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AIM2 Engages Active but Unprocessed Caspase-1 to Induce Noncanonical Activation of the NLRP3 Inflammasome

Graphical Abstract

Highlights

- AIM2 triggers caspase-1 activation, but not cleavage, in response to Legionella infection
- AIM2 engages active but unprocessed caspase-1 to induce pore formation and K⁺ efflux
- AIM2 amplifies infection signals to trigger activation of the NLRP3 inflammasome
- AIM2 and caspase-11 cooperate to induce host resistance to Legionella infection

Authors

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In Brief

Cunha et al. find that the AIM2 inflammasome is activated in response to Legionella and cooperates with caspase-11 to trigger host resistance. Mechanistically, AIM2 engages active but unprocessed caspase-1 to trigger pore formation and K⁺ efflux, which also converges into NLRP3 activation. Thus, the AIM2 inflammasome cooperates with caspase-11 to amplify the signals of infection and triggers noncanonical activation of the NLRP3 inflammasome.

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AIM2 Engages Active but Unprocessed Caspase-1 to Induce Noncanonical Activation of the NLRP3 Inflammasome

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SUMMARY

Inflammasomes are multimeric protein complexes that initiate inflammatory cascades. Their activation is a hallmark of many infectious or inflammatory diseases. Their composition and activity are specified by proinflammatory stimuli. For example, the NLRP3 inflammasome is activated in response to cell damage and K⁺ efflux, whereas the AIM2 inflammasome is activated in response to cytosolic DNA. We used *Legionella pneumophila*, an intracellular bacterial pathogen that activates multiple inflammasomes, to elucidate the molecular mechanisms regulating inflammasome activation during infection. Upon infection, the AIM2 inflammasome engaged caspase-1 to induce pore formation in the cell membrane, which then caused K⁺-efflux-mediated activation of NLRP3. Thus, the AIM2 inflammasome amplifies signals of infection, triggering noncanonical activation of NLRP3. During infection, AIM2 and caspase-11 induced membrane damage, which was sufficient and essential for activating the NLRP3 inflammasome. Our data reveal that different inflammasomes regulate one another’s activity to ensure an effective immune response to infection.

INTRODUCTION

Inflammation is a protective response triggered by the innate immune system in response to pathogens or damage caused by potentially harmful stimuli. A key contributor to the inflammatory response is the intracellular activation of inflammasomes, which are large multimeric protein complexes that serve primarily as an activating scaffold for caspase-1. Upon sensing harmful stimuli such as pathogens or irritants, pattern-recognition receptors assemble distinct inflammasome structures that recruit and activate caspase-1. Activated caspase-1 then initiates a large proinflammatory cascade by cleaving various molecules, including inflammatory cytokines, into their bioactive forms. Inflammasome function and activity is dictated by the particular pattern-recognition receptor sensing the stimulus. Although the mechanisms leading to activation of inflammasomes are well characterized, the mechanisms by which distinct inflammasome types are able to compartmentalize their activity are not.

Inflammasomes are often studied in genetically engineered mice lacking a single gene. High concentrations of synthetic or purified molecules that activate distinct inflammasome types are also used. This approach provides valuable information about the specific functions of different inflammasome types; however, it does not provide a comprehensive understanding of the cooperation between inflammasomes during infection or the effect of one inflammasome type on the regulation of another. In this context, the use of relevant pathogens that target inflammasomes to model the physiologic response to infection is essential to assess the role of inflammasomes in infection and immunity. This is particularly important for activation of the pattern-recognition receptor absent in melanoma 2 (AIM2), which recruits the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) to promote caspase-1 activation in response to cytosolic DNA (Burckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). The AIM2 inflammasome is broadly involved in pathogen recognition, but the physiologic functions of this inflammasome type remain obscure.

*Legionella pneumophila* is a gram-negative bacterium that causes a severe form of pneumonia called Legionnaires disease. *L. pneumophila* evolved in freshwater environments, where it replicates in several protozoa species. Mammalian infection is inadvertent and considered a “dead end” for the bacteria. Therefore, *L. pneumophila* has not adapted a mechanism to bypass/inhibit innate immune signaling pathways that are not present in amoeba. Because *L. pneumophila* initiates the activation of multiple inflammasome types, it is an appropriate model of pathogenic microbial infection to investigate functions of the innate immune system (Cunha and Zamboni, 2014; Fontana and Vance, 2011; Massis and Zamboni, 2011).
L. pneumophila expresses Dot/Icm, a type IV secretion system that delivers bacterial proteins into the cytoplasm of host cells. Dot/Icm is essential for bacterial replication in macrophages, which also enables the innate immune system to identify pathogens and activate multiple innate immune pathways (Shin, 2012; Vance, 2011). Dot/Icm facilitates the delivery of bacterial flagellin into the cytoplasm, which is an agonist that stimulates intracellular Nod-like receptors (NLRs) such as NAI5 that initiate the assembly an inflammasome composed of NLRC4 and caspase-1 (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006). L. pneumophila flagellin mutants ( flaA ) bypass NAI5/NLRC4 inflammasome-mediated growth restriction and freely multiply in restrictive macrophages. Therefore, NAI5 and NLRC4 inflammasomes are required to restrict bacterial replication in macrophages (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006).

The AIM2 inflammasome is also activated in response to L. pneumophila infection, which also depends on Dot/Icm. SdhA, an effector protein secreted by Dot/Icm, stabilizes L. pneumophila-containing vacuoles and consequently reduces AIM2 activation (Creasey and Isberg, 2012; Ge et al., 2012). Deletion of the sdhA gene strongly activates AIM2-mediated cell death. Nevertheless, AIM2 is activated in wild-type (WT) L. pneumophila containing SdhA (Ge et al., 2012).

Caspase-11 is activated in response to L. pneumophila infection (Aachoui et al., 2013; Akhter et al., 2012; Case et al., 2013; Casson et al., 2013; Pilla et al., 2014) through a mechanism independent of bacterial flagellin, NLRC4, or caspase-1. However, this occurs after NLRC4 inflammasome activation (Case et al., 2013) most likely because caspase-11 activation requires macrophage priming to trigger its transcriptional upregulation (Kayagaki et al., 2011). Once activated, caspase-11 triggers macrophage pyroptosis (i.e., inflammation-associated programmed cell death) and noncanonical activation of the NACHT-, LRR-, and PYD-domain-containing protein 3 (NLRP3) inflammasome. Caspase-11-induced NLRP3 inflammasome formation requires ASC and culminates in caspase-1 activation and inflammatory cytokine secretion (Case et al., 2013; Casson et al., 2013; Kayagaki et al., 2011).

Lipopolysaccharide (LPS), flagellin, and bacterial DNA are agonists for caspase-11, NAI5/NLRC4/caspase-1, and AIM2/ASC/caspase-1 inflammasomes, respectively. However, the specific agonist and mechanisms underlying the activation of NLRP3/ASC/caspase-1 remain obscure, which is surprising given caspase-11 is the most thoroughly studied inflammasome type. NLRP3 is also involved in the pathogenesis of several inflammatory and infectious diseases (reviewed in Wen et al., 2012). Although several molecules activate NLRP3 (e.g., reactive oxygen species, lysosomal cathepsins, bacterial RNA, and mitochondrial DNA), K+ efflux appears to be essential for NLRP3 activation (Baker et al., 2015; Muñoz-Planillo et al., 2013; Perregaux and Gabel, 1994; Pétrilli et al., 2007; Rühl and Broz, 2015; Schmid-Burgk et al., 2015).

We used the bacterial pathogen L. pneumophila as a relevant model of infection to assess the coordination of inflammasome functions during infection. We hypothesize that the AIM2 inflammasome does not directly cooperate with NLRP3 to trigger caspase-1 activation. Instead, AIM2 indirectly activates the NLRP3 inflammasome during the physiologic response to infection by inducing membrane damage and K+ efflux, thereby amplifying the signals of infection. Thus, AIM2 induces noncanonical activation of the NLRP3 inflammasome, a process that ensures robust activation of inflammasomes and an effective innate immune response to infection.

RESULTS

Pore Formation Can Occur Independently of Flagellin/NLRC4 and LPS/Caspase-11 Inflammasomes in L. pneumophila-Infected Macrophages

Infection of mouse macrophages with WT L. pneumophila activates the NAI5/NLRC4 inflammasome, which induces pore formation via mechanisms dependent on caspase-1 and flagellin (Case et al., 2009; Silveira and Zamboni, 2010; Zamboni et al., 2006). In addition, caspase-11 mediates pore formation and pyroptosis independently of flagellin/NLRC4 and caspase-1 but requires upregulation of Casp11 through macrophage priming (Case et al., 2013). To assess the role of caspase-1 and caspase-11 in pyroptosis, we examined pore formation by evaluating the loss of membrane integrity in Legionella-infected bone-marrow-derived macrophages (BMDMs) using a real-time fluorimetric assay of propidium iodide (PI) uptake. We primed the cells with LPS and observed that pore formation induced by WT L. pneumophila (JR32 WT) partially depended on caspase-1 and was abolished in BMDMs derived from Casp11−/− deficient mice (Figures 1A and 1C). As previously reported (Case et al., 2013), pore formation was reduced overall and depended on caspase-11, but not caspase-1, in response to flaA L. pneumophila (Figure 1B). Experiments performed using L. pneumophila dotA mutants, which have a defective Dot/Icm system, indicated that the bacterial type IV secretion system is essential for pore formation in macrophages (Figure 1D). We obtained similar results for BMDMs primed with tumor necrosis factor alpha (TNF-α), indicating that regardless of the molecule used for priming, caspase-1 activation requires flagellin and caspase-11 does not (Figures 1E–1G). Furthermore, flaA L. pneumophila triggered pore formation in Casp11−/− BMDMs, suggesting that a pathway independent of flagellin/NLRC4 and caspase-11 can mediate pore formation (Figures 1B and 1F). This unidentified pore-formation pathway presumably depends on caspase-1, because it was abolished in Casp11−/− deficient BMDMs and reduced in Casp1−/− BMDS compared to WT BMDMs (C57BL/6) (Figures 1B and 1F).

These data support the idea that Legionella-induced pore formation and pyroptosis involve multiple pathways. One pathway depends on flagellin/NLRC4 and caspase-1, another depends on caspase-11, but not flagellin, and a third is independent of flagellin and caspase-11.

The AIM2 Inflammasome Promotes Pore Formation, Pyroptosis, and Caspase-1 Activation in Response to L. pneumophila

We determined the molecules comprising the pathway that functions independently of flagellin/NLRC4 and LPS/caspase-11 with flaA L. pneumophila. The AIM2 inflammasome is activated...
Figure 1. AIM2 Induces NLRC4 and Caspase-11-Independent Pore Formation and Pyroptosis in Response to L. pneumophila Infection

(A–K) Fluorometric quantification of PI uptake over time, expressed in relative fluorescence units (RFUs). BMDMs BL/6, Casp11–/–, Casp1–/–, and Casp1/11–/– pretreated with LPS (500 ng/mL) for 3 hr were either not infected (NI) (A) or infected at MOI 20 with flaA mutant strain of L. pneumophila (JR32 flaA) (B), WT L. pneumophila (J232 WT) (C), or dotA mutant strain (JR32 dotA) (D). BMDMs BL/6, Casp11–/–, Casp1–/–, and Casp1/11–/– pretreated with TNF-α (10 ng/mL) for 3 hr were either NI (E), infected with JR32 flaA at MOI 20 (F), or JR32 WT (G). BMDMs BL/6, Aim2–/–, Casp11–/–, and Casp1/11–/– pretreated with LPS (500 ng/mL) for 3 hr were either NI (H) or infected at MOI 20 with JR32 flaA (I), BMDMs BL/6, Casp1/1–/–, Aim2–/–/Casp11–/–, and Casp1/11–/– pretreated with LPS (500 ng/mL) for 3 hr were NI (J) or infected with JR32 flaA at MOI 20 (K). Data are representative of four independent experiments and are expressed as the average of triplicate wells.

(L and M) Cytotoxicity was measured in an LDH-release assay. BMDMs pretreated with LPS (500 ng/mL) for 3 hr were either NI (L) or infected with JR32 flaA at MOI 20 (M). Data are representative of three independent experiments and are expressed as the mean of triplicate wells. Error bars depict SEM. *p < 0.05 versus Casp1/11–/– BMDMs.

See also Figure S1.
BMDMs from induced pore formation. We found that the absence of AIM2 in we tested whether AIM2 was required for Dot/Icm and activates caspase-1 (in response to L. pneumophila-induced pore formation culminates in macrophage pyroptosis. Therefore, we measured lactate dehydrogenase (LDH) release to assess macrophage pyroptosis in response to flaA L. pneumophila. In agreement with our pore-formation findings, AIM2 and caspase-11 induced pyroptosis in response to L. pneumophila (Figures 1L and 1M). In immortalized C57BL/6 macrophages that express an AIM2-citrine fusion construct, flaA+ L. pneumophila induced AIM2 oligomerization (Figures S1C and S1D). This corroborates previous reports of AIM2 activation in response to L. pneumophila infections (Ge et al., 2012).

Caspase-11 activates the noncanonical pathway of NLRP3 inflammasome activation (Kayagaki et al., 2011; Rathnam et al., 2012). Therefore, we tested whether AIM2 cooperates with caspase-11 to trigger the noncanonical pathway, which induces caspase-1 proteolysis and proteolytic maturation and secretion of interleukin-1 beta (IL-1β). Compared to that in WT C57BL/6 BMDMs, Casp11−/− BMDMs exhibited reduced caspase-1 cleavage in response to infection with flaA+ L. pneumophila in cells primed with LPS or TNF-α (Figures 2A and 2B). Deficiency of Aim2 alone had little effect on caspase-1 processing; however, caspase-1 proteolysis was strongly reduced in BMDMs that were double deficient in Aim2 and Casp11. Caspase-11-independent pore formation was abolished in these macrophages (Figures 1J and 1K). Similar results were obtained by infection with Lp01, a widely used laboratory strain of L. pneumophila that was isolated from a historic outbreak of Legionnaires disease in Philadelphia in 1976 (Figures S1A and S1B).

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NLRP3 Activation and K⁺ Efflux Are Required for Caspase-1 Proteolysis, but Not Pore Formation, in Response to L. pneumophila

Caspase-11 activation facilitates the maturation of caspase-1 and the secretion of IL-1β in response to flaA⁻⁻ L. pneumophila (Case et al., 2013). This suggests that caspase-11 plays a role in mediating the noncanonical activation of NLRP3, a process that culminates with caspase-1 cleavage and IL-1β secretion in response to gram-negative bacteria (Kayagaki et al., 2011; Rathinam et al., 2012). Our findings indicated that AIM2 or caspase-11 is required for caspase-1 proteolysis. Therefore, we tested whether AIM2 participates in the noncanonical pathway to activate NLRP3. We initially used flaA⁺⁻ L. pneumophila to assess whether NLRP3 inflammasome activation was required for caspase-1 proteolysis in response to infection in the presence of AIM2 and caspase-11. NLRP3 activation was essential for caspase-1 proteolysis and IL-1β secretion, regardless of the presence of caspase-11 and AIM2 (Figures 3A and 3B). In the absence of both, caspase-1 proteolysis and IL-1β secretion were abolished, regardless of the presence of NLRP3. These results are consistent with a model in which caspase-11 or AIM2 is required to activate NLRP3. To determine whether AIM2 and NLRP3 function in two independent inflammasome types or in the same type, we infected BMDMs with flaA⁻⁻ L. pneumophila and performed immunostaining for endogenous NLRP3 and AIM2. Both AIM2 and NLRP3 foci formed in response to infection, but they did not colocalize (Figures 3C–3F). Therefore, AIM2 and NLRP3 function in discrete molecular complexes. These results support the hypothesis that AIM2 mediates a process resulting in noncanonical activation of NLRP3 via a mechanism similar to yet distinct from that of caspase-11.

To investigate whether the AIM2 inflammasome is sufficient to trigger caspase-1 proteolysis in the absence of NLRP3, we evaluated caspase-1 proteolysis in high extracellular K⁺ conditions, which inhibits the activation of NLRP3, but not that of AIM2. Caspase-1 proteolysis and IL-1β maturation and secretion were abrogated in the presence of KCl, but not NaCl, in all BMDMs tested (Figures 3C–3F). These data support a model in which NLRP3 is a key factor in caspase-1 proteolysis and IL-1β secretion. According to this model, caspase-11 or AIM2 is required to trigger NLRP3-mediated caspase-1 cleavage and IL-1β secretion.

Figure 3. AIM2 and Caspase-11 Cooperate to Induce K⁺ Efflux that Enables NLRP3-Induced Cleavage of Caspase-1 in Response to Infection

(A) Immunoblot of caspase-1 in the supernatant (SN) and cell extract (CE) of BMDMs. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were either not infected (NI) or infected for 2 hr with JR32 flaA (MOI 20). Data are representative of three independent experiments.

(B) IL-1β released in BMDM SN, as measured by ELISA. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were infected for 4 hr with JR32 flaA (MOI 20), in the presence of NaCl (C) or KCl (D). Data shown are representative of three independent experiments.

(C and D) Immunoblot of caspase-1 and IL-1β in the SN and CE of BMDMs. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were NI or infected for 2 hr with JR32 flaA (MOI 20), in the presence of NaCl (C) or KCl (D). Data shown are representative of three independent experiments.

(E and F) IL-1β released in BMDM SN, as measured by ELISA. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were infected for 4 hr with JR32 flaA (MOI 20), in the presence of NaCl (E) or KCl (F). Data shown are representative of three independent experiments and are expressed as the mean of trypical wells. Error bars depict ± SEM. *p < 0.05 versus NI.

(G) Assessment of intracellular K⁺ levels, as measured by the fluorescent K⁺ probe APG-2. BMDMs were NI or were infected for 2 hr with JR32 flaA (MOI 5). Each dot represents the percentage of APG-2 fluorescence intensity in relation to the average fluorescence of control cells, and the bars represent the mean of all analyzed cells. Error bars depict ± SEM. *p < 0.05 versus NI. Data shown are representative of three independent experiments.

See also Figures S3 and S4.
secretion. To further test this hypothesis, we determined whether AIM2 and caspase-11 are required for K\textsuperscript{+} efflux in macrophages infected with flaA\textsuperscript{−/−} L. pneumophila. The intracellular K\textsuperscript{+} concentration decreased in response to infection, and this was independent of ASC and NLRP3 and partially dependent on AIM2 and caspase-11 (Figure 3G). We detected no K\textsuperscript{+} efflux in Aim2\textsuperscript{−/−}/Casp11\textsuperscript{−/−} or Casp11\textsuperscript{−/−}/BMDMs (Figure 3G). These data are consistent with the hypothesis that AIM2 or caspase-11 is required for pore formation, leading to the noncanonical activation of NLRP3 via K\textsuperscript{+} efflux.

Our data demonstrate that NLRP3 is required to induce caspase-1 proteolysis via either AIM2 or caspase-11. Therefore, we investigated whether NLRP3 is likewise required for the functions of caspase-11 and AIM2 inflammasomes (i.e., pore formation, pyroptosis, and inflammasome assembly) in infected BMDMs. NLRP3 was not required for pore formation model in which AIM2 or caspase-11 is required to induce pore formation and activate NLRP3. Conversely, NLRP3 is dispensable for the functions of AIM2 and caspase-11.

Activation of AIM2 Inflammasome Preferentially Induces Pore Formation, and NLRP3 Is Essential for Caspase-1 Cleavage

To evaluate the requirement of NLRP3 for caspase-1 cleavage and pore formation, we established mouse strains that are double deficient for Nlrp3 and Casp11 or Nlrp3 and Aim2. Measuring pore formation in response to flaA\textsuperscript{−/−} L. pneumophila, we found that Nlrp3\textsuperscript{−/−}/Casp11\textsuperscript{−/−} BMDMs phenocopied Casp11\textsuperscript{−/−} cells, confirming that Nlrp3 does not affect pore formation in response to infection (Figures 4A and 4B). We treated BMDMs with nigericin or transfected them with poly(dA:dT) to confirm that pore formation is induced by canonical activation (Figures S4A and S4B). Conversely, attenuating K\textsuperscript{+} efflux did not affect pore formation in response to infection in either WT or Casp11\textsuperscript{−/−}/BMDMs (Figures S4C–S4F), which confirmed that NLRP3 is not required for this process. In agreement with these findings, pyroptosis was not affected by the absence of NLRP3, as LDH levels and K\textsuperscript{+} efflux were unaffected in Nlrp3\textsuperscript{−/−}/BMDMs (Figures S4G–S4I). These findings also support the hypothesis that caspase-11- or AIM2-mediated pore formation does not require NLRP3. Finally, reducing K\textsuperscript{+} efflux did not affect the induction of the AIM2 inflammasome, as indicated by AIM2-citrine oligomerization in response to infection with flaA\textsuperscript{−} L. pneumophila or transfection with double-stranded DNA (Figures S4J and S4K). Collectively, our data support a
of NLRP3 (Figure 4C) or AIM2 (Figure 4D), as previously established (Kayagaki et al., 2011). We also confirmed that caspase-1 cleavage in response to infection depends on NLRP3. Nlrp3−/−/Casp11−/− and Aim2−/−/Nlrp3−/− BMDMs did not process caspase-1 (Figure 4B), whereas NLRC4 activation triggered caspase-1 cleavage in WT BMDMs (Figure 4F). Furthermore, caspase-1 cleavage induced by nigericin depended only on NLRP3, whereas cleavage induced by poly(dA:dT) depended only on AIM2 (Figure 4F). These data support the hypothesis that AIM2 inflammasome promotes the activation and cleavage of caspase-1 in the presence of high amounts of cytosolic DNA. However, during the physiologic response to infection, AIM2 preferentially triggers pore formation to induce caspase-1 cleavage by NLRP3.

AIM2-Induced Pore Formation Requires Caspase-1 Activity, but Not Proteolysis

NLRP3 is essential for caspase-1 proteolysis in the physiologic response to L. pneumophila infection. AIM2-mediated pore formation requires caspase-1 but occurs in the absence of NLRP3. Therefore, we hypothesized that AIM2 requires caspase-1 but triggers pyroptosis independently of caspase-1 proteolysis. An uncleavable yet catalytically active variant of caspase-1 but triggers pyroptosis independently of caspase-1 (Broz et al., 2010). To test the hypothesis that AIM2 employs an active but uncleaved form of caspase-1 to trigger pore formation independently of caspase-1, we transduced Casp1/11−/− BMDMs with a lentivirus encoding WT caspase-1 (Casp1 WT), Casp1 6DN, or empty virus (vector) and then assayed pore formation. Western blot analysis confirmed that Casp1 WT, but not Casp1 6DN or Casp1 C285A, underwent autoproteolysis in response to infection with flaA-L. pneumophila (Figure 5A). We then verified the caspase-1 requirements for pore formation in immortalized Casp1/11−/− BMDMs that stably overexpress Casp1 WT or Casp1 6DN. Overexpression of either variant failed to induce spontaneous pore formation in uninfected cells. Likewise, infection with dotA-L. pneumophila did not trigger pore formation (Figures 5B–5D). In contrast, infection with WT or flaA-L. pneumophila readily induced pore formation in BMDMs expressing Casp1 WT or Casp1 6DN, but not empty virus (Figures 5B–5D). Similar results were obtained when we used a lentivirus to express Casp1 WT and Casp1 6DN in primary macrophages from Casp1/11−/− mice (Figures S5A–S5D). Overexpression of Casp1 WT or Casp1 6DN did not restore caspase-1 activity in triple-knockout BMDMs in response to infection, suggesting that the activation of uncleavable caspase-1 requires ASC (Figures S5E–S5G).

To determine whether AIM2 engages unprocessed caspase-1 to trigger pore formation in the absence of caspase-1, we transduced BMDMs from Casp1/11−/− or Aim2−/−/Casp1/11−/− mice with a lentivirus encoding Casp1 WT, Casp1 6DN, or vector and then assayed the cells for pore formation. We also included a lentivirus encoding the catalytically inactive form of caspase-1 (Casp1 C285A) (Figure 5A). Overexpression of Casp1 WT and Casp1 6DN restored pore formation in Casp1/11−/− BMDMs infected with flaA-L. pneumophila (Figures 5E–5G). In contrast, Casp1/11−/− BMDMs expressing Casp1 C285A did not trigger pore formation (Figure 5F). Therefore, cleavage is dispensable, but the C285 active site is essential for caspase-1-induced pore formation.

When we used Aim2−/−/Casp1/11−/− BMDMs, no pores formed in response to flaA-L. pneumophila (Figures 5H and 5I). In this experiment, we included infections with WT L. pneumophila as a positive control, because the NLRC4 inflammasome triggers caspase-1-dependent pore formation. WT L. pneumophila induced pore formation in Casp1/Casp11−/− and Aim2−/−/Casp1/Casp11−/− BMDMs when the cells expressed Casp1 WT or Casp1 6DN, but not Casp1 C285A (Figure 5J). These results indicate that AIM2 is dispensable for NLRC4-induced pore formation, and caspase-1 activation without proteolysis is sufficient to induce pore formation via AIM2 or NLRC4 inflammasomes. These results imply that these inflammasomes engage an active, uncleaved form of caspase-1 to trigger pore formation. This process is also necessary and sufficient to induce the noncanonical activation of the NLRP3 inflammasome.

AIM2- and Caspase-11-Induced Pore Formation Contributes to the Restriction of Pathogen Replication In Vivo

We next evaluated the consequences of activating inflammasomes on the infection ability of L. pneumophila in macrophages and in vivo. In Legionnaires disease, NAP5/NLRC4 (but not NLRP3) inflammasome mediates resistance to pulmonary infection (Amer et al., 2006; Case et al., 2009; Molofsky et al., 2006; Pereira et al., 2011; Zamboni et al., 2008). Therefore, we used flaA-L. pneumophila to evaluate whether mice deficient in the signaling pathways that mediate pore formation are more susceptible to infection. WT and Aim2−/−/Casp11−/− mice were infected with flaA-L. pneumophila at a very low MOI (103 per mouse). Bacterial replication was higher in Aim2−/−/Casp11−/− mice than in WT mice (Figure 6A). Although loss of AIM2 or caspase-11 individually in littermate control mice did not influence the restriction of L. pneumophila replication, Aim2−/−/Casp11−/− mice were more susceptible than WT mice (Figure 6B). Because the innate immune pathways that restrict L. pneumophila infections in vivo are extensively redundant, these data indicate that pore formation induced by caspase-11 and AIM2 contributes to the restriction of pulmonary bacterial replication in a murine model of Legionnaires disease.

DISCUSSION

L. pneumophila is a genetically tractable bacterial pathogen that targets multiple inflammasome types. We determined that the activation of NLRP3 by L. pneumophila is regulated by other inflammasome types. Cell damage induced by at least one inflammasome type (NLRC4, caspase-11, or AIM2) is required and sufficient to engage NLRP3 and to independently mediate resistance to L. pneumophila infection.

Membrane-disrupting pathogens (e.g., Listeria, Shigella, Francisella, and Rickettsia) and many toxin-producing bacteria damage host cell membranes. Likewise, certain fungi and the protozoan parasite Trypanosoma cruzi cause cell damage (Silva et al., 2013; Wellington et al., 2014; Zamboni and Lima-Junior, 2015). Therefore, these pathogens may activate NLRP3 via a...
Figure 5. Caspase-1 Activity, but Not Proteolysis, Is Necessary and Sufficient to Trigger AIM2-Dependent Pore Formation in Response to L. pneumophila Infection

(A) Immunoblot of caspase-1 in the precipitated supernatant (SN) and cell extract (CE) of BMDMs. The BMDMs from Casp1/11<sup>−/−</sup> Aim2<sup>−/−</sup>/Casp1/11<sup>−/−</sup> and Casp1/11<sup>−/−</sup> Aim2<sup>−/−</sup>/Casp1/11<sup>−/−</sup> mice were transduced with viruses encoding empty vector (Vector), WT caspase-1 (Casp1<sup>WT</sup>), 6DN mutant (Casp1<sup>6DN</sup>), or catalytically inactive C285A mutant (Casp1<sup>C285A</sup>). Cells pretreated for 3 hr with LPS (500 ng/mL) were either not infected (NI) or infected for 2 hr with JR32 flaA (MOI 20).

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process that does not first require inflammasomes to damage host cells. This hypothesis is consistent with the fact that crystals and pore-forming molecules are bona fide activators of NLRP3. Of note, the crystal- and toxin-mediated canonical activation of the NLRP3 inflammasome requires host cell priming to regulate the transcription of Nlrp3 and the pro-forms of inflammatory cytokines (e.g., Il1b and Il18), in addition to the posttranscriptional mechanisms of NLRP3 degradation (Bauerfeind et al., 2009; Gohonine et al., 2014; Juliana et al., 2012; Mariathasan et al., 2004; Perregaux et al., 2003). Therefore, host cell priming in combination with a membrane-disrupting agent may mimic host cell infection by virulent pathogens by damaging membranes. In contrast, bona fide intracellular pathogens are usually nonlytic to host cells and may even protect cells from damage (Ashida et al., 2011; Cunha and Zamboni, 2013; Galluzzi et al., 2008; Rodrigues et al., 2012). In this case, innate immune cells may employ additional cellular components to induce cell damage and K⁺ efflux, which is essential to activate NLRP3. As shown in this study, additional inflammasome types are activated by intracellular pathogen-associated molecular patterns and can trigger pore formation, thereby facilitating the engagement of the NLRP3 inflammasome. We have shown previously and here that flagellin/NLRC4, LPS/caspase-11, and DNA/AIM2 can induce cell damage, which enables K⁺ efflux and activates NLRP3 and caspase-1 proteolysis (Baker et al., 2015; Rühl and Broz, 2015; Schmid-Burgk et al., 2015). We found that caspase-11, NLRC4, AIM2, or a combination thereof is necessary and sufficient to trigger NLRP3 inflammasome in response to L. pneumophila infection. Therefore, several inflammasome types provide innate immune cells with multiple platforms from which to detect distinct cytosolic pathogens and trigger the canonical activation of NLRP3, a process that culminates in caspase-1 cleavage and inflammatory cytokine induction.

Cell membrane permeabilization and consequent pyroptosis is required for inflammasome-mediated restriction of infection by intracellular pathogens (Miao et al., 2010). Although recognition of flagellin by NAIP/NLRC4 is the main pathway triggering pyroptosis and control of L. pneumophila replication, NLRP3 is dispensable for restricting bacterial replication in vivo (Case et al., 2009). Our data reveal a role for AIM2 and caspase-11 in control of infection by L. pneumophila in vivo, suggesting that the induction of pyroptosis by these inflammasomes ensures bacterial restriction independently of their role in the down-stream activation of NLRP3 (Figure 7).

Our results suggest that AIM2 operates via a different multi-protein platform than does NLRP3. We did not detect colocalization of NLRP3 and AIM2 in infected cells, indicating that two distinct inflammasome types are assembled in response to infection. These data are consistent with the fact that NLRP3 is dispensable for either the assembly of AIM2 inflammasome or AIM2-induced pore formation. Recent reports have shown the colocalization of proteins from different inflammasome types (Karki et al., 2015; Man et al., 2014). Therefore, the lytic activities of pathogens may simultaneously activate the two types of inflammasomes, leading to the colocalization of AIM2 and NLRP3. In contrast, L. pneumophila did not activate NLRP3 in the absence of caspase-11, AIM2, or NLRC4, which suggests that at least one of these inflammasome types is required to activate NLRP3. This temporal delay in inflammasome activation may not favor the recruitment of AIM2 and NLRP3 in the same protein complex. Therefore, our data support a model in which AIM2 and NLRP3 are assembled in two distinct inflammasome types.

An important aspect of caspase-1 activation that we have demonstrated here and others have shown is that caspase-1 can function in an active but uncleaved form (Broz et al., 2010; Guey et al., 2014; Van Opdenbosch et al., 2014). Active, (B–J) Fluorometric quantification of PI uptake over time, expressed in RFUs. Casp1/11−/− immortalized bone-marrow-derived macrophages (BMDMs) transduced with vector (B), Casp1 WT (C), or Casp1 ΔBDN (D) and pretreated with LPS (500 ng/mL) for 3 hr were infected with L. pneumophila (MOI 20 with JR32 flaA), or JR32 ΔdoA (MOI 20). Casp1/11−/−BMDMs transduced with the different caspase-1 constructs were pretreated with LPS (500 ng/mL) and either NI (E) or were infected at MOI 20 with JR32 flaA (F), or JR32 WT (G). Aim2−/−/Casp1/11−/− BMDMs transduced with the different caspase-1 constructs were pretreated with LPS (500 ng/mL) and either NI (H) or were infected at MOI 20 with JR32 flaA (I) or JR32 WT (J). Data shown are representative of three independent experiments.
uncleaved caspase-1 is recruited by NLRC4 and AIM2 to trigger pore formation and pyroptosis. These inflammasomes function in cells that express a catalytically null form of caspase-1. In contrast, once recruited by the NLRP3 inflammasome, caspase-1 undergoes proteolytic cleavage and may become less available to AIM2. In addition to producing inflammatory cytokines, NLRP3 may negatively regulate the activation of AIM2, which is important for the induction of pyroptosis. Therefore, NLRP3 may represent a key regulator of the balance between AIM2 and caspase-1. Activation of AIM2 inflammasome during infection occurs via membrane damage caused by upstream inflammasomes, including the AIM2 and caspase-11. Activation of AIM2 inflammasome during infection leads to pore formation via a process that requires the activation, but not cleavage of caspase-1. Pore formation and pyroptosis lead to infection control. The membrane damage that is induced by the AIM2 inflammasome further triggers K⁺-efflux-mediated activation of NLRP3 inflammasome, a process that culminates in caspase-1 proteolysis and the secretion of the inflammatory cytokines IL-1β and IL-18.

**EXPERIMENTAL PROCEDURES**

Further details and an outline of resources used in this work can be found in Supplemental Experimental Procedures.

**Experimental Model Details**

**Institutional Permission**

All mice were bred and maintained under specific-pathogen-free conditions in the Animal Facilities of the Medical School Ribeirão Preto (FMRP-USP). The care of the mice was in compliance with institutional guidelines on ethics in animal experiments approved by CETEA (Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto; protocol number 130/2014).

**Mice**

We used male and female 8- to 12-week-old C57BL/6 mice, including WT (Jackson Laboratory, stock number 000664), Casp11⁻/⁻ (Kagayaki et al., 2011), Casp11⁻/⁻/Casp11⁻/⁻ (Kudia et al., 1999), Casp11⁻/⁻/Casp11⁻/⁻ (Kagayaki et al., 2011) (herein called Casp1⁻/⁻), Aim2⁻/⁻ (Jones et al., 2010), Aim2⁻/⁻/Casp1⁻/⁻ (Rathinam et al., 2010), Asc⁻/⁻ (Sutterwala et al., 2008), Nlrp3⁻/⁻ (Kara-Turkoğlu et al., 2006), and Nlrp3⁻/⁻ (Marathasan et al., 2009) mice in these experiments. Asc⁻/⁻/Casp11⁻/⁻, Aim2⁻/⁻/Casp11⁻/⁻, Nlrp3⁻/⁻/Casp1⁻/⁻, and Aim2⁻/⁻/Nlrp3⁻/⁻ mice were generated in-house by intercrosses. To ensure a similar genetic background between experimental mice, we crossed Aim2⁻/⁻/Casp1⁻/⁻ mice with C57BL/6 mice and with Aim2⁻/⁻ and Casp1⁻/⁻ mice to obtain Aim2⁻/⁻/Casp11⁻/⁻, Aim2⁻/⁻/Casp1⁻/⁻, Aim2⁻/⁻/Casp1⁻/⁻, and Aim2⁻/⁻/Casp1⁻/⁻ mice, respectively.

**Bone-Marrow-Derived Macrophages**

BMDMs were obtained as previously described (Marin et al., 2010). Briefly, mice were euthanized and bone marrow cells were harvested from the femurs and differentiated in RPMI 1640 (Sigma) containing 20% fetal bovine serum (FBS), 30% L-929 cell-conditioned media (LCCM), 1% L-glutamine (200 mM), and 1% penicillin-streptomycin (100 U/mL; Sigma). BMDMs were harvested and seeded on tissue culture plates 1 day before infection and maintained in RPMI 1640 media containing 10% FBS and 5% LCCM. All cells used in this study were cultivated at 37°C with 5% CO₂.

**In Vivo Infections**

For L. pneumophila infections, mice were anesthetized with ketamine and xylazine (50 mg/kg and 10 mg/kg, respectively) by intraperitoneal injection, followed by intranasal inoculation with 40 µL PBS containing 10⁶ JR32 flaA−/− per mouse. To determine the number of colony-forming units, mice were euthanized, the lungs harvested and homogenized in sterile water. Lung homogenates were then diluted in sterile water and plated on CYE agar plates on C with 5% CO₂. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grants 401577/2014-7 and 445881/2014-3), the Instituto Nacional de Ciencia y Tecnologia de Vacunas (INCT/VCPN), and the Núcleo de Apoio à Pesquisa em Doenças Inflamatórias (NAPDIN; grant 11.121625.01.0). D.S.Z. is a recipient of the Noncanonical Activation of NLRP3 Inflammasome. Cell Reports (2017), http://dx.doi.org/10.1016/j.celrep.2017.06.086

**Supplemental Information**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.06.086.

**AUTHOR CONTRIBUTIONS**

L.D.C. and D.S.Z. designed the experiments. L.D.C., A.L.N.S., and J.M.R. performed the experiments and analyzed the data. P.A.M., G.F.S.Q., and L.L.S. performed and analyzed specific experiments. R.A.F. provided reagents and discussed hypotheses. L.D.C. and D.S.Z wrote the manuscript. All authors reviewed and approved the final text.

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**SUPPLEMENTAL INFORMATION**

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L.D.C. and D.S.Z. designed the experiments. L.D.C., A.L.N.S., and J.M.R. performed the experiments and analyzed the data. P.A.M., G.F.S.Q., and L.L.S. performed and analyzed specific experiments. R.A.F. provided reagents and discussed hypotheses. L.D.C. and D.S.Z wrote the manuscript, and all authors reviewed and approved the final text.


