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IL-18 Triggered by the Nlrp3 Inflammasome Induces Host Innate Resistance in a Pulmonary Model of Fungal Infection

Natália Ketelut-Carneiro,* Grace Kelly Silva,* Fernanda Agostini Rocha,* Cristiane Maria Milanzei,* Florêncio Figueiredo Cavalcanti-Neto,† Dario Simões Zamboni,‡ and João Santana Silva*

Pathogens are sensed by innate immune receptors that initiate an efficient adaptive immune response upon activation. The elements of the innate immune recognition process for Paracoccidioides brasiliensis include TLR-2, TLR-4, and dectin-1. However, there are additional receptors necessary for the host immune responses to P. brasiliensis. The nucleotide-binding oligomerization domain–like receptor (NLRs), which activate inflammasomes, are candidate receptors that deserve renewed investigation. After pathogen infection, the NLRs form large signaling platforms called inflammasomes, which lead to caspase-1 activation and maturation of proinflammatory cytokines (IL-1β and IL-18). In this study, we showed that NLR family pyrin domain–containing 3 (Nlrp3) is required to induce caspase-1 activation and further secretion of IL-1β and IL-18 by P. brasiliensis–infected macrophages. Additionally, potassium efflux and lysosomal acidification induced by the fungus were important steps in the caspase-1 activation mechanism. Notably, Nlrp3 and caspase-1 knockout mice were more susceptible to infection than were the wild-type animals, suggesting that the Nlrp3-dependent inflammasomes contribute to host protection against P. brasiliensis. This protective effect occurred owing to the inflammatory response mediated by IL-18, as shown by an augmented fungus burden in IL-18 knockout mice. Taken together, our results show that the Nlrp3 inflammasome is essential for resistance against P. brasiliensis because it orchestrates robust caspase-1 activation and triggers an IL-18–dependent proinflammatory response. The Journal of Immunology, 2015, 194: 4507–4517.

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Abbreviations used in this article: Asc, apoptosis-associated speck-like protein containing a caspase recruitment domain; BMM, bone marrow macrophage; CFP, cyan fluorescent protein; dpi, day postinfection; FLICA, fluorescent-labeled inhibitor of caspasases; MOL, multiplicity of infection; Nac, N-acyctethylcysteine; NLR, nucleotide-binding oligomerization domain–like receptor; Nlrp3, nucleotide-binding oligomerization domain–like receptor family pyrin domain–containing 3; Pb18, Paracoccidioides brasiliensis varulente strain 18; PCM, paracoccidioidomycosis; PPR, pattern recognition receptor.

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(20). Recently, it was suggested that P. brasilensis induces Nlrp3 inflammasome–dependent IL-1β production (21). However, the signaling pathway, the mechanisms, and the role of the inflammasome in the control of this infection remain unknown. Thus, because macrophages are essential to the resolution of PCM and because there are additional PRRs involved in the activation of these cells during P. brasilensis infection, we hypothesized that inflammasomes could be engaged in innate immune responses against this fungus. Our results indicated that P. brasilensis is sensed by the Nlrp3 inflammasome, which orchestrates a vigorous caspase-1 activation and proinflammatory response. Additionally, we showed that IL-18 production, controlled by the Nlrp3 inflammasome, is required to control the fungal loads and to protect the host from death during experimental infection with P. brasilensis. Thus, we revealed a new signaling pathway involved in the regulation of pulmonary antifungal defenses.

Materials and Methods

Mice

For experimental infection and isolation of bone marrow macrophages (BMMs), male 6- to 7-wk-old caspase-1−/−, apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc−/−), Nlrp3−/−, IL-1R−/−, and IL-18−/−deficient mice (Casp1−/−, Asc−/−, Nlrp3−/−, IL1R−/−, and IL18−/−) and strain-matched wild-type (C57BL/6) mice were obtained from the Isogenic Breeding Unit at Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil. The mice were bred and maintained under specific pathogen-free conditions and provided with clean food and water ad libitum in the animal housing facility of the Department of Biochemistry and Immunology, University of São Paulo, Brazil, according to the recommendations of the Commission on Ethics in Animal Experiments. The experiments were conducted according to the ethical principles in animal research adopted by the Brazilian Society of Science in Animals Laboratory and approved by the Ethical Commission in Animal Research (protocol 046/2012).

Fungus, experimental infection, and mortality

The yeast cells of P. brasilensis virulent strain 18 (Pb18) were cultured for 7–10 d at 35–37˚C in brain heart infusion agar medium (Difco Laboratories) supplemented with gentamicin (100 µg/ml) and 5% FCS (Life Technologies). To prepare the inocula, the Pb18 cells were harvested and washed once in sterile PBS (pH 7.2–7.4), and their viability was determined as previously described (22). Only fungal suspensions containing >90% viable cells were used. Then, each animal was inoculated i.v. with 1 × 107 yeast cells (23). The survival of the Pb18-infected wild-type and knockout mice (10–12 mice of each group) was verified daily for 120 d.

Assay for CFUs

The numbers of viable yeast cells in the organs (lungs, liver, and spleen) of the Pb18-infected mice were determined at 7, 15, and 30 d postinfection (dpi) by counting the CFUs. Fragments of the lungs, liver, and spleen were aseptically collected, weighed, and homogenized using a sterile tissue grinder (IKA-Werke). The resulting macerate was diluted 10-fold in sterile PBS and plated onto Petri dishes containing brain heart infusion agar enriched with 5% FBS. The plates were incubated at 37˚C for 7 d, and the number of CFUs per gram of tissue was calculated.

Histopathological analysis

Animals selected at random from each group were sacrificed at 15 and 30 dpi. The lungs were excised, fixed with 10% formalin for 48 h, and embedded in paraffin. Tissue sections (5 µm) were stained with H&E for analysis of the lesions or impregnated with silver for demonstration of the fungus in the lung tissue using standard protocols.

Differentiation and culture of bone marrow–derived macrophages

Murine BMMs from wild-type, Asc−/−, Casp1−/−, and Nlrp3−/− mice were differentiated. The BMMs were obtained as previously described (24). Briefly, the total bone marrow cells were cultured for 7 d in RPMI 1640 medium (Sigma-Aldrich), supplemented with 20% FBS (Invitrogen, Carlsbad, CA) and 30% L-929 cell–conditioned media, at 37˚C and 5% CO2. The cells were infected with viable fungi at a multiplicity of infection (MOI) of 1 or 5. Prior to infection, the BMMs were treated for 2 h with one of the following reagents: 50 or 130 mM potassium chloride, 50 or 130 mM sodium chloride, 50 or 100 µM glibenclamide, 0.1, 1, or 10 µM chloroquine, 1, 10, or 25 mM N-acetylcysteine (Nac), or 20 or 50 µM CA-074Me (all from Sigma-Aldrich).

Measurement of cytokines by ELISA

BMMs (2 × 105 cells/well) from the wild-type or knockout mice were infected with P. brasilensis (MOI of 5) for 48 h. The levels of IL-1β, IL-18, and TNF-α in the culture supernatants were measured using standard sandwich ELISAs according to the manufacturer’s recommendations (BD Biosciences, San Jose, CA).

Detection of activated caspase-1

The BMMs (105 cells/well) were infected with P. brasilensis as described above. After 12 h of incubation, the cells were stained with a caspase-1 fluorescent-labeled inhibitor of caspases (FLICA) kit (Immunochemistry Technologies) according to the manufacturer’s instructions. Flow cytometric analysis to assess intracellular caspase-1 activity in the infected BMMs was performed in a BD FACSCanto II machine (BD Biosciences), and the data were analyzed using FlowJo software. The cell-free supernatants were collected after 48 h of incubation, and the IL-1β (Sigma-Aldrich) was detected by Western blotting, as previously described (25). The bands were quantified densitometrically using the ImageTool 2.0 software (University of Texas), and the results were expressed as arbitrary units. In the Western blotting experiment we also used a combination of LPS and nigericin as a positive control. BMMs were primed with 500 ng/ml ultrapure LPS (InvivoGen, itrl-pkps) for 6 h and then stimulated with 20 µg nigericin (Sigma-Aldrich) for 40 min.

Inflammasome PCR array

At the 30 dpi time point, the lungs from three uninfected (controls) and three infected C57BL/6 mice were collected, and the total RNA was purified using PureLink microarray mRNA isolation kit (Invitrogen). The RT2 first-strand kit was used to convert the RNA into cDNA (Qiagen). SYBR Green qPCR Master mix a and a 96-well plate precoated with preamplified gene-specific cDNA targets were used, both of which were provided by the manufacturer (SABiosciences). A StepOnePlus PCR system (Applied Biosystems) was used for RT-PCR. The data were uploaded onto the manufacturer’s Web site (Applied Biosystems) for analysis.

Inflammasome assembly assay

The cyan fluorescent protein (CFP)-Asc− and citrine-Nlrp3–expressing macrophages were generated as described previously (26, 27). Briefly, 1.5 × 105 cells were seeded into 35-mm confocal dishes and infected with P. brasilensis (MOI of 5) that had previously been stained with 1 µM caspase-1 fluorescent-labeled inhibitor of caspases (FLICA) kit (Immunochemistry Technologies) according to the manufacturer’s instructions. For the intracellular cytokine staining, the cells were previously permeabilized using 1 × PBS containing 1% FBS, 0.1% sodium azide, and 0.2% saponin. The cellular data were acquired using a Neubauer camera and a light microscope.

Flow cytometry

The cells were stained with Abs specific for the surface molecules CD3 and CD4 for the intracellular cytokines IFN-γ and IL-17. The Abs were conjugated to PerCP, PE-Cy7, aliphophycocyanin, Alexa Fluor 647, or aliphophycocyanin-Cy7 fluorochromes (BD Biosciences, eBioscience, and Santa Cruz Biotechnology). For the intracellular cytokine staining, the cells were previously permeabilized using 1 × PBS containing 1% FBS, 0.1% sodium azide, and 0.2% saponin. The cellular data were acquired using a FACSCanto II flow cytometer and FACSDiva software (BD Biosciences). The data were plotted and analyzed using FlowJo software (Tree Star, Ashland, OR).

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**Results**

*P. brasiliensis* upregulates inflammasome genes and induces caspase-1–dependent IL-1β production

To identify additional receptors that are engaged during the innate immune recognition of *P. brasiliensis*, we first performed a PCR array analysis to evaluate the expression of a panel of inflammasome genes in the lungs of wild-type mice at 30 dpi. The array allowed us to determine the expression of 84 known signaling molecules downstream of the NLRs and showed that *P. brasiliensis* alters the expression profiles of the target genes. Several genes related to inflammasome function were upregulated compared with the uninfected control mice. In particular, we found >3-fold changes in the mRNA levels of *Nlpr3*, *Pycard/Asc*, *Casp1*, and *Ilb* (Supplemental Table I). Curiously, the expression levels of the chemokine genes associated with the recruitment of monocytes and neutrophils were dramatically elevated at the site of infection compared with the uninfected mice. The expression of *Nlrp6* and *Nlrp12* was not affected (Fig. 1A). Because *P. brasiliensis* infection induced the expression of caspase-1 and *Nlrp3*, we asked whether the fungus was able to trigger IL-1β secretion (indirect caspase-1 activation). We found that BMMs exposed to viable *P. brasiliensis* yeast for 48 h produced significant levels of IL-1β when compared with the nonstimulated BMMs. Additionally, only live *P. brasiliensis* stimulated significant IL-1β production (Fig. 1B). Consistent with these results, the Western blotting analysis showed that the mature form of IL-1β (p17) was being produced in response to *P. brasiliensis* in the supernatants of the BMMs incubated with viable yeast cells and that the release of IL-1β started at 12 h and increased markedly at 48 h postinfection. As expected, IL-1β was processed after activation of the *Nlrp3* inflammasome induced by nigericin after priming with LPS (positive control) (Fig. 1C).

Because active caspase-1 is essential for the cleavage of pro–IL-1β to its mature and biologically active form, we speculated that *P. brasiliensis* could trigger the production of the IL-1β–activating caspase-1. We quantified the activated caspase-1 in the *P. brasiliensis*–infected macrophages using a fluorescent marker, FAM-YVAD-FMK (FLICA<sup>Casp1</sup>), which can specifically bind to the active form of caspase-1. Flow cytometric analysis revealed that caspase-1 was activated at 12 h postinfection (Fig. 1D). Taken together, these data indicate that *P. brasiliensis* promotes caspase-1 activation to release the bioactive IL-1β, suggesting the requirement of the inflammasome during experimental PCM.

**P. brasiliensis**–induced caspase-1 and IL-1β release by infected macrophages is Asc inflammasome–dependent

To define the molecular mechanisms that regulate caspase-1–mediated processing of IL-1β, we investigated which inflammasome could be involved in *P. brasiliensis* recognition. Because Asc is a common adaptor for a diverse set of inflammasomes, we infected wild-type, Asc<sup>−/−</sup>, and Casp1<sup>−/−</sup> BMMs with *P. brasiliensis* and evaluated the IL-1β levels. In the absence of these inflammasome components, the secretion of IL-1β was suppressed when compared with the infected wild-type BMMs (Fig. 2A). The abrogation of IL-1β production observed in the infected Asc<sup>−/−</sup> and Casp1<sup>−/−</sup> BMMs was specifically related to the inflammasome because the release of inflammasome-independent proinflammatory cytokines such as TNF-α was similar in all groups analyzed (Fig. 2A). Moreover, we showed that the mature form of IL-1β was present in the wild-type BMMs following exposure to *P. brasiliensis* infection or activation of the Nlrp3 inflammasome with LPS plus nigericin but was diminished in Asc<sup>−/−</sup> or Casp1<sup>−/−</sup> BMMs at 48 h postinfection (Fig. 2B). We also found that the Asc pathway was engaged in the activation of caspase-1 because the percentage of Fica<sup>+</sup> cells was significantly diminished in the Asc<sup>−/−</sup> and Casp1<sup>−/−</sup> mice compared with the wild-type group (Fig. 2C). The participation of the Asc inflammasome in *P. brasiliensis* infection was confirmed when an immortalized macrophage cell line that expresses ectopic Asc tagged with CFP was analyzed by confocal microscopy. In the absence of stimuli, these cells exhibit diffuse fluorescence in the cytoplasm, and the activation by certain stimuli causes a cluster of ASC-CFP molecules, which is visible under confocal microscopy as a single bright fluorescent “speck” in the cytosol (26, 27). After incubating these cells for 72 h with *P. brasiliensis* that had previously been stained with a red fluorescent dye (CMPTX), we visualized the formation of fluorescent clusters of ASC-CFP in the infected macrophages, indicating that the Asc inflammasome is activated during *P. brasiliensis* infection (Fig. 2D). Altogether, the results indicate that IL-1β release and caspase-1 activation during *P. brasiliensis* infection are dependent on the Asc inflammasome.

*P. brasiliensis* infection induces Nlrp3 inflammasome activation

Because Asc interacts with several NLRs, including Nlrp3 (12, 28, 29), we investigated whether this receptor contributes to inflammasome formation in response to *P. brasiliensis* infection. We observed that similar to the Asc<sup>−/−</sup> and Casp1<sup>−/−</sup> macrophages, the Nlrp3<sup>−/−</sup> macrophages also demonstrated a significant reduction in IL-1β production (Fig. 3A) and caspase-1 activation (Fig. 3B) following Pb18 infection. Importantly, note that the Western blotting results (Fig. 3C) support the ELISA data, which showed that IL-1β production was diminished in Asc<sup>−/−</sup>, Casp1<sup>−/−</sup>, and Nlrp3<sup>−/−</sup> *P. brasiliensis*–infected BMMs but was not abolished. Furthermore, we observed an accumulation of pro–IL-1β in Asc<sup>−/−</sup>, Casp1<sup>−/−</sup>, and Nlrp3<sup>−/−</sup> BMMs compared with the positive control, demonstrating that IL-1β was not promptly cleaved (Fig. 3C). To monitor the Nlrp3 activation, we infected the Nlrp3–citrine–overexpressing macrophages with CMPTX-stained *P. brasiliensis* and observed the speck formation. At 72 h postinfection, we found that foci of Nlrp3 had formed in the infected cells, indicating that the inflammasomes assembled in response to *P. brasiliensis* infection (Fig. 3D). We conclude that the caspase-1 activation and IL-1β secretion induced by *P. brasiliensis* depend on the Nlrp3 inflammasome. Next, we investigated whether treatment with inhibitors of potassium efflux, lysosomal acidification, generation of reactive oxygen species, or lysosomal de-stabilization would abrogate the IL-1β production induced by *P. brasiliensis*. We found a significant reduction in the release of IL-1β after treatment with glibenclamide (an inhibitor of K⁺ efflux) (Fig. 3E). However, because this drug can directly interact with Asc and impair the IL-1β release (30, 31), we pretreated the BMMs with KCl (50 and 150 mM) and assayed the IL-1β production. Similar to pretreatment with glibenclamide, pretreatment with KCl, but not with NaCl, inhibited IL-1β production by wild-type BMMs infected with *P. brasiliensis* in a dose-dependent manner (Fig. 3E). Moreover, the addition of chloroquine (an inhibitor of endosomal-lysosomal acidification) also interfered with IL-1β secretion (Fig. 3F). No effect was observed after treatment with Nac (Supplemental Fig. 1A) or CA-074Me (Supplemental Fig. 1B). Collectively, the above results demonstrated that potas-
sium efflux and lysosomal acidification are important mechanisms involved in *P. brasiliensis*-induced inflammasome activation.

**Caspase-1 mediates resistance to *P. brasiliensis* infection**

To determine the roles of Asc and caspase-1 during PCM, the survival rates of the infected wild-type, Asc$^{-/-}$, and Casp1$^{-/-}$ mice were analyzed for 120 d. We found that the Casp1$^{-/-}$ mice were more susceptible to experimental infection with *P. brasiliensis* than were wild-type animals: 100% of the knockout mice succumbed to the fungal challenge within 100 dpi (Fig. 4A). Interestingly, the Asc$^{-/-}$ and wild-type mice exhibited an intermediate phenotype in terms of mortality rates, suggesting that other adapter molecules are engaged in the caspase-1 signaling pathway responsible for protection against *P. brasiliensis* infection (Fig. 4A). To investigate the roles of Asc and caspase-1 in the control of fungal replication, we determined the CFUs in the infected wild-type, Asc$^{-/-}$, and Casp1$^{-/-}$ mice. The Asc- and caspase-1–deficient mice exhibited higher numbers of CFUs in their lungs than did the wild-type mice at 30 dpi (Fig. 4B). Additionally, the histological investigation also demonstrated more *P. brasiliensis* in the lungs of the Asc$^{-/-}$ and Casp1$^{-/-}$ mice at 30 dpi when compared with the wild-type mice (Fig. 4C). Furthermore, in the same postinfection period, we observed a significantly increased number of fungi recovered from the livers of the Casp1$^{-/-}$ mice and equivalent fungal counts in the spleen tissues of both knockout mouse strains (data not shown). Curiously, at 15 dpi, only the Casp1$^{-/-}$ mice demonstrated high fungal burdens compared with the infected Asc$^{-/-}$ and wild-type animals (Fig. 4B). These results indicate that caspase-1 modulates additional mechanisms, independent of the Asc inflammasome, responsible for fungal growth in the early stages of infection. Taken together, our data indicate that a completely caspase-1–dependent and partially Asc-dependent mechanism are required to control *P. brasiliensis* infection.

**Caspase-1 modulates granuloma formation independently of the Asc inflammasome**

To address whether the protection dependent on Asc and caspase-1 is associated with the formation of compact granulomas and reduced pulmonary injury, histological lung sections from wild-type, Asc$^{-/-}$, and Casp1$^{-/-}$ mice were analyzed at 15 and 30 dpi. At 15 dpi, the wild-type and Asc$^{-/-}$–infected mice exhibited a diffuse pattern of inflammation with few foci of well-organized granulomas. Additionally, the photomicrographs reveal that the alveolar

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**FIGURE 1.** *P. brasiliensis* upregulates the expression of inflammasome component genes and triggers IL-1β production by macrophages through caspase-1 signaling. (A) Gene expression profile of lung tissue from three wild-type mice infected for 30 d with *P. brasiliensis*. The heat map represents the relative fold changes of inflammasome-induced genes normalized to heat shock protein 90α (Hsp90ab1). (B) BMMs from wild-type mice were either nonstimulated (medium) or stimulated with live or heat-killed Pb18 yeast (MOI of 5). After 48 h of incubation in the presence of the fungus, the culture supernatants from the infected BMMs were collected to measure the concentrations of secreted IL-1β by ELISA. (C) Kinetics of IL-1β release at 12, 24, and 48 h postinfection to detect the p17 subunit of the cytokine by Western blotting. The intensities of immunoreactive bands in the Western blotting were quantified through densitometric analysis. Wild-type BMMs stimulated for 6 h with LPS (500 ng/ml) and treated with 20 μM nigericin for 40 min were used as the positive control. (D) Caspase-1 activation was verified by measuring the fluorescence of BMMs via flow cytometry after 12 h of culture with the fungus. NI, noninfected. The bars represent the average ± SEM of three animals tested individually. The experiments were performed in triplicate. The data shown are representative of at least three independent experiments. *p < 0.05 relative to the medium.
spaces in the lung tissue of the infected Asc\(^{-/-}\) mice were filled with neutrophils, monocytes, and lymphocytes (Fig. 4D). Curiously, the Casp1\(^{-/-}\) infected mice showed early formation of lung granulomas. The granulomatous lesions were surrounded by a rim composed of high numbers of monocytes, histiocytes, epithelioid cells, and giant cells (Fig. 4D). After 30 dpi, the lung injury assumed a more compact aspect, with well-defined and confluent granulomas in the wild-type and Casp1\(^{-/-}\) mice. However, granuloma formation in the Casp1\(^{-/-}\) mice was not able to control fungal growth. Therefore, although a lack of caspase-1 does not affect granuloma formation, it also does not prevent P. brasiliensis dissemination.

Nlrp3 is the pivotal upstream receptor involved in the control of P. brasiliensis infection

To assess the significance of the Nlrp3 pathway in the susceptibility to PCM, the wild-type and Nlrp3\(^{-/-}\) mice were infected with 2 \times 10^6 yeast cells, and survival was monitored for up to 120 dpi. The Nlrp3\(^{-/-}\) mice showed decreased survival in response to the fungal challenge, as indicated by the deaths of 100% of these mice within 120 dpi, whereas the wild-type mice were resistant (Fig. 5A). To determine whether the failure of the Nlrp3-deficient mice to survive was associated with a defect in the clearance of the fungi from the lungs, we determined the fungal counts at 15 and 30 d after P. brasiliensis infection. Although at 15 dpi, similar numbers of fungi were observed in the lungs of the wild-type and Nlrp3\(^{-/-}\) mice, at 30 dpi, the Nlrp3\(^{-/-}\) mice carried considerably higher CFUs compared with the wild-type mice. Moreover, at 30 dpi, Nlrp3\(^{-/-}\) mice, as well as Casp1\(^{-/-}\) mice, harbored an augmented fungal load in the liver (Fig. 5B) and similar fungus counts in the spleen compared with wild-type infected mice (data not shown). Thus, the Nlrp3\(^{-/-}\) mice have a defect in fungal clearance, which most likely explains the decreased survival of the Nlrp3\(^{-/-}\) mice following the P. brasiliensis infection. Additionally, the absence of the Nlrp3 receptor did not prevent granuloma formation, based on the observation that the infected Nlrp3\(^{-/-}\) mice exhibited organized granulomas in their lung tissue at 30 dpi, and the lung parenchyma was not consolidated with inflammatory cells (Fig. 5C). Taken together, these results support the concept that Nlrp3 is the upstream receptor required for fungal growth restriction after P. brasiliensis infection.

IL-18 production by the Nlrp3 inflammasome is a critical mechanism for protection from PCM

Because IL-1\beta and IL-18 are the effector molecules of the inflammasome signaling pathway, we analyzed the role of the inflammasome-dependent cytokines during murine PCM. To assess the influence of IL-1 and IL-18 deficiency on the disease outcome, the mortality rates of P. brasiliensis–infected Il1r1\(^{-/-}\), Il18\(^{-/-}\), and wild-type mice were evaluated. We observed that whereas the IL-18–deficient mice were extremely susceptible to P. brasiliensis infection (Fig. 6A), the Il1r1\(^{-/-}\) mice displayed a slightly increased resistance to P. brasiliensis infection compared with the wild-type mice (Fig. 6B). To elucidate the mechanisms modulated by IL-18 and IL-1 following P. brasiliensis infection, we evaluated the fungal loads and local inflammatory lesions during the course of infection in the Il1r1\(^{-/-}\) and Il18\(^{-/-}\) infected mice. The difference in susceptibility between the Il18\(^{-/-}\) and Il1r1\(^{-/-}\) mice to PCM may be explained by the disseminated infection observed in the absence of IL-18. In the lungs of both strains of knockout mice, the fungal burdens were dramatically higher than in the infected wild-type animals. However, significantly lower amounts of the fungus were recovered at 30 dpi in the livers and spleens of the Il1r1\(^{-/-}\) mice compared with the
IL18−/− mice (Fig. 6C, 6D). Histopathological lung analysis was conducted at week 4 of infection, and as shown in Fig. 6E, the granulomas had not formed in the IL18−/− mice in contrast to the wild-type mice, leading to a disorganized inflammatory process. The lack of granulomas correlated with the increase in the number of fungi recovered. In contrast, in the II1R1−/− mice, the fungus was contained in organized and compact granulomas (Fig. 6F). Furthermore, examination of the liver sections showed that the II1R1−/− and IL18−/− mice formed larger granulomas than did the wild-type mice (Fig. 6E, 6F). These results suggest that the two cytokines investigated are important in controlling the fungus, but only IL-18 is required to contain the fungus in the granulomas and to promote resistance to experimental P. brasiliensis infection.

Considering these results, we suggest that the susceptibility of the Asc−/− and Casp1−/− mice could be due to their impaired IL-18 production. To test this hypothesis, we quantified IL-18 in the lung homogenates obtained from the infected Asc−/−, Casp1−/−, and Nlrp3−/− mice. At 30 dpi, the IL-18 levels per milligram of tissue were severely reduced in the Casp1−/− and Nlrp3−/− mice compared with the wild-type mice (Fig. 7A). Thus, we conclude that the IL-18 produced by the Nlrp3 inflammasome plays a critical role in the host resistance to the granulomatous disease caused by P. brasiliensis infection. Moreover, we also demonstrated that in infected lung tissue samples, IFN-γ production, which is induced by P. brasiliensis infection in the wild-type mice, was diminished in the Asc−/−, Casp1−/−, and Nlrp3−/− mice at 30 dpi, a finding consistent with the established function of IL-18 as an IFN-γ–inducing cytokine (Fig. 7B). In accordance with the decreased IFN-γ levels, at 30 dpi, the Asc−/−, caspase-1−/−, and Nlrp3−/− deficient mice were unable to generate IFN-γ–producing Th1 cells in their lungs in contrast to wild-type mice, which showed an effective Th1 response (Fig. 7C). However, although CD4+ T cells are the major source of IFN-γ during PCM, this cytokine can also be produced by other cell types, including NK cells and CD8+ T cells (32, 33). When NK cells and CD8+ T cells were phenotyped, we did not note any change in IFN-γ production, either by NK cells or CD8+ T cells in the absence of Asc, caspase-1, and Nlrp3 in week 2 of infection (data not shown). As IL-1β is an inflammasome-induced cytokine that triggers Th17 differentiation, which plays a critical role in host protection, we decided to analyze the generation of Th17 in lungs obtained from Asc−/−, Casp1−/−, and Nlrp3−/− mice that had been infected with
We found that even in the absence of the inflammasome signaling pathway, a normal pulmonary Th17 response was mounted after *P. brasiliensis* infection (Supplemental Fig. 2), reinforcing the conclusion that in *P. brasiliensis* infection, the Nlrp3 inflammasome is associated with Th1 but not Th17.

Taken together, these data reveal that the effector mechanism in vivo, which is modulated by the inflammasomes and associated with resistance to this infection, correlates with IFN-γ levels.

Finally, to confirm the idea that the production of IL-18 is the key mechanism modulated by caspase-1 and is responsible for resist-

![FIGURE 4](image)

**FIGURE 4.** Asc and caspase-1 control fungal growth during experimental infection with *P. brasiliensis*. (A) The mortality was evaluated daily in the wild-type, Asc<sup>−/−</sup>, and Casp1<sup>−/−</sup> mice infected i.v. with 2 × 10<sup>6</sup> viable Pb18 yeast (10–12 mice/group). (B) At 15 and 30 dpi, the lung lobes and liver were collected from the wild-type, Asc<sup>−/−</sup>, and Casp1<sup>−/−</sup> mice for evaluation of the recovery of CFUs. (C) Microscopic analysis of the histological sections stained using silver impregnation (Gomori method). (D) H&E staining of the lung tissue at days 15 and 30 dpi. Each point represents the mean ± SEM of the organs from four mice in three independent experiments. *p < 0.05 compared with the wild-type mice.

![FIGURE 5](image)

**FIGURE 5.** The Nlrp3 inflammasome is crucial for host innate immune responses against *P. brasiliensis*. (A) Wild-type and Nlrp3<sup>−/−</sup> mice were infected i.v. with 2 × 10<sup>6</sup> viable yeast cells, and the survival of the Pb18-infected mice (10 mice of each group) was verified daily for 120 d. (B) The number of CFUs was determined in the lungs and liver at days 15 and 30 dpi with 1 × 10<sup>6</sup> Pb18 yeast. (C) Photomicrographs (original magnification, ×200) of granuloma lesions from the wild-type and Nlrp3<sup>−/−</sup> mice at 30 dpi. The lung sections were fixed in formalin, paraffin embedded, stained with H&E, and analyzed by light microscopy. Each point represents the mean ± SEM of the organs from five to seven mice. *p < 0.05 for the knockout versus the C57BL/6 mice.
tance during *P. brasiliensis* infection, Casp1<sup>−/−</sup> mice were infected i.v. with 1 × 10<sup>6</sup> fungal cells, and 1 μg rIL-18 was injected i.p. (Fig. 7D). The evolution of the disease in the IL-18–treated and untreated Casp1<sup>−/−</sup> mice was monitored by CFU counts in the lungs at 15 dpi. The administration of rIL-18 altered the infection pattern in the lung, resulting in a significant reduction in the fungal burden in the treated Casp1<sup>−/−</sup> mice compared with the untreated Casp1<sup>−/−</sup> mice (Fig. 7E). Thus, we conclude that the Nlrp3 inflammasome promotes a protective Th1 response by inducing IL-18 secretion. Our findings demonstrate a novel innate immune pathway involved in host resistance to infection by *P. brasiliensis*.

**Discussion**

The innate immune response to *P. brasiliensis* infection involves several PRRs, including TLR-2 and TLR-4 as well as dectin-1 (5, 6). However, cytokine production was not completely eliminated in MyD88-deficient mice (7), suggesting that additional PRRs may be involved in the host immune responses to *P. brasiliensis*. Specifically, the NLRs that activate caspase-1 and form the platform molecular structure called the inflammasome may be involved in the efficient innate immune response against *P. brasiliensis*. In this study, we reveal that *P. brasiliensis* triggered caspase-1 activation and IL-1β production in vitro. Interestingly, only live *P. brasiliensis* efficiently induced the IL-1β release and caspase-1 maturation, indicating that virulence factors are necessary to generate the cellular damage recognized by the NLRs and cause caspase-1 activation. These data are similar to those from several other studies showing that live pathogens are required to activate caspase-1 (34).

We then investigated the receptors and inflammasome components involved in *P. brasiliensis*–mediated caspase-1 activation and showed that Asc inflammasomes were required to promote IL-1β induction during *P. brasiliensis* infection. Moreover, in this study, we demonstrated that Nlrp3 is a central receptor engaged in this signaling pathway on the basis that profound suppression of IL-1β production was observed in macrophages from Nlrp3-deficient mice. Our results also showed that Nlrp3<sup>−/−</sup> macrophages are still able to produce a small amount of IL-1β. However, no significant difference was observed when we compared medium- and *P. brasiliensis*–infected samples. Therefore, these data suggest that IL-1β activation did not occur via the non-canonical pathway, revealing a significant and exclusive role of caspase-1 signaling in the production of IL-1β during *P. brasili-
P. brasiliensis infection. Additionally, a detectable amount of pro–IL-1β was observed in supernatants obtained from Casp1−/− and Nlrp3−/− macrophages compared with the positive control, suggesting that caspase-1 signaling is essential to control the formation of pro–IL-1β, possibly through regulating the transcription factor NF-κB, which is necessary for pro–IL-1β production (35).

The question arising from these data are how Nlrp3 senses P. brasiliensis. Because activation of the Nlrp3 inflammasome induced by P. brasiliensis was inhibited by adding KCl and chloroquine to the cultures, it is conceivable that the postinfection Nlrp3 inflammasome activation induced by Pb18 depends on K⁺ efflux and lysosomal acidification. Consistent with this hypothesis, the addition of glibenclamide to the cell cultures resulted in a significant decrease in the secretion of IL-1β from the wild-type macrophages infected with P. brasiliensis. The generation of reactive oxygen species and cathepsin B release from damaged lysosomal compartments are additional mechanisms that have been proposed for the activation of the Nlrp3 inflammasome. Our investigations with wild-type macrophages treated with Nac or CA-074Me indicate that neither of these mechanisms is responsible for triggering Nlrp3 activation in P. brasiliensis–infected cells. Additionally, we detected a significant amount of IL-1β in Nlrp3−/− BMMs compared with uninfected BMMs, which suggests that additional receptors are involved in P. brasiliensis–mediated caspase-1 activation. Because Asc interacts with several NLRs, including Nlrp6 (36), Nlrp10 (37), and the intracellular AIM2 receptor (38–41), we speculated that these receptors may interact with Asc and cooperate with Nlrp3 in stimulating an efficient innate immune response against P. brasiliensis.

Notably, our study using an experimental model of P. brasiliensis infection showed a significant abnormal phenotype in the inflammasome-deficient mice, providing in vivo support to our in vitro findings. The infected Casp1−/− and Nlrp3−/− mice demonstrated a significantly greater mortality and fungal burden than did the infected wild-type animals. Therefore, we conclude that inflammasomes are required for resistance to experimental P. brasiliensis infection. Interestingly, the P. brasiliensis–infected Casp1−/− mice demonstrated a higher susceptibility than did the Asc−/− animals, revealing that caspase-1 is a bona fide pathway...
involved in the PCM response. Additionally, we also conclude that caspase-1 orchestrates additional mechanisms responsible for the control of this fungus in vivo because the Casp1−/− animals failed to eliminate the P. brasiliensis in the early phases of this mycosis compared with the infected Asc−/− animals. Because granuloma formation is the crucial process for restricting the expansion of the fungi during chronic P. brasiliensis infection, the absence of an appropriate granulomatous response could be the cause of the fungal persistence. However, we found that the Nlrp3−/− and Casp1−/− mice did not eradicate the P. brasiliensis infection even after the development of granulomas. Supporting these findings, it has also been demonstrated that Nlrp3 and caspase-1 are not required in granuloma formation in tuberculosis (42). Importantly, histological examination also showed that the size of the granulomas found in the lungs of Nlrp3−/− mice was larger than that in WT, which is correlated with the increased fungal burden. Thus, Nlrp3−/− mice harbored higher fungal counts, which are proportional to granuloma size. Furthermore, as there were no inflammatory cells observed in the lung parenchyma of Nlrp3−/− mice, we suggest that these animals die because they present uncontrolled fungal growth.

The caspase-1–dependent cytokines exert important effects on the initiation of the adaptive Th1 and Th17 cellular responses to fungal infection (43). No studies have previously assessed the impact of the inflammusomes on the Th1 defense mechanisms during PCM. Because IFN-γ is an important cytokine involved in the resistance to P. brasiliensis and other several fungi (11, 27, 44–50), we speculated that IFN-γ could play a role in the central mechanism regulated by caspase-1 after P. brasiliensis infection. Confirming this hypothesis, IFN-γ production and the Th1 response were lower in infected Asc−/−, Casp1−/−, and Nlrp3−/− mice compared with the wild-type mice. Additionally, treatment of the infected Casp1−/− mice with IL-18 reduced the increase in CFUs at 15 dpi. Alternatively, when we investigated whether Asc−/−, Casp1−/−, and Nlrp3−/− mice presented reduced generation of Th17 cells, we did not observe a significant difference in the number of IL-17–producing T cells. Furthermore, the production of IFN-γ by NK cells and CD8+ T cells remained unchanged between the analyzed groups (data not shown). Therefore, we suggest that the Asc−/−, Casp1−/−, and Nlrp3−/− mice were susceptible to experimental PCM because they exhibited reduced Th1 (and not Th17) immunity. It has been established that mice lacking a functional IFN-γ gene, in addition to forming incipient granulomas, are unable to contain a virulent P. brasiliensis infection and produce less TNF-α (II), which is thought to be responsible for attracting and activating effector cells as well as for macrophage accumulation and differentiation into epithelioid cells (51). Further studies are necessary to define whether there is a differential influx of other cell populations, to characterize the cellular profile in situ, and to determine the participation of the inflammusome in the migration of lung-infiltrating leukocytes in our model of infection. Thus, caspase-1, via the Nlrp3 inflammusome, is required to produce IL-18, which then induces IFN-γ and promotes efficient killing of this pathogen. Consistent with this process, Asc and caspase-1 also effectively regulate the IL-18/IFN-γ axis upon Anaplasma phagocytophilum infection (52).

Remarkably, our results showed that whereas the IL18−/− mice are profoundly vulnerable to PCM, the II1R1−/− mice unexpectedly demonstrated mortality rates similar to the wild-type mice. However, despite both genotypes having higher amounts of fungi in their lungs compared with wild-type, the localization of P. brasiliensis is substantially different between the infected II1R1−/− and IL18−/− mice. The lungs from the II18−/− infected mice showed a large amount of P. brasiliensis in nongranulomatous tissue. Conversely, almost no fungus was found outside of the lung granulomas in the II1R1−/− mice at 30 dpi, demonstrating that these animals were able to generate a granulomatous response that contained the fungal growth. Therefore, the protective role of IL-18 during PCM, which appears to be related to its ability to induce IFN-γ, is important for the resistance to infection by many pathogens, including Burkholderia pseudomallei and Streptococcus pneumoniae (53, 54). It was interesting and surprising that the Nlrp3−/− and Casp1−/− mice, which are defective in IL-1β and IL-18 production, were more resistant to P. brasiliensis than were the mice that lack IL-18. Consistent with these data, IL-18 has been shown to be upregulated in a caspase–1–dependent manner in response to C. albicans as well as to mediate the development of protective Th1 immunity (55, 56).

In summary, the present data define a signaling pathway that leads to the integration of innate and adaptive host immune responses against infection with P. brasiliensis. These results indicate that the inflammusome is activated and that Nlrp3, Asc, and caspase-1 mediate P. brasiliensis–induced IL-18 production, which promotes a host antifungal defense that elicits a strong Th1-mediated immune response.

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Disclosures

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References


36. Ceballos-Olvera, L. M., S. Ahmad, M. A. Miller, D. Del Barrio, and F. Re. 2011. Inflammation-dependent pyroptosis and IL-1β protect against Bubulcusia pseudomallei lung infection while IL-1β is deleterious. PLoS Pathog. 7: e1002452.

