Non-canonical NLRP3 inflammasome activation and IL-1β signaling are necessary to *L. amazonensis* control mediated by P2X7 receptor and leukotriene B$_4$

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Abstract

Leishmaniasis is a neglected tropical disease affecting millions of individuals worldwide. P2X7 receptor has been linked to the elimination of *Leishmania amazonensis*. Biological responses evoked by P2X7 receptor activation have been well-documented, including apoptosis, phagocytosis, cytokine release, such as IL-1β. It was demonstrated that NLRP3 inflammasome activation and IL-1β signaling participated in resistance against *L. amazonensis*. Furthermore, our group has shown that *L. amazonensis* elimination through P2X7 receptor activation depended on leukotriene B$_4$ (LTB$_4$) production and release. Therefore, we investigated whether *L. amazonensis* elimination by P2X7 receptor and LTB$_4$ involved NLRP3 inflammasome activation and IL-1β signaling. We showed that macrophages from NLRP3$^{-/-}$, ASC$^{-/-}$, Casp-1/11$^{-/-}$, gp91$^{phox-/-}$, and IL-1R$^{-/-}$ mice treated with ATP or LTB$_4$ did not decrease parasitic load as was observed in WT mice. When ASC$^{-/-}$ macrophages were treated with exogenous IL-1β, parasite killing was noted, however, we did not see parasitic load reduction in IL-1R$^{-/-}$ macrophages. Similarly, macrophages from P2X7 receptor-deficient mice treated with IL-1β also showed decreased parasitic load. In addition, when we infected Casp-11$^{-/-}$ macrophages, neither ATP nor LTB$_4$ were able to reduce parasitic load, and Casp-11$^{-/-}$ mice were more susceptible to *L. amazonensis* infection than were WT mice. Furthermore, P2X7$^{-/-}$ *L. amazonensis*-infected mice locally treated with exogenous LTB$_4$ showed resistance to infection, characterized by lower parasite load and smaller lesions compared to untreated P2X7$^{-/-}$ mice. A similar observation was noted when infected P2X7$^{-/-}$ mice were treated with IL-1β, i.e., lower parasite load and smaller lesions compared to P2X7$^{-/-}$ mice. These data suggested that *L. amazonensis* elimination mediated by P2X7 receptor and LTB$_4$ was dependent on non-canonical NLRP3 inflammasome activation, ROS production, and IL-1β signaling.

Author summary

Leishmania spp. is a protozoan parasite that infects human and causes several diseases.
Leishmania amazonensis causes cutaneous leishmaniasis (CL) and mucocutaneous leish-
maniasis (MCL). *Leishmania* parasites preferentially infect macrophages. In macrophages,
several mechanisms have been described as controlling *L. amazonensis* infection. Here,
we showed that P2X7 receptor and LTB$_4$ eliminated *L. amazonensis* in macrophages by a
pathway dependent on non-canonical NLRP3 inflammasome activation and IL-1β
signaling.

Introduction

Leishmaniases are a group of neglected human infectious diseases that affect more than 12 mil-
lion people worldwide, with 1.5 million of new cases per year [1,2]. The protozoan parasites of
Leishmania spp. cause several clinical manifestations, from skin lesions (cutaneous leishmania-
sis) to visceral injuries (visceral leishmaniasis) that may be fatal [3]. In the South America,
Leishmania amazonensis is an important causative agent of Leishmaniasis.

Leishmania infect phagocytic cells in host mammalian cells, including macrophages. Ironi-
cally, these cells are responsible for parasite control upon membrane receptor activation via
various effector mechanisms [4]. Among the several mediators that affect macrophage func-
tion, purinergic receptor activation has been described as important for *L. amazonensis* infec-
tion control [5,6]. Purinergic receptors are activated by extracellular nucleotides and are
divided in two families: P2Y and P2X. P2Y receptors are metabotropic receptors coupled to G
proteins, while P2X receptors are ionotropic receptors activated by extracellular ATP (eATP)
[7]. The subtype P2X7 receptor was implicated in the control of several intracellular pathogens,
including *T. gondii* [8–10], *Chlamydia spp*. [11,12] and *Mycobacterium tuberculosis* [13,14].
Our previous work reported that P2X7 receptor was important for *L. amazonensis* control by a
mechanism dependent on leukotriene (LT) B$_4$ [15].

Pathogen recognition by cells of the immune system occurs through a large number of
extra and intracellular receptors. This process can lead to the synthesis of inflammatory lipid
mediators, such as LTs [16]. LTs constitute a family of inflammatory mediators formed from
arachidonic acid metabolism by 5-lipoxygenase (5-LO) [17]. Among 5-LO products, LTB$_4$ is
recognized as a pivotal neutrophil chemotactic factor. However, several reports also pointed
to LTs as immunomodulators, participating in the control of infections by pathogens such
as *Trypanosoma spp*. [18]. In addition, Serezani and collaborators [19] demonstrated LTB$_4$
participation in *L. amazonensis* elimination. Furthermore, other studies have suggested the
participation of LTs in the production of IL-1β-mediated inflammation by the NLRP3 inflam-
masome [20].

Stimulation of pattern-recognition receptors (PRRs) such as PAMPs and DAMPs (*patho-
gen- and danger-associated molecular patterns*, respectively) in the immune system were associ-
ated with an inflammatory cellular response that included the production of cytokines and
chemokines [21]. One of the cellular systems activated by PRRs is the inflammasome platform,
a cytoplasmic multiprotein complex that mediates IL-1β and IL-18 secretion [22,23]. The most
well-characterized inflammasome is NLRP3. NLRP3 inflammasome activation may be accom-
plished by a wide variety of structurally varied agonists, including pathogenic organisms, pore-
forming toxins, and DAMPs [24]. NLRP3 activation requires two signals, where P2X7 receptor
is recognized as one of the major secondary signals for NLRP3 inflammasome activation

[25,26]. Furthermore, NLRP3 inflammasome activation results in canonical or non-canonical activation: canonical NLRP3 inflammasomes convert pro-caspase-1 into active enzyme caspase-1 (Casp-1) [27], and the undefined non-canonical inflammasome promotes activation of pro-caspase-11 (Casp-11) [28,29]. The participation of the NLRP3 inflammasome in the elimination of *L. amazonensis* in a nitric oxide-dependent manner has already been demonstrated [30]. Also, a recent paper showed non-canonical NLRP3 inflammasome activation by lipophosphoglycan (LPG) from *Leishmania* membrane and casp-11 is important to the infection control [31].

Based on this rationale, it is reasonable to suggest that the P2X7 receptor, LTB₄, and IL-1β may participate in the same pathway, leading to the control of *L. amazonensis*. Therefore, the objective of this study was to investigate the mechanisms of elimination of *L. amazonensis* when P2X7 receptor is activated by eATP.

Results

Control of *L. amazonensis* infection via P2X7 receptor depended on NLRP3 inflammasome activation and IL-1R signaling

A number of studies demonstrated the role of the pro-inflammatory cytokine IL-1β in protection against pathogens such as *Toxoplasma gondii* and *Trypanosoma cruzi* [32–34]. Therefore, we hypothesized that the P2X7 receptor may mediate the elimination of *L. amazonensis* in a manner dependent on NLRP3 inflammasome activation.

Our data demonstrated that control of *L. amazonensis* via ATP was dependent on the NLRP3 inflammasome, because ATP did not reduce parasite burden in infected macrophages from NLRP3$^{-/-}$ mice (Fig 1D–1F), ASC$^{-/-}$ mice (Fig 1G–1I), and Casp-1/11$^{-/-}$ mice (Fig 1J–1L); however, it did reduce parasite burden in WT mice (Fig 1A–1C). These results suggested that the assembly of the NLRP3 inflammasome is an important mechanism in the elimination of parasites triggered by the P2X7 receptor. Furthermore, when we treated IL-1R$^{-/-}$ infected macrophages with ATP, we did not observe reductions in parasitic load (Fig 1M–1O), demonstrating that IL-1R signaling was important to *L. amazonensis* control, mediated by the P2X7 receptor.

L. amazonensis control via LTB₄ depended on NLRP3 inflammasome activation and IL-1R signaling

We previously demonstrated that the elimination of *L. amazonensis* mediated by the P2X7 receptor depended on LTB₄ production and release [15]. It had been demonstrated that LTB₄ modulated activation of NLRP3-dependent inflammation following monosodium urate stimulation [20]. Therefore, using the same approach previously used, we tested whether LTB₄ leishmanicidal activity was NLRP3 inflammasome-dependent. Indeed, the treatment of infected macrophages from NLRP3$^{-/-}$ mice (Fig 2D–2F), ASC$^{-/-}$ mice (Fig 2G–2I), Casp-1/11$^{-/-}$ mice (Fig 2J–2L), and IL-1R$^{-/-}$ mice (Fig 2M–2O) with LTB₄ did not reduce parasite load; however, LTB₄ reduced parasite load when infected macrophages from WT mice were treated (Fig 2A–2C), suggesting the importance of NLRP3 inflammasome and IL-1R signaling in elimination of *L. amazonensis* mediated by LTB₄.

L. amazonensis control via IL-1β depended on IL-1R signaling

Infected macrophages from C57BL/6, ASC$^{-/-}$, and IL-1R$^{-/-}$ mice were treated with exogenous IL-1β and parasite load was determined. Infected cells from ASC$^{-/-}$ mice (Fig 3D–3F) reduced parasite load following treatment with IL-1β, in similar fashion as IL-1β-treated WT cells. As
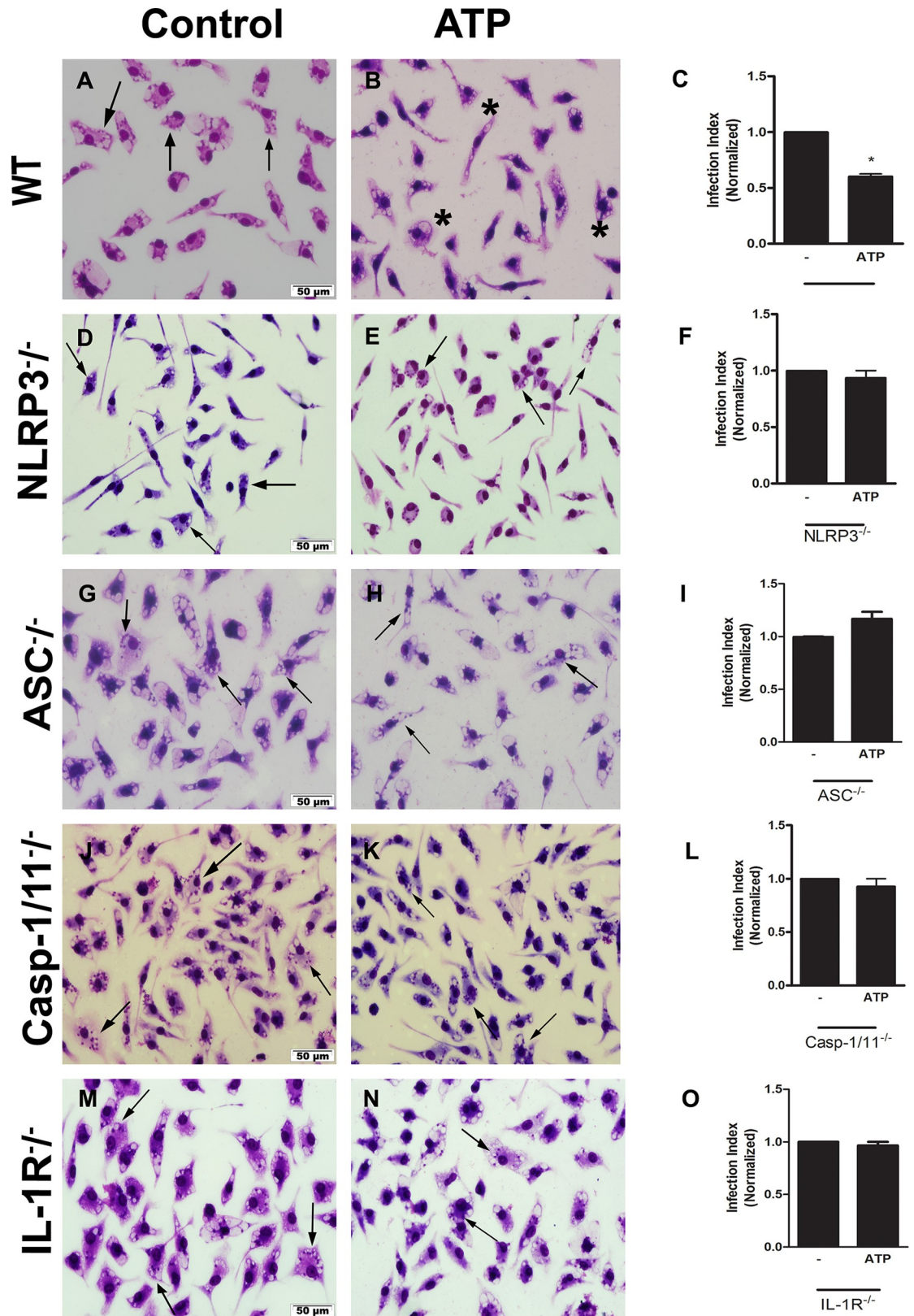
Fig 1. *L. amazonensis* **control via P2X7 receptor was dependent on NLRP3 inflammasome and IL-1R signaling.** Peritoneal macrophages from C57BL/6 (A-C), NLRP3$^{-/-}$ (D-F), ASC$^{-/-}$ (G-I), Casp-1/11$^{-/-}$ (J-L), and IL-1R$^{-/-}$ (M-O) mice were infected with

L. amazonensis promastigotes at the ratio of 10:1 (Leishmania:macrophage). After 4 hours, the free parasites were washed and after 24 hours, infected cells were treated (B, E, H, K, and N) or not (A, D, G, J, and M) with 500 μM of ATP. Twenty-four hours later, cells were stained with May-Grunwald-Giemsa and the infection index was determined by direct counting under light microscopy. Normalized values represent means ± SEM of 3–4 independent experiments performed in triplicate. Arrows correspond to vacuoles with *L. amazonensis* and asterisks represent empty vacuoles. ($^{*}P < 0.05$) compared to the control group (without treatment).

expected, infected macrophages from IL-1R$^{-/-}$ mice did not demonstrate an IL-1β effect (Fig 3G–3I).

To demonstrate the capacity of *L. amazonensis* to induce IL-1β release, we infected peritoneal macrophages with *Leishmania* and treated with or without ATP or LTB₄. As seen in S1A Fig, only in infected macrophages was ATP or LTB₄ able to induce IL-1β secretion. The infection by itself induced IL-1β secretion. However, when infected macrophages from Casp-11$^{-/-}$ mice were stimulated with ATP or LTB₄, we did not observe IL-1β release, suggesting that IL-β induced by P2X7 receptor and LTB₄ during infection are dependent on Casp-11 (S1B Fig).

It is known that pannexin-1 is essential to Casp-11 activation mediated by P2X7 receptor [35]; therefore, we performed experiments blocking the pannexin-1 channel. We observed that neither ATP nor LTB₄ reduced the parasitic load of infected macrophages when pannexin-1 inhibitor was added, suggesting that *L. amazonensis* control mediated by P2X7 receptor and LTB₄ are dependent on pannexin-1 activation (S2 Fig).

L. amazonensis control via P2X7 receptor and LTB₄ depended on non-canonical NLRP3 inflammasome

A previous study implicated Casp-11 in the elimination of intracellular pathogens [36]. Moreover, a recent work has showed that parasite membrane LPG from different species of Leishmania is able to activate casp-11 and consequent NLRP3 inflammasome in a non-canonical-dependent manner [31]. Therefore, we evaluated the importance of Casp-11 in the control of *L. amazonensis* mediated by P2X7 receptor and LTB₄. We found that Casp-11 was essential for parasite burden reduction, because infected macrophages from Casp-11-deficient mice did not reduce parasite burden neither in the presence of ATP (Fig 4E and 4F) nor of LTB₄ (Fig 5E and 5F). In addition, we used pharmacological inhibitors specific for Casp-1 and Casp-11, Z-YVAD-FMK and Z-LEVD-FMK, respectively, at concentrations of 2 μM prior to treatment with ATP (Fig 4G and 4H) or LTB₄ (Fig 5G and 5H). Infected macrophages pre-treated with Z-YVAD-FMK and Z-LEVD-FMK did not reduce parasitic load after ATP exposure. These data suggest that both Casp-1 and Casp-11 are important for control of *L. amazonensis* via the P2X7 receptor and LTB₄.

ROS is important for *Leishmania* control [37] as well as for non-canonical NLRP3 inflammasome activation; therefore, we performed experiments using peritoneal macrophages from gp91$^{phox-/-}$ mice. Treatment of infected macrophages with ATP and LTB₄ did not reduce parasitic load in macrophages from gp91$^{phox-/-}$ mice, suggesting the importance of ROS in the P2X7 receptor-LTB₄-NLRP3 activation axis during *L. amazonensis* infection (S3 Fig).

One of hallmarks of non-canonical inflammasome activation is the pyroptosis effect. We determined whether pyroptosis was involved in ATP and LTB₄ anti-Leishmania effects. We found that ATP and LTB₄ did not induce pyroptosis in infected macrophages (S4 Fig).

Casp-11$^{-/-}$ mice were susceptible to *L. amazonensis* infection

To confirm the importance of Casp-11 during *L. amazonensis* infection, WT, Casp-1/11$^{-/-}$, and Casp-11$^{-/-}$ mice were infected in the footpad with *L. amazonensis*, and lesion development was
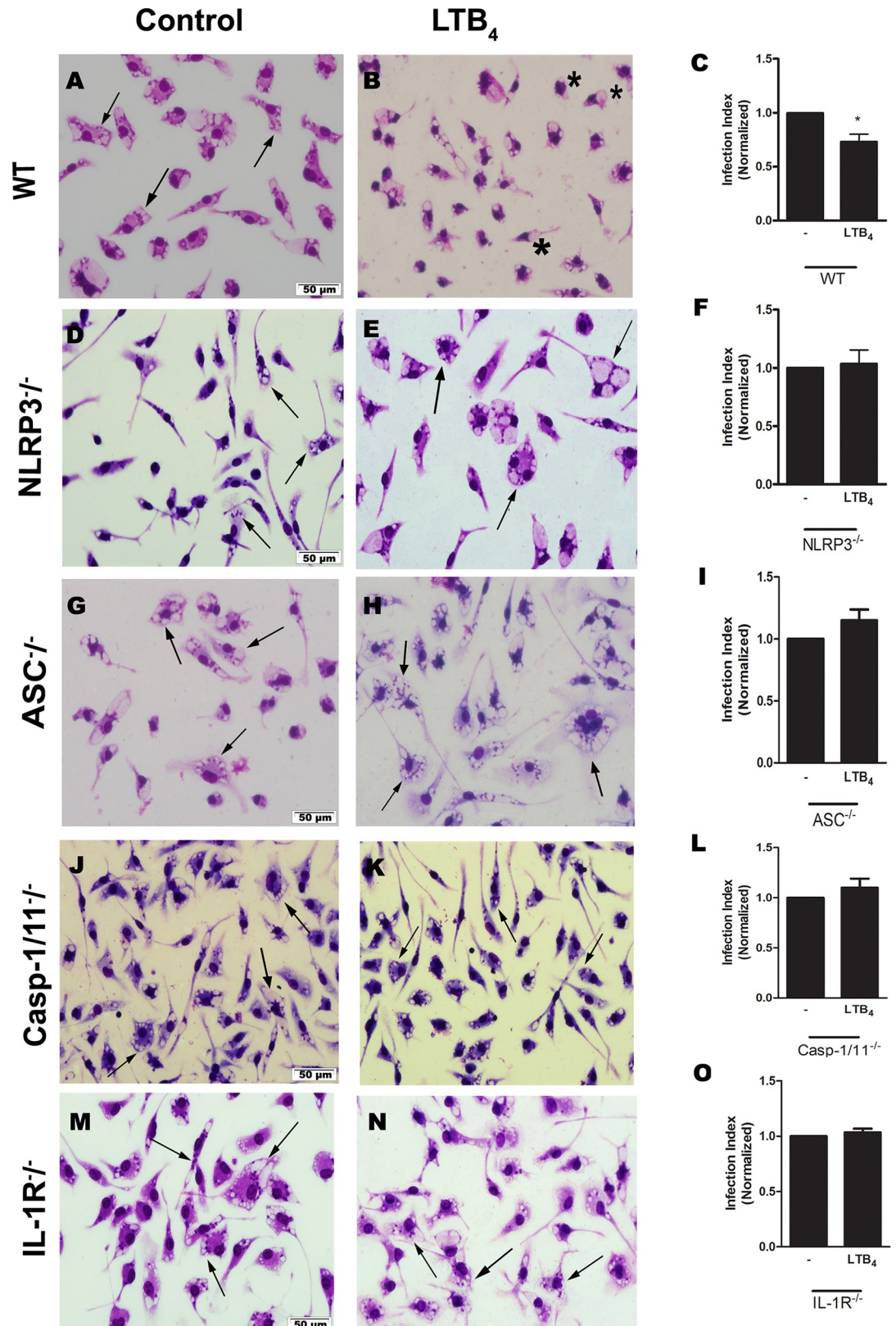
Fig 2. *L. amazonensis* **control via LTB$_4$ was dependent on the NLRP3 inflammasome and IL-1R signaling.** Peritoneal macrophages from C57BL/6 (A-C), NLRP3$^{-/-}$ (D-F), ASC$^{-/-}$ (G-I), Casp-1/11$^{-/-}$ (J-L), and IL-1R$^{-/-}$ (M-O) mice were infected

with *L. amazonensis* promastigotes at a ratio of 10:1 (Leishmania:macrophage). After 4 hours, the free parasites were washed and after 24 hours, infected cells were treated (B, E, H, K, and N) or not (A, D, G, J, and M) with 100 nM of LTB₄. Twenty-four hours later, cells were stained with May-Grunwald-Giemsa and the infection index was determined by direct counting under light microscopy. Normalized values represent means ± SEM of 3–4 independent experiments performed in triplicate. Arrows correspond to vacuoles with *L. amazonensis* and asterisks represent empty vacuoles. ($^{*}P < 0.05$) compared to the control group (without treatment).

followed for 28 days. As can be observed in Fig 6, Casp-11$^{-/-}$ mice were susceptible to infection, presenting with larger lesions (6A) and larger parasitic loads than WT mice (6B). Casp-1/11$^{-/-}$
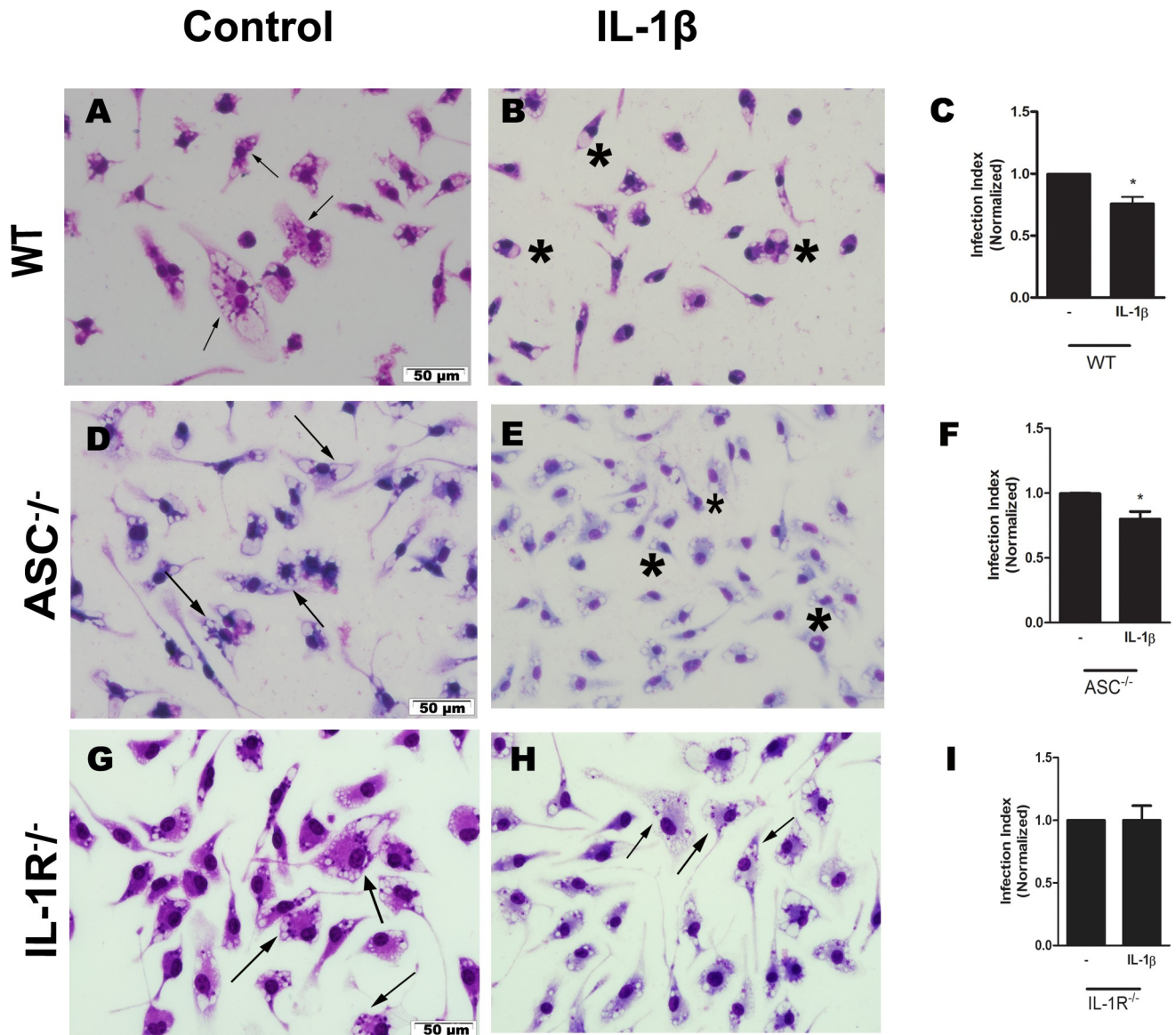
Fig 3. *L. amazonensis* control via IL-1β was dependent on IL-1R signaling. Peritoneal macrophages from C57Bl/6 (A-C), ASC$^{-/-}$(D-F), and IL-1R$^{-/-}$ (G-I) were infected with *L. amazonensis* promastigotes at the ratio of 10:1 (Leishmania:macrophage). After 4 hours, the free parasites were washed and after 24 hours, infected cells were treated with 100 pg/ml of IL-1β. Twenty-four hours later, cells were stained and the infection index was obtained. Normalized values represent means ± SEM of 3–4 independent experiments performed in triplicate. Arrows correspond to vacuoles with *L. amazonensis* and asterisks represent empty vacuoles. ($^{*}P < 0.05$) compared to the control group (without treatment).
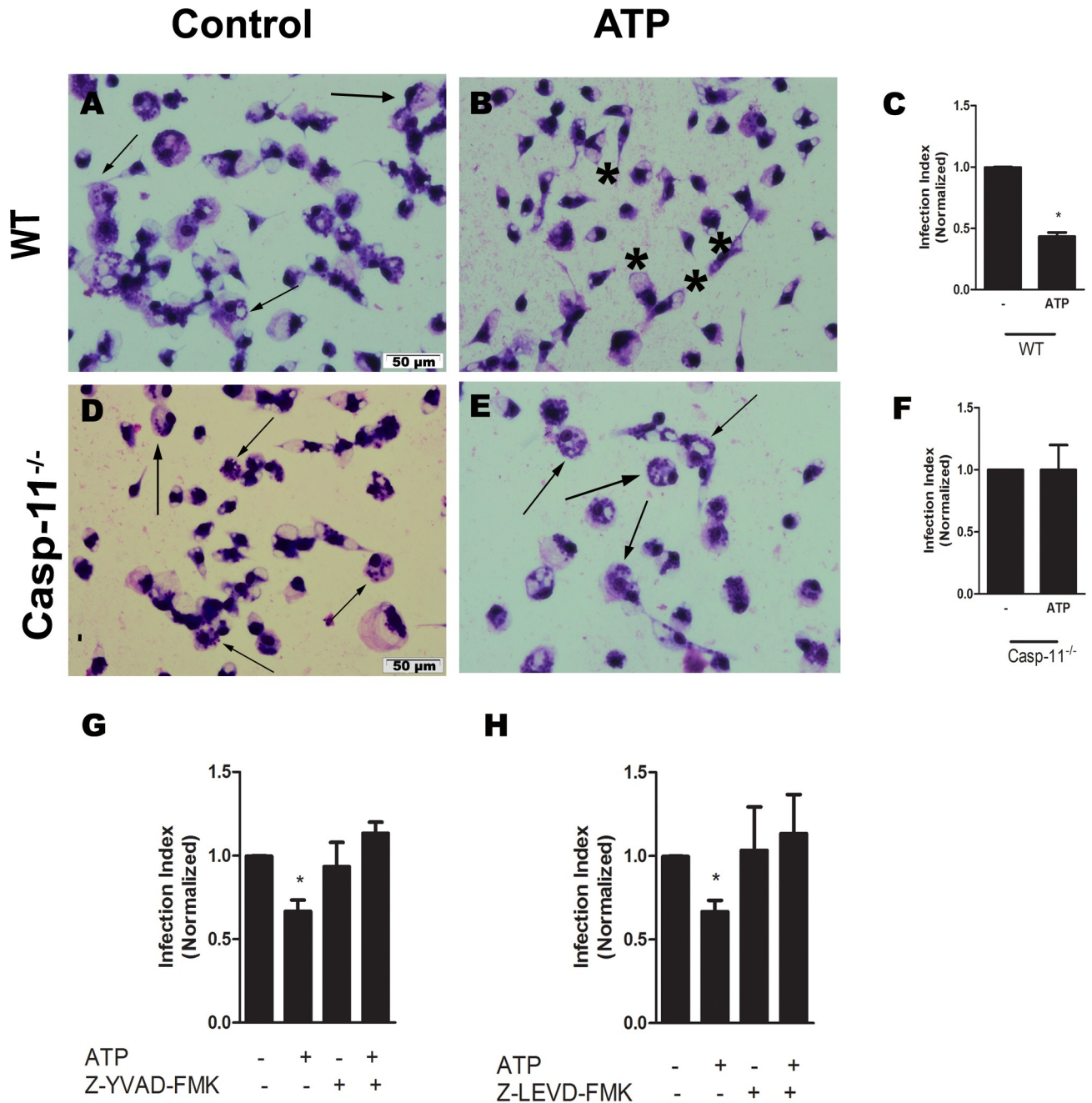
Fig 4. *L. amazonensis* control via ATP/P2X7 receptor was Casp-11 dependent. Peritoneal macrophages from C57Bl/6 (panels A-C, G, and H) and Casp-11$^{-/-}$ (D-F) mice were infected with promastigotes of *L. amazonensis* at a ratio of 10:1 (Leishmania:macrophage). After 4 hours, the free parasites were washed and after 24 hours, infected cells were treated with ATP (500 μM; B and E). Infected macrophages were also treated with Z-YVAD-FMK and Z-LEVD-FMK, Casp-1 and Casp-11 inhibitors respectively, at the concentration of 2 μM 30 minutes before ATP treatment. Twenty-four hours later, the infection index was determined. Standard values represent means ± SEM of 3 independent experiments performed in triplicate. Arrows correspond to vacuoles with *L. amazonensis* and asterisks represent empty vacuoles. ($^*P$ < 0.05) compared to the control group (without treatment).

mice also showed larger lesions and parasite loads compared to infected WT, agreeing with as study in which the importance of Casp-1/11 in resistance to *L. amazonensis* infection was demonstrated for the *L. amazonensis* PH8 strain [30].

Exogenous LTB$_4$ restored resistance in P2X7$^{-/-}$ mice during *L. amazonensis* infection

Recent data from our group demonstrated that P2X7$^{-/-}$ mice more susceptible to *L. amazonensis* infection than were WT mice [38]. We also demonstrated that macrophages from P2X7$^{-/-}$
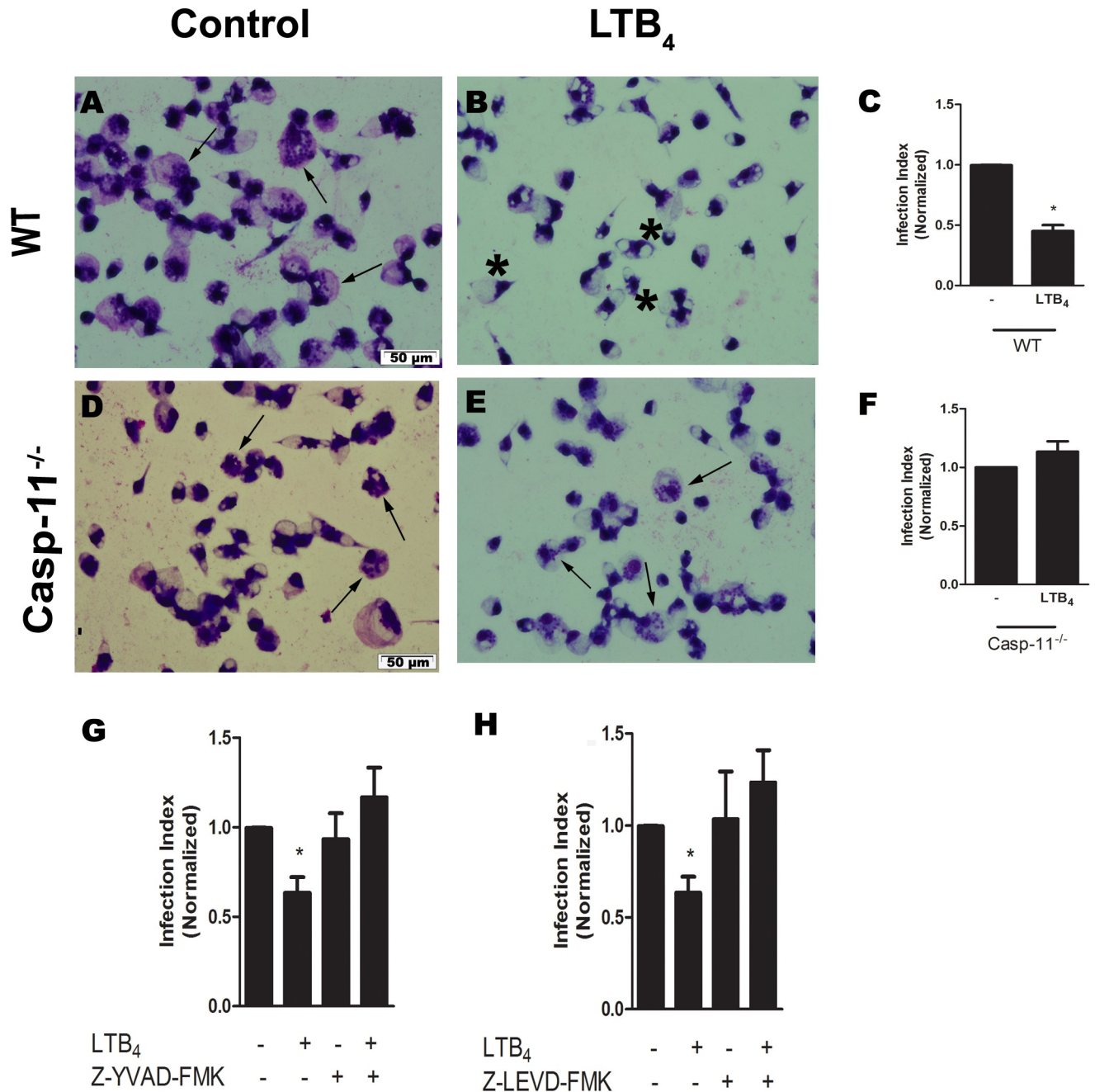
Fig 5. *L. amazonensis* control via LTB$_4$ was Casp-11 dependent. Peritoneal macrophages from C57Bl/6 (panels A-C, G, and H) and Casp-11$^{-/-}$ (D-F) mice were infected with promastigotes of *L. amazonensis* at a ratio of 10:1 (Leishmania:macrophage). After 4 hours, the free parasites were washed and after 24 hours infected cells were pretreated with 2 μM of Z-YVAD-FMK (G) or Z-LEVD-FMK (H) for 30 min. Subsequently, cells were treated with LTB$_4$ (100 nM). Twenty-four hours later, the infection index was determined. Standard values represent means ± SEM of 3 independent experiments performed in triplicate. Arrows correspond to vacuoles with *L. amazonensis* and asterisks represent empty vacuoles. (*$P < 0.05$) compared to the control group (without treatment).
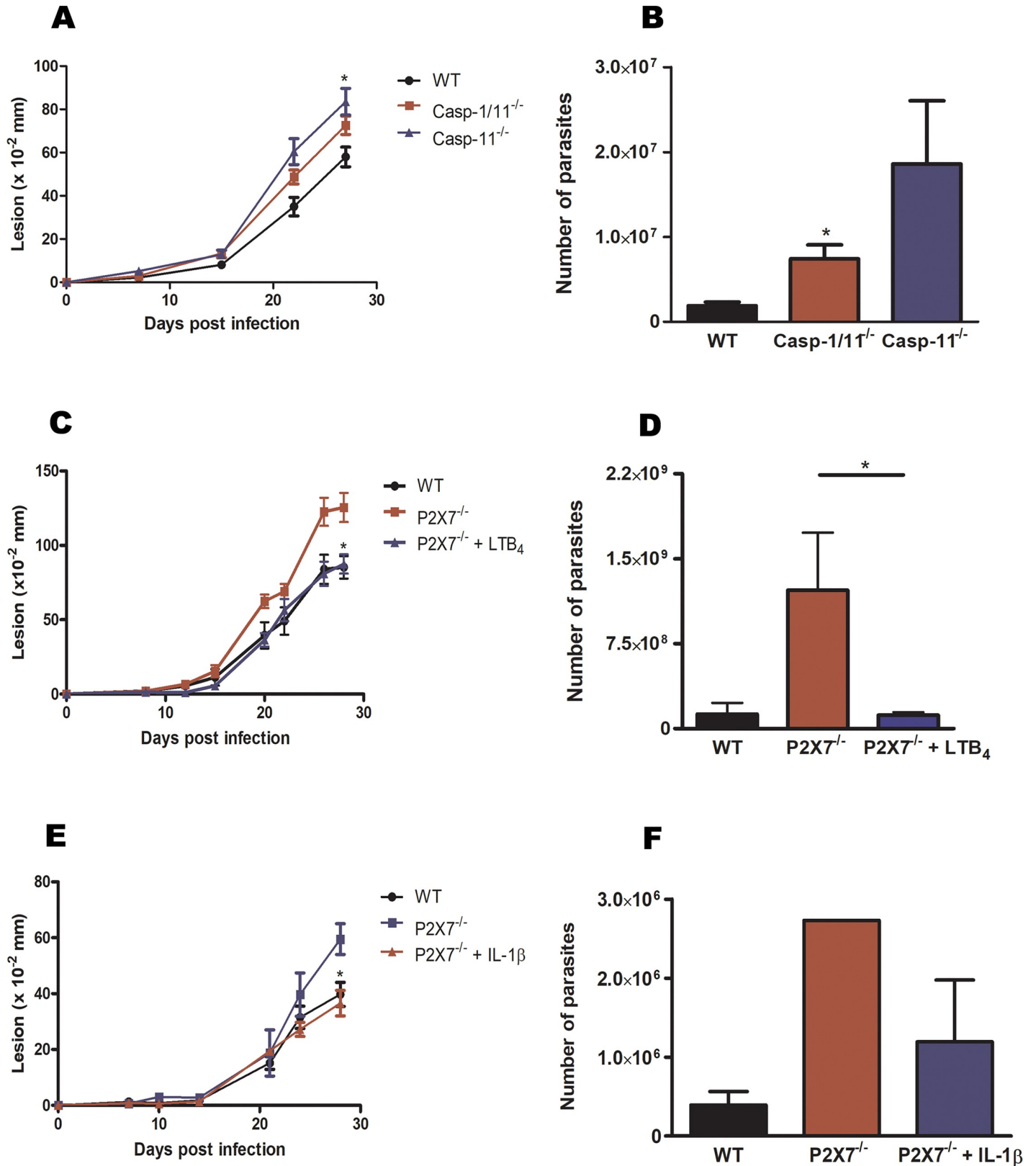
Fig 6. Casp-11$^{-/-}$ mice were more susceptible infection and LTB$_4$ and IL-1β restored resistance of P2X7 receptor$^{-/-}$ mice to *L. amazonensis*. C57Bl/6 (A, B, C, D, E, F), P2X7$^{-/-}$ (C, D, E, F), Casp-1/11$^{-/-}$ (A, B), and Casp-11$^{-/-}$ (A, B) mice were infected with 10^6 promastigotes of *L. amazonensis* for 28 days and were treated with or

without 5 ng of LTB$_4$ (C, D) or 300 pg of IL-1β (E, F) twice weekly, for 3 weeks. Lesions was determined by thickening (A, C, E) and number of parasites by LDA as described (B, D, F). A, B correspond to mean ± SEM of a group of 9 animals; C, D correspond to mean ± SEM n = 8 mice in two independent experiments; E, F correspond to mean ± SEM of n = 5–4 animals (*$P < 0.05$).

mice infected with *L. amazonensis* did not produce LTB$_4$ after ATP stimulation [15]. Therefore, we hypothesized that the susceptibility of P2X7$^{-/-}$ mice was due to ineffective LTB$_4$ production. Therefore, when infected P2X7$^{-/-}$ mice were treated with local LTB$_4$ injections, we were able to restore resistance in these animals, demonstrated by smaller lesions and parasitic loads (6C, 6D) compared to those of vehicle-treated deficient mice (PBS). In addition, both lesion and parasite load were very similar to those of WT infected mice.

Exogenous IL-1β restored resistance in P2X7$^{-/-}$ mice during *L. amazonensis* infection

As previously mentioned, LTB$_4$ induced IL-1β release. Therefore, we hypothesized that the susceptibility of P2X7$^{-/-}$ mice to *L. amazonensis* infection could be due to deficient IL-1β production. To test this hypothesis, we treated infected P2X7$^{-/-}$ mice with exogenous IL-1β and found that the treatment caused reduction of lesion size and parasite load to the same magnitude as was observed in WT mice (6E, 6F).

Taken together, these data suggest that physiological ATP, through P2X7 activation, leads to LTB$_4$ production and release. LTB$_4$, per se, induced non-canonical NLRP3 inflammasome activation and IL-1β maturation, activating IL-1R to control *L. amazonensis* infection (Fig 7).

Discussion

Previous data from our group showed that LTB$_4$ was important to *L. amazonensis* control mediated by the P2X7 receptor [15]. However, the mechanism by which the P2X7 receptor together with LTB$_4$ led to the elimination of *L. amazonensis* had not been elucidated. Purinergic receptors were important for elimination of several intracellular pathogens, including *T. gondii* [8–10], *Chlamydia spp.* [11,12] and *Mycobacterium tuberculosis* [13,14]. Furthermore, LTB$_4$ was shown to be important in the control of *Histoplasma capsulatum* [39], *Klebsiella pneumoniae* [40], among other parasites. In the specific case of *L. amazonensis* infection, both P2X7 receptor and LTB$_4$ were shown to be essential for parasite control [5,15,19,41]. The importance of the purinergic receptor-lipid mediator axis during leishmaniasis has been described [42]. In the present study, we demonstrated that ATP/P2X7 and LTB$_4$ decreased *L. amazonensis* infection by a mechanism-dependent on non-canonical NLRP3 inflammasome activation, ROS production, and IL-1R signaling.

We showed that the NLRP3 inflammasome and IL-1R signaling were important for P2X7 and LTB$_4$ receptor-dependent *L. amazonensis* elimination. Data in the literature point to the role of the NLRP3 inflammasome in *L. amazonensis* host resistance [30]. Furthermore, other pathogens also induced immune responses via inflammasome activation, including *T. gondii*, *T. cruzi* and *Paracoccidioides brasiliensis* [32–34,43]. However, in *L. major* infections, NLRP3 inflammasome activation appeared to be associated with infection susceptibility [44,45]. Therefore, activation of the NLRP3 inflammasome promoting resistance appeared to be species-specific in leishmaniasis. Our data suggested that NLRP3 inflammasome components, including NLRP3, ASC, and Casp-1/11 are important in *L. amazonensis* infection control, because, in the absence of these components, P2X7 activation and LTB$_4$ did not decrease parasite load in infected macrophages.
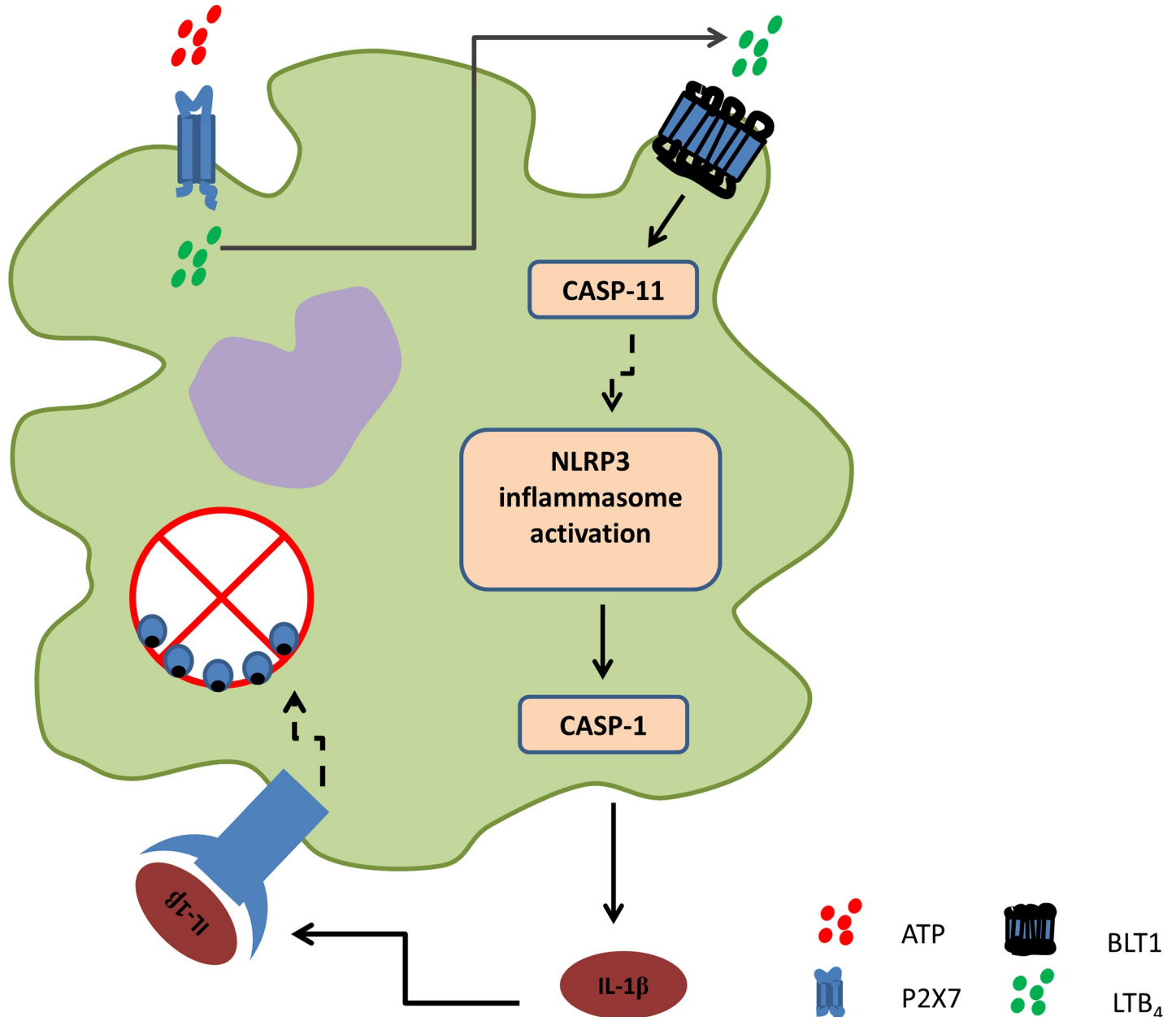
Fig 7. Proposed Mechanism. ATP activates the P2X7 receptor, which in turn leads to the production and release of LTB$_4$. LTB$_4$, through a mechanism as yet unknown, activates Casp-11. Casp-11 activates the NLRP3 inflammasome, leading to maturation of Casp-1. Casp-1 cleaves pro-IL-1β into mature IL-1β as it is released into extracellular medium and activates IL-1 receptors. By a mechanism as yet unknown, activation of IL-1R leads to the control of *L. amazonensis* in macrophages.

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The concept of P2X7 receptor activating the NLRP3 inflammasome is not new [46]. Other studies have also demonstrated the participation of LTB$_4$ in NLRP3 inflammasome activation [20]. Moreover, a recent article demonstrated the importance of membrane permeabilization for IL-1β release [47]. Inhibition of IL-1β secretion in *L. donovani*-infected macrophages has been reported [48–50]. Furthermore, several species of *Leishmania* inhibited IL-1β production through GP63-dependent mechanisms [51]. Our previous study showed that macrophages infected with *L. amazonensis* released lower amounts of LTB$_4$ when stimulated with ATP [15]. Therefore, it is reasonable to deduce that IL-1β and the NLRP3 inflammasome are important

for the control of the infection caused by *Leishmania*, and also that the parasite has developed mechanisms to subvert the immune system, interfering with IL-1β synthesis/response.

Our data also suggested the participation of the non-canonical NLRP3 inflammasome in elimination of *L. amazonensis* mediated by the P2X7 receptor and LTB₄, because, in the absence of Casp-11 or the use of a specific Casp-11 inhibitor, no reduction in the parasite load was detected after ATP or LTB₄ treatment. Casp-11 was activated by LPS from Gram-negative bacteria [52,53]. Furthermore, other pathogens that do not express LPS, including *Candida albicans*, also led to Casp-11 activation [54]. Moreover, the importance of Casp-11 in control of enteric bacterial pathogens has been demonstrated [55]. It is also important to highlight that during the revision process of this manuscript, it was published an elegant study showing all hallmarks of caspase-11 activation in response to *Leishmania* infection, fact mediated by LPG molecule presented in parasite membrane [31]. Moreover, the same paper showed that non-canonical NLRP3 inflammasome activation is important to the control of Leishmania infection in vitro and in vivo. Recent work showed the importance of ROS for expression and activation of Casp-11 during infection [56]; another study showed that pyroptosis induced by Casp-11 activation was P2X7 receptor-dependent [35]. Moreover, the P2X7 receptor and LTB₄ induced ROS release [57–62]. Furthermore, ROS is important to NLRP3 inflammasome assembly and control of the parasite during *Leishmania* infection [37]. Our data demonstrated that gp91^{phox}, a NADPH oxidase component is important for *L. amazonensis* control mediated by P2X7 receptor and LTB₄. However, caspase-11-dependent pyroptosis is not P2X7R-dependent because we did not observe pyroptosis 24 h after ATP treatment, suggesting that the parasite may inhibit this mechanism initially in order to evade host defense.

ATP is an intracellular molecule, however, it can actively and passively reach extracellular medium during inflammation [63]. Moreover, it is important to notice that in the immune response in vivo during *L. amazonensis* infection, several cells are recruited and infected that may even be a niche for replication of the parasite. The growth of Leishmania inside cells leads to rupture of these cells with consequent release of the intracellular contents to the extracellular medium. One of the molecules released after cell lysis is ATP, at millimolar intracellular concentrations. It is worth remembering that this happens simultaneously in several different cell types during in vivo infection, and that ATP is release actively during *L. amazonensis* phagocytosis by peritoneal macrophages [15]. Among the possible ways by which ATP is released to the extracellular compartment is through pannexin-1, a membrane channel/pore [64], and/or as a consequence of the death of infected cells could increase the extracellular ATP concentration, thereby controlling the infection through the activation of P2X7 receptors. Furthermore, Thorstenberg et al., 2018 [65] demonstrated in vivo release of ATP in lymph nodes during infection by *L. amazonensis* and showed that low doses of ATP (50 μM) decreased parasite burden in infected macrophage, in a pannexin-1- and P2X7 receptor-dependent mechanism. It is also worth mentioning that when released ATP is rapidly hydrolyzed by enzymes present in the host and parasite cell membranes, including CD39 and CD73.

We showed that lack of the Casp-11 enzyme caused susceptibility to *L. amazonensis* infection, because lesions and parasite loads were higher in these animals, as well as in Casp-1/11$^{-/-}$ mice. Conversely, recent work from our group showed that during *T. gondii* control mediated by P2X7 receptor, Casp-11 was not important [66], suggesting that non-canonical NLRP3 inflammasome activation mediated by P2X7 receptor and LTB₄ during infection is species-specific.

Conclusions

Taken together, these results suggest that *L. amazonensis* control mediated by P2X7 receptor and LTB₄ is dependent on production and release of IL-1β via non-canonical NLRP3

inflammasome activation. The understanding of this mechanism is of extreme importance for development of new therapeutic strategies in order to combat leishmaniasis.

Materials and methods

Ethics statement

All animal experiments were performed in accordance with Brazilian regulations conduct by Conselho Nacional de Experimentação Animal (CONCEA). All procedures using animals were approved by Comissão de Ética no Uso de Animais da Universidade Federal do Rio de Janeiro (CEUA-UFRJ) under number 077/15.

Experimental animals

We used mice C57Bl/6, P2X7$^{-/-}$ (Pfizer, USA), NLRP3$^{-/-}$ (Genentech, USA), ASC$^{-/-}$ (Genentech, USA), caspase (Casp)-1/11$^{-/-}$ (Genentech, USA), Casp-11$^{-/-}$ (Genentech, USA), and IL-1R$^{-/-}$ (JAX Mice, USA) that were housed in a temperature-controlled room with a light/dark cycle and received food and water *ad libitum*. The P2X7 receptor$^{-/-}$ and Casp-11$^{-/-}$ mice were maintained at the Laboratory of Transgenic Animals (LAT) of the Institute of Biophysics Carlos Chagas Filho. The animals that were NLRP3$^{-/-}$, ASC$^{-/-}$, Casp-1/11$^{-/-}$ were kindly provided by Dr. Dario Zamboni of the Medical School of USP-Ribeirão Preto, while the IL-1R$^{-/-}$ animals were donated by Dr. Maria Bellio of the Institute of Microbiology Paulo de Góes of UFRJ. The mice used were of both genders, aged 6 to 16 weeks for the removal of peritoneal macrophages, and 6 to 8 weeks for in vivo experiments.

Cell culture

All mice were euthanized in a CO$_2$ chamber, followed by cervical dislocation as described in the report submitted and approved by the IBCCF ethics committee. Macrophages were obtained from the peritoneal cavity by inoculation and subsequent aspiration of 5 mL of cold PBS. The solution obtained was then centrifuged at 300 g for 10 minutes. Cells were counted by exclusion of dead cells using Trypan Blue (Sigma); 2 x 10^5 cells per well were cultured with or without cover slips at 37˚C and 5% CO$_2$ for 1 hour. Non-adherent cells were then removed by washing twice with sterile PBS at 37˚C. Macrophages were cultured in DMEM supplemented with 2 mM L-glutamine, penicillin (10 units/mL), streptomycin (10 μg/mL), and 10% inactivated fetal bovine serum.

Parasites

Amastigote forms of *L. amazonensis* (MHOM/BR/75/Josefa) were obtained from popliteal lymph nodes of infected BALB/c mice for the maintenance of infectivity. Axenic promastigotes were transformed at 27˚C into 199 medium supplemented with 2 mM L-glutamine, 10 units penicillin, 10 μg/ml streptomycin, 10% inactivated fetal bovine serum, 0.25% hemin, and 2% male sterile urine. Promastigotes were maintained until the tenth passage to maintain infective potential.

In vitro infection

For macrophage infection, we used an MOI ratio of 10:1 (Leishmania:macrophage). The parasites were counted using a Neubauer chamber in an optical microscope. Infection was performed for 4 hours at 37˚C and 5% CO$_2$. After this time, the non-internalized parasites were removed by washing twice with PBS sterile. Infected macrophages were maintained in an incubator at 37˚C and 5% CO$_2$ for 24 hours.

ATP, LTB$_4$, and IL-1β treatment

The physiological agonist of P2X7 receptor, ATP (Sigma), LTB$_4$ (Calbiochem), and IL-1β (R&D systems) were added at final concentrations of 500 μM, 100 nM, and 100 pg/mL, respectively. ATP and LTB$_4$ were added for 30 min and IL-1β was added for 24 h.

Infection index

The infection index was obtained by direct counting of infected cells under light microscopy. Cells were infected, and after 24 hours were stimulated with 500 μM eATP, 100 nM of LTB$_4$ or 100 pg/mL of IL-1β. Twenty-four hours after the treatments, the infected macrophages, treated or not, were fixed and stained with a Panotico Fast kit (Laborclin) and mounted on slides for analysis by optical microscope. The infection index was determined from the infected macrophages count and also by the mean number of parasites per infected macrophage. This number was obtained by counting at least 100 cells in a total of five fields. The results were expressed as the infection index, which was the percentage of infected macrophages multiplied by the mean number of amastigotes per infected macrophage, divided by 100, as described previously. [67].

In vivo infection

Mice were infected in the dermis of the right footpad by intradermal injection of 10^6 parasites. The growth of the lesion was accompanied by measurement of the thickness of the infected paw compared to the uninfected paw. After 28 days, the animals were euthanized and their footpads were removed and macerated for parasite load determination by the limiting dilution test (LDA) [68]. Briefly, serial fourfold dilutions were performed in 96-well microtiter plates. After 7–14 days at 27˚C, the presence or the absence of promastigotes in the wells was determined. The final titer was the last well in which it was possible to detect the presence of at least one parasite. In addition, C57Bl/6 and P2X7$^{-/-}$ mice were infected and after 7 days. Deficient mice were locally treated with 300 pg of IL-1β or 5 ng of LTB$_4$ twice a week for three weeks. Subsequently, the animals were euthanized and their paws were removed for parasitic load determination.

Statistical analysis

Data were analyzed using the program GraphPad Prism 5.0 and the determination of the significance among the various experimental groups was performed by determining the mean and standard error of the mean from the student t test or ANOVA post-test Tukey (more than two groups). The results were considered statistically significant if $P < 0.05$.

For detailed experimental protocols used in supporting information figures, please refer to S1 Methods.

Supporting information

S1 Methods. Supporting information methods.
(DOC)

S1 Fig. LTB$_4$ and P2X7 increase IL-1β production in a CASP-11$^{-/-}$ dependent-manner.
Peritoneal macrophages (2.0 x 10^5) from WT (A) and CASP-11$^{-/-}$ (B) mice were infected with stationary-phase *L. amazonensis* promastigotes for 1h. Quickly ATP and LTB were added in culture by 30 minutes. Following 4 h cells cultures were centrifuged by 10 minutes at 1200 RPM and supernatants were harvest to measured IL-1β by ELISA. Data correspond to the mean ± SEM values of n = 2 experiments performed in triplicate, with pooled cells from 4 to 5

animals.
(TIF)

S2 Fig. ROS from NADPH-oxidase is involved in anti-amastigote response by ATP and LTB$_4$. Peritoneal macrophages from WT (A) and gp91phox$^{-/-}$ (B) were infected with *L. amazonensis*. Infected cells were treated by 30 minutes with ATP and LTB$_4$ 24 h post infection. After, macrophages were fixed 30h post treatment, stained with panoptic, and the parasite load in infected macrophages was quantified as the "infection index" (% of infection x number of amastigote/total number of cells/100). Data correspond to the mean ± SEM values of n = 2 experiments performed in triplicate, with pooled cells from 4 to 5 animals.
(TIF)

S3 Fig. Pannexin-1 is important to anti-parasitic effects by ATP and LTB$_4$ treatment. Peritoneal macrophages from C57Bl/6 were infected with stationary-phase *L. amazonensis* promastigotes for 4h. Post 24 h infected cells were treated with Pannexin-1 antagonist CBX (50μM) for 30 minutes, following by stimulation with ATP and LTB$_4$ for 30 minutes. Infected macrophages were fixed 30h post treatment, stained with panoptic, and the parasite load in infected macrophages was quantified as the "infection index" (% of infection x number of amastigote/total number of cells/100). Data correspond to the mean ± SEM values of n = 2 experiments performed in triplicate, with pooled cells from 4 to 5 animals.
(TIF)

S4 Fig. Pyroptosis is not triggered after Casp-11 activation mediated by P2X7 receptor and LTB$_4$ during L. amazonensis infection. Peritoneal macrophages from C57Bl/6 mice were infected with stationary-phase *L. amazonensis* promastigotes for 4h (MOI 10:1). Followed 24 h of *L. amazonensis* infection, the macrophages were treated or not with 500μM of ATP; or 100 nM of LTB4, during 30 minutes. As positive control, macrophages were treated with 0.1% triton X-100 in a cell culture media. The supernatant was collected after 24 h of treatment. The free lactate dehydrogenase (LDH) levels were measured using the LDH enzymatic Kit (Bioclin-BRA), according to the manufactured instructions). Data correspond to the mean ± SEM values of n = 2 experiments performed in triplicate, with pooled cells from 4 to 5 animals.
(TIF)

Acknowledgments

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References

1. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den BM (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7: e35671. https://doi.org/10.1371/journal.pone.0035671 PONE-D-11-24894 [pii]. PMID: 22693548

2. Desjeux P (2004) Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis 27: 305–318. https://doi.org/10.1016/j.cimid.2004.03.004 S0147-9571(04)00023-2 [pii]. PMID: 15225981

3. Murray HW, Berman JD, Davies CR, Saravia NG (2005) Advances in leishmaniasis. Lancet 366: 1561–1577. S0140-6736(05)67629-5 [pii];https://doi.org/10.1016/S0140-6736(05)67629-5 PMID: 16257344

4. Moradin N, Descoteaux A (2012) Leishmania promastigotes: building a safe niche within macrophages. Front Cell Infect Microbiol 2: 121. https://doi.org/10.3389/fcimb.2012.00121 PMID: 23050244

5. Chaves SP, Torres-Santos EC, Marques C, Figliuolo VR, Persechini PM, Coutinho-Silva R, Rossi-Bergmann B (2009) Modulation of P2X(7) purinergic receptor in macrophages by Leishmania amazonensis and its role in parasite elimination. Microbes Infect 11: 842–849. S1286-4579(09)00103-8 [pii]; https://doi.org/10.1016/j.micinf.2009.05.001 PMID: 19439191

6. Marques-da-Silva C, Chaves MM, Chaves SP, Figliuolo VR, Meyer-Fernandes JR, Corte-Real S, Lameu C, Ulrich H, Ojcius DM, Rossi-Bergmann B, Coutinho-Silva R (2011) Infection with Leishmania amazonensis upregulates purinergic receptor expression and induces host-cell susceptibility to UTP-mediated apoptosis. Cell Microbiol 13: 1410–1428. https://doi.org/10.1111/j.1462-5822.2011.01630.x PMID: 21740498

7. Burnstock G (2007) Purine and pyrimidine receptors. Cell Mol Life Sci 64: 1471–1483. https://doi.org/10.1007/s00018-007-6497-0 PMID: 17375261

8. Correa G, Marques da SC, de Abreu Moreira-Souza AC, Vommaro RC, Coutinho-Silva R (2010) Activation of the P2X(7) receptor triggers the elimination of Toxoplasma gondii tachyzoites from infected macrophages. Microbes Infect 12: 497–504. S1286-4579(10)00073-0 [pii];https://doi.org/10.1016/j.micinf.2010.03.004 PMID: 20298798

9. Lees MP, Fuller SJ, McLeod R, Boulter NR, Miller CM, Zakrzewski AM, Mui EJ, Witola WH, Coyne JJ, Hargrave AC, Jamieson SE, Blackwell JM, Wiley JS, Smith NC (2010) P2X7 receptor-mediated killing of an intracellular parasite, Toxoplasma gondii, by human and murine macrophages. J Immunol 184: 7040–7046. jimmunol.1000012 [pii];https://doi.org/10.4049/jimmunol.1000012 PMID: 20488797

10. Miller CM, Zakrzewski AM, Robinson DP, Fuller SJ, Walker RA, Ikin RJ, Bao SJ, Grigg ME, Wiley JS, Smith NC (2015) Lack of a Functioning P2X7 Receptor Leads to Increased Susceptibility to Toxoplasmic Ileitis. PLoS One 10: e0129048. https://doi.org/10.1371/journal.pone.0129048 PONE-D-14-51051 [pii]. PMID: 26053862

11. Coutinho-Silva R, Stahl L, Raymond MN, Jungas T, Verbeke P, Burnstock G, Darville T, Ojcius DM (2003) Inhibition of chlamydial infectious activity due to P2X7R-dependent phospholipase D activation. Immunity 19: 403–412. S1074-7613(03)00235-8 [pii]. PMID: 14499115

12. Darville T, Welter-Stahl L, Cruz C, Sater AA, Andrews CW Jr., Ojcius DM (2007) Effect of the purinergic receptor P2X7 on Chlamydia infection in cervical epithelial cells and vaginally infected mice. J Immunol 179: 3707–3714. 179/6/3707 [pii]. https://doi.org/10.4049/jimmunol.179.6.3707 PMID: 17785807

13. Placido R, Auricchio G, Falzoni S, Battistini L, Colizzi V, Brunetti E, Di VF, Mancino G (2006) P2X(7) purinergic receptors and extracellular ATP mediate apoptosis of human monocytes/macrophages infected with Mycobacterium tuberculosis reducing the intracellular bacterial viability. Cell Immunol 244: 10–18. S0008-8749(07)00018-4 [pii];https://doi.org/10.1016/j.cellimm.2007.02.001 PMID: 17433275

14. Fairbairn IP, Stober CB, Kumararatne DS, Lammas DA (2001) ATP-mediated killing of intracellular mycobacteria by macrophages is a P2X(7)-dependent process inducing bacterial death by phagosome-lysosome fusion. J Immunol 167: 3300–3307. https://doi.org/10.4049/jimmunol.167.6.3300 PMID: 11544318

15. Chaves MM, Marques-da-Silva C, Monteiro AP, Canetti C, Coutinho-Silva R (2014) Leukotriene B4 modulates P2X7 receptor-mediated Leishmania amazonensis elimination in murine macrophages. J

Immunol 192: 4765–4773. jimmunol.1301058 [pii];https://doi.org/10.4049/jimmunol.1301058 PMID: 24729618

16. Astudillo AM, Balgoma D, Balboa MA, Balsinde J (2012) Dynamics of arachidonic acid mobilization by inflammatory cells. Biochim Biophys Acta 1821: 249–256. S1388-1981(11)00249-6 [pii];https://doi.org/10.1016/j.bbalip.2011.11.006 PMID: 22155285

17. Samuelsson B (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 220: 568–575. https://doi.org/10.1126/science.6301011 PMID: 6301011

18. Talvani A, Machado FS, Santana GC, Klein A, Barcelos L, Silva JS, Teixeira MM (2002) Leukotriene B (4) induces nitric oxide synthesis in Trypanosoma cruzi-infected murine macrophages and mediates resistance to infection. Infect Immun 70: 4247–4253. https://doi.org/10.1128/IAI.70.8.4247-4253.2002 PMID: 12117933

19. Serezani CH, Perrela JH, Russo M, Peters-Golden M, Jancar S (2006) Leukotrienes are essential for the control of Leishmania amazonensis infection and contribute to strain variation in susceptibility. J Immunol 177: 3201–3208. 177/5/3201 [pii]. https://doi.org/10.4049/jimmunol.177.5.3201 PMID: 16920959

20. Amaral FA, Costa VV, Tavares LD, Sachs D, Coelho FM, Fagundes CT, Soriani FM, Silveira TN, Cunha LD, Zamboni DS, Quesniaux V, Peres RS, Cunha TM, Cunha FQ, Ryffel B, Souza DG, Teixeira MM (2012) NLRP3 inflammasome-mediated neutrophil recruitment and hypernociception depend on leukotriene B(4) in a murine model of gout. Arthritis Rheum 64: 474–484. https://doi.org/10.1002/art.33355 PMID: 21952942

21. Kumar H, Kawai T, Akira S (2011) Pathogen recognition by the innate immune system. Int Rev Immunol 30: 16–34. https://doi.org/10.3109/08830185.2010.529976 PMID: 21235323

22. Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: guardians of the body. Annu Rev Immunol 27: 229–265. https://doi.org/10.1146/annurev.immunol.021908.132715 PMID: 19302040

23. Martinon F, Burns K, Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10: 417–426. S1097-2765(02)00599-3 [pii]. PMID: 12191486

24. Ratsimandresy RA, Dorfleutner A, Stehlik C (2013) An Update on PYRIN Domain-Containing Pattern Recognition Receptors: From Immunity to Pathology. Front Immunol 4: 440. https://doi.org/10.3389/fimmu.2013.00440 PMID: 24367371

25. Kahlenberg JM, Lundberg KC, Kertesy SB, Qu Y, Dubyak GR (2005) Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF-kappaB-driven protein synthesis. J Immunol 175: 7611–7622. 175/11/7611 [pii]. https://doi.org/10.4049/jimmunol.175.11.7611 PMID: 16301671

26. Kahlenberg JM, Dubyak GR (2004) Mechanisms of caspase-1 activation by P2X7 receptor-mediated K + release. Am J Physiol Cell Physiol 286: C1100–C1108. https://doi.org/10.1152/ajpcell.00494.2003 [pii]. PMID: 15075209

27. Gross O, Thomas CJ, Guarda G, Tschopp J (2011) The inflammasome: an integrated view. Immunol Rev 243: 136–151. https://doi.org/10.1111/j.1600-065X.2011.01046.x PMID: 21884173

28. Kayagaki N, Warming S, Lamkanfi M, Vande WL, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, Zhang J, Lee WP, Roose-Girma M, Dixit VM (2011) Non-canonical inflammasome activation targets caspase-11. Nature 479: 117–121. nature10558 [pii];https://doi.org/10.1038/nature10558 PMID: 22002608

29. Gurung P, Malireddi RK, Anand PK, Demon D, Vande WL, Liu Z, Vogel P, Lamkanfi M, Kanneganti TD (2012) Toll or interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-beta (TRIF)-mediated caspase-11 protease production integrates Toll-like receptor 4 (TLR4) protein- and Nlrp3 inflammasome-mediated host defense against enteropathogens. J Biol Chem 287: 34474–34483. M112.401406 [pii];https://doi.org/10.1074/jbc.M112.401406 PMID: 22898816

30. Lima-Junior DS, Costa DL, Carregaro V, Cunha LD, Silva AL, Mineo TW, Gutierrez FR, Bellio M, Bortoluci KR, Flavell RA, Bozza MT, Silva JS, Zamboni DS (2013) Inflammasome-derived IL-1beta production induces nitric oxide-mediated resistance to Leishmania. Nat Med 19: 909–915. nm.3221 [pii]; https://doi.org/10.1038/nm.3221 PMID: 23749230

31. de Carvalho RVH, Andrade WA, Lima-Junior DS, Dilucca M, de Oliveira CV, Wang K, Nogueira PM, Rugani JN, Soares RP, Beverley SM, Shao F, Zamboni DS (2019) Leishmania Lipophosphoglycan Triggers Caspase-11 and the Non-canonical Activation of the NLRP3 Inflammasome. Cell Rep 26: 429–437. S2211-1247(18)31977-6 [pii];https://doi.org/10.1016/j.celrep.2018.12.047 PMID: 30625325

32. Goncalves VM, Matteucci KC, Buzzo CL, Miollo BH, Ferrante D, Torrecilhas AC, Rodrigues MM, Alvarez JM, Bortoluci KR (2013) NLRP3 controls Trypanosoma cruzi infection through a caspase-1-dependent IL-1R-independent NO production. PLoS Negl Trop Dis 7: e2469. https://doi.org/10.1371/journal.pntd.0002469 PNTD-D-13-00595 [pii]. PMID: 24098823

33. Silva GK, Costa RS, Silveira TN, Caetano BC, Horta CV, Gutierrez FR, Guedes PM, Andrade WA, De NM, Gazzinelli RT, Zamboni DS, Silva JS (2013) Apoptosis-associated speck-like protein containing a caspase recruitment domain inflammasomes mediate IL-1beta response and host resistance to Trypanosoma cruzi infection. J Immunol 191: 3373–3383. jimmunol.1203293 [pii];https://doi.org/10.4049/jimmunol.1203293 PMID: 23966627

34. Gorfu G, Cirelli KM, Melo MB, Mayer-Barber K, Crown D, Koller BH, Masters S, Sher A, Leppla SH, Moayeri M, Saeij JP, Grigg ME (2014) Dual role for inflammasome sensors NLRP1 and NLRP3 in murine resistance to Toxoplasma gondii. MBio 5. mBio.01117-13 [pii];https://doi.org/10.1128/mBio.01117-13 PMID: 24549849

35. Yang D, He Y, Munoz-Planillo R, Liu Q, Nunez G (2015) Caspase-11 Requires the Pannexin-1 Channel and the Purinergic P2X7 Pore to Mediate Pyroptosis and Endotoxic Shock. Immunity 43: 923–932. S1074-7613(15)00409-4 [pii];https://doi.org/10.1016/j.immuni.2015.10.009 PMID: 26572062

36. Aachoui Y, Leaf IA, Hagar JA, Fontana MF, Campos CG, Zak DE, Tan MH, Cotter PA, Vance RE, Aderem A, Miao EA (2013) Caspase-11 protects against bacteria that escape the vacuole. Science 339: 975–978. science.1230751 [pii];https://doi.org/10.1126/science.1230751 PMID: 23348507

37. Lima-Junior DS, Mineo TWP, Calich VLG, Zamboni DS (2017) Dectin-1 Activation during Leishmania amazonensis Phagocytosis Prompts Syk-Dependent Reactive Oxygen Species Production To Trigger Inflammasome Assembly and Restriction of Parasite Replication. J Immunol 199: 2055–2068. jimmunol.1700258 [pii];https://doi.org/10.4049/jimmunol.1700258 PMID: 28784846

38. Figliuolo VR, Chaves SP, Savio LEB, Thorstenberg MLP, Machado SE, Takiya CM, D'Imperio-Lima MR, de Matos Guedes HL, Rossi-Bergmann B, Coutinho-Silva R (2017) The role of the P2X7 receptor in murine cutaneous leishmaniasis: aspects of inflammation and parasite control. Purinergic Signal 13: 143–152. https://doi.org/10.1007/s11302-016-9544-1 [pii]. PMID: 27866341

39. Secatto A, Soares EM, Locachevic GA, Assis PA, Paula-Silva FW, Serezani CH, de Medeiros AI, Faccioli LH (2014) The leukotriene B(4)/BLT(1) axis is a key determinant in susceptibility and resistance to histoplasmosis. PLoS One 9: e85083. https://doi.org/10.1371/journal.pone.0085083 PONE-D-13-25916 [pii]. PMID: 24465479

40. Bailie MB, Standiford TJ, Laichalk LL, Coffey MJ, Strieter R, Peters-Golden M (1996) Leukotriene-deficient mice manifest enhanced lethality from Klebsiella pneumonia in association with decreased alveolar macrophage phagocytic and bactericidal activities. J Immunol 157: 5221–5224. PMID: 8955165

41. Tavares N, Afonso L, Suarez M, Ampuero M, Prates DB, Araujo-Santos T, Barral-Netto M, DosReis GA, Borges VM, Brodskyn C (2016) Degranulating Neutrophils Promote Leukotriene B4 Production by Infected Macrophages To Kill Leishmania amazonensis Parasites. J Immunol 196: 1865–1873. jimmunol.1502224 [pii];https://doi.org/10.4049/jimmunol.1502224 PMID: 26800873

42. Chaves MM, Canetti C, Coutinho-Silva R (2016) Crosstalk between purinergic receptors and lipid mediators in leishmaniasis. Parasit Vectors 9: 489. https://doi.org/10.1186/s13071-016-1781-1 [pii]. PMID: 27595742

43. Feriotti C, Bazan SB, Loures FV, Araujo EF, Costa TA, Calich VL (2015) Expression of dectin-1 and enhanced activation of NALP3 inflammasome are associated with resistance to paracoccidioidomycosis. Front Microbiol 6: 913. https://doi.org/10.3389/fmicb.2015.00913 PMID: 26388856

44. Gurung P, Karki R, Vogel P, Watanabe M, Bix M, Lamkanfi M, Kanneganti TD (2015) An NLRP3 inflammasome-triggered Th2-biased adaptive immune response promotes leishmaniasis. J Clin Invest 125: 1329–1338. 79526 [pii];https://doi.org/10.1172/JCI79526 PMID: 25689249

45. Charmoy M, Hurrell BP, Romano A, Lee SH, Ribeiro-Gomes F, Riteau N, Mayer-Barber K, Tacchini-Cottier F, Sacks DL (2016) The Nlrp3 inflammasome, IL-1beta, and neutrophil recruitment are required for susceptibility to a nonhealing strain of Leishmania major in C57BL/6 mice. Eur J Immunol 46: 897–911. https://doi.org/10.1002/eji.201546015 PMID: 26689285

46. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440: 228–232. nature04515 [pii];https://doi.org/10.1038/nature04515 PMID: 16407890

47. Martin-Sanchez F, Diamond C, Zeitler M, Gomez AI, Baroja-Mazo A, Bagnall J, Spiller D, White M, Daniels MJ, Mortellaro A, Penalver M, Paszek P, Steringer JP, Nickel W, Brough D, Pelegrin P (2016) Inflammasome-dependent IL-1beta release depends upon membrane permeabilisation. Cell Death Differ 23: 1219–1231. cdd2015176 [pii];https://doi.org/10.1038/cdd.2015.176 PMID: 26868913

48. Frankenburg S, Leibovici V, Mansbach N, Turco SJ, Rosen G (1990) Effect of glycolipids of Leishmania parasites on human monocyte activity. Inhibition by lipophosphoglycan. J Immunol 145: 4284–4289. PMID: 2147940

49. Reiner NE, Ng W, Wilson CB, McMaster WR, Burchett SK (1990) Modulation of in vitro monocyte cytokine responses to Leishmania donovani. Interferon-gamma prevents parasite-induced inhibition of interleukin 1 production and primes monocytes to respond to Leishmania by producing both tumor necrosis

factor-alpha and interleukin 1. J Clin Invest 85: 1914–1924. https://doi.org/10.1172/JCI114654 PMID: 2112157

50. Gupta AK, Ghosh K, Palit S, Barua J, Das PK, Ukil A (2017) Leishmania donovani inhibits inflamma-some-dependent macrophage activation by exploiting the negative regulatory proteins A20 and UCP2. FASEB J 31: 5087–5101. fj.201700407R [pii];https://doi.org/10.1096/fj.201700407R PMID: 28765172

51. Shio MT, Christian JG, Jung JY, Chang KP, Olivier M (2015) PKC/ROS-Mediated NLRP3 Inflamma-some Activation Is Attenuated by Leishmania Zinc-Metalloprotease during Infection. PLoS Negl Trop Dis 9: e0003868. https://doi.org/10.1371/journal.pntd.0003868 PNTD-D-15-00312 [pii]. PMID: 26114647

52. Meunier E, Dick MS, Dreier RF, Schurmann N, Kenzelmann BD, Warming S, Roose-Girma M, Bumann D, Kayagaki N, Takeda K, Yamamoto M, Broz P (2014) Caspase-11 activation requires lysis of patho-gen-containing vacuoles by IFN-induced GTPases. Nature 509: 366–370. nature13157 [pii];https://doi.org/10.1038/nature13157 PMID: 24739961

53. Pilla DM, Hagar JA, Haldar AK, Mason AK, Degrandi D, Pfeffer K, Ernst RK, Yamamoto M, Miao EA, Coers J (2014) Guanylate binding proteins promote caspase-11-dependent pyroptosis in response to cytoplasmic LPS. Proc Natl Acad Sci U S A 111: 6046–6051. 1321700111 [pii];https://doi.org/10.1073/pnas.1321700111 PMID: 24715728

54. Gabrielli E, Pericolini E, Luciano E, Sabbatini S, Roselletti E, Perito S, Kasper L, Hube B, Vecchiarelli A (2015) Induction of caspase-11 by aspartyl proteinases of Candida albicans and implication in promot-ing inflammatory response. Infect Immun 83: 1940–1948. IAI.02895-14 [pii];https://doi.org/10.1128/IAI.02895-14 PMID: 25712931

55. Knodler LA, Crowley SM, Sham HP, Yang H, Wrande M, Ma C, Ernst RK, Steele-Mortimer O, Celli J, Vallance BA (2014) Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithe-lial defenses against enteric bacterial pathogens. Cell Host Microbe 16: 249–256. S1931-3128(14)00256-X [pii];https://doi.org/10.1016/j.chom.2014.07.002 PMID: 25121752

56. Lupfer CR, Anand PK, Liu Z, Stokes KL, Vogel P, Lamkanfi M, Kanneganti TD (2014) Reactive oxygen species regulate caspase-11 expression and activation of the non-canonical NLRP3 inflammasome during enteric pathogen infection. PLoS Pathog 10: e1004410. https://doi.org/10.1371/journal.ppat.1004410 PPATHOGENS-D-14-01592 [pii]. PMID: 25254654

57. Perez R, Balboa MA, Balsinde J (2006) Involvement of group VIA calcium-independent phospholipase A2 in macrophage engulfment of hydrogen peroxide-treated U937 cells. J Immunol 176: 2555–2561. 176/4/2555 [pii]. https://doi.org/10.4049/jimmunol.176.4.2555 PMID: 16456017

58. Perez R, Melero R, Balboa MA, Balsinde J (2004) Role of group VIA calcium-independent phospholi-pase A2 in arachidonic acid release, phospholipid fatty acid incorporation, and apoptosis in U937 cells responding to hydrogen peroxide. J Biol Chem 279: 40385–40391. https://doi.org/10.1074/jbc.M402562200 M402562200 [pii]. PMID: 15252038

59. Hewinson J, Mackenzie AB (2007) P2X(7) receptor-mediated reactive oxygen and nitrogen species for-mation: from receptor to generators. Biochem Soc Trans 35: 1168–1170. BST0351168 [pii];https://doi.org/10.1042/BST0351168 PMID: 17956304

60. Pfeiffer ZA, Guerra AN, Hill LM, Gavala ML, Prabhu U, Aga M, Hall DJ, Bertics PJ (2007) Nucleotide receptor signaling in murine macrophages is linked to reactive oxygen species generation. Free Radic Biol Med 42: 1506–1516. S0891-5849(07)00115-3 [pii];https://doi.org/10.1016/j.freeradbiomed.2007.02.010 PMID: 17448897

61. Noguchi T, Ishii K, Fukutomi H, Naguro I, Matsuzawa A, Takeda K, Ichijo H (2008) Requirement of reac-tive oxygen species-dependent activation of ASK1-p38 MAPK pathway for extracellular ATP-induced apoptosis in macrophage. J Biol Chem 283: 7657–7665. M708402200 [pii];https://doi.org/10.1074/jbc.M708402200 PMID: 18211888

62. Yun MR, Park HM, Seo KW, Lee SJ, Im DS, Kim CD (2010) 5-Lipoxygenase plays an essential role in 4-HNE-enhanced ROS production in murine macrophages via activation of NADPH oxidase. Free Radic Res 44: 742–750. https://doi.org/10.3109/10715761003758122 PMID: 20370567

63. Giuliani AL, Sarti AC, Di VF (2018) Extracellular nucleotides and nucleosides as signalling molecules. Immunol Lett. S0165-2478(18)30354-7 [pii];https://doi.org/10.1016/j.imlet.2018.11.006 PMID: 30439478

64. Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, Armstrong AJ, Penuela S, Laird DW, Salvesen GS, Isakson BE, Bayliss DA, Ravichandran KS (2010) Pannexin 1 channels medi-ate 'find-me' signal release and membrane permeability during apoptosis. Nature 467: 863–867. nature09413 [pii];https://doi.org/10.1038/nature09413 PMID: 20944749

65. Thorstenberg ML, Rangel Ferreira MV, Amorim N, Canetti C, Morrone FB, Alves Filho JC, Coutinho-Silva R (2018) Purinergic Cooperation Between P2Y2 and P2X7 Receptors Promote Cutaneous

Leishmaniasis Control: Involvement of Pannexin-1 and Leukotrienes. Front Immunol 9: 1531. https://doi.org/10.3389/fimmu.2018.01531 PMID: 30038612

66. Moreira-Souza ACA, Almeida-da-Silva CLC, Rangel TP, Rocha GDC, Bellio M, Zamboni DS, Vommaro RC, Coutinho-Silva R (2017) The P2X7 Receptor Mediates Toxoplasma gondii Control in Macrophages through Canonical NLRP3 Inflammasome Activation and Reactive Oxygen Species Production. Front Immunol 8: 1257. https://doi.org/10.3389/fimmu.2017.01257 PMID: 29075257

67. Lonardoni MV, Russo M, Jancar S (2000) Essential role of platelet-activating factor in control of Leishmania (Leishmania) amazonensis infection. Infect Immun 68: 6355–6361. https://doi.org/10.1128/iai.68.11.6355-6361.2000 PMID: 11035745

68. Titus RG, Marchand M, Boon T, Louis JA (1985) A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. Parasite Immunol 7: 545–555. PMID: 3877902