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J Immunol published online 4 March 2013
http://www.jimmunol.org/content/early/2013/03/03/jimmunol.1202817
Critical Role of ASC Inflammasomes and Bacterial Type IV Secretion System in Caspase-1 Activation and Host Innate Resistance to *Brucella abortus* Infection

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Pathogens are detected by innate immune receptors that, upon activation, orchestrate an appropriate immune response. Recent studies revealed the intracellular signaling cascades involved in the TLR-initiated immune response to *Brucella abortus* infection. However, no report has elucidated the role of inflammasome receptors in *Brucella* recognition. Therefore, we decided to investigate the function of NLRC4, NLRP3, and AIM2 in sensing *Brucella*. In this study, we showed that NLRC4 is not required to induce caspase-1 activation and further secretion of IL-1β by *B. abortus* in macrophages. In contrast, we determined that AIM2, which senses *Brucella* DNA, and NLRP3 are partially required for caspase-1 activation and IL-1β secretion. Additionally, mitochondrial reactive oxygen species induced by *Brucella* were implicated in IL-1β production. Furthermore, AIM2, NLRP3, ASC, and caspase-1 knockout mice were more susceptible to *B. abortus* infection than were wild-type animals, suggesting that multiple ASC-dependent inflammasomes contribute to host protection against infection. This protective effect is due to the inflammatory response caused by IL-1β and IL-18 rather than pyroptosis, because we observed augmented bacterial burden in IL-1R and IL-18 knockout mice. Finally, we determined that bacterial type IV secretion system VirB and live, but not heat-killed, *Brucella* are required for full inflammasome activation in macrophages during infection. Taken together, our results indicate that *Brucella* is sensed by ASC inflammasomes that collectively orchestrate a robust caspase-1 activation and proinflammatory response. The Journal of Immunology, 2013, 190: 000–000.

Innate immune cells sense pathogens through pattern-recognition receptors and respond rapidly by producing cytokines and antimicrobial intermediates (1). Macrophages mediate crucial innate immune responses through caspase-1–dependent processing and secretion of IL-1β and IL-18 (2). TLRs respond to extracellular or vacuolar activation, whereas Nod-like receptors (NLRs) respond to cytosolic stimuli. However, in the case of pleiotropic cytokines IL-1β and IL-18, TLRs and NLRs function in concert. TLRs induce expression of the precursor forms of these cytokines (pro–IL-1β and pro–IL-18), after which NLR-dependent activation of caspase-1 regulates their proteolytic processing and release (3).

The NLR family is composed of several receptors, such as NLRP1, NLRP3, and NLRC4, which can assemble inflammasomes that are molecular platforms responsible for activation of the proinflammatory cytosolic protease caspase-1 (4). More recently, AIM2 was identified as a novel intracellular receptor involved in inflammasome activation in response to the recognition of cytosolic DNA during viral and bacterial infection (5–10).

The NLR family of cytosolic receptors responds to a variety of bacterial pathogens. For instance, NLRC4 is required to activate caspase-1 after macrophage infection with *Salmonella* spp., *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Legionella pneumophila* (11–15). NLRC4 recognizes bacterial flagellin that is inserted into the cytoplasm of host cells by bacterial type III or type IV secretion systems (T4SS). More recently, it was reported that NLRC4 also detects the rod subunit of certain type 3 secretion systems (16), which explains how NLRC4 can detect nonflagellated bacteria, such as *Shigella flexneri* (17). Many bacterial pathogens, such as *L. monocytogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*, are able to induce the NLRP3 inflammasome (18–21). The most common mechanism involves the secretion of pore-forming bacterial toxins that promote NLRP3 inflammasome activation. Additionally, a recently identified pattern-recognition receptor termed AIM2 was found to recognize cytoplasmic dsDNA through its HIN-200 domain and ASC via its pyrin domain. *Franciscella tularensis* and *L. monocytogenes* DNA
activate AIM2 inflammasome by their interaction with ASC to in-duce caspase-1 (6, 22). Further, studies using knockout (KO) mice revealed the importance of AIM2 in host defense against cytosolic bacteria, such as Francisella spp. (6, 10). *Brucella abortus* is a Gram-negative, facultative intracellular coccobacillus that causes brucellosis in humans and in cattle. In humans, *B. abortus* causes undulant fever, endocarditis, arthritis, and osteomyelitis; in animals, it leads to abortion and infertility, resulting in serious economic losses (23, 24). The innate immune response against *B. abortus* infection begins with the recognition of molecular structures related to this pathogen by receptors, such as TLRs (25). It was shown that *Brucella* is recognized by several TLR-associated pathways, triggering proinflammatory responses that impact both the nature and the intensity of the immune re-sponse (26–28). Recently, we demonstrated that NOD1 and NOD2 cytosolic receptors play no role in host control of *B. abortus* in vivo (29). However, no further studies have been performed to determine the role of inflammasome receptors in the recognition of *Brucella* components. In this study, we examined the mechanisms underlying caspase-1 activation and IL-1β production upon NLR recognition of *Brucella*.

**Materials and Methods**

**Mice**

Wild-type C57BL/6 mice were purchased from the Federal University of Minas Gerais (UFMG). ASC, NLRC4, NLRP3, AIM2, caspase-1, MyD88, IL-1R, and IL-18 KO mice were described previously (10, 30–34). The animals were maintained at UFMG and used at 6–9 wk of age. All animal experiments were preapproved by the Institutional Animal Care and Use Committee of UFMG (CTEA #103/2011).

**Bacterial strains**

Bacteria used included *B. abortus* S2308 strain obtained from our laboratory collection and the *B. abortus* virulent mutant strain kindly provided by Dr. Renato de Lima Santos (UFMG). They were grown in *Brucella* broth medium (BD Pharmingen, San Diego, CA) for 3 d at 37°C under constant agitation.

**Infection and B. abortus counts in spleens**

Five mice from each group (C57BL/6, NLRC4−/−, NLRP3−/−, AIM2−/−, caspase-1−/−, ASC−/−, IL-18−/−, IL-1R−/−, and MyD88−/−) were infected i.p. with 1 × 10^6 CFU *B. abortus* virulent strain S2308 and sacrificed at 4 wk postinfection. To count residual *Brucella* CFU, the spleen harvested from each animal was macerated in 10 ml saline (NaCl 0.9%), serially diluted, and plated in duplicate on *Brucella* Broth agar. After 3 d of incubation on 25°C, the number of CFU was determined as described previously (26). Blood samples were collected from wild-type and KO mice at 1 wk postinfection to determine IFN-γ levels by ELISA using the DuoSet kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

**Histopathology**

The liver medial lobes of *Brucella*-infected mice were collected at 4 wk postinfection, fixed in 10% buffered formaldehyde solution, dehydrated, diaphanized, and embedded in paraffin. Four-micrometer-thick tissue sections were stained with H&E. The granulomas present in liver histo-logical sections were analyzed using an Olympus CX31 microscope (Tokyo, Japan) with a 40× objective lens. Digital images of 15 granulomas/mouse were captured using an Olympus SC30 camera (Tokyo, Japan), and the total area of each section was measured with Image Tool 3.0 software (The University of Texas Health Science Center at San Antonio, San Antonio, TX). The total number of granulomas present in histological liver sections was determined using the same microscope with a 10× objective lens, and the granuloma numbers were normalized for a 50-mm^2 tissue area. Five animals/group were analyzed.

**Generation and in vitro stimulation of bone marrow–derived macrophages**

Macrophages were derived from bone marrow of C57BL/6 and NLRC4, NLRP3, AIM2, ASC, caspase-1, and MyD88 KO mice. Briefly, bone marrow cells were removed from the femurs and tibias of the animals and cultured in DMEM (Life Technologies, Carlsbad, CA) containing 10% FBS (HyClone, Logan, UT), 1% HEPES, and 10% L-1529 cell-conditioned medium (LCCM), as the source of M-CSF, in 24-well plates (5 × 10^4 cells/well). After 4 d, 100 µl well LCCM was added. At day 10 of culture, when the cells had completely differentiated into macrophages, the medium was harvested, and we added DMEM (500 µl/well) containing 1% FBS with or without Escherichia coli LPS (1 µg/ml). After 4 h of incubation, the medium was replaced by DMEM (500 µl/well) supplemented with 1% FBS containing *B. abortus* S2308 (multiplicity of infection [MOI] 100:1), virb mutant strain (MOI 100:1), heat-killed *B. abortus* (HKB2), and monosodium urate (MSU) crystals (250 µg/ml). Culture supernatants were collected after 17 h of stimulation and assayed for the concentrations of IL-1β by ELISA (R&D Systems) and IL-1β (p17 subunit) or caspase-1 (p20 subunit) by Western blot.

**Purification of B. abortus DNA and transfection experiments**

*B. abortus* were grown as indicated above, and DNA was purified using the illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare), according to the manufacturer’s instructions with minor modifications. Following lysis, the bacterial pellet was treated with 10 µl Proteinase K (200 mg/ml) for 1 h at 55°C under gentle agitation. After the purification step, the sample was resuspended in 100 µl nuclease-free water and treated with DNase I (1 µg/ml DNA; Fermentas, Hanover, MD). *Brucella* DNA was measured spectrophotometrically. Transient transfections of bone marrow-derived macrophages (BMDMs) were carried out using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. Cells were cultured in 10% DMEM and incubated with MitoSOX Red at a final concentration of 2.5 µM. Three hours later, the cells were washed twice with ice-cold aceton, air dried, and subjected to electrophoresis on 12% SDS-PAGE sample buffer. Samples were sonicated for 15 min and then boiled for 5 min. Protein content from 5 × 10^5 BMDMs was loaded on 15% SDS-PAGE gel. Western blot was performed with rat anti-p20 caspase-1 (Genentech, South San Francisco, CA) diluted 1:500 and goat anti-rat IgG alkaline phosphatase conjugated (Promega, Madison, WI) diluted 1:100. Goat anti–IL-1β (Sigma-Aldrich, St. Louis, MO) were diluted 1:200 and rabbit anti-goat IgG HRP conjugated diluted 1:2000. Con-sequently, BMDMs were lysed with a cell lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 10 mM β-glycerophosphate, 0.1 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with 1:100 protease inhibitor mixture (Sigma-Aldrich). The lysates (equivalent of 5 × 10^5 cells) were subjected to electrophoresis on 12% SDS-PAGE gel, followed by Western blotting according to standard techniques. The mAb used was anti–β-actin (Cell Signaling Technology, Danvers, MA). Immunoreactive bands were visualized using Luminol chemiluminescent HRP substrate (Millipore) and analyzed using the Storm System 860 (Amersham Biosciences).

**Measurement of mitochondrial reactive oxygen species production**

Mitochondrial reactive oxygen species (ROS) were detected in cells by MitoSOX (Invitrogen, Carlsbad, CA) staining, which is a fluorescent dye specific for the detection of O_2− in the mitochondria of live cells. Macrophages grown on four-well chamber slides were stimulated with *B. abortus* (MOI 100:1) plus ATP (5 mM), MitoSOX, or medium alone for 30 min at 37°C. Then, the cells were washed with DMEM and incubated with MitoSOX Red at a final concentration of 2.5 µM for 5 min. After removing MitoSOX Red and washing cells with HBSS, macrophages were stained with Hoechst 33342 at a final concentration of 0.5 µg/ml for 20 min. Cells were then fixed with freshly prepared 4% formaldehyde in HBSS for 20 min; after repeated washing with HBSS, the cells were mounted with Prolong Gold Antifade (Invitrogen) and visualized by fluorescence microscopy with identical exposure settings. The images were analyzed with ImageJ open source software (http://rsweb.nih.gov/ij/) to determine the fluorescent intensity of individual cells. Average cell intensity was determined for 100 cells for each treatment. The results were obtained from two independent experiments performed in triplicate. To evaluate the effect of mitochondrial ROS in IL-1β secretion, BMDMs were preincubated for 1 h with the mitochondrial superoxide scavenger Mito-TEMPO (500 µM) and then treated or not with LPS (1 µg/ml) for 4 h. Primed cells were stimulated with ATP (5 mM) for 30 min, and nonprimed cells were infected with *B. abortus* (MOI 100:1) for 17 h.
Culture supernatants were then harvested, and IL-1β was measured by ELISA.

Lactate dehydrogenase measurement

BMDMs of C57BL/6 and NLRC4, NLRP3, ASC, AIM2, and caspase-1 KO mice were cultured and infected with B. abortus, as described above. After *Brucella* infection, cell culture supernatants from BMDMs were harvested, and the remaining cells were lysed. The lactate dehydrogenase (LDH) activity in supernatant and lysates was measured using a CytoTox96 LDH-release kit (Promega), according to the manufacturer’s instructions.

Retroviral transduction of macrophages with GFP-ASC

Murine ASC was cloned into the pEgFP(N2) vector (Clontech) using Xhol and BamH restriction sites. Further, GFP-ASC and GFP alone were cloned into the pMSCV2.2 murine-specific retroviral vector (Clontech). Then, the pCL vector system (35) was used for packaging of retroviruses in transfected monolayers of Peak cells (ATCC CRL-2828), and these cells were maintained in RPMI 1640 with 10% FBS. The supernatant of Peak cells was collected 3 d after transfection, passed through a 0.45-μm filter, and used for BMDM transduction. BMDMs were obtained from the femur of caspase-1–deficient mice and seeded at 5 × 105 cells/well in differentiation media (RPMI 1640 media supplemented with 30% LCCM, 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin). At day 3 of differentiation, BMDMs were harvested and spun down, and the media were replenished by retroviral-containing Peak cell supernatants complemented with RPMI 1640 containing 20% FBS and 25% LCCM. BMDMs were fed with differentiation media and cultured for an additional 4 d. BMDMs were seeded at 2.0 × 106 cells in 24-well plates containing 12-mm glass cover slides and infected with *B. abortus* S2308 or virB mutant (T4SS-deficient strain) or stimulated with HKBa at an MOI of 100:1 in RPMI 1640 containing 10% FBS. Plates were centrifuged at 600 × g for 10 min, and penicillin (100 U/ml) and streptomycin (100 μg/ml) were added 30 min after the infection. At 0.5, 2, 6, and 24 h postinfection, infected cells were fixed with 4% paraformaldehyde and immunostained against *B. abortus* using a polyclonal Ab anti-LPS diluted 1:1000 (kindly provided by Dr. Diego J. Comerci, Universidad Nacional de San Martin, San Martin, Argentina) and the secondary Ab labeled with Alexa Fluor 594 diluted 1:4000 (Molecular Probes). Cells were counterstained with DAPI (35 μM), mounted using Prolong Gold Antifade Reagent (Invitrogen), and analyzed under epifluorescence using a Leica DMI 4000B microscope with a 100× oil objective. Images were processed using LAS AF software (Leica Microsystems), and the number of GFP-ASC+ cells was counted.

Statistical analysis

The results of this study were analyzed using the Student *t* test or ANOVA, as indicated, with GraphPad Prism 4 computer software (GraphPad Software). The level of significance in the analysis was *p < 0.05, p < 0.01, or p < 0.001.

**FIGURE 1.** ASC and MyD88 are critical for IL-1β secretion and caspase-1 activation induced by *B. abortus*. BMDMs derived from indicated KO mice were primed or not with LPS (1 μg/ml) for 4 h and then infected with *B. abortus* (MOI 100:1) or treated with MSU (250 μg/ml) as a positive control. (A) Culture supernatants were harvested 17 h after treatment, and IL-1β secretion was determined by ELISA. (B) The same culture supernatants were harvested 17 h postinfection, and the caspase-1 or IL-1β processing was determined by Western blot. Equal loading was controlled by measuring β-actin in the corresponding cell lysates. *p < 0.001, versus wild-type mice for infection with *B. abortus*, **p < 0.001, versus wild-type mice for treatment with MSU, two-way ANOVA.

Results

ASC and MyD88 are required for *Brucella*-induced caspase-1 activation and IL-1β secretion by macrophages independently of NLRC4

Genetic studies demonstrated that ASC is an adaptor protein for NLRC4, NLRP3, and AIM2 inflammasomes, bridging microbial signals to caspase-1 activation and IL-1β production (36). To assess whether NLRC4 and ASC are essential for the activation of caspase-1 and IL-1β secretion during infection, C57BL/6 and NLRC4, ASC, and MyD88 KO BMDMs were infected with *B. abortus* for 17 h. IL-1β secretion induced by *B. abortus* infection was completely abolished in ASC and MyD88 KO macrophages (Fig. 1A). However, deficiency in NLRC4 did not affect the production of IL-1β induced by *B. abortus*. Additionally, we decided to investigate the role of NLRC4, ASC, and MyD88 in caspase-1 activation by *Brucella* in the absence or presence of LPS priming. Caspase-1 activation results in its autoproteolytic cleavage into two subunits, p10 and p20, which are released into the extracellular milieu (37). We did not detect the caspase-1 p20 subunit in supernatants from ASC or MyD88 KO macrophages, indicating that caspase-1 cleavage was dependent on the presence of ASC and MyD88 (Fig. 1B). In contrast, NLRC4 is not involved in caspase-1 activation after *Brucella* infection. In parallel with these findings, IL-1β processing (subunit p17) requires ASC but not NLRC4 (Fig. 1B). Furthermore, the absence of LPS priming did not reduce caspase-1 activation and IL-1β processing and secretion after *B. abortus* infection. Taken together, these results suggested that ASC is essential for the caspase-1 response to *B. abortus* and that a receptor(s) other than NLRC4 is also involved in ASC-dependent caspase-1 activation induced by this pathogen.

Live *Brucella* and bacterial T4SS are necessary for inflammasome activation

Pathogenic bacteria can modulate host cell–signaling pathways by delivering virulence factors to the cytosol of host cells. For instance, *Brucella* T4SS (VirB) is essential to subvert lysosome fusion and to create an organelle permissive for replication. One possible role for VirB is to translocate effector proteins that modulate host cellular functions for the biogenesis of the replicative organelle (38). To address the role of *Brucella* T4SS in inflammasome activation, we infected C57BL/6 macrophages...
with wild-type bacteria or virB mutant and determined IL-1β secretion. As demonstrated in Fig. 2, Brucella lacking the T4SS induced much lower secretion of IL-1β in C57BL/6 macrophages compared with wild-type bacteria. These findings suggest that Brucella uses the T4SS to translocate effector proteins into host cytosol to further activate the ASC-dependent inflammasome. Additionally, reduced numbers of virB mutant compared with wild-type Brucella at 17 h postinfection in macrophages may also account for the reduction in IL-1β secretion. Furthermore, these results demonstrate that live, but not heat-killed, Brucella activated the inflammasome to secrete IL-1β.

Brucella infection of macrophages recruits ASC

Spatial relocalization of ASC into punctate structures has been described during inflammasome activation in response to different stimuli (39, 40). To gather further insight about the role of ASC in inflammasome activation in response to Brucella, the cytosolic distribution of ASC in response to bacterial infection was assessed in situ. BMDMs were transduced with a retroviral vector encoding the GFP-ASC fusion protein so that ASC recruitment and localization could be visualized in macrophages. In uninfected or HKBa-treated macrophages, the GFP-ASC protein was dispersed throughout the cytosol (Fig. 3A). In contrast, overexpressed GFP-ASC protein reorganizes in a single punctate structure following infection of macrophages with wild-type B. abortus or virB mutant. However, the percentage of transduced cells containing GFP-ASC punctate structures was significantly reduced in virB-infected macrophages compared with wild-type Brucella (Fig. 3B). These data are in agreement with a dramatic reduction in IL-1β production in virB mutant–treated macrophages compared with cells infected with B. abortus S2308 (Fig. 2). Taken together, these data confirm that B. abortus infection triggers formation of ASC puncta in the process of inflammasome activation.

Brucella-induced IL-1β secretion by macrophages requires AIM2 and NLRP3

To address whether NLRP3 and AIM2 are important for inflammasome activation and IL-1β secretion during infection, C57BL/6 and NLRC4, NLRP3, AIM2, ASC, and caspase-1 KO BMDMs were infected with B. abortus. IL-1β secretion induced by B. abortus infection was completely abolished in ASC and caspase-1 KO macrophages and was strongly reduced in AIM2 and NLRP3 KO cells (Fig. 4A). These results demonstrated that activation of ASC-dependent inflammasomes and production of IL-1β are partially dependent on NLRP3 and AIM2. Additionally, we assessed the role of Brucella T4SS in IL-1β production in these KO macrophages. As demonstrated in Fig. 4A, Brucella T4SS is important to activate the inflammasome in wild-type mouse cells.

To dissect the mechanisms underlying inflammasome activation after Brucella infection, we decided to investigate the role of pyroptosis. BMDMs were infected with B. abortus at the indicated MOI, and cell death was monitored by measuring the amount of LDH released into the culture supernatant by cell permeabilization and/or lysis. B. abortus strain S2308 did not induce significant BMDM death, even at a higher MOI of 100 bacteria/host cell, which was <10% of LDH release (Fig. 4B). This result indicates that pyroptosis is not a major mechanism induced by Brucella in macrophages.

AIM2 inflammasome is involved in caspase-1 activation induced by Brucella DNA

AIM2 was shown to recognize DNA and to be responsible for ASC-dependent caspase-1 activation in response to DNA molecules (41). Therefore, we tested whether Brucella genomic DNA can be a ligand for AIM2. Brucella DNA was transfected into C57BL/6
and ASC, NLRC4, caspase-1, and AIM2 KO macrophages using Lipofectamine, and the activation of caspase-1 and IL-1β secretion were determined. Bacterial DNA induced caspase-1 activation and IL-1β secretion in C57BL/6 and NLRC4 cells but not in ASC and caspase-1 KO macrophages (Fig. 5). Additionally, DNase I treatment abrogated Brucella DNA–induced IL-1β secretion, demonstrating that bacterial DNA is a major agonist that activates the inflammasome. Furthermore, macrophages deficient in AIM2 transfected with Brucella DNA resulted in a major reduction in IL-1β secretion and caspase-1 activation. Taken together, these results indicated that Brucella genomic DNA is recognized by AIM2, and this cytosolic receptor is partially required for caspase-1 activation and IL-1β secretion induced by bacterial DNA.

**IL-1β secretion induced by Brucella is partially dependent on mitochondrial ROS**

Although the exact mechanism involved in NLRP3 inflammasome activation by different stimuli remains elusive, a common role for ROS has been widely implicated (42). In this study, we investigated whether mitochondrial ROS play a role in Brucella-induced IL-1β secretion. First, we assessed mitochondrial ROS produced in macrophages infected with Brucella or stimulated with LPS and ATP. As shown by fluorescence microscopy, Brucella induced mitochondrial ROS production at 30 min or 17 h (data not shown) postinfection compared with cells treated with medium alone (Fig. 6A). To quantitatively measure mitochondrial ROS, the average intensity in an individual cell was determined for 100 cells from each treatment group, and images were analyzed by ImageJ software. As observed in Fig. 6B, mitochondrial ROS was increased dramatically following Brucella infection or LPS and ATP stimulation compared with medium-treated macrophages. We next examined whether mitochondria-specific generation of ROS contributed to IL-1β secretion induced by Brucella. Treatment of macrophages with the mitochondria-targeted antioxidant MitoTEMPO, a scavenger specific for mitochondrial ROS, inhibited the secretion of IL-1β in macrophages in response to Brucella or LPS and ATP (Fig. 6C). Taken together, these data indicate that secretion of IL-1β by Brucella is partially dependent on mitochondrial generation of ROS.

**AIM2, NLRP3, ASC, caspase-1, IL-1R, and IL-18 KO mice are more susceptible to B. abortus infection**

To determine whether ASC-dependent inflammasome formation plays a role in vivo, we infected C57BL/6 and ASC, NLRC4, caspase-1, NLRP3, and AIM2 KO mice. Consistent with the in vitro findings using macrophages when we determined a critical role for AIM2, NLRP3, and ASC in caspase-1 activation and IL-1β secretion, AIM2, NLRP3, ASC, and caspase-1 KO mice showed a reduced resistance to Brucella at 4 wk postinfection, as determined by bacterial numbers in the spleens (Fig. 7A). Bacterial load recovery was 0.99, 1.41, 0.70, and 0.51 logs higher in the ASC, caspase-1, AIM2, and NLRP3 KO mice, respectively, compared with C57BL/6 animals. Additionally, our data demonstrate that NLRC4 appears to be dispensable for in vivo innate immune defense against B. abortus. Because Brucella-induced inflammasome activation did not result in pyroptosis, we decided to investigate whether enhanced susceptibility to infection in ASC, caspase-1, AIM2, and NLRP3 KO mice was attributable to a defect in IL-1β and/or IL-18 production. C57BL/6 and IL-1R, IL-18, and MyD88 KO mice were infected with 1 × 10^6 B. abortus, and bacterial numbers were determined at 4 wk postinfection. IL-1R and IL-18 KO mice showed a reduced resistance to Brucella compared with C57BL/6 animals (Fig. 7A). These results demonstrate that IL-18 and IL-1β are important components of the ASC inflammasomes to enhance resistance to Brucella infection. Protective immunity against infection by B. abortus is directly related to the induction of a type 1 pattern of immune response. Thus, to evaluate the role of inflammasome receptors in inducing

**FIGURE 4. B. abortus triggers AIM2 and NLRP3 activation in macrophages to secrete IL-1β but not to induce pyroptosis.** (A) BMDMs derived from the indicated KO mice were treated with MSU (250 µg/ml) or infected with B. abortus or the virB mutant (both at MOI 100:1). Culture supernatants were harvested 17 h postinfection, and cytokine secretion was determined by ELISA. (B) BMDMs from wild-type animals and mice deficient for inflammasome components were infected with B. abortus (MOI 25:1, 50:1, or 100:1). Cell death was determined by measuring the release of LDH in the supernatant at 17 h postinfection. The graph is representative of two independent experiments. *p < 0.001, versus wild-type mice in relation to B. abortus, **p < 0.001, versus wild-type mice in relation to virB mutant, #p < 0.001, versus wild-type mice in relation to MSU, two-way ANOVA. "*" indicates a statistically significant difference between B. abortus and virB mutant treatment.

**FIGURE 5. DNA from B. abortus elicited IL-1β secretion and caspase-1 activation in BMDMs via AIM2.** BMDMs derived from the indicated KO mice were transfected with DNA isolated from B. abortus (2 µg/ml) encapsulated with Lipofectamine or Lipofectamine alone as control. Culture supernatants were harvested 17 h after treatment to measure IL-1β by ELISA (A) and caspase-1 processing by Western blot (B). *p < 0.001, versus wild-type mice, **p < 0.001, in relation to untreated DNA, two-way ANOVA.
IFN-γ response to *B. abortus* infection, serum IFN-γ was assessed in wild-type and KO mice. IFN-γ production in IL-18, ASC, caspase-1, and MyD88 KO mice was greatly reduced at 1 wk postinfection compared with wild-type animals (Fig. 7B). This result suggests that IL-18, ASC, and caspase-1 are involved in IFN-γ synthesis during *Brucella* infection. Additionally, all KO mice tested in this study were monitored daily for survival for 55 d postinfection. However, no mortality was observed for any inflammasome KO animals analyzed (data not shown). Finally, we investigated the contribution of these inflammasome components to weight loss in KO mice after *Brucella* infection. NLRC4, NLRP3, AIM2, ASC, caspase-1, IL-18, and IL-1R KO animals displayed similar body weight compared with wild-type mice, demonstrating that a lack of these inflammasome receptors did not alter this clinical condition following bacterial infection (data not shown).

Lack of inflammasome receptors does not influence liver granuloma formation induced by Brucella

The liver is another important organ that harbors *B. abortus* during infection. To assess whether the increased bacterial burden in KO mice alters liver pathology, the number and area of granulomas in this organ were determined. As demonstrated in Fig. 8, the most prominent reduction in granuloma size and number was observed in MyD88 KO mice compared with C57BL/6 mice. In contrast, ASC, NLRC4, caspase-1, NLRP3, AIM2, IL-18, and IL-1R KO mice did not exhibit alterations in liver granuloma at 4 wk post-*Brucella* infection.

**Discussion**

Pathogenic bacteria can use many strategies to enter and establish an infection inside the host. Because of that, the immune system displays mechanisms to detect and to eliminate a broad range of these microbes. Innate immunity can detect components’ so-called “pathogen-associated molecular patterns” (PAMPs) to elicit a host-protective response (43). Pathogen recognition is triggered by pattern recognition receptors, including TLRs (44), NLRs (45), and RIG-I-like receptors (46), and is followed by proinflammatory responses characteristic of infection. Recent studies by our group (25, 47) and other investigators (28) revealed the intracellular signaling cascades involved in the TLR-initiated immune response to *Brucella* spp. infection. However, a piece of the puzzle is missing: the role of non-TLRs in innate immunity. Inflammasomes have emerged as critical signaling molecules of the innate immune system. In this study, we investigated the mechanism of caspase-1 activation and IL-1β secretion in macrophages infected...
with *B. abortus*. We found that ASC inflammasome is indispensable for inducing the activation of caspase-1 and the maturation and secretion of IL-1β during *Brucella* infection.

Several NLRs, such as NLRP1, NLRP3, NLRC4, AIM2, and IFI16, form multiprotein complexes that induce caspase-1 activation by functioning as sensors of PAMPs or danger-associated molecular patterns (4). NLRC4 inflammasome is activated during infection with a broad range of bacterial pathogens, such as *Salmonella* (48), *Legionella* (49), *Pseudomonas* (15), *Yersinia* (50), and *Shigella* (17). NLRP3 inflammasome is also known to be activated by *L. monocytogenes* (51), *S. pneumoniae* (19), *S. aureus* (20), and *M. tuberculosis* (21), among others. However, we showed in this study that NLRC4 is not involved in sensing *Brucella* infection. NLRC4 is a receptor dispensable in the process of caspase-1 activation and IL-1β secretion by this pathogen. In contrast, we observed that NLRP3 KO macrophages secreted much lower levels of IL-1β, and NLRP3 KO mice were more susceptible to *Brucella* infection compared with C57BL/6 mice. However, the mechanism involved in this susceptibility needs to be characterized further. An inflammatory response initiated by the NLRP3 inflammasome is triggered by a variety of situations of host danger, including infection and metabolic dysregulation. It is unclear how these highly varied stress signals can be detected by a single inflammasome, but one of the crucial elements for NLRP3 activation is the generation of ROS. In this study, we determined that *Brucella* genomic DNA induces type I IFN production through a cytoplasmic receptor that signals via STING (52). Further, secretion of IFN-β and signaling through type I IFN are required for inflammasome activation in macrophages (53). However, it remains to be defined how such immune sensing of *Brucella* DNA occurs intracellularly.

*B. abortus* expresses the virB T4SS that plays a crucial role in the bacterial replication (32). Bacteria use T4SS for genetic exchange and to deliver effector molecules to eukaryotic target cells. In this study, we showed that a *Brucella virB* mutant strain lacking the T4SS induced much lower secretion of IL-1β in C57BL/6 and NLRC4 KO macrophages compared with wild-type bacteria. Additionally, the data shown in Fig. 4 indicated that *Brucella* T4SS may deliver effector molecules via AIM2 or NLRP3 to activate the ASC inflammasomes. Taken together, these findings suggest that *Brucella* uses the T4SS to translocate effector proteins into host cytosol or even nucleic acids to further activate the inflammasome. Furthermore, in this study we compared the inflammasome activation by live or dead *Brucella*. As demonstrated in Fig. 2, IL-1β secretion was totally abrogated in macrophages stimulated with heat-killed *Brucella* compared with live bacteria. Additionally, *Brucella* infection recruits the ASC complexes to assemble a well-defined puncta structure in GFP-ASC–transduced macrophages that was not observed in uninfected or HKBa-treated cells. The scaffold assembled by ASC plays an important role in cellular homeostasis and caspase-1–dependent processing of cytokines. Therefore, the innate immune system actively senses the infectious risk by searching for PAMPs present in live microbes.

Inflammasome-dependent caspase-1 activity can result in a highly inflammatory form of cell death, known as pyroptosis (54).
Pyroptosis occurs most frequently upon infection with intracellular pathogens (17, 55, 56) and is likely to be part of the antimicrobial response. Active caspase-1 allows the host to control various microbial infections, so it is not surprising that pathogens have evolved mechanisms to limit caspase-1 activation in response to infection. In this study, we demonstrated that caspase-1 activation induced by \textit{B. abortus} does not lead to pyroptosis. Chen et al. (57) demonstrated that rough attenuated \textit{Brucella suis} induces caspase-2–mediated cell death, but not caspase-1–mediated cell death, in infected macrophages. It is tempting to suggest that \textit{Brucella} has developed a strategy to avoid pyroptosis, therefore limiting inflammation and allowing continued intracellular replication of the bacteria. However, this hypothesis has to be proven. \textit{Mycobacterium} and \textit{Franciscella} possess mechanisms for inhibiting caspase-1 activation, and mutants of these bacteria that cannot control caspase-1 activation are attenuated in vivo (58, 59). These findings are consistent with the idea that increased levels of active caspase-1 and pyroptosis limit bacterial replication.

Caspase-1 and ASC are critical host molecules for innate immune defense against several bacterial pathogens, such as \textit{Franciscella} (60), \textit{S. pneumoniae} (61), and \textit{S. typhimurium} (30, 62). In this study, we showed that mice deficient for inflammasome components ASC, caspase-1, AIM2, and NLRP3 are more susceptible to \textit{Brucella} infection. These mice displayed higher bacterial counts in the spleen at 4 wk postinfection. Further, we speculate that enhanced susceptibility to \textit{Brucella} infection in caspase-1, ASC, AIM2, and NLRP3 KO mice was partially attributed to reduced IL-1β, and possibly IL-18, production and/or

\textbf{FIGURE 8.} Lack of inflammasome receptors does not affect liver granuloma formation. Medial liver lobes from \textit{B. abortus}–infected wild-type and KO mice were collected at 4 wk postinfection, fixed in 10% buffered formaldehyde solution, and embedded in paraffin. Tissue sections were processed and stained with H&E. (A) Digital images of representative granulomas (original magnification ×400). Scale bars, 20 μm. (B) Granuloma areas from each sample were measured (in μm²). (C) Granuloma numbers were normalized for 50 mm² of hepatic tissue. Data are mean ± SD of 15 granulomas/mouse for five animals/strain and are representative of two independent experiments. *p < 0.05, versus C57BL/6 mice, one-way ANOVA.
other caspase-1–dependent processes. This hypothesis was con- 
formed by enhanced susceptibility to Brucella infection observed in IL-18 and IL-1R KO mice. Additionally, we also observed 
reduced serum IFN-γ production in ASC, caspase-1, IL-18, and 
MyD88 KO mice. Because IFN-γ is critical to host control of 
Brucella infection, we speculate that limited production of this 
cytokine may account for reduced resistance to bacterial infection 
in these KO mice. The formation of granulomas is an important 
component of coordinated antibacterial defenses, in which lym-
phocytes cooperate with macrophages to restrain bacterial growth. 
In this study looking at liver granulomas during Brucella infec-
tion, we observed that a lack of NLRC4, NLRP3, AIM2, ASC, 
caspase-1, IL-1R, and IL-18 was not involved in granuloma for-
mation in KO mice. Reduced numbers and area of granulomas 
were detected only in MyD88 KO mice compared with C57BL/6j 
mouse. Remarkably, the inflammatory parameters tested in 
this study revealed a nearly absolute requirement for MyD88, 
because reduced levels of cytokines and granuloma areas and numbers 
were observed in MyD88 KO mice; these confirm the pivotal role 
of this molecule in host innate immunity to Brucella infection. 
The results from this study clearly indicate that ASC inflammas-
omes, mainly AIM2 sensing DNA and NLRP3, are essential for 
the secretion of caspase-1–dependent IL-1β and resistance to mice 
infected with B. abortus.

Acknowledgments

We thank Dr. Richard A. Flavell (Howard Hughes Medical Institute Inves-
tigator at the Department of Immunobiology, Yale University School of 
Medicine, New Haven, CT), Dr. Katherine Fitzgerald (University of Mas-
sachusetts, Worcester, MA), Dr. Vishva Dixit (Genentech), Dr. Shizuo Akira 
(Osaka University, Osaka, Japan), Dr. Maria Bellio (Federal University of 
Río de Janeiro, Río de Janeiro, Brazil), and Dr. Leda Q. Vieira (UFMG) for 
providing the mice used in these studies.

Disclosures

The authors have no financial conflicts of interest.

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