (**g**,**i**).

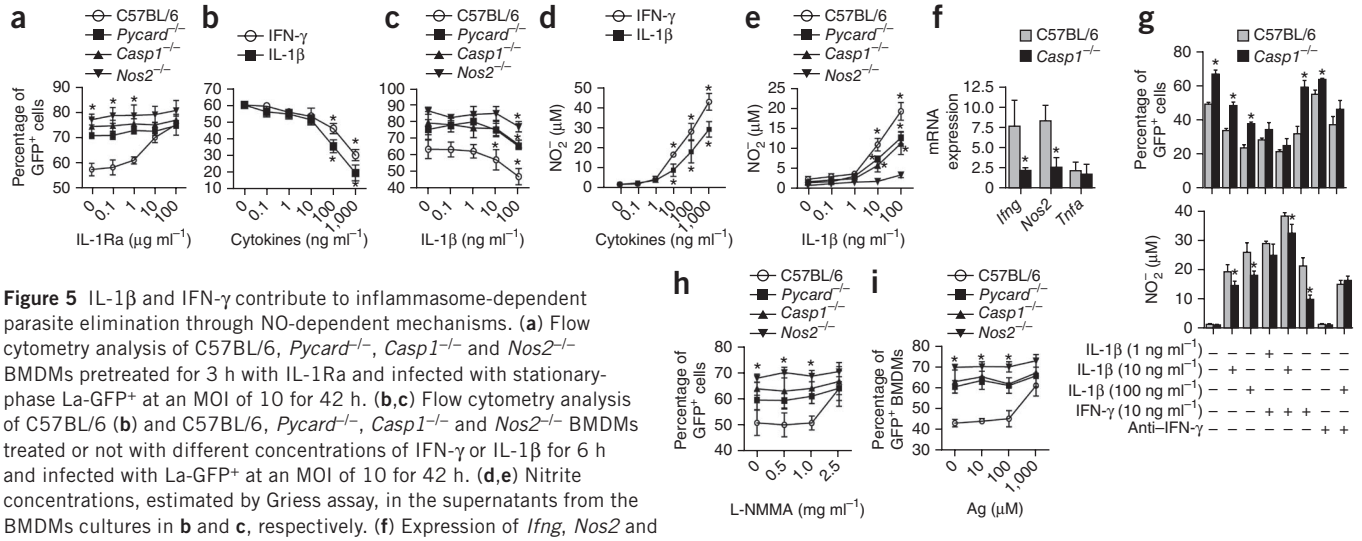

Figure 5 IL-1β and IFN-γ contribute to inflammasome-dependent parasite elimination through NO-dependent mechanisms. (**a**) Flow cytometry analysis of C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nos2*$^{-/-}$ BMDMs pretreated for 3 h with IL-1Ra and infected with stationary-phase La-GFP^{+} at an MOI of 10 for 42 h. (**b**,**c**) Flow cytometry analysis of C57BL/6 (**b**) and C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nos2*$^{-/-}$ BMDMs treated or not with different concentrations of IFN-γ or IL-1β for 6 h and infected with La-GFP^{+} at an MOI of 10 for 42 h. (**d**,**e**) Nitrite concentrations, estimated by Griess assay, in the supernatants from the BMDMs cultures in **b** and **c**, respectively. (**f**) Expression of *Ifng*, *Nos2* and *Tnfa* in C57BL/6 and *Casp1*$^{-/-}$ BMDMs infected with stationary-phase *L. amazonensis* promastigotes at an MOI of 10 for 12 h. The values are shown as the average fold change over uninfected controls. (**g**) Flow cytometry analysis and nitrite production of C57BL/6 and *Casp1*$^{-/-}$ BMDMs treated or not with the indicated concentrations of IFN-γ, IL-1β or both for 6 h and subsequently infected with La-GFP^{+} at an MOI of 10 for 42 h. Where indicated, 2 μg ml^{-1} of antibody to IFN-γ (anti–IFN-γ) was added to the cultures. (**h**,**i**) Flow cytometry analysis of C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nos2*$^{-/-}$ BMDMs pretreated for 3 h with different concentrations of L-NMMA (**h**) or aminoguanidine (Ag) (**i**) and subsequently infected with La-GFP^{+} at an MOI of 10 for 42 h. One representative of four (**a**), three (**c**,**e**–**i**) or two (**b**,**d**) independent experiments performed in triplicate is shown. Error bars (**a**–**i**), s.d. *$P < 0.05$ (two-way ANOVA with Bonferroni's post test (**a**,**c**,**e**,**g**–**i**), one-way ANOVA with Bonferroni's post test (**b**,**d**) or Student's *t* test (**f**)) for *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ and *Nos2*$^{-/-}$ compared to C57BL/6 (**a**,**h**,**i**) or for *Casp1*$^{-/-}$ compared to C57BL/6 (**f**,**g**) or untreated cultures (**b**–**e**).

Ifng, but not *Tnfa* (tumor necrosis factor-α) RNA, in response to infection (**Fig. 5f**). These data indicate that the inflammasome is required for the efficient production of IFN-γ in BMDMs.

Next, we treated BMDMs with IFN-γ in combination with IL-1β and observed that the addition of both cytokines fully complemented *Casp1*$^{-/-}$ BMDMs for NO production and restriction of *L. amazonensis* infection (**Fig. 5g**). Accordingly, when we added antibodies specific to IFN-γ to the cultures (that is, blocking the endogenous IFN-γ), IL-1β alone induced similar production of NO and restriction of *L. amazonensis* infection in C57BL/6 and *Casp1*$^{-/-}$ BMDMs (**Fig. 5g**). To further evaluate the role of NO in leishmanicidal mechanisms induced through IL-1β and inflammasomes, we used the inhibitors L-NMMA and aminoguanidine to block the endogenous production of NO in *L. amazonensis*–infected BMDMs. We observed that both drugs affected the leishmanicidal activity of C57BL/6 BMDMs but did not alter the parasitism of cells deficient in *Asc*, *Casp1* or *Nos2* (**Fig. 5h**,**i**). Collectively these data indicate that the inflammasome is important for NO-dependent restriction of parasite replication in BMDMs through mechanisms involving the production of IL-1β and IFN-γ.

The role of the inflammasome in the induction of NO production in response to *L. amazonensis* was also evident *ex vivo*. Cells obtained from the lymph nodes and spleens of infected C57BL/6, but not *Pycard*$^{-/-}$ or *Casp1*$^{-/-}$, mice induced NOS2 expression and NO production after stimulation with *L. amazonensis* antigens (**Supplementary Fig. 4a**–**d**). This process was dependent on the IFN-γ produced in response to the *L. amazonensis* antigens, as the *ex vivo* cultures from *Ifng*$^{-/-}$ mice did not produce IL-1β and NO in response to antigen stimulation (**Supplementary Fig. 5**).

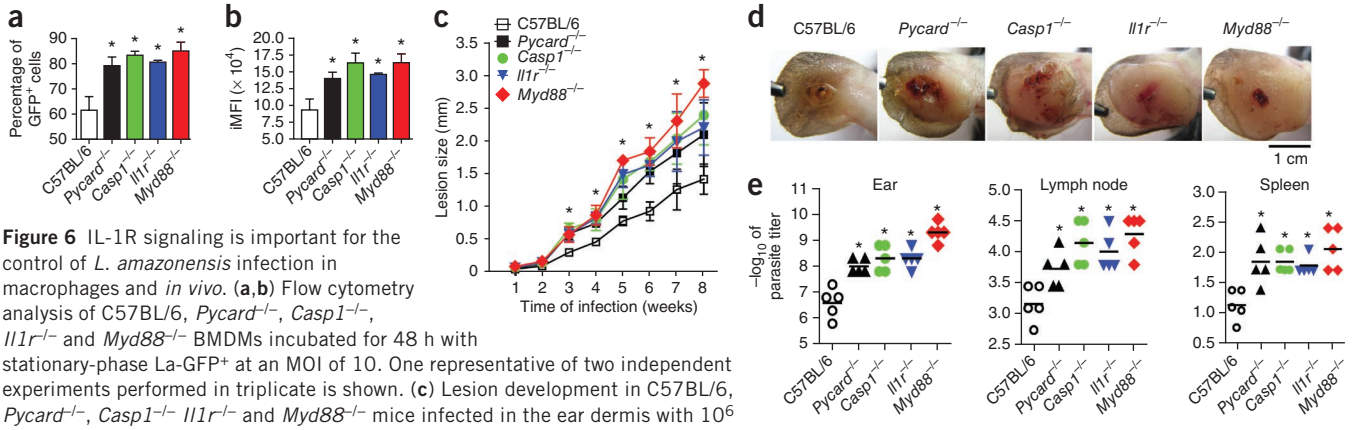

Figure 6 IL-1R signaling is important for the control of *L. amazonensis* infection in macrophages and *in vivo*. (**a**,**b**) Flow cytometry analysis of C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$, *Il1r*$^{-/-}$ and *Myd88*$^{-/-}$ BMDMs incubated for 48 h with stationary-phase La-GFP^{+} at an MOI of 10. One representative of two independent experiments performed in triplicate is shown. (**c**) Lesion development in C57BL/6, *Pycard*$^{-/-}$, *Casp1*$^{-/-}$ *Il1r*$^{-/-}$ and *Myd88*$^{-/-}$ mice infected in the ear dermis with 10^6 stationary-phase *L. amazonensis* promastigotes. (**d**) Representative images of ear lesions after 8 weeks of infection in the mice in **c**. (**e**) Limiting dilution analysis of parasite burden in the infected ear, draining lymph node and spleen measured 8 weeks after infection in the mice in **c**. One representative of three independent experiments using five mice per group is shown in **c** and **e** (each symbol in **e** represents an individual mouse). Error bars (**a**–**c**), s.d. *$P < 0.05$ (Student's *t* test (**a**,**b**,**e**) or two-way ANOVA with Bonferroni's post test (**c**)) for *Pycard*$^{-/-}$, *Casp1*$^{-/-}$, *Il1r*$^{-/-}$ and *Myd88*$^{-/-}$ compared to C57BL/6.

To further investigate the role of IL-1 signaling in the restriction of macrophage infection, we performed experiments with BMDMs from mice deficient in IL-1R or MyD88, which are both required for IL-1 signaling. We observed that the BMDMs obtained from *Il1r*$^{-/-}$ and *Myd88*$^{-/-}$mice were as susceptible to infection as those obtained from *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice (**Fig. 6a**,**b**). Accordingly, *Il1r*$^{-/-}$ and *Myd88*$^{-/-}$ mice were as susceptible to infection as mice deficient in *Asc* and *Casp1* (**Fig. 6c**–**e**).

We then examined the activation and role of the inflammasome in response to infection with other species of the *Leishmania* genus, namely *Leishmania (Leishmania) major, L. (V.) braziliensis* and *L. (L.) infantum chagasi*. FAM-YVAD staining and IL-1β production showed that the inflammasome is activated in BMDMs infected with *L. major, L. braziliensis* or *L. amazonensis* (**Supplementary Fig. 6a**,**b**). However, we detected no difference in lesion development or parasite burden in the ears and lymph nodes of C57BL/6 and inflammasome-deficient mice after intradermal inoculation of *L. major* (**Supplementary Fig. 7**). In contrast, the inflammasome was important for restriction of *in vivo* infection with *L. braziliensis* or *L. infantum chagasi* (**Supplementary Fig. 8**). Taken together, these results indicate that activation of the inflammasome is an important process in host resistance against infection with several species of the *Leishmania* genus.

DISCUSSION

The generation of an appropriate immune response, which requires an effective innate immune recognition of parasites, is essential for human resistance against infectious diseases, including leishmaniasis. Although MyD88-dependent Toll-like receptor (TLR) signaling has been reported to be important for parasite recognition[34,35], the species of *Leishmania* are known to be immunologically silent and bypass recognition by innate immune receptors during infection[36–39]. In this study we identified the inflammasome as a crucial innate immune platform for the recognition of *Leishmania* spp. Notably, the activation of the inflammasome leads to autonomous macrophage mechanisms that culminate with the restriction of intracellular parasite replication. These processes involve the regulation of IFN-γ and processing of IL-1β, which facilitates the expression of NOS2, an enzyme that is required for NO-mediated restriction of *Leishmania* replication in macrophages[6–8]. These data explain recent reports indicating that IL-1β signaling is crucial for the determination of the severity of disease in humans[28,29]. Polymorphisms within the human *IL1B* gene are associated with severity of disease, which can lead to either the development of severe cases of visceral leishmaniasis in patients infected with *L. infantum chagasi* or uncontrolled diffuse forms of cutaneous leishmaniasis in patients infected with *Leishmania mexicana*[28,29]. In this study we found that macrophages and mice that were deficient in IL-1R were just as susceptible to infection as those deficient in *Casp1*, *Asc* or *Nlrp3*, which suggests that IL-1 signaling is important for the inflammasome-dependent restriction of *Leishmania* spp. replication.

Although the inflammasome is activated in BMDMs infected with *L. major*, it was dispensable for host resistance to infection. It is important to highlight that the C57BL/6 background is the canonical restrictive background for *L. major* infection (reviewed in ref. 40). Thus, in such a restrictive genetic background, additional resistance mechanisms may be sufficient to limit parasite replication regardless of the presence of the inflammasome–IL-1 axis. The dispensable role of the inflammasome in the restriction of *L. major* infection corroborates previous reports indicating that IL-1 signaling is dispensable in resistance against *L. major* infection in mice with the C57BL/6 genetic background[41–43]. In this context, future studies will be important to test the role of the inflammasome and IL-1 signaling in a mouse background that is susceptible to *L. major* infection. Nonetheless, regardless of genetic background, infections performed with highly virulent species, which bypass the C57BL/6-mediated natural resistance to infection, supported an important role of the inflammasome and IL-1 signaling in host resistance to infection. Our data favor a model that couples molecules and cellular process that are known to be important in host resistance, including IFN-γ, NO, MyD88 and IL-1 signaling. The identification of this pivotal pathway in host resistance to leishmaniasis may enable the development of future therapeutic strategies for the modulation of the inflammasome, IL-1 signaling or both to facilitate the treatment of chronic, life-threatening and neglected infectious diseases such as leishmaniasis.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.S.L.-J. designed and performed experiments, analyzed data, generated figures and wrote the manuscript. D.L.C., V.C., L.D.C. and A.L.N.S. designed and performed experiments and analyzed data. T.W.P.M., F.R.S.G. and M.T.B. helped with data interpretation, discussed the hypotheses and participated in manuscript preparation. M.B., K.R.B., R.A.F. and J.S.S. provided reagents and discussed the hypotheses. D.S.Z. supervised the project, designed the experiments, helped with data interpretation, participated in data analysis and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Reithinger, R. *et al.* Cutaneous leishmaniasis. *Lancet Infect. Dis.* **7**, 581–596 (2007).
2. Chappuis, F. *et al.* Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat. Rev. Microbiol.* **5**, 873–882 (2007).
3. Bosque, F., Saravia, N.G., Valderrama, L. & Milon, G. Distinct innate and acquired immune responses to *Leishmania* in putative susceptible and resistant human populations endemically exposed to *L. (Viannia) panamensis* infection. *Scand. J. Immunol.* **51**, 533–541 (2000).
4. Liu, D. & Uzonna, J.E. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. *Front. Cell Infect. Microbiol.* **2**, 83 (2012).
5. Mosser, D.M. & Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8**, 958–969 (2008).
6. Green, S.J., Crawford, R.M., Hockmeyer, J.T., Meltzer, M.S. & Nacy, C.A. *Leishmania major* amastigotes initiate the l-arginine–dependent killing mechanism in IFN-(–stimulated macrophages by induction of tumor necrosis factor-α. *J. Immunol.* **145**, 4290–4297 (1990).
7. Green, S.J., Meltzer, M.S., Hibbs, J.B. Jr. & Nacy, C.A. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an l-arginine–dependent killing mechanism. *J. Immunol.* **144**, 278–283 (1990).

8. Mukbel, R.M. *et al.* Macrophage killing of *Leishmania amazonensis* amastigotes requires both nitric oxide and superoxide. *Am. J. Trop. Med. Hyg.* **76**, 669–675 (2007).
9. Chen, G., Shaw, M.H., Kim, Y.G. & Nunez, G. NOD-like receptors: role in innate immunity and inflammatory disease. *Annu. Rev. Pathol.* **4**, 365–398 (2009).
10. Martinon, F., Mayor, A. & Tschopp, J. The inflammasomes: guardians of the body. *Annu. Rev. Immunol.* **27**, 229–265 (2009).
11. Wen, H., Ting, J.P. & O'Neill, L.A. A role for the NLRP3 inflammasome in metabolic diseases—did Warburg miss inflammation? *Nat. Immunol.* **13**, 352–357 (2012).
12. Franchi, L., Kanneganti, T.D., Dubyak, G.R. & Nunez, G. Differential requirement of P2X7 receptor and intracellular K^+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J. Biol. Chem.* **282**, 18810–18818 (2007).
13. Pétrilli, V. *et al.* Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ.* **14**, 1583–1589 (2007).
14. Dostert, C. *et al.* Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* **320**, 674–677 (2008).
15. Hornung, V. *et al.* Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat. Immunol.* **9**, 847–856 (2008).
16. Mariathasan, S. *et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440**, 228–232 (2006).
17. Mariathasan, S. *et al.* Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* **430**, 213–218 (2004).
18. Sutterwala, F.S. *et al.* Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. *J. Exp. Med.* **204**, 3235–3245 (2007).
19. Suzuki, T. *et al.* Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in *Shigella*-infected macrophages. *PLoS Pathog.* **3**, e111 (2007).
20. Zamboni, D.S. *et al.* The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of *Legionella pneumophila* infection. *Nat. Immunol.* **7**, 318–325 (2006).
21. Hamon, M.A. & Cossart, P. K^+ efflux is required for histone H3 dephosphorylation by *Listeria monocytogenes* listeriolysin O and other pore-forming toxins. *Infect. Immun.* **79**, 2839–2846 (2011).
22. Lamkanfi, M. *et al.* Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *J. Cell Biol.* **187**, 61–70 (2009).
23. Montaser, M., Lalmanach, G. & Mach, L. CA-074, but not its methyl ester CA-074Me, is a selective inhibitor of cathepsin B within living cells. *Biol. Chem.* **383**, 1305–1308 (2002).
24. Liew, F.Y. *et al.* Resistance to *Leishmania major* infection correlates with the induction of nitric oxide synthase in murine macrophages. *Eur. J. Immunol.* **21**, 3009–3014 (1991).
25. Stenger, S., Thuring, H., Rollinghoff, M. & Bogdan, C. Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major*. *J. Exp. Med.* **180**, 783–793 (1994).
26. Belkaid, Y. *et al.* A natural model of *Leishmania major* infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J. Immunol.* **165**, 969–977 (2000).
27. Andrade, Z.A., Reed, S.G., Roters, S.B. & Sadigursky, M. Immunopathology of experimental cutaneous leishmaniasis. *Am. J. Pathol.* **114**, 137–148 (1984).
28. Fernandez-Figueroa, E.A. *et al.* Disease severity in patients infected with *Leishmania mexicana* relates to IL-1β. *PLoS Negl. Trop. Dis.* **6**, e1533 (2012).
29. Moravej, A. *et al.* IL-1β (–511T/C) gene polymorphism not IL-1β (+3953T/C) and LT-α (+252A/G) gene variants confers susceptibility to visceral leishmaniasis. *Mol. Biol. Rep.* **39**, 6907–6914 (2012).
30. Ferreira, S.H., Lorenzetti, B.B., Bristow, A.F. & Poole, S. Interleukin-1 β as a potent hyperalgesic agent antagonized by a tripeptide analogue. *Nature* **334**, 698–700 (1988).
31. Moncada, S., Palmer, R.M. & Higgs, E.A. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109–142 (1991).
32. Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**, 3051–3064 (1992).
33. Werner-Felmayer, G. *et al.* Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. *J. Exp. Med.* **172**, 1599–1607 (1990).
34. Debus, A., Glasner, J., Rollinghoff, M. & Gessner, A. High levels of susceptibility and T helper 2 response in MyD88-deficient mice infected with *Leishmania major* are interleukin-4 dependent. *Infect. Immun.* **71**, 7215–7218 (2003).
35. Muraille, E. *et al.* Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to *Leishmania major* infection associated with a polarized Th2 response. *J. Immunol.* **170**, 4237–4241 (2003).
36. Gregory, D.J. & Olivier, M. Subversion of host cell signalling by the protozoan parasite *Leishmania*. *Parasitology* **130** (suppl.), S27–S35 (2005).
37. Shio, M.T. *et al.* Host cell signalling and *Leishmania* mechanisms of evasion. *J. Trop. Med.* **2012**, 819512 (2012).
38. Soong, L. Modulation of dendritic cell function by *Leishmania* parasites. *J. Immunol.* **180**, 4355–4360 (2008).
39. Xin, L., Li, K. & Soong, L. Down-regulation of dendritic cell signaling pathways by *Leishmania amazonensis* amastigotes. *Mol. Immunol.* **45**, 3371–3382 (2008).
40. Sacks, D. & Noben-Trauth, N. The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat. Rev. Immunol.* **2**, 845–858 (2002).
41. Satoskar, A.R. *et al.* Enhanced Th2-like responses in IL-1 type 1 receptor-deficient mice. *Eur. J. Immunol.* **28**, 2066–2074 (1998).
42. Kostka, S.L., Knop, J., Konur, A., Udey, M.C. & von Stebut, E. Distinct roles for IL-1 receptor type I signaling in early versus established *Leishmania major* infections. *J. Invest. Dermatol.* **126**, 1582–1589 (2006).
43. Kautz-Neu, K. *et al.* IL-1 signalling is dispensable for protective immunity in *Leishmania*-resistant mice. *Exp. Dermatol.* **20**, 76–78 (2011).

ONLINE METHODS

Animals. Six- to 8-week-old-female mice were used for the infection experiments. *Pycard*$^{-/-}$ and *Casp1*$^{-/-}$ mice were previously described and backcrossed to C57BL/6 mice for nine and eight generations, respectively, to ensure similar genetic backgrounds[44,45]. The *Nlrp3*$^{-/-}$ mice were generated in the C57BL/6 background[16]. A/J, C57BL/6, *Myd88*$^{-/-}$, *Il1r*$^{-/-}$ and *Ifng*$^{-/-}$ mice in the C57BL/6 background were obtained from the animal facilities of the Faculdade de Medicina de Ribeirão Preto, FMRP/USP. Caspase-1–deficient mice in an A/J background (A/J-*Casp1*$^{-/-}$ mice) were constructed by backcrossing mice carrying the caspase-1–deficient allele with A/J mice for ten generations. The mice were bred and maintained under specific pathogen-free conditions at the animal facilities of the University of São Paulo, FMRP/USP. All of the mice experiments were conducted according to the guidelines of the institutional committee for animal care at the Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto, FMRP/USP.

Parasites and infection. The parasite used were *L. (L.) amazonensis* PH8 strain (IFLA/BR/67/PH8), *L. (L.) amazonensis* 73M2269 strain (MHOM/BR/73M2269), which constitutively expresses GFP (La-GFP$^+$), *L. (V.) braziliensis* M2903 strain (MHOM/BR/75/M2903), *L. (L.) major* LV39 strain (MRHO/SU/59/P) and *L. (L.) infantum chagasi* (HU-USF 8). The parasites were cultured at 26 °C in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA), pH 7.0, supplemented with 20% heat-inactivated FCS (GIBCO BRL), 100 U ml^{-1} penicillin G potassium (USB Corporation, Cleveland, OH, USA), 2 mM L-glutamine and 2% human urine, pH 6.5. After seven culture passages, the parasites were serially passed in C57BL/6 mice to ensure that their virulence was maintained. The infective-stage metacyclic promastigotes of *L. amazonensis* were isolated from stationary cultures through density-gradient centrifugation as described previously[46]. For the *L. amazonensis*, *L. braziliensis* and *L. major* infections, the mice were infected with either 1×10^6 stationary-phase or 1×10^5 metacyclic promastigotes, which were suspended in 10 µl of PBS, through an intradermal injection into the left ear. The ear lesions were measured weekly with a dial gauge caliper and compared to the thickness of the uninfected contralateral ear. For the *L. infantum chagasi* infection, the mice were inoculated with 1×10^7 stationary-phase promastigotes through the intraperitoneal route. The parasite burdens were determined in the ear, spleen and liver and in retromaxilar lymph nodes draining from the side of the infection as described previously[47].

BMDMs and infection. BMDMs were prepared as previously described[48]. Briefly, isolated femurs and tibia were flushed with PBS, and precursor cells were cultured in RPMI supplemented with 30% L929 cell-conditioned medium and 20% FBS[48]. After 7 d in culture, mature BMDMs were harvested and infected with stationary-phase promastigotes at an MOI of 10 or with metacyclic promastigotes at an MOI of 1 (unless otherwise indicated). After 6 h of infection, the free parasites were washed, and fresh medium was added to the infected cultures. In some experiments, the free parasites were washed, and fresh medium was added to the infected cultures after 1 h of infection. The leishmanicidal activity of the cells was determined at 24, 48, 72 or 96 h after infection by flow cytometry using a FACSCanto II flow cytometer (BD Biosciences) and counting the Giemsa-stained cytospin preparations under a light microscope with a ×40 objective. The flow cytometric data were analyzed using FlowJo software (Tree Star). In this analysis, two parameters were considered: the percentage of infected cells and the iMFI, which reflects the total functional response toward the infection and is calculated by multiplying the number of infected cells by the MFI[49]. In the analysis of Giemsa staining, the infection rate was determined by scoring the infected and uninfected cells (100–200 BMDMs) and the number of intracellular amastigotes per infected BMDM. Where indicated, the BMDMs were pretreated for 6 h with recombinant mouse IL-1β (eBioscience) or IFN-γ (Invitrogen) in the range of 0.1–1000 ng ml^{-1} or with both cytokines at the indicated concentrations. For the inhibition of IL-1R, BMDMs were pretreated with 0.1–100 µg ml^{-1} of IL-1Ra[30] for 3 h before infection for 48 h. For the inhibition of IFN-γ, BMDMs were pretreated with 2 µg ml^{-1} of antibodies to IFN-γ (BioXCell, clone XMG1.2) with or without IL-1β (100 ng ml^{-1}) for 6 h and subsequently infected with *L. amazonensis* for 42 h. For the inhibition of cathepsins and the K^+ efflux, the BMDMs were primed for 4 h with 500 ng ml^{-1} ultrapure LPS (InvivoGen, tlrl-peklps), treated with glibenclamide, CA-074-ME, KCl or NaCl at the indicated concentrations for 2 h and subsequently stimulated with *L. amazonensis* for 42 h.

ELISA and nitrite determination. IL-1β and IFN-γ production was assessed using IL-1β (BD Biosciences) and IFN-γ (BD Biosciences) ELISA kits. *In vitro* IL-1β production was analyzed in supernatants harvested from the BMDMs that were prestimulated with 500 ng ml^{-1} of ultrapure LPS (InvivoGen, tlrl-peklps) or 10 ng ml^{-1} of IFN-γ (Invitrogen) for 6 h and subsequently infected with stationary-phase promastigotes at an MOI of 10 or metacyclic promastigotes at an MOI of 1 for 42 h. For the *ex vivo* analysis of IL-1β and IFN-γ production, single-cell suspensions were prepared from the lymph nodes or spleen of mice infected for 2 or 5 weeks. The cell concentrations were adjusted to 5×10^6 cells ml^{-1} and plated at 0.5 ml per well in 48-well tissue-culture plates. The cells were stimulated with 50 µg ml^{-1} of *L. amazonensis* particulate antigens. The supernatants were harvested after 48 h, and the amounts of IL-1β and IFN-γ were measured using ELISA. NO_2^- accumulation, as an indicator of NO production, was measured using Griess reagent[50].

Endogenous caspase-1 staining using FAM-YVAD–fluoromethyl ketone (FMK). BMDMs were cultured and infected with stationary-phase *L. amazonensis* at an MOI of 10. After 6, 12, 24 or 48 h of infection, the BMDMs were stained for 1 h with FAM-YVAD-FMK (Immunochemistry Technologies) as recommended by the manufacturer's instructions. Active caspase-1 was then measured by flow cytometry. The data were acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Western blot analyses. A total of 5×10^6 BMDMs were seeded per well, primed with 500 ng ml^{-1} ultrapure LPS (InvivoGen, tlrl-peklps) for 4 h and then infected with *L. amazonensis* for 48 h or stimulated with 20 µM nigericin (Sigma-Aldrich) for 40 min in the absence of FBS. The supernatants were collected and precipitated with 50% trichloroacetic acid and acetone. After their clarification by centrifugation, the cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate and 0.1% SDS) in the presence of a protease inhibitor cocktail (Roche). The lysates and supernatants were resuspended in Laemmli buffer, boiled, resolved by SDS-PAGE and transferred (Semidry Transfer Cell, Bio-Rad) to a 0.22-µm nitrocellulose membrane (GE Healthcare). The membranes were blocked in Tris-buffered saline (TBS) with 0.01% Tween-20 and 5% nonfat dry milk. The rat monoclonal antibody to caspase-1 p20 (1:250, Genentech, 4B4), the goat antibody to IL-1β p-17 (1:200, Sigma Aldrich, I3767) and specific horseradish peroxidase–conjugated antibodies (1:3,000, KPL, 14-16-06 and 14-13-06) were diluted in blocking buffer for the incubations. The enhanced chemiluminescence luminol reagent (GE Healthcare) was used for antibody detection.

Retroviral transduction and quantification of Asc foci. Mouse *Asc* was cloned into the pEGFP(N2) vector (Clontech) using XhoI and BamHI restriction sites. Asc-GFP and GFP were cloned into the pMSCV2.2 mouse-specific retroviral vector (Clontech). The pCL vector system[51] was used for packaging retroviruses in transfected monolayers of Peak cells (ATCC CRL-2828, maintained in RPMI 1640 with 10% FBS). The supernatant of the Peak cells was collected at 3 d after transfection, passed through a 0.45-µm filter and used for BMDM transduction. BMDMs were obtained from the femurs of *Casp1*$^{-/-}$ mice and seeded at 5×10^5 cells per well in differentiation medium (RPMI 1640 medium supplemented with 30% L929 cell-conditioned medium, 20% FBS, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin) as previously described[48]. After differentiation for 3 d, the BMDMs were harvested and spun down, and the medium was replenished with retroviral-containing Peak cell supernatants complemented with RPMI 1640 containing 20% FBS and 25% LCCM. The BMDMs were fed with differentiation medium and cultured for an additional 4 d. The BMDMs were seeded at 2.0×10^5 cells in 24-well plates containing 12-mm glass cover slides. The infection was performed as described above with promastigotes of *L. amazonensis* (MOI 5) and fixed with 4% paraformaldehyde at 2, 6 or 24 h after infection. *L. amazonensis* was stained with in house–generated rabbit polyclonal antibody to *L. amazonensis* (1:3,000) and secondary antibody to rabbit IgG conjugated with Alexa 594 (1:300, Invitrogen, A11012). The cells were counterstained

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with DAPI, mounted using Prolong Gold Antifade Reagent (Invitrogen) and analyzed under fluorescence using a Leica DMI 4000B inverted microscope with a ×100 oil objective. The images were processed using LAS AF software (Leica Microsystems), and the number of Asc-GFP foci in the infected cells was quantified.

Real-time PCR. Total RNA was extracted from 1×10^6 *Leishmania*-infected BMDMs (MOI 10) using TRIzol reagent (Invitrogen). Contaminating DNA was removed with an RNAse-free DNAse set (Promega). cDNA was synthesized from 1 μg of RNA using the SuperScript II reverse transcriptase (Invitrogen). Subsequent real-time PCR was performed on an ABI PRISM 7000 sequence detector (Applied Biosystems) using SYBR Green (Invitrogen). The following primer sequences were used: *Actb* forward (5′-AGCTGCGTTTTACACCCTTT-3′), reverse (5′-AAGCCATGCCAATGTTGTCT-3′); *Nos2* forward (5′-CGAA ACGCTYCACTTCCAA-3′), reverse (5′-TGAGCCTATATTGCTGTGGCT-3′); *Ifng* forward (5′-GCATCTTGGCTTTGCAGCT-3′), reverse (5′-CCTTTTT CGCCTTGCTGTTG-3′); and *Tnfa* forward (5′-TGTGCTCAGAGCTTTCAA CAA-3′), reverse (5′-CTTGATGGTGGTGCATGAGA-3′). The mRNA expression levels were normalized to β-actin. The adjusted values were calculated using the following formula: $2^{-(\text{CT target} - \text{CT } \beta\text{-actin})}$, where CT is the cycle threshold. The fold change in the expression was calculated as the *n*-fold difference in expression in the infected cells compared to the uninfected cells.

Statistical analyses. For comparisons of multiple groups, two-way analysis of variance (ANOVA) followed by the Bonferroni's post test were used. The differences in values obtained for two different groups were determined using Student's *t* test. Analyses were performed using Prism 5.0 software (GraphPad). A difference was considered statistically significant when $P \leq 0.05$.

44. Kuida, K. *et al.* Altered cytokine export and apoptosis in mice deficient in interleukin-1β converting enzyme. *Science* **267**, 2000–2003 (1995).
45. Lara-Tejero, M. *et al.* Role of the caspase-1 inflammasome in *Salmonella typhimurium* pathogenesis. *J. Exp. Med.* **203**, 1407–1412 (2006).
46. Späth, G.F. & Beverley, S.M. A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. *Exp. Parasitol.* **99**, 97–103 (2001).
47. Afonso, L.C. & Scott, P. Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. *Infect. Immun.* **61**, 2952–2959 (1993).
48. Marim, F.M., Silveira, T.N., Lima, D.S. Jr. & Zamboni, D.S. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. *PLoS ONE* **5**, e15263 (2010).
49. Darrah, P.A. *et al.* Multifunctional T_H1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat. Med.* **13**, 843–850 (2007).
50. Green, L.C., Tannenbaum, S.R. & Goldman, P. Nitrate synthesis in the germfree and conventional rat. *Science* **212**, 56–58 (1981).
51. Naviaux, R.K., Costanzi, E., Haas, M. & Verma, I.M. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J. Virol.* **70**, 5701–5705 (1996).

Supplementary Information Titles

Journal: Nature Medicine

Article Title:	Inflammasome-derived IL-1β production induce nitric oxide–mediated resistance to *Leishmania*
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Supplementary Item & Number	**Title or Caption**
Supplementary Figure 1	Cathepsins and potassium efflux are required for the *L. amazonensis*-induced inflammasome activation
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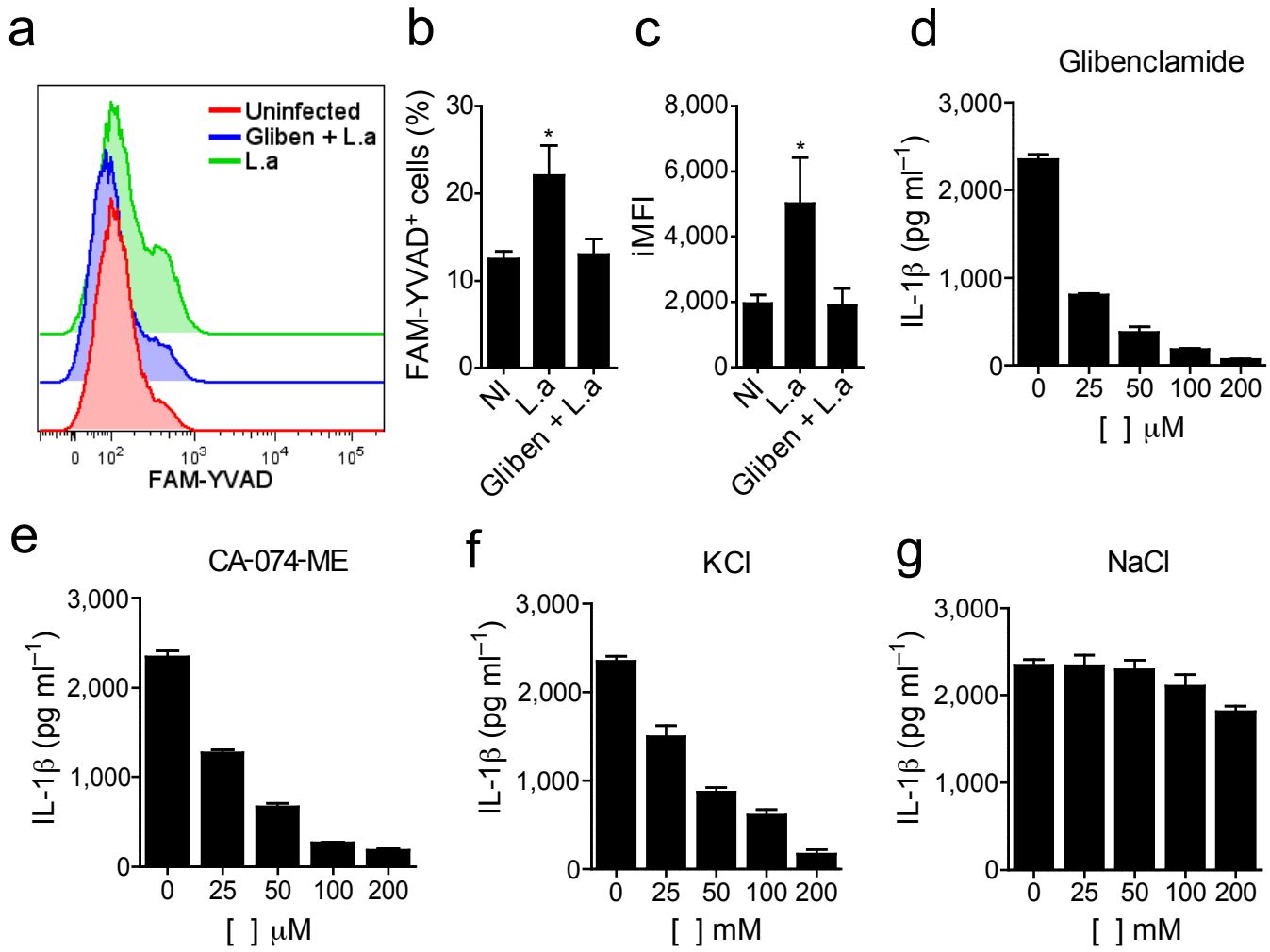
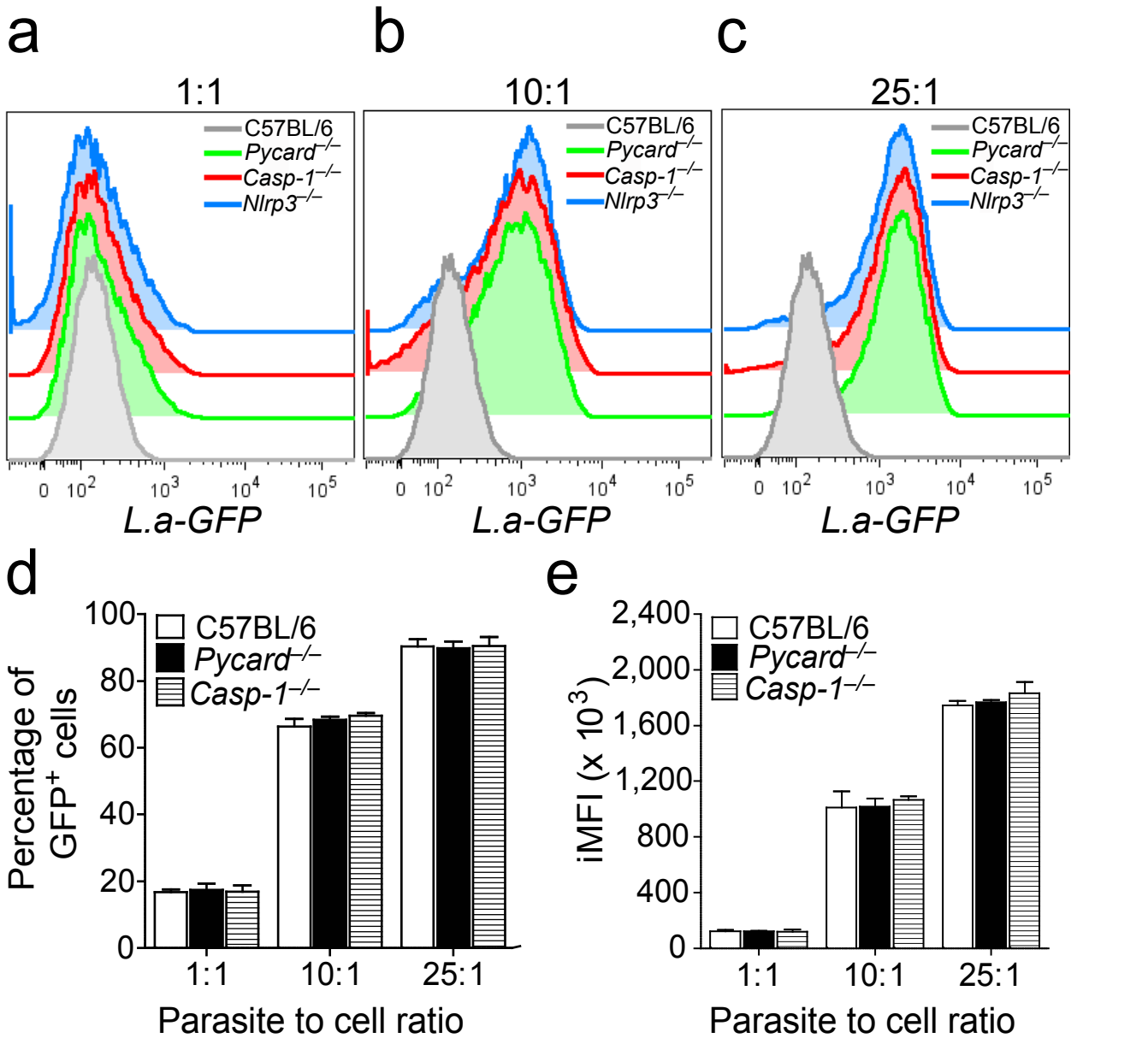
Supplementary Methods	Online Methods

Inflammasome-derived IL-1β production induce nitric oxide–mediated resistance to *Leishmania*

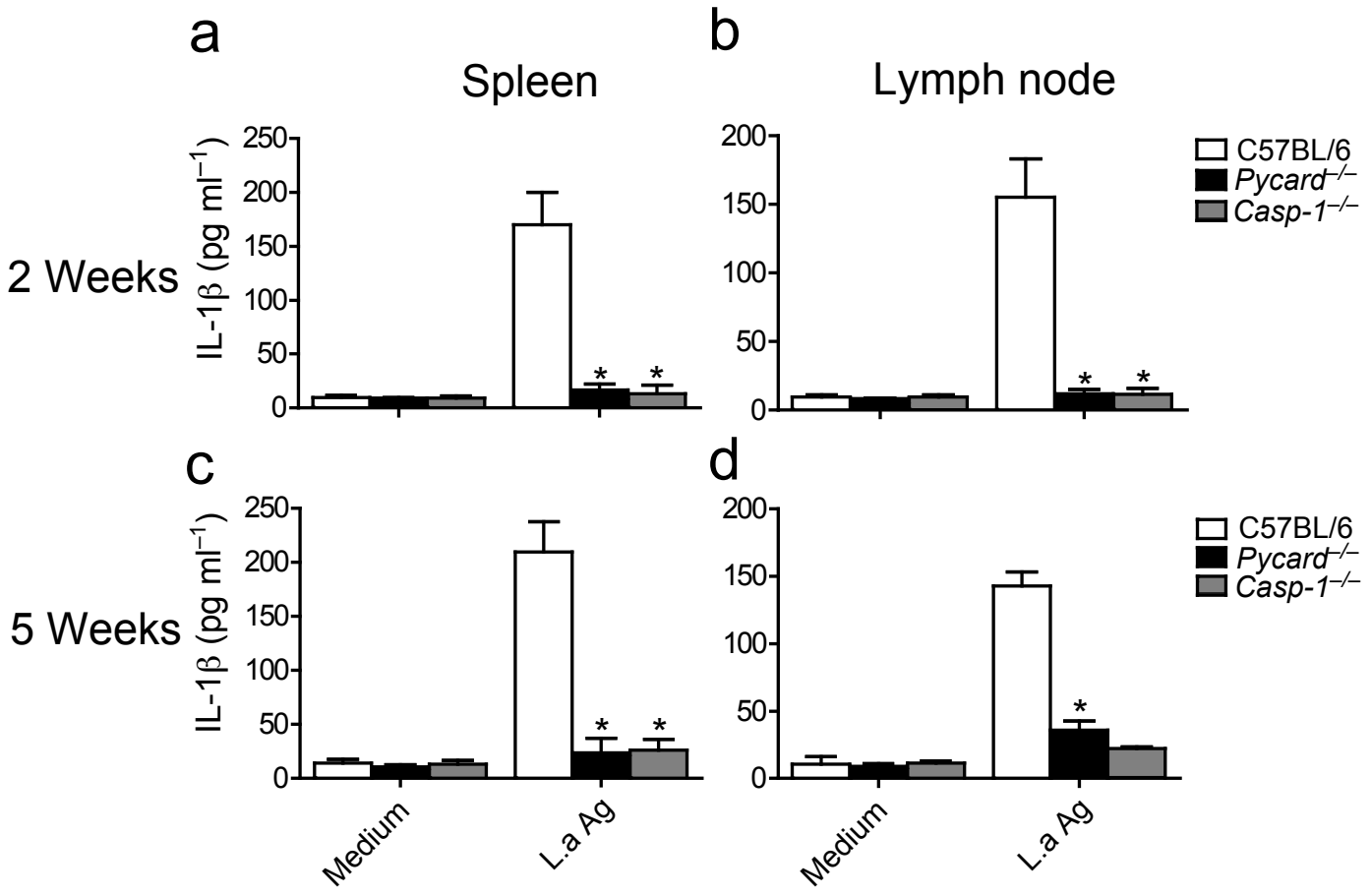
Djalma de S. Lima-Junior, Diego L. Costa, Vanessa Carregaro, Larissa D. Cunha, Alexandre L.N. Silva, Tiago W.P. Mineo, Fredy R.S. Gutierrez, Maria Bellio, Karina Bortoluci, Richard A. Flavell, Marcelo T. Bozza, João S. Silva, and Dario S. Zamboni



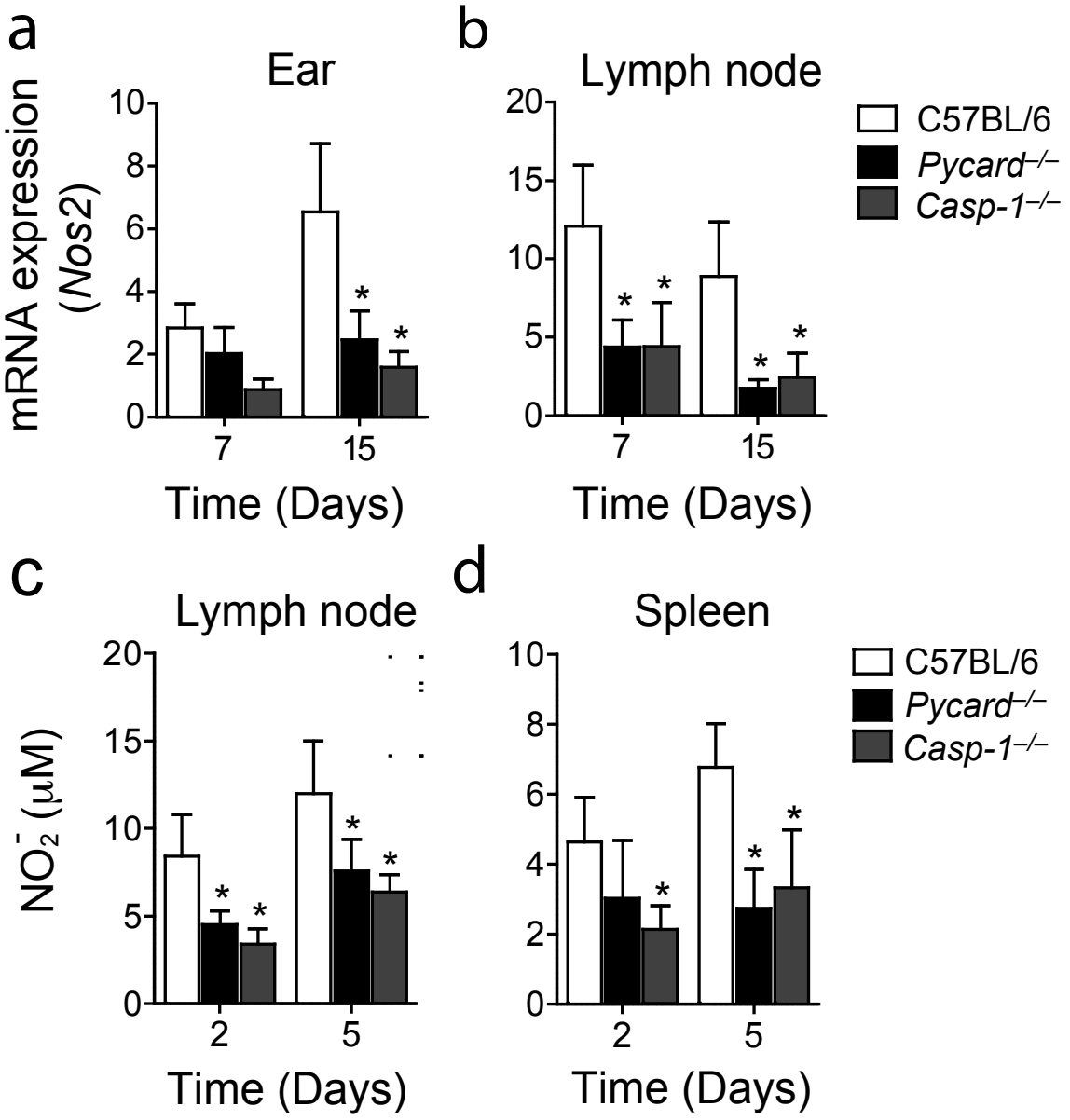
Supplementary Figure 1. Cathepsins and potassium efflux are required for the *L. amazonensis*-induced inflammasome activation. (**a-c**) Bone marrow-derived macrophages (BMDMs) obtained from C57BL/6 mice were pretreated for 2 h with 100 μM glibenclamide (Gliben), incubated with stationary phase *L. amazonensis* promastigotes (L.a) at an MOI of 10 and assessed for caspase-1 activation by FAM-YVAD staining. (**a**) The blue- and green-filled histograms represent the staining of infected macrophage that were treated (Gliben+L.a) or not treated (L.a) with glibenclamide, respectively. The red-filled histograms represent the untreated and uninfected BMDMs (NI). The percentage of FAM-YVAD positive cells (**b**) and the integrated mean fluorescence intensity (iMFI) (**c**) are shown. (**d-g**) BMDMs were primed with LPS (500 ng/ml) for 6 h, then treated for 2 h with different concentrations of glibenclamide (**d**), CA-074-ME (**e**), KCl (**f**) or NaCl (**g**) and subsequently incubated with *L. amazonensis* promastigotes at an MOI of 10. Forty hours after infection, the IL-1β released was assessed in the supernatant by ELISA. The data shown represent the means ± SD of triplicate samples and are representative of the data obtained from two independent experiments. *P < 0.05 (Student's *t* test) compared to NI.



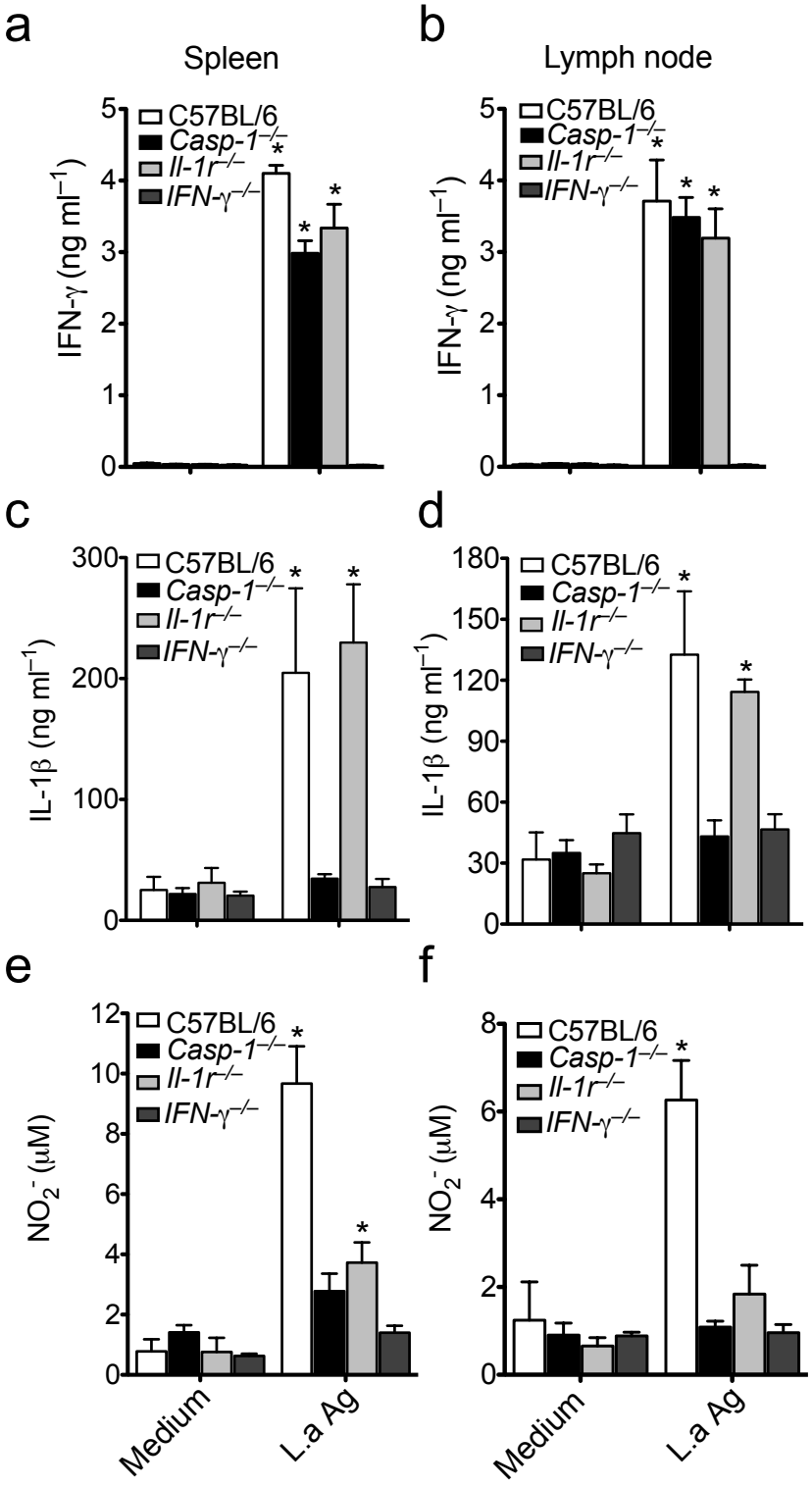
Supplementary Figure 2. The inflammasome components are not required for the internalization of *L. amazonensis* by macrophages. Bone marrow-derived macrophages (BMDMs) from C57BL/6, *Pycard*$^{-/-}$, and *Casp-1*$^{-/-}$ mice were incubated for 3 hours with stationary phase *L. amazonensis* promastigotes that constitutively express GFP (La-GFP) at MOIs of 1 (**a**), 10 (**b**), and 25 (**c**). The green-, red-, and blue-filled histograms represent the staining of infected WT, *Pycard*$^{-/-}$, and *Casp-1*$^{-/-}$ BMDMs, respectively. The gray-filled histograms indicate the uninfected BMDMs. (**d,e**) The flow cytometry quantification of the experiment shown in a-c indicates the percentage of La-GFP^{+} cells (**d**) and the integrated mean fluorescence intensity (iMFI) (**e**). The data shown represent the means ± SD of triplicate samples and are representative of the data obtained from two independent experiments.



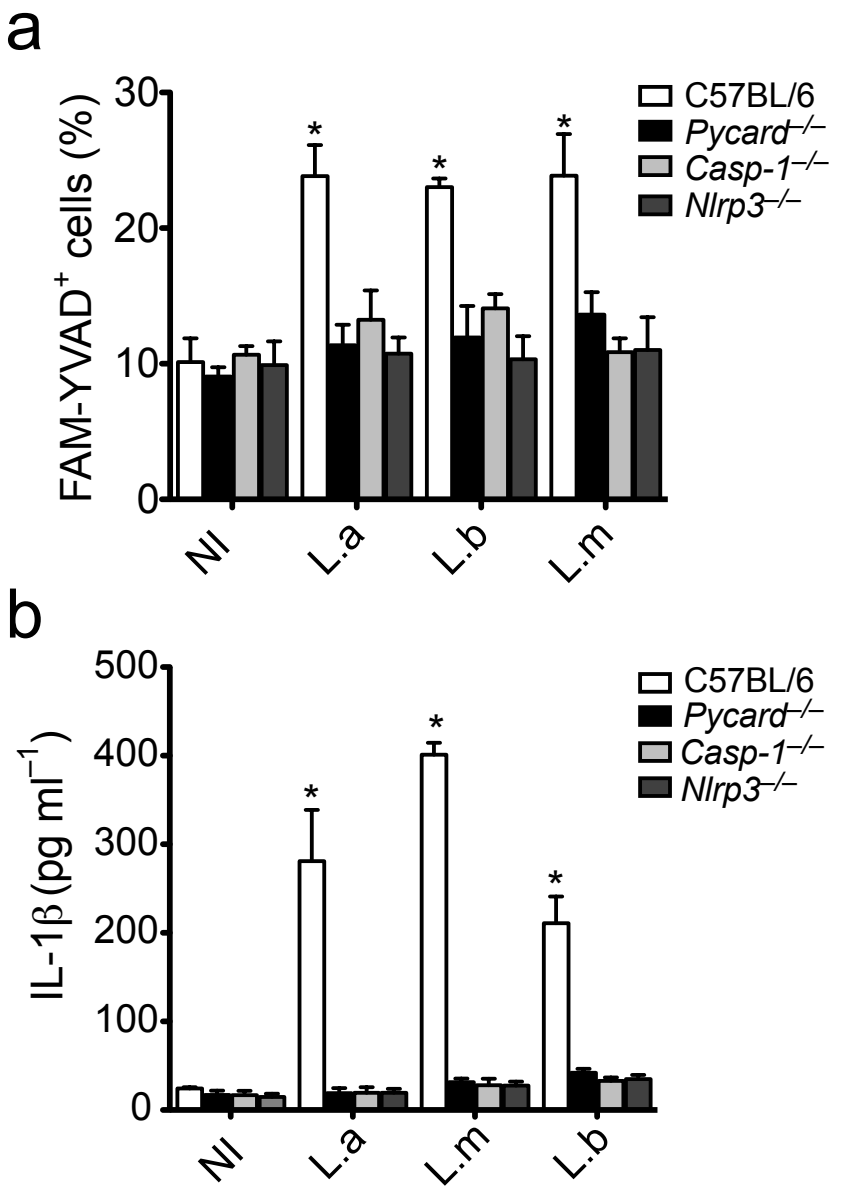
Supplementary Figure 3. The inflammasome is required for IL-1β production *ex vivo*. C57BL/6, *Pycard*$^{-/-}$, and *Casp-1*$^{-/-}$ mice were inoculated with 10^6 stationary phase *L. amazonensis* promastigotes and sacrificed 2 weeks (**a,b**) and 5 weeks (**c,d**) after infection. The cells from the spleen (**a,c**) and lymph node (**b,d**) were cultured and stimulated with 50 µg/ml of *L. amazonensis* particulate antigen (L.a Ag) for 48 h. The amount of IL-1β contained in the culture supernatants was estimated by ELISA. The datashown represent the means ± SD of 4 mice per group and are representative of the data obtained from three independent experiments. *P < 0.05 (two-way ANOVA with Bonferroni's post test) compared with C57BL/6 cultures.



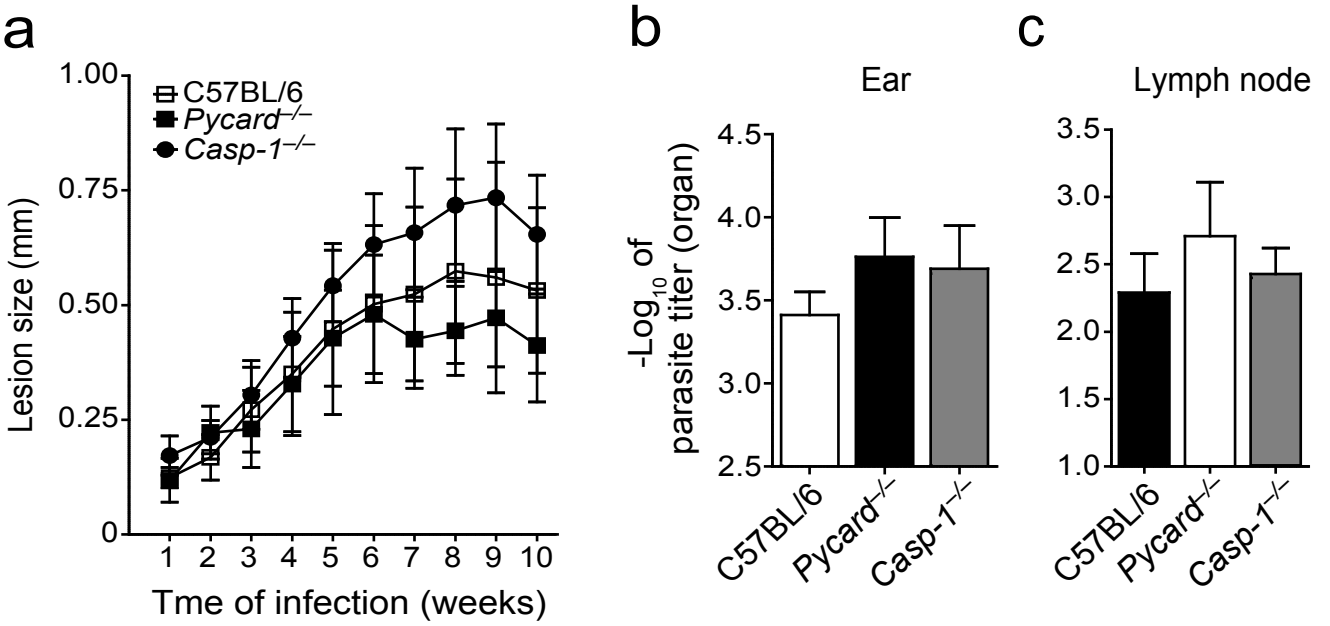
Supplementary Figure 4. The inflammasome is important for NOS2 expression and NO production *in vivo* **and** *ex vivo* **in response to *L. amazonensis* stimulation.** C57BL/6, *Pycard*$^{-/-}$, and *Casp-1*$^{-/-}$ mice were inoculated with 10^6 stationary phase *L. amazonensis* promastigotes. (**a,b**) After 7 and 15 days of infection, the expressions of *Nos2* in the ear (**a**) and lymph node (**b**) were determined by qRT-PCR and normalized to the β-actin gene. The levels of mRNA expression were calculated as the fold induction compared to uninfected mice from the corresponding strain. (**c,d**) Mice that were infected for 2 or 5 weeks were used to generate *ex vivo* cultures of the lymph node (**c**) and spleen (**d**). The cultures were stimulated with 50 μg/ml of *L. amazonensis* particulate antigen for 48 h. The amount of $NO2^-$ present in the culture supernatants was estimated by the Griess assay. The data shown represent the means ± SD of triplicate samples and are representative of the data obtained from two independent experiments. *P < 0.05 (two-way ANOVA with Bonferroni's post test) compared with C57BL/6 cultures.



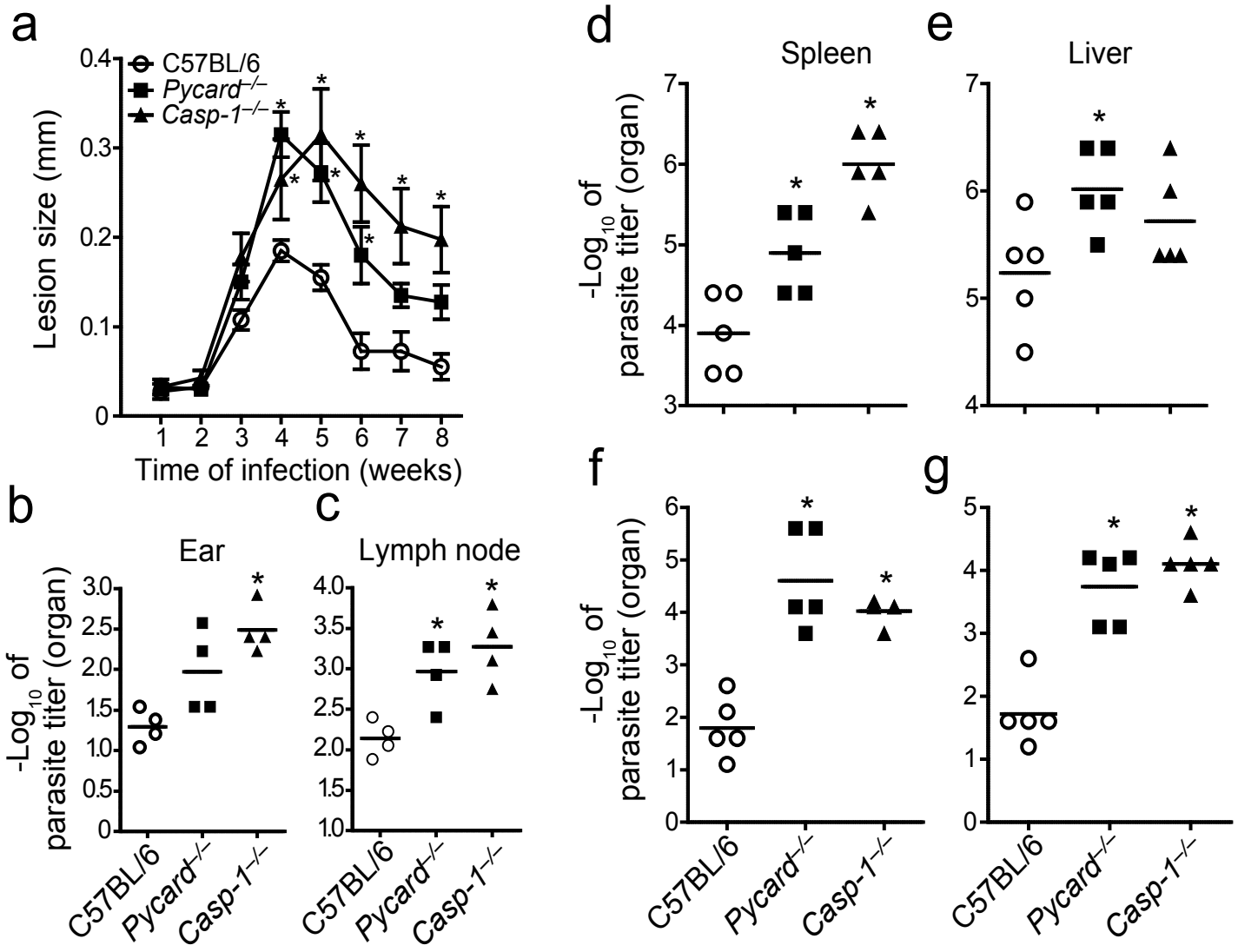
Supplementary Figure 5. IFN-γ is important for IL-1β and nitric oxide production *ex vivo*. C57BL/6, *Casp-1*$^{-/-}$, *IL-1R*$^{-/-}$, and *IFN-γ*$^{-/-}$ mice were inoculated with 10^6 stationary phase *L. amazonensis* promastigotes and sacrificed at 5 weeks after infection. The cells from the spleen (**a,c,e**) and lymph node (**b,d,f**) were cultured and stimulated with 50 μg/ml of *L. amazonensis* particulate antigen (L.a Ag) for 48 h.The amount of IFN-γ (**a,b**), and IL-1β (**c,d**) contained in the culture supernatants was estimated using ELISA. The amount of $NO2^-$ present in the culture supernatants was estimated using the Griess assay (**e,f**). The data shown represent the means ± SD of 4 mice per group and are representative of the data obtained from two independent experiments. *P < 0.05 (two-way ANOVA with Bonferroni's post test) compared with non-stimulated cultures (medium).



Supplementary Figure 6. The inflammasome is activated in macrophages infected with *L. amazonensis*, *L. major*, and *L. braziliensis*. Bone marrow-derived macrophages (BMDMs) obtained from C57BL/6, *Pycard*$^{-/-}$, *Casp-1*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice were infected with stationary phase promastigotesof *L. amazonensis* (L.a), *L. braziliensis* (L.b), and *L. major* (L.m) at an MOI of 10. (**a**) The cells were stained for FAM-YVAD after 48 h of infection and analyzed through flow cytometry to determine the percentages of FAM-YVAD positive cells. (**b**) BMDMs were treated with LPS (500 ng/ml) for 6 h and infected for 42 h. The IL-1β production was assessed in the cell-free supernatants using ELISA. The data shown representthe means ± SD of triplicate samples and are representative of the data obtained from two independent experiments. *P < 0.05 (two-way ANOVA with Bonferroni's post test) compared with non-infected (NI).



Supplementary Figure 7. Inflammasome activation is not necessary for the restriction of *L. major* infection. C57BL/6, *Pycard*$^{-/-}$, and *Casp-1*$^{-/-}$ mice were infected in the ear dermis with 10^6 stationary phase *L. major* promastigotes. (**a**) The lesion size (in mm; for each mouse, the size of the infected ear was subtracted by the size of the uninfected control ear) was monitored weekly. (**b**) The parasite burdensin the infected ear and draining lymph node were measured 8 weeks post infection using limiting dilutionanalysis. The data shown represent the means ± SD of 5 mice per group and are representative of the data obtained from three independent experiments.



Supplementary Figure 8. The inflammasome accounts for the host resistance to *L. braziliensis* and *L. infantum chagasi*. C57BL/6, *Pycard*$^{-/-}$, and *Casp-1*$^{-/-}$ mice were infected in the ear dermis with 10^6 stationary phase *L. braziliensis* promastigotes (**a-c**) or infected intraperitoneally with 10^7 stationary phase *L. infantum chagasi* promastigotes (**d-g**). (**a**) The lesion size (in mm; for each mouse, the size of the infected ear was subtracted by the size of the uninfected control ear) was monitored weekly. (**b**) The parasite burdens in the infected ear and draining lymph node were measured 8 weekspost infection using limiting dilution analysis. (**d-g**) The parasite burdens in the spleen (**d,f**) and liver (**e,g**) were measured 4 (**d,e**) and 8 (**f,g**) weeks post infection using limiting dilution analysis. The data shown represent the means ± SD of 4 or 5 mice per group and are representative of the data obtained from three independent experiments. *P < 0.05 (two-way ANOVA with Bonferroni's post test (**a**) or Student's *t* test (**d-g**)) compared with C57BL/6.