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AIM2 senses *Brucella abortus* DNA in dendritic cells to induce IL-1 β secretion, pyroptosis and resistance to bacterial infection in mice

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Abstract

Absent in melanoma 2 (AIM2) is a sensor of cytosolic dsDNA and is responsible for the activation of inflammatory and host immune responses to DNA viruses and intracellular bacteria. AIM2 is a member of the hematopoietic interferon-inducible nuclear proteins with a 200 amino-acid repeat (HIN200) family, containing a pyrin domain (PYD) at the N-terminus. Several studies have demonstrated that AIM2 is responsible for host defense against intracellular bacteria such as *Francisella tularensis*, *Listeria monocytogenes* and *Mycobacterium tuberculosis*. However, the role of AIM2 in host defenses against *Brucella* is poorly understood. In this study, we have shown that AIM2 senses *Brucella* DNA in dendritic cells to induce pyroptosis and regulates type I IFN. Confocal microscopy of infected cells revealed co-localization between *Brucella* DNA and endogenous AIM2. Dendritic cells from AIM2 KO mice infected with *B. abortus* showed impaired secretion of IL-1 β as well as compromised caspase-1 cleavage. AIM2 KO mice displayed increased susceptibility to *B. abortus* infection in comparison to wild-type mice, and this susceptibility was associated with defective IL-1 β production together with reduced IFN- γ responses. In summary, the increased bacterial burden observed *in vivo* in AIM2 KO animals confirmed that AIM2 is essential for an effective innate immune response against *Brucella* infection.

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Conflict of interest statement

The authors declare that this work was not performed in the presence of any personal, professional or financial conflict of interest.

Keywords

Innate immunity; inflammasome; AIM2; dendritic cells; *Brucella abortus*; DNA sensing

1. Introduction

Intracellular bacterial pathogens infect cells such as macrophages and dendritic cells and subvert them to propagate and disseminate within the host. Some bacteria, including *Listeria monocytogenes* and *Shigella flexneri*, escape from their phagocytic vacuole, and by mechanisms still not completely understood release components such as bacterial DNA into the cytosol [1]. Other pathogens such as *Salmonella typhimurium* and *Francisella tularensis* induce the expression of members of the IFN-inducible GTPase family, which have the ability to target the vacuolar membrane encapsulating intracellular parasites, thus enabling bacterial products to reach cytosolic compartments [2]. Bacterial ligands must secure entry into the cytoplasm to activate inflammasome receptors such as AIM2; however, the mechanisms by which concealed ligands are liberated in the cytoplasm remains unclear.

The NLR family is composed of several receptors such as NLRP3, NLRC4, NLRP1 and AIM2. AIM2 was identified as the receptor involved in inflammasome activation in response to the recognition of cytosolic DNA during bacterial infections [3]. This receptor engages its DNA ligand directly via the DNA-binding HIN domain, and activation directs maturation of the proinflammatory cytokines IL-1 β and IL-18 and an inflammatory form of cell death termed pyroptosis [4]. Additionally, a recent report indicated a link between DNA vaccine-induced pyroptotic cell death via the AIM2 receptor and vaccine immunogenicity, which is instrumental in shaping the antigen-specific immune response to DNA vaccines [5].

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, and in animals, it leads to abortion and infertility, resulting in serious economic losses [6, 7]. The innate immune response against *B. abortus* infection begins with the recognition of molecular structures related to this pathogen by receptors such as Toll-like receptors (TLRs) [8]. It has been shown that *Brucella* is recognized by several TLR-associated pathways which trigger proinflammatory responses that impact both the nature and intensity of the immune response [9–11]. Recently, we demonstrated in macrophages that NLRP3 and AIM2 are important sensors to detect *Brucella* components in the host cell cytosol [12]. However, in-depth investigation is required to determine the role of AIM2 in *Brucella* DNA sensing *in vitro* and *in vivo*. In this study, we examined the mechanisms underlying caspase-1 activation and IL-1 β production in dendritic cells upon AIM2 recognition of *Brucella* and its DNA and determined how this cytosolic receptor operates to induce protection against infection.

2. Material and Methods

2.1. Mice

Wild-type C57BL/6 was purchased from the Federal University of Minas Gerais (UFMG). AIM2 KO mice were described previously [4]. The animals were maintained at UFMG and used at 8–12 weeks of age. Food and water were provided *ad libitum*, and all animal experiments were preapproved by the Institutional Animal Care and Use Committee of UFMG (CETEA #128/2014).

2.2. Bacterial strain

The bacteria used in this work included the *B. abortus* virulent strain S2308, obtained from our laboratory collection, and the *B. abortus* *virB* operon mutant strain, kindly provided by Dr. Renato de Lima Santos (UFMG). Before their use for cell infection or DNA extraction, the bacteria were grown in *Brucella* Broth Medium (BD Pharmingen) for 72 hours at 37°C under constant agitation.

2.3. Purification of *B. abortus* DNA

B. abortus was grown for 72 hours at 37°C under constant agitation, and DNA was purified using an Illustra bacteria genomic Prep Mini Spin Kit (GE Healthcare) according to the manufacturer's instructions.

2.4. Cell culture and in vitro stimulation

Bone marrow cells were removed from the femurs and tibias of C57BL/6 and AIM2 KO mice. Bone marrow-derived dendritic cells (BMDCs) were generated and cultured in DMEM medium as previously described [9]. Cells were differentiated for 10 days and maintained in DMEM (Gibco) containing 10% fetal bovine serum (FBS) (HyClone), 1% HEPES (Gibco), 1% penicillin G sodium (100 U/ml), and streptomycin sulfate (100 mg/ml). Differentiation into dendritic cells was induced by supplementation of culture media with 20 ng/ml murine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and cells were cultured at 37°C in a 5% CO₂ atmosphere. Petri dishes containing 1×10⁷ cells were incubated at 37°C with an atmosphere of 5% CO₂. After 3 days of incubation, 5 mL of complete DMEM supplemented with recombinant murine GM-CSF was added to each plate. After 5 and 7 days of incubation, 3 mL of medium from each plate was replaced with freshly prepared complete DMEM supplemented with recombinant murine GM-CSF. After 10 days of incubation, the cells were submitted to flow cytometry and approximately 90% of the cells were dendritic cells. One day prior to stimulation by bacterial infection or DNA transfection, cells were harvested and seeded onto 24-well plates (5 × 10⁵ cells/well) for cytokine assays, western blot analysis and real-time RT-PCR. BMDC stimulation was performed by adding supplemented DMEM (500 µl/well) containing *B. abortus* S2308 (MOI 100:1). Monosodium urate (MSU) crystals (250 µg/ml) was used as positive control for IL-1β secretion. Transient transfections of BMDCs were carried out using Lipofectamine 2000 (Invitrogen) at a ratio (in ml) of 1:0.25, following the manufacturer's instructions. Cells were cultured in DMEM and transfected with *Brucella* DNA (1 µg/well), DNase and

poly-dA:dT (1 µg/well) (Invivogen). To block inflammasome activation, a specific AIM2 inhibitor, A151 (3 µM), was applied 1 hour prior to transfection with the DNA.

2.5. Western blotting to detect activated caspase-1

Cells were lysed with M-PER Protein Extraction Reagent (Thermo Scientific) supplemented with protease inhibitor cocktail (Sigma-Aldrich). An equal amount were loaded onto 12% SDS polyacrylamide gel, transferred to nitrocellulose membrane (Amersham Biosciences) and blocked for 1 hour at room temperature with TBS containing 0.1% Tween-20 and 5% nonfat dry milk. Subsequently, membranes were incubated overnight with the primary antibody anti-β-actin (clone 13E5, rabbit mAb, Cell Signaling) at 4°C. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature and Luminol chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) were used for antibody detection using an Amersham Imager 600 imager (GE Healthcare). Additionally, the supernatants of cultured cells were loaded onto a 15% SDS polyacrylamide gel to perform the Western Blot similarly as describe above, but with anti-Caspase-1 (p20) as the primary antibody (Clone Casper-1, mouse mAb, Adipogen).

2.6. Cytokine measurements

After stimulation, IL-1β (17 hours) and IFN-γ (72 hours) levels in the cell culture supernatants were measured using ELISA kits (R&D Systems), in accordance with the manufacturer's instructions.

2.7. Real-time RT-PCR

RNA was extracted with Trizol reagent (Invitrogen) to isolate total RNA in accordance with the manufacturer's instructions. Reverse transcription of 2 µg of total RNA was performed using Illustra Ready-To-Go RT-PCR Beads (GE Healthcare) according to the manufacturer's instructions. Real-time RT-PCR was performed using 2x SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7900 real-time PCR instrument (Applied Biosystems). Appropriate primers were used to amplify the following specific fragments corresponding to specific gene targets: β-actin F: 5'-GGC TGT ATT CCC CTC CAT CG-3'; β-actin R: 5'-CCA GTT GGT AAC AAT GCC ATG T-3'; IFN-β F: 5'-GCC TTT GCC ATC CAA GAG ATG C-3'; IFN-β R: 5'-ACA CTG TCT GCT GGT GGA GTT C-3'; GBP2 F: 5'-CTG CAC TAT GT G ACG GAG CTA-3'; GBP2 R: 5'-CGG AAT CGT CTA CCC CAC TC-3'; GBP3 F: 5'-CTG ACA GTA AAT CTG GAA GCC AT-3'; GBP3 R: 5'-CCG TCC TGC AAG A CG ATT CA-3'; GBP5 F: 5'-CTG AAC TCA GAT TTT GTG CAG GA-3'; and GBP5 R: 5'-CAT CGA CAT AAG TCA GCA CCA G-3'. All data are presented as relative expression units after normalization to the β-actin gene, and measurements were conducted in triplicate.

2.8. Propidium iodide uptake assay and antibody generation

Cells derived from C57BL/6 and AIM2 KO mice were seeded onto black 96-well plates (1×10⁵ cells/well). The cells were infected with *B. abortus* 2308 (MOI 100:1), transfected with *Brucella* DNA (as described above), or treated with nigericin (20 µM). All treatments

were carried out in RPMI 1640 medium lacking phenol-red containing 15 mM HEPES, 0.38 g/l NaHCO₃, 10% FBS and 6 µg/ml propidium iodide. Pore formation in cells was determined by quantifying propidium iodide uptake. Throughout infection, plates were incubated at 37°C in a SpectraMax i3 microplate reader (Molecular Devices), and propidium iodide fluorescence was measured every 5 min. To ensure greater efficiency of bacterial phagocytosis during infection, bacteria were opsonized with a mouse polyclonal antibody (1:1000 dilution). This polyclonal antibody was generated by injecting mice with 1×10⁶ heat-killed bacteria/mouse. Animals were injected three times at 15-day intervals, and the sera of each mouse were tested for the presence of the specific antibody and stored at -80 °C.

2.9. Lactate dehydrogenase measurement (LDH)

BMDCs of C57BL/6 and AIM2 KO mice were cultured, transfected with bacterial DNA (1 µg/well), or infected with *B. abortus* (MOI 100:1), as described above. After 17 hours, cell culture supernatants from BMDCs 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.

2.10. Immunofluorescence

Dendritic cells were seeded onto 12-mm glass coverslips (1 × 10⁵ cells/well) 24 hours prior to infection with *Brucella*-RFP (MOI 1:100). Cells were then infected with *Brucella* for 16 hours and after that washed twice with PBS and fixed in 4% paraformaldehyde at room temperature for 30 min. After fixation, the coverslips were washed 3X, and permeabilization was carried out in PBS containing 0.3% Triton X-100 for 15 min. Subsequently, the cells were blocked for 1 hour with 1% BSA in PBS at room temperature prior to incubation with anti-AIM2 (1:200; Santa Cruz) at 4°C overnight. Detection was performed with an anti-rabbit secondary antibody conjugated to Alexa-488 (Jackson Immuno Research). Coverslips were mounted on slides using Prolong Gold with DAPI mounting medium (Invitrogen). Confocal microscopy analyses were performed in a Zeiss 880 confocal system.

2.11. In vivo infection and *B. abortus* counts in spleens

Five mice from each group (C57BL/6 or AIM2 KO) were infected intraperitoneally (i.p.) with 1 × 10⁶ CFU of the *B. abortus* virulent strain S2308 in 0,1 mL of PBS. At 1 and 3 weeks postinfection, mice were sacrificed, and the spleens were used to determine bacterial numbers. To count residual *Brucella* CFU, spleens were collected, macerated in 10 mL of saline (NaCl 0.9%), serially diluted, and plated in duplicate on *Brucella* Broth agar. After 72 hours of incubation at 37°C, CFU numbers were determined as described previously [9].

2.12. Spleen cell culture

Cells obtained from the spleens of C57BL/6 and AIM2 KO infected mice were washed with saline, and erythrocytes were lysed with a hemolytic solution (155 mM NH₄Cl, 10 mM KHCO₃). Spleen cells were seeded into 96-well plates (1 × 10⁶ cells/well) in RPMI 1640 (Life Technologies) supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% FBS (Life Technologies), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 mg/ml). The

cells were stimulated with purified *Brucella* DNA (1 µg/well), *Brucella* DNA treated with DNase (1 µg/well), or Con A (5 µg /ml, Sigma-Aldrich), and unstimulated cells were used as a negative control. After 72 hours, the supernatants were collected to measure IFN-γ levels by ELISA.

2.13. Histopathology

Liver medial lobes of C57BL/6 or AIM2 KO mice infected with *B. abortus* were collected at 1 and 3 weeks postinfection, and tissue sections were stained with H&E. Granulomas present in liver histological sections were analyzed using an Olympus CX31 microscope (Tokyo, Japan) with a 40X objective lens. Digital images of 15 granulomas/animal were captured using an Olympus SC30 camera (Tokyo, Japan). The total number of granulomas present in histological liver sections was determined using the same microscope with a 10X objective lens, and granuloma numbers were normalized to a 50-mm² tissue area. Five-animals/group were analyzed.

3. Results

3.1. *Brucella abortus* genomic DNA is co-localized with cytosolic AIM2

Confocal experiments were carried out to investigate whether an association could be observed between AIM2 and *Brucella* DNA in infected cells. BMDCs derived from C57BL/6 mice were infected with RFP-expressing *B. abortus* for 16 hours after that immunofluorescence with anti-AIM2 was performed to visualize any alterations in the cytosolic localization of AIM2. Dendritic cells infected with *Brucella* showed an increased presence of AIM2 aggregates and this finding represents an indication of inflammasome assembly (Fig. 1). Colocalization between AIM2 and RFP-*Brucella* could be frequently observed in most infected cells. Stronger labelling of anti-AIM2 is frequently associated with *Brucella*-containing vacuole (BCV) showing high bacterial DNA content (arrows in Fig. 1).

3.2. *B. abortus* DNA elicits IL-1β secretion and caspase-1 activation in dendritic cells via AIM2

To evaluate the activation of AIM2 in dendritic cells, C57BL/6 and AIM2 KO bone marrow-derived dendritic cells (BMDCs) were infected with *B. abortus* (MOI:100) or transfected with *Brucella* DNA. IL-1β secretion (Fig. 2A) and caspase-1 activation (Fig. 2B) induced by *B. abortus* infection or DNA transfection were strongly reduced in AIM2 KO dendritic cells. DNase I treatment abrogated *Brucella* DNA induced IL-1β secretion and caspase-1 activation, demonstrating that bacterial DNA is an important agonist that activates the inflammasome. Additionally, the *B. abortus* mutant for the type IV secretion system (virB1) was used and reduced IL-1β secretion was also observed in wild-type cells, demonstrating that the T4SS is critical to activate the inflammasome pathway. We next explored the inhibitory potential of the suppressive synthetic oligodeoxynucleotide A151 on activation of the AIM2 inflammasome. BMDCs pretreated with A151 were exposed to an AIM2 ligand, poly(dA:dT) or *Brucella* DNA and IL-1β secretion measured by ELISA was reduced (Fig. 2C). Taken together, our results demonstrate that *B. abortus* DNA induces AIM2 inflammasome activation in dendritic cells.

3.3. AIM2 activation in response to *B. abortus* DNA leads to pore formation in bone marrow-derived cells

Pyroptosis is a form of inflammatory cell death that is triggered by inflammatory caspases in response to PAMPs and DAMPs [13]. Once the AIM2 receptor recognizes cytoplasmic DNA, it activates caspase-1, which readily triggers pore formation [14]. Therefore, we decided to investigate whether AIM2 activation in response to *B. abortus* also leads to pyroptosis. Cells from C57BL/6 and AIM2 KO mice were infected with *B. abortus* or transfected with *B. abortus* DNA and incubated in medium containing propidium iodide (PI). To assess pore formation, we quantified the influx of propidium iodide into the nuclei of the cells in real time during infection. We found that AIM2-deficient cells were as able to form pores in response to *B. abortus* as observed in C57BL/6 cells (Fig. 3A). However, when we transfected cells with *B. abortus* DNA, we observed that whereas C57BL/6 cells readily triggered pore formation in response to *B. abortus* DNA, AIM2 KO cells failed to trigger pore formation (Fig. 3B). As expected, non-infected cells or cells treated only with the transfection reagent (lipofectamine) did not exhibit pore formation. As a control, we also treated cells with nigericin, which is a well-known stimulus of pore formation. After treatment with nigericin, both C57BL/6 and AIM2 KO cells readily triggered pore formation, as expected (Fig. 3C). To confirm these results we also perform LDH release assay. As shown in Figure 3D, *B. abortus* induces LDH release in the dendritic cells of wild-type mice and in the cells of AIM2-deficient animals. On the other hand, under transfection with *Brucella* DNA or pdAdT there was significant release of LDH in the supernatant of wild-type dendritic cells when compared to cells from AIM2 KO mice. Together, these studies indicate that AIM2 is dispensable for pore formation and LDH release in response to *B. abortus* but is required for pore formation in response to *B. abortus* DNA.

3.4. Negative regulatory effects of AIM2 on type I interferon responses

In the absence of AIM2, when dendritic cells from AIM2-deficient animals were infected by *Brucella* or transfected with *Brucella* DNA, we observed greater expression of *IFN- β* , *guanylate-binding protein 2 (GBP2)*, *GBP3* and *GBP5* compared with cells from wild-type animals, suggesting that AIM2 has a negative regulatory effect on the type I interferon responses (Fig. 4).

3.5. IFN- γ production by splenocytes stimulated with DNA is partially dependent on AIM2

A previous study published by our group has shown that protection against *B. abortus* infection requires the induction of a Th1-type immune response, in which IFN- γ is a pivotal cytokine for host control of brucellosis [15–17]. Thus, to investigate the role of AIM2 in regulating IFN- γ responses upon *B. abortus* infection, the level of this cytokine was measured in splenocytes stimulated with *Brucella* DNA. A dramatic reduction in IFN- γ production was observed in AIM2 KO mice compared to that of wild-type animals at 1, 3 and 6 weeks after *Brucella* DNA stimulation (Fig. 5). These results demonstrate that the Th1 cytokine profile is compromised in AIM2 KO mice during *Brucella* DNA activation.

3.6. AIM2 is partially required for control of *B. abortus* infection

To determine the role of AIM2 *in vivo* following *B. abortus* infection, C57BL/6 and AIM2 KO mice were infected intraperitoneally with virulent *B. abortus* S2308, and bacterial burden in the spleen was determined after 1 and 3 weeks of infection. AIM2 KO mice were more susceptible to *B. abortus* infection and presented a significant increase in bacterial load compared to WT controls at all evaluated time points (Fig. 6A). Infection with *B. abortus* resulted in the formation of liver granulomas, in which inflammatory cells aggregate to control bacterial growth. After the first week of infection, AIM2 KO mice displayed a significant reduction in granuloma numbers compared to their WT counterparts (Fig. 6C and D). At the same time point of infection, it was possible to observe macroscopically the formation of granulomas in the livers of wild-type mice but not in the livers of AIM2 KO mice (Fig. 6B), indicating that granuloma formation is an important component of coordinated antibacterial defenses in which cells cooperate to restrain bacterial growth. These data corroborate the fact that AIM2 KO mice are more susceptible to infection and also suggest that AIM2 modulates host liver pathology during the early stages of *Brucella* infection.

4. Discussion

AIM2 is a cytosolic double-stranded DNA receptor that contributes to host defenses against bacterial and viral pathogens. Recognition of DNA by pathogens via the AIM2 receptor leads to protective inflammasome-mediated host responses [14, 3]. Activation of the inflammasome has been shown to play a critical role in the recognition and containment of various microbial pathogens; however, several steps that trigger inflammasome activation remain poorly understood. *Brucella* is sensed by ASC inflammasomes, primarily AIM2 and NLRP3 in macrophages, which coordinate robust caspase-1 activation and pro-inflammatory responses [12]. Our study expands this observation and demonstrates an increase in AIM2 inflammasome formation in dendritic cells cytosol which shows some co-localization with *B. abortus* and its DNA. *Brucella* enters the host cell and prevents the fusion of the phagosome with lysosomal compartments by varying the intracellular traffic of the early phagosome vesicle. Ultimately, a *Brucella* replicative vacuole is formed with structures that resemble the endoplasmic reticulum membrane [18]. Gomes *et al* determined that the bacterial type IV secretion system virB and live, but not heat-killed, *Brucella* are required for full inflammasome activation in macrophages during infection [12]. Therefore, *Brucella* DNA is available in the endoplasmic reticulum-like organelle and/or escapes to the cytosolic compartment in order to be available to bind cytosolic DNA sensors. We believe that these are the steps required for the recognition of *Brucella* DNA by AIM2. Activation of caspase-1 leads to the proteolytic processing of pro-inflammatory cytokines and triggers pyroptosis [13]. Accordingly, the AIM2 receptor recognizes cytoplasmic *Brucella* DNA and activates caspase-1, which readily triggers pore formation. In our study, we observed that *B. abortus* and its DNA were able to activate the AIM2 inflammasome, causing caspase-1 activation and the release of large amounts of IL-1 β in dendritic cells. Nucleic acids are highly immunostimulatory when internalized or delivered into the cytoplasm of cells, and their role in driving immune defenses during infection with viral, bacterial and parasitic pathogens has gained appreciation over the last few years [19]. We observed that AIM2 was required for

pore formation in response to *B. abortus* DNA or poly-dAdT, but it was dispensable for pore formation in response to *B. abortus*. As shown in previous studies, pyroptosis plays an important role in controlling microbial infections; however, many pathogens, like *Brucella*, inhibit programmed cell death pathways using different approaches [20–22]. This indicates that pyroptosis is not a major mechanism induced by live *Brucella* via AIM2.

The majority of inflammasome studies have been performed in murine macrophages, and only a few studies have described the mechanisms of AIM2 activation triggered by bacterial infection in dendritic cells. A study performed with intracellular *F. tularensis* showed that this bacterium was able to activate the AIM2 inflammasome in dendritic cells, causing the release of IL-1 β and host cell death [23]. Similarly to macrophages infected by *F. tularensis*, BMDCs derived from AIM2, ASC, or caspase-1 KO mice are defective for cell death, IL-1 β secretion, and caspase-1 processing in response to infection [14, 23]. *Brucella* replicates within dendritic cells and hinders their functional activation [24]. Similar to observations in other cell types, such as macrophages, *Brucella* is dependent on the type IV secretion system (T4SS) to replicate within dendritic cells in an endoplasmic reticulum-derived compartment that evades the lysosomal degradative pathway. *Brucella* also inhibits dendritic cell maturation, leading to a reduction in cytokine secretion and antigen presentation [24]. Recently, we showed that *B. abortus* is able to activate the AIM2 inflammasome in bone marrow-derived macrophages (BMDMs), leading to caspase-1 activation and the release of IL-1 β [12]. In this study, we observed that AIM2 KO dendritic cells infected with *B. abortus* or transfected with *Brucella* DNA showed a major reduction in caspase-1 activation and impaired secretion of IL-1 β . Furthermore, we observed that type I IFN signaling was required for robust expression of guanylate-binding proteins (GBPs), which are involved in the intracellular killing of *Brucella* [25]. We believe that GBPs act on the *Brucella*-containing vacuole (BCV), and take part on processes that release bacterial components such as DNA into the cytoplasm to activate the AIM2 inflammasome [25]. Members of the guanylate-binding protein family are essential mediators of AIM2 activation during infection with the cytosolic bacterial pathogen *F. tularensis*. It was reported that GBPs act downstream of signaling via type I interferon receptors and bind *F. tularensis* to liberate its DNA. Thus, the mobilization of GBPs during infection facilitates the exposure of otherwise hidden bacterial ligands [26, 27]. Herein, we observed a negative regulatory effect of AIM2 on the interferon response in a *Brucella* infection assay. Dendritic cells derived from mice deficient for AIM2 after infection with *B. abortus* showed high expression of IFN- β and the molecules *GBP2*, *GBP3* and *GBP5* relative to their expression in wild-type BMDCs. Corrales *et al.* have demonstrated in tumor cells model, that pyroptosis induced by AIM2 activation reduces IFN- β production [28]. They concluded that *in vitro* activation of the AIM2 inflammasome in murine macrophages and dendritic cells leads to reduced activation of the type I IFN pathway via STING, in part through promoting caspase-1-dependent cell death [28].

AIM2 leads to the activation of caspase-1 and the production of IL-1 β and IL-18, which are potent pro-inflammatory cytokines and are crucial components of the defense against infection mediated by the activation of Th1 immune responses [29]. Th1-type lymphocytes secrete IFN- γ as the major effector cytokine against intracellular bacterial infections, including *Brucella*. In a *B. abortus* infection model, CD4⁺ and CD8⁺ T lymphocytes secrete

a Th1-type cytokine pattern responsible for control of the infection. Therefore, interferon-gamma is crucial for host control of *B. abortus* infection in mice [30, 17]. Saiga *et al* observed an impaired production of IL-1 and IL-18 as well as Th1 responses after *M. tuberculosis* infection in AIM2-deficient mice [31]. We also assessed IFN- γ responses following *Brucella* DNA stimulation. Splenocytes were isolated from *Brucella*-infected wild-type and AIM2 KO mice and subjected to *in vitro* stimulation with *Brucella* genomic DNA. The antigen-specific production of IFN- γ was severely reduced in splenocytes derived from AIM2 KO mice. These results indicate that the absence of AIM2 results in the impaired activation of Th1 responses.

In this study, we determined the important role of AIM2 in infection control, since we observed that AIM2 KO mice are more susceptible to *B. abortus* infection than wild-type animals in the first and third weeks after infection. Additionally, we observed that wild-type mice develop a significant granulomatous response in the first week of infection when compared to AIM2 KO animals, suggesting this response plays an important role in the control of infection. Therefore, these studies establish AIM2 as an important antimicrobial sensor and determinant of protective immunity to bacterial pathogens. Collectively, we have demonstrated that AIM2 acts as an important receptor for *Brucella* DNA in dendritic cells and plays a critical role in caspase-1-mediated cytokine processing and control of infection.

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Fig. 1. AIM2 associates with *Brucella* in infected BMDCs.

Dendritic cells were infected with RFP-expressing *B. abortus* (shown in red) (MOI: 10) for 16 hours and fixed to be processed for immunofluorescence using an anti-AIM2 antibody (shown in green). Only weak and disperse cytoplasmic AIM2 staining can be observed in uninfected cells (right panels) whereas pronounced aggregates of AIM2 can be frequently observed in association with *Brucella* in infected DCs (left panels). Dendritic cells and bacterial DNA is labelled with DAPI (shown in blue). Data are representative of three

independent experiments. White arrows show clear association between AIM2 and *Brucella*. Scale bar corresponds to 5µm.

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Fig. 2. *Brucella* and its DNA elicited IL-1 β secretion and caspase-1 activation in dendritic cells via AIM2.

BMDCs derived from AIM2 KO or C57BL/6 mice were infected with *B. abortus* or the virB mutant strain (both at an MOI of 100:1) or treated with MSU (250 μ g/ml). BMDCs were transfected with DNA isolated from *B. abortus* (1 μ g/well) or with poly-dAdT (1 μ g/well) complexed with lipofectamine or lipofectamine alone as a control. DNase-treated *Brucella* DNA was also used as control. Culture supernatants were harvested 17 h after treatment to measure (A) IL-1 β levels in an ELISA assay or caspase-1 processing (B) was evaluated by western blotting. * $p < 0.05$, AIM2 KO versus C57BL/6 mice, two-way ANOVA. (C)

BMDCs from wild-type mice were transfected with DNA isolated from *B. abortus* (1 µg/well) or with poly-dAdT (1 µg/well) complexed with Lipofectamine. The A151 AIM2 inhibitor was added at 3 µM 1 h prior to transfection. Culture supernatants were harvested 17 h after treatment to measure IL-1β by ELISA. * $p < 0.05$, DNA or poly-dAdT transfecte cells compared to DCs pretreated with A151 inhibitor. Data are representative of three independent experiments and three replicates in each experimental group.

Fig. 3. *Brucella abortus* DNA leads to pore formation and pyroptosis via AIM2.

Cells obtained from C57BL/6 and AIM2 KO mice were left uninfected (NI), infected with *B. abortus* at an MOI of 100 (A), transfected with *Brucella* DNA (1 μ g/well) (B) or treated with nigericin (20 μ M) (C). Pore formation was assessed fluorimetrically in real time based on the uptake of propidium iodide (relative fluorescence units). (D) Cell death was also determined by measuring the release of LDH in the supernatant at 17 h postinfection or stimulation. DCs were infected with *B. abortus* (MOI 100:1) or transfected with *Brucella* DNA (1 μ g/well) or with poly-dAdT (1 μ g/well) encapsulated with lipofectamine. Statistically significant differences are denoted by an asterisk for $p < 0.05$, AIM2 KO versus wild-type mice, two way ANOVA. Data are representative of three independent experiments.

Fig. 4. Negative regulatory effects of AIM2 on type I interferon responses.

AIM2 KO BMDCs showed elevated expression of the genes encoding for (A) *IFN-β*, (B) *GBP2*, (C) *GBP3* and (D) *GBP5* relative to their expression in wild-type BMDCs. Real-time quantitative RT-PCR analysis of *IFN-β* and *GBP*-encoding genes in BMDCs 16 hrs after infection with *B. abortus*, transfected with *B. abortus* genomic DNA (1 μg/well) or genomic DNA pretreated with DNase as a negative control (1 μg/well), presented relative to the expression of the gene encoding β-actin. * $p < 0.05$, AIM2 KO versus C57BL/6 mice, two-way ANOVA. Data are representative of three independent experiments and three replicates in each experimental group.

Fig. 5. The production of IFN- γ by spleen cells is partially dependent on AIM2.

We evaluated IFN- γ production induced by *B. abortus* genomic DNA in AIM2 KO and C57BL/6 splenocytes. Groups of 5 mice were injected intraperitoneally with 10^6 CFU of *B. abortus* for 1 (A), 3 (B) and 6 (C) weeks. After this period of priming, spleens cells (1×10^6 cells) were stimulated with *B. abortus* genomic DNA (1 μ g/well), genomic DNA pretreated with DNase as a negative control (1 μ g/well) and ConA (5 μ g/mL). IFN- γ levels were measured by ELISA after 72 hrs. Statistically significant differences in relation to C57BL/6 mice are indicated by *** for $p < 0.001$ in relation to AIM2 KO mice. Data are

representative of three independent experiments and three replicates in each experimental group.

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Fig. 6. AIM2 is critical for the efficient control of *B. abortus* infection *in vivo*.

(A) Residual *B. abortus* CFU in the spleens of wild-type and AIM2 KO mice (n=5) were determined at 1 and 3 weeks post-infection. * $p < 0.05$ or *** $p < 0.001$, AIM2 KO versus wild-type mice, two-way ANOVA. (B) Medial liver lobes from *B. abortus*-infected wild-type and AIM2 KO mice were collected and the arrows show granulomatous lesion at 1 week postinfection. (C) Tissue sections were processed and stained with H&E to evaluate granuloma formation. Digital images of representative granulomas (original magnification 40x). (D) Granuloma numbers were normalized to 50 mm² of hepatic tissue at 1 and 3 weeks postinfection (wpi). *** $p < 0.001$, AIM2 KO versus C57BL/6 mice, two-way ANOVA. Data are representative of three independent experiments.