Gasdermin-D and Caspase-7 are the key Caspase-1/8 substrates downstream of the NAIP5/NLRC4 inflammasome required for restriction of *Legionella pneumophila*

Augusto V. Gonçalves1☯, Shally R. Margolis2☯, Gustavo F. S. Quirino1, Danielle P. A. Mascarenhas1, Isabella Rauch2a, Randilea D. Nichols2ab, Eduard Ansaldo2, Mary F. Fontana2c, Russell E. Vance2,3*, Dario S. Zamboni1*

1 Department of Cell Biology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil, 2 Division of Immunology and Pathogenesis, Department of Molecular and Cell Biology, and Cancer Research Laboratory, University of California, Berkeley, California, United States of America, 3 Howard Hughes Medical Institute, University of California, Berkeley, California, United States of America

☯ These authors contributed equally to this work.

¤ Current address: Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR, United States of America

¤ Current address: Department of Microbiology, Immunology, Molecular Genetics, University of California, Los Angeles, Los Angeles, CA, United States of America

¤ Current address: Department of Immunology, University of Washington School of Medicine, Seattle, WA, United States of America

* rvance@berkeley.edu (REV); dszamboni@fmrp.usp.br (DSZ)

Abstract

Inflammasomes are cytosolic multi-protein complexes that detect infection or cellular damage and activate the Caspase-1 (CASP1) protease. The NAIP5/NLRC4 inflammasome detects bacterial flagellin and is essential for resistance to the flagellated intracellular bacterium *Legionella pneumophila*. The effectors required downstream of NAIP5/NLRC4 to restrict bacterial replication remain unclear. Upon NAIP5/NLRC4 activation, CASP1 cleaves and activates the pore-forming protein Gasdermin-D (GSDMD) and the effector caspase-7 (CASP7). However, *Casp1*–/– (and *Casp1/11*–/–) mice are only partially susceptible to *L. pneumophila* and do not phenocopy *Nlrc4*–/– mice, because NAIP5/NLRC4 also activates CASP8 for restriction of *L. pneumophila* infection. Here we show that CASP8 promotes the activation of CASP7 and that *Casp7*1/11−/− and *Casp8*1/11−/− mice recapitulate the full susceptibility of *Nlrc4*−/− mice. *Gsdmd*−/− mice exhibit only mild susceptibility to *L. pneumophila*, but *Gsdmd*−/−*Casp7*−/− mice are as susceptible as the *Nlrc4*−/− mice. These results demonstrate that GSDMD and CASP7 are the key substrates downstream of NAIP5/NLRC4/CASP1/8 required for resistance to *L. pneumophila*.

Author summary

Inflammasomes are multi-protein complexes that detect infection and other stimuli and activate the Caspase-1 (CASP1) protease. The effectors required downstream of NAIP5/
NLRC4 to restrict bacterial replication remain unclear. Active CASP1 cleaves and activates the pore-forming protein gasdermin D (GSDMD) to induce inflammation and cell death. We have previously shown that CASP8 is activated by the NAIP5/NLRC4 inflammasome independently of CASP1 and functions to restrict replication of the intracellular bacterium *Legionella pneumophila*. Here, we show that CASP7 is activated downstream of CASP8 and is required for CASP8-dependent restriction of *L. pneumophila* replication in macrophages and in vivo. In addition, CASP7 is also activated by CASP1. Taken together, these results imply that CASP7 and GSDMD are the two key caspase substrates downstream of NAIP5/NLRC4. In support of this hypothesis, we found that mice double deficient in CASP7 and GSDMD are more susceptible than the single knockouts and are as susceptible as the *Nlrc4* deficient mice for restriction of *L. pneumophila* replication in vivo. Collectively, our data indicate that GSDMD and CASP7 are activated by CASP1 and induce cell death and restriction of bacterial infection. Therefore, GSDMD and multiple caspases (CASP1, CASP7 and CASP8) operate downstream of the NAIP5/NLRC4 inflammasome for restriction of infection by pathogenic bacteria.

**Introduction**

Inflammasomes are multi-protein complexes that assemble in the cytosol of infected or damaged cells and initiate host defense by functioning as a platform for the recruitment and activation of caspase proteases [1,2]. The NAIP/NLRC4 family of inflammasomes is especially well-characterized and has been shown to be activated upon detection of specific bacterial proteins, such as flagellin, via direct binding to various NAIP family members [3]. Ligand-activated NAIPs recruit and co-oligomerize with NLRC4, which in turn recruits and activates Caspase-1 directly, or indirectly via the adaptor protein ASC. Recently it was shown that ASC can also recruit and activate Caspase-8 downstream of NAIP/NLRC4 [4,5].

The NAIP5/NLRC4 inflammasome was originally discovered as an essential host component to restrict intracellular replication of several bacterial pathogens, including *Legionella pneumophila*, the causative agent of a severe pneumonia called Legionnaires’ Disease [6,7]. NAIP5 binds directly to *L. pneumophila* flagellin [8–10], resulting in NLRC4 and CASP1/8 activation. Flagellin-deficient *L. pneumophila* evade detection by NAIP5/NLRC4 [11,12] and NAIP5-deficient or NLRC4-deficient cells or mice also fail to detect or restrict the intracellular replication of flagellated *L. pneumophila* [13–15], but the underlying effectors downstream of NAIP5/NLRC4 required for resistance to *L. pneumophila* remain unclear. Caspase-1 cleaves dozens of host proteins [16–18], but two key substrates suggested to participate in host defense are Gasdermin-D (GSDMD) (reviewed in [19]) and the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. Cleaved Gasdermin-D oligomerizes and inserts into the plasma membrane to form large pores [20,21], leading to release of IL-1β/-18, as well as to a characteristic form of cell death called pyroptosis. In *vitro*, pyroptotic cell death, but not IL-1β/-18, is believed to restrict the intracellular replication of *L. pneumophila* in macrophages, presumably by elimination of the intracellular niche required for bacterial replication. However, CASP1-deficient macrophages are only partially susceptible to *L. pneumophila* [4,22] and the CASP8 substrates that contribute to inflammasome-mediated host defense remain unclear.

Caspase-11 (CASP11) and Caspase-7 (CASP7) are additional caspases previously implicated in resistance to *L. pneumophila*. CASP11 detects *L. pneumophila* lipopolysaccharide (LPS) and triggers GSDMD cleavage to activate pyroptosis independent of NAIP5/NLRC4 activation. Although CASP11 is activated by *L. pneumophila* [23–26], CASP11 does not appear...
to play a major role in restricting bacterial replication in bone marrow macrophages, as Casp11−/− macrophages fully restrict *L. pneumophila* replication, and *Nlrc4*−−/− macrophages, which still harbor functional CASP11, are fully permissive [27]. The lack of a discernable role for CASP11 in restricting *L. pneumophila* is likely due to a requirement for ‘priming’ signals to induce CASP11 expression, as well as to redundancy with the NAIP5/NLRC4 inflammasome.

CASP7 has also been reported to be activated downstream of flagellin detection and CASP1 activation by the NAIP5/NLRC4 inflammasome [28]. NAIP5/NLRC4-dependent CASP7 activation was reported to require CASP1 and, consistent with previous work [17], CASP7 was suggested to be cleaved directly by CASP1. In fact, Casp7−/− macrophages were reported to phenocopy the susceptibility of Casp1−/− macrophages, and CASP7 was thus proposed to be required for CASP1-dependent resistance to *L. pneumophila* [28]. Although GSDMD was not known at the time of this work, in retrospect it is surprising Casp7−/− cells would recapitulate the susceptibility of Casp1−/− cells given the clear role for GSDMD as a direct CASP1 substrate that is sufficient to mediate pyroptosis. Moreover, given that NAIP5/NLRC4 activates CASP8 [4,5,29], and that CASP8 cleaves CASP7 [30], it is surprising that CASP1 would be required for CASP7 activation.

Here we sought to define the key effectors downstream of the NAIP5/NLRC4 inflammasome that are required to restrict bacterial replication. Consistent with prior studies, we find that both CASP1 and CASP8 are activated by the NAIP5/NLRC4 inflammasome. Importantly, we find that mice doubly deficient in both enzymes fully recapitulate the susceptibility of Casp1−/− cells given the clear role for GSDMD as a direct CASP1 substrate that is sufficient to mediate pyroptosis. Moreover, given that NAIP5/NLRC4 activates CASP8 [4,5,29], and that CASP8 cleaves CASP7 [30], it is surprising that CASP1 would be required for CASP7 activation.

Results

CASP8 is activated in the absence of GSDMD and CASP1/11 for restriction of *L. pneumophila* replication in macrophages

We have previously shown that CASP8 is activated in response to *L. pneumophila* infection when we silence GSDMD or in the absence of CASP1 [4]. To confirm these data, we infected macrophages deficient in GSDMD (*Gsdmd*−/−) with *L. pneumophila* and measured CASP8 activation using western blot and a substrate that detects CASP8 activity. We found that infection with wild type bacteria and *fliI* mutants (that express cytosolic flagellin, but do not assemble the flagellum), but not with *flaA* mutants, triggers robust CASP8 activation in Casp1/11−−/− and Gsdmd/Casp7−/− mice (Fig 1A and 1B). CASP8 did not appear to be robustly activated in C57BL/6 or Casp11−/− macrophages, possibly because these macrophages undergo rapid pyroptosis. In addition, CASP8 activation was not observed in Asc/Casp1/11−−/− mice (Fig 1A and 1B). These data support previous findings indicating that CASP8 is activated in inflammasomes, particularly when CASP1 or GSDMD is absent, in a process that requires ASC [4,5,31].

Next, we tested the role of CASP8 in restricting *L. pneumophila* replication in macrophages in the absence of CASP1/11. We have previously shown that *Nlrc4*−/− macrophages are more permissive than Casp1/11−−/− [22], implying that CASP1 is not the sole caspase activated by NAIP5/NLRC4. Thus, to discern a role for CASP8, we compared macrophages deficient in Casp8/1/11/Ripk3 with those deficient in Casp1/11/Ripk3. Experiments were conducted in a Ripk3 mutant background because Casp8-deficiency is embryonically lethal except in the
absence of Ripk3 [32]. We found that whereas Casp1/11/Ripk3−/− macrophages are slightly more permissive to L. pneumophila replication than the C57BL/6, the Casp8/1/11/Ripk3−/− cells are highly susceptible, similar to Nlrc4−/− macrophages. This was shown using both very low
MOI (MOI = 0.015) and high MOI (MOI = 10) infections (Fig 1C and 1D). We also assessed bacterial replication in macrophages using a *L. pneumophila* strain stably expressing the *Photobacterium luminescens* luxCDABE (*lux*) operon as described previously [33]. We generated a JR32 strain of *L. pneumophila* expressing the *lux* operon and detected robust bacterial replication in Casp8/1/11/Ripk3−/− and Nlrc4−/− macrophages (Fig 1E). In contrast, C57BL/6 and Casp1/11/Ripk3−/− macrophages were restrictive to bacterial replication (Fig 1E). This was observed using wild type JR32 *L. pneumophila* (Fig 1E) and *flil* mutants (Fig 1F). As expected, isogenic flaA mutants expressing the *lux* operon robustly replicate in all macrophages used (Fig 1G). We also tested the importance of Casp8 for bacterial growth restriction using the wild type *L. pneumophila* and obtained comparable results (S1A Fig). As expected, flagellin mutants of *L. pneumophila* effectively replicated in all macrophages used (S1B Fig), whereas dotA mutants were defective for intracellular replication (S1C Fig).

**In the absence of CASP1/11, CASP7 is activated downstream of CASP8 and accounts for macrophage cell death**

CASP7 was previously proposed to require Casp1 for activation downstream of NAIP5/NLRC4 [28]. However, since CASP7 is also known to be a substrate of CASP8 [30,34], we decided to re-assess CASP7 activation in CASP1-deficient cells where CASP8 is still active. We infected Casp1/11−/− macrophages with wild type *L. pneumophila* (or with flaA mutants as control) and assessed CASP7 processing by western blot using an antibody specific for CASP7 p18. We found that CASP7 is processed in response to flagellin in Casp1/11−/− macrophages (Fig 2A). In Casp1/11−/− cells, CASP7 processing in response to flagellin requires CASP8 because we detect no CASP7 p18 in Casp8/1/11/Ripk3−/− macrophages (Fig 2A). In this experiment, Casp7/1/11−/− macrophages were used to confirm the specificity of the anti-CASP7 p18 antibody (Fig 2A). Next, we used the same macrophages to test if CASP7 is required for CASP8 activation. By assessing CASP8 activation by western blot and by the chemiluminescence assay, we found that CASP8 is activated in Casp1/11−/− and in Casp7/1/11−/− macrophages, but not in Asc/Casp1/11−/− or Casp8/1/11/Ripk3−/− macrophages (Fig 2B and 2C). These data indicate that, as expected, CASP7 is not required for CASP8 activation in the absence of CASP1/11. Experiments performed with immortalized macrophages confirmed that ASC but not CASP7 is important for CASP8 activation in the absence of CASP1/11 (S2 Fig).

We and others have previously shown that *L. pneumophila* infection triggers pore formation and pyroptosis that is dependent on Casp1 and Casp11 [11,12,25,35]. We also showed that in the absence of CASP1 and CASP11, *L. pneumophila* induces pore formation dependent on flagellin, ASC and CASP8 [4]. Thus, we tested if CASP7 accounts for pore formation downstream of CASP8. We measured pore formation in real time by assessing the uptake of propidium iodide into the nuclei of permeabilized macrophages as described [25]. We confirmed that pore formation occurs in Casp1/11−/− macrophages in response to *L. pneumophila* infection (Fig 3A and 3B). The Caspase-1/11-independent pore formation is abolished in Asc/Casp1/11−/− and Casp8/1/11/Ripk3−/− macrophages and is reduced in Casp7/1/11−/− macrophages (Fig 3B). As previously demonstrated, Fig 3B shows that pore formation in wild type C57BL/6 cells is very robust because it occurs through CASP1 and CASP11 [25]. We also measured LDH release after infection as a readout for membrane leakage. As suggested by the pore formation assay, we found that LDH release occurs in C57BL/6 and Casp1/11−/− but not in Asc/Casp1/11−/− or Casp8/1/11/Ripk3−/− macrophages (Fig 3C). Although reduced, we still detected cell death in Casp7/1/11−/− cells in response to infection (Fig 3C). We measured CASP3 activation by western blot and confirmed that CASP3 is activated in response to flagellin in Casp7/1/11−/− cells, but not in C57BL/6, Casp1/11−/− and Nlrc4−/− macrophages (Fig 3D). These data
suggest that CASP3 does not play a significant role downstream of NAIP5/NLRC4 inflammasome as previously reported [13–15], but can account to explain the modest levels of cell death detected in Casp7/1/11−/− cells. Collectively, these data suggest that in the absence of CASP1/11, macrophages are still able to respond to flagellin and trigger pore formation and pyroptosis via ASC and CASP8 and partially via CASP7.

**CASP7 activation downstream of CASP8 is important for restriction of *L. pneumophila* replication in macrophages and in vivo**

Next, we tested the importance of CASP7 for restriction of *L. pneumophila* replication in macrophages. To address this we used macrophages from Casp7−/− single mutants and also from mice triple deficient for CASP7/1/11 (Casp7/1/11−/−). In contrast to a previous report [28], we found that Casp7−/− macrophages efficiently restrict the replication of wild type *L. pneumophila*
Fig 3. CASP7 accounts for pore formation and pyroptosis. Bone marrow-derived macrophages from C57BL/6, Casp1/11\(^{-/-}\), Casp7/11\(^{-/-}\), Casp8/11/Ripk3\(^{-/-}\) and Asc/Casp1/11\(^{-/-}\) mice were left uninfected (NI) or infected with wild type \textit{L. pneumophila} (WT Lp) or flaA mutants at an MOI of 10. (A and B) Pore formation in uninfected (A) and WT Lp-infected (B) macrophages was assessed by Propidium Iodide (PI) uptake. Data are expressed as a percentage of total PI uptake (estimated using Triton-X100). (C) LDH release was assessed 7 hours after infection using CytoTox 96 Non-Radioactive Cytotoxicity Assay. (D) Lysates from C57BL/6, Casp1/11\(^{-/-}\), Casp7/11\(^{-/-}\) and Nlrc4\(^{-/-}\) macrophages were assessed for CASP3

GSDMD and CASP7 are the key CASP1 and CASP8 substrates downstream of the NAIP5/NLRC4 inflammasome.
cleavage using anti-Casp3 antibodies. Data are expressed as a percentage of LDH release induced by Triton-X100. Statistical significance was calculated using Student’s t test. *P<0.05. ns: non-significant. Data are presented for one representative experiment of four (A-B) and two (C-D) experiments performed with similar results.

as measured by luciferase and CFU (Fig 4A and 4B). In contrast, Casp7/1/11−/− macrophages are highly permissive for bacterial replication and phenocopy cells deficient for Nlrc4−/− and Casp8/1/11/Ripk3−/− (Fig 4A and 4B). These data support a role of CASP8 and CASP7 for restriction of bacterial replication in the absence of CASP1/11. As expected, flaA mutants evaded NAIP5/NLRC4 and effectively replicated in all macrophages regardless of the genotype (Fig 4C and 4D). We performed additional experiments comparing the replication of wild type L. pneumophila and isogenic flaA mutants in each macrophage genotype and found that flaA replicated significantly better than flagellin-positive bacteria in C57BL/6, Casp1/11−/−, Casp7−/− macrophages (S3A–S3C Fig). The flaA mutants phenocopy the wild type bacteria in Nlrc4−/− and Casp8/1/11/Ripk3−/− (S3D and S3E Fig) and flaA replicated slightly better than wild type bacteria in Casp7/1/11−/− macrophages (S3F Fig). These data indicate that NLRC4, CASP1, CASP8 and CASP7 are important for flagellin-mediated restriction of bacterial replication.

We have previously shown that Nlrc4−/− mice are more susceptible than Casp1/11−/− mice in vivo [22,36]. Thus, we tested if CASP7 accounts for the CASP1/11-independent mechanisms of restriction of bacterial replication. To test this, we infected mice with L. pneumophila and measured CFU in the lungs after 48 and 96h. Strikingly, we found that Casp7/1/11−/− mice are as susceptible as the Nlrc4−/− mice and significantly more susceptible than Casp1/11−/− mice (Fig 5A). As expected the C57BL/6 and heterozygote control Casp7−/−/1−/−/1−/− are highly restrictive (Fig 5A). Next, we crossed the Casp7/1/11−/− x Casp1/11−/− to generate Casp7−/−/1−/−/1−/− progeny and infected these mice together with Casp7/1/11−/− mice. We confirmed that Casp7/1/11−/− mice are more susceptible than the Casp7−/−/1−/−/1−/− mice, suggesting that CASP7 accounts for restriction of L. pneumophila replication in the absence of CASP1/11 (Fig 5B).

CASP7 and GSDMD operate downstream of CASP1 for restriction of L. pneumophila replication in macrophages and in vivo

Our data show that CASP7 operates downstream of CASP8 for restriction of L. pneumophila replication in Casp1/11−/− macrophages. Thus, we investigated if CASP7 is also activated in wild type macrophages. To test this we measured CASP7 cleavage in response to infection of C57BL/6 and Casp1/11−/− macrophages. We detected CASP7 processing both in C57BL/6 and Casp1/11−/− macrophages infected with wild type L. pneumophila (Fig 6A). In contrast, we only detected processing and activation of CASP8 in the Casp1/11−/− macrophages (Fig 6B and 6C). These data suggest that CASP7 can be activated in conditions where CASP8 is inactive, possibly by CASP1 as previously reported [17,28]. To test this hypothesis we performed experiments in macrophages that are deficient in CASP8 and sufficient in CASP1 and confirmed that CASP7 is cleaved in Casp8/Ripk3−/− cells (Fig 6D). In this experiment we detected a weak CASP7 cleavage in response to flaA and in Nlrc4−/− cells, suggesting the participation of another pathway for CASP7 activation.

We next performed growth curves in macrophages that are CASP1 positive and CASP8 negative to test if CASP1 activation is sufficient to trigger restriction of L. pneumophila replication. We found that Casp8/Ripk3−/− macrophages are as restrictive as C57BL/6 (and Ripk3−/−) macrophages (Fig 6E). As expected, Casp1/Ripk3−/− macrophages exhibit intermediate susceptibility and Casp8/1/11/Ripk3−/−, Casp8/1/11/Ripk3−/− macrophages are as susceptible as Nlrc4−/− macrophages (Fig 6E). The flaA mutants replicated in all macrophages tested, as expected (Fig
6F). These data confirm that CASP1 activation is sufficient to restrict *L. pneumophila* replication via the NAIP5/NLRC4 inflammasome despite the deficiency in CASP8.

In addition to CASP7, GSDMD is also downstream of CASP1. We tested by western blot if GSDMD is activated in response to *L. pneumophila* and found that GSDMD is cleaved in response to wild type *L. pneumophila* but not flaA mutants after 6 hs of infection (S4 Fig). Gsdmd–/– macrophages were included to control antibody specificity. GSDMD activation was found in C57BL/6 and Asc–/– macrophages but not in Casp1/11–/– and Nlrc4–/– mice (S4 Fig), indicating that GSDMD is cleaved by CASP1 independent of CASP8 via the NAIP5/NLRC4 inflammasome. We further generated mice double deficient in GSDMD and CASP8 to investigate if the CASP8-independent, CASP1-mediated restriction of *L. pneumophila* replication requires GSDMD. We found that Gsdmd/Casp8/Ripk3–/– cells are as restrictive as the Gsdmd/Ripk3–/– and C57BL/6 macrophages (Fig 6G and 6H). These data indicate that CASP1 activation triggers restriction of *L. pneumophila* replication in the absence of CASP8 and GSDMD, consistent with the participation of CASP7 downstream of CASP1.
Therefore, we reasoned that in wild type macrophages, CASP1 activates both GSDMD and CASP7 when the NAIP5/NLRC4 inflammasome is activated and that GSDMD and CASP7 can both mediate restriction of \( \text{L. pneumophila} \) independently of each other. The involvement of both CASP7 and GSDMD would explain why \( \text{Casp7}^{-/-} \) or \( \text{Gsdmd}^{-/-} \) singly deficient cells are able to restrict \( \text{L. pneumophila} \) replication in macrophages (Figs 4A, 4B and 6G). To test this hypothesis, we generated \( \text{Casp7/Gsdmd}^{-/-} \) double deficient mice and tested the requirement of these molecules downstream of NAIP5/NLRC4 inflammasome. Initially, we tested pore formation in response to wild type \( \text{L. pneumophila} \) and \( \text{flaA} \) mutants and found that \( \text{Casp7/Gsdmd}^{-/-} \), \( \text{Casp7/1/11}^{-/-} \) and \( \text{Nlrc4}^{-/-} \) mice induced low pore formation in comparison to \( \text{Casp7}^{-/-}, \text{Gsdmd}^{-/-} \) and \( \text{C57BL/6} \) macrophages (Fig 7A–7C). Of note, the pore formation assay performed with live \( \text{L. pneumophila} \) in presence of CASP11 may be difficult to interpret because LPS effectively triggers CASP11-mediated pore formation [25,27]. Therefore, we tested pore formation and LDH release in response to flagellin using FlaTox, a reagent that selectively activates NAIP5/NLRC4 without the confounding activation of CASP11 [37]. We found that cytosolic flagellin triggers a response that is CASP1-dependent and requires both CASP7 and GSDMD. This can be observed by pore formation (Fig 7D) and LDH release (Fig 7E). To evaluate the effect of CASP7 and GSDMD for restriction of \( \text{L. pneumophila} \) replication
Fig 6. CASP7 is activated and contributes to restriction of *L. pneumophila* replication in the absence of CASP8. (A–D) Bone marrow-derived macrophages were left uninfected (NI) or were infected with wild type (WT Lp) or *flaA* mutants (*flaA* L. pneumophila) for the indicated time points to assay CASP7 and CASP8 activation. (A) Lysates from C57BL/6 and Casp1/11−/− macrophages infected with WT Lp for 1 to 8 h were assessed by
Western blot using anti-Casp7 p18 antibodies and anti-α-actin. (B) C57BL/6 and Casp1/11−/− macrophages were infected with WT Lp for 5, 6, 7 and 8 h and Caspase-8 cleavage was measured by western blot anti-Casp8 p18 antibody and anti-α-actin. (C) C57BL/6 and Casp1/11−/− macrophages were infected for 8 h and CASP8 activation was measured using the Caspase-Glo 8 Assay kit. (D) Lysates from C57BL/6, Casp1/11−/−, Casp8/Ripk3−/− and Nlrc4−/− macrophages were assessed for CASP7 cleavage using anti-Casp7 p18 antibodies and anti-α-actin after 6 h infection. (E–H) Macrophages from C57BL/6, Ripk3−/−, Casp1/Ripk3−/−, Casp8/Ripk3−/−, Casp8/1/Ripk3−/−, Casp8/1/1/Ripk3−/− and Nlrc4−/− mice (E–F) or from C57BL/6, Gsdmd/Casp8/Ripk3−/− and Nlrc4−/− mouse (G–H) were infected with wild type L. pneumophila (WT Lp) or flaA mutants (flaA) expressing luciferase at an MOI of 0.015 and bacterial replication was estimated by measurement of luminescence (RLU). Student’s t-test, *P<0.05 compared to C57BL/6. **P<0.05 compared to Casp1/Ripk3−/−. Data are presented for one representative experiment of three (C) and two (A, B, E–H) experiments performed with similar results.

https://doi.org/10.1371/journal.ppat.1007886.g006

we performed macrophage infections and found that Casp7/Gsdmd−/− cells are significantly more permissive than the single KOs Casp7−/− and Gsdmd−/− (Fig 7F and 7G). Casp7/Gsdmd−/− macrophages phenocopied Casp7/11−/− and Nlrc4−/− for restriction of L. pneumophila replication (Fig 7F). We tested bacterial infection in vivo and found that the Casp7/Gsdmd−/− mice are as susceptible as the Nlrc4−/−/− mice (Fig 7H). In contrast, the Casp7−/− and Gsdmd−/− mice were significantly more restrictive, while still slightly more susceptible than C57BL/6, suggesting that these molecules are important for host resistance in vivo. Together, these data support a model that both CASP7 and GSDMD are required for restriction of L. pneumophila replication downstream of CASP1. When CASP1 or GSDMD is missing, CASP8 is activated in the NLR4 inflammasome and triggers activation of CASP7, which also accounts for restriction of L. pneumophila replication in macrophages and in vivo (Fig 8).

Discussion

CASP1 activation downstream of the NAIP5/NLRC4 inflammasome is critical for restriction of L. pneumophila replication in macrophages and in the lungs of infected mice [25,27], but the CASP1 substrates required for restriction of bacterial replication are still obscure. Previous work showed CASP7 is activated downstream of CASP1 [17,28] and is required for restriction of L. pneumophila replication via the NAIP5/NLRC4 inflammasome [28]. Although these data provide a direct link between CASP1 and CASP7, our experiments performed with macrophages and mice deficient only in CASP7 do not suggest an essential role of CASP7 for restriction of L. pneumophila replication in vivo. Indeed, we detected only a modest (less than one log) increase in bacterial loads in Casp7−/− compared to C57BL/6 macrophages during L. pneumophila growth curves in macrophages (Figs 4A, 4B, 7F and 7G). In this study we sought to identify the redundant pathways that operate downstream of CASP1 for host resistance to infection. Our data support the hypothesis that redundancy between CASP7 and GSDMD explains the minor phenotypes of singly deficient Gsdmd−/− and Casp7−/− cells for restriction of L. pneumophila infection. This hypothesis is strongly supported by our data indicating that Gsdmd/Casp7−/− double-deficient mice are highly susceptible to L. pneumophila and phenocopy the Nlrc4−/− mice (Fig 7).

We and others have previously shown that CASP8 is also activated by the NAIP5/NLRC4 inflammasome, particularly when CASP1 or GSDMD is inhibited or missing [4,5,31]. As with CASP1, the CASP8 substrates required for bacterial restriction have been unclear. Interestingly, our data support a model indicating that CASP7 also operates downstream of CASP8 when the NAIP5/NLRC4 inflammasome is activated by cytosolic flagellin. We speculate that the kinetics of CASP8 activation in this inflammasome is slow compared with the quick and robust induction of pyroptosis that occurs via CASP1 and GSDMD. Therefore, CASP8 activation is preferentially detected in the absence of CASP1 and GSDMD. In these conditions, CASP7 is also activated downstream of CASP8 and our data using the Casp7/11−/− (and the Casp8/1/11−/−) mice and their macrophages strongly support the role of CASP7 downstream...
Fig 7. CASP7 and GSDMD are important for NAIP5/NLRC4/CASP1-dependent pore formation and restriction of *L. pneumophila* replication in vivo. (A-C) Bone marrow-derived macrophages from C57BL/6, Casp7−/−, Gsdmd−/−, Gsdmd/Casp7−/−, Casp7/1/11−/− and Nlrc4−/− mice were left uninfected (NI) or infected with wild type *L. pneumophila* (WT Lp) or flaA mutants (flaA) at an MOI of 10. Pore formation was assessed by Propidium Iodide (PI) uptake. Data are expressed as a percentage...
of PI uptake induced by Triton-X100. (D and E) Bone marrow-derived macrophages from C57BL/6, Casp1−/−, Casp7−/−, Gsdmd−/− and Gsdmd/Casp7−/− mice were stimulated with 4μg/ml Pa and 2μg/ml LFn-Fla (FlaTox). (D) The percentage of propidium iodide uptake was estimated by assessing the fluorescence (RFU). Data is shown as percentage of the total PI uptake (estimated using Triton-X100). (E) LDH release was assessed 4 hours after FlaTox treatment using CytoTox 96 Non-Radioactive Cytotoxicity Assay. (F and G) Bone marrow-derived macrophages from C57BL/6, Casp7−/−, Gsdmd−/−, Gsdmd/Casp7−/−, Casp7/8−/− and Nlrc4−/− mice were infected with WT Lp (F) or flaA (G) expressing luciferase at an MOI of 0.015 and bacterial replication was estimated by measuring the luminescence (RLU) of each well. * P<0.05: compared to C57BL/6. Data are presented for one representative experiment of two (A-C), one (D-E) and two (F-G) experiments performed with similar results. (H) C57BL/6, Casp7−/−, Gsdmd−/−, Gsdmd/Casp7−/− and Nlrc4−/− mice were infected intranasally with 10⁴ wild type L. pneumophila, lungs were harvested at the indicated time points and homogenates were plated for CFU determination. Each dot represents a single animal, and the horizontal lines represent the averages. +, P<0.05. ns, P>0.05. Data presented in (H) are a pool of two independent experiments performed.

https://doi.org/10.1371/journal.ppat.1007886.g007

GSDMD and CASP7 are the key CASP1 and CASP8 substrates downstream of the NAIP5/NLRC4 inflammasome of CASP8 in the NAIP5/NLRC4 inflammasome. Together, our data indicate that CASP1 and CASP8 are the primary caspases activated by the NAIP5/NLRC4 inflammasome. Downstream of CASP1/8 we have now identified CASP7 and GSDMD as the key substrates required for restriction of bacterial replication (Fig 8). Our data therefore suggest that there is considerable redundancy built into the signaling outputs of the NAIP5/NLRC4 inflammasome: NAIP5/NLRC4 activates both CASP1 and CASP8; CASP1 can activate both GSDMD and CASP7; and CASP8 can activate CASP7 [38]. We speculate that this redundancy may be a mechanism for hosts to ensure responses even to pathogens that inhibit specific arms of the response. The identification of the critical substrates involved in the restriction of bacterial infection via the NAIP/NLRC4 inflammasomes provides important information for our understanding of the biology of these important platforms that operate for host protection against pathogenic bacteria. In response to Yersinia spp. infection, CASP8 was reported to trigger GSDMD activation [38,39]. Interestingly, it was recently reported that the GSDMD-mediated proinflammatory function of CASP8 is counteracted by CASP3-dependent cleavage and inactivation of GSDMD [40], suggesting that CASP3 suppresses GSDMD-mediated cell lysis during CASP8-induced apoptosis. We speculate that this may explain why we did not detect a CASP8-mediated GSDMD cleavage in Casp1/11−/− macrophages (S4 Fig) and a robust pore formation and LDH release in Casp7/11−/− (Figs 3 and 7B).

A question that arises from our studies is how CASP7 and GSDMD operate to restrict L. pneumophila replication. GSDMD is known to trigger pore formation and pyroptosis [20,21], a process that results in host cell death and thereby likely eliminates the intracellular replicative niche. In addition, pyroptosis has been proposed to result in formation of pore-induced intracellular traps (PITs) in macrophages infected with intracellular bacteria. The formation of PITs may help sequester bacteria and lead to their clearance by efferocytosis [41]. Our data demonstrate that CASP7 also promotes pore formation and host cell death. Although CASP7 appears to be involved in induction of cell death, the CASP7 substrates operating to trigger pore formation are still unclear. However, our data showing that CASP7 deficiency exerts a significant effect on bacterial replication even on a Gsdmd−/− background implies that CASP7 substrates other than GSDMD contribute to host defense [28,42]. It will be of interest to identify these substrates and their mechanisms of action in future studies.

Material and methods

Animals

Mice used in this study were bred and maintained in institutional animal facilities at FMRP/USP or at UC Berkeley. Mice used were C57BL/6 (Jax 000664), Casp7−/− (Jax 006237), Nlrc4−/− [43], Casp1/11−/− [44], Asc−/− [45], Casp1−/− [46], Asc/Casp1/11−/− [4], Casp8−/− [5], Gsdmd−/− [5], Casp7−/− [5], Ripk3−/− mice were originally from Xiaodong Wang [47] and backcrossed to C57BL/6 by Astar Winoto. Mice deficient in more than one gene not described above were generated in this study by intercrossing a F1 progeny of the parental strains.
Legionella

→ Dot/Icm-dependent

→ Flagellin

→ NAIP5/NLRC4

→ Caspase-1

GSDMD

→ Pore formation and cell death

Caspase-7

→ Pore formation and cell death (GSDMD-independent)

→ Restriction of bacterial replication

Caspase-8

Late response (strong when CASP1 or GSDMD is inhibited)

Fig 8. Schematic model illustrating that Gasdermin-D (GSDMD) and Caspase-7 are the key substrates of Caspase-1 and Caspase-8 downstream of the NAIP5/NLRC4 inflammasome.
Ethics statement
The care of the mice was in compliance with the institutional guidelines on ethics in animal experiments; approved by CETEA (Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto, approved protocol number 218/2014). CETEA follow the Brazilian national guidelines recommended by CONCEA (Conselho Nacional de Controle em Experimentação Animal). Animal experiments at UC Berkeley were approved by the institutional animal care and use committee. Mice were euthanized by CO₂ asphyxiation with cervical dislocation as a secondary method.

Bone marrow-derived macrophages
Bone marrow-derived macrophages were obtained as previously described [48]. Briefly, mice were euthanized and bone marrow cells were obtained from femurs and tibias. The cells were cultivated in RPMI 1640 (Gibco, Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10–20% Fetal Bovine Serum (FBS) (Gibco) and 30% L929-Cell Conditioned Medium (LCCM) and 2 mM L-glutamine (Sigma-Aldrich) for 7 days, at 37°C, 5% CO₂. In some experiments, instead of 20% LCCM it was used 10% of a conditional medium from 3T3 cells stably expressing mouse MCSF as a source of macrophage colony stimulation factor. Cells were detached with cold PBS, resuspended in RPMI 1640 supplemented with 10% FBS (R10) and plated as indicated. For all in vitro experiments, the plates were centrifuged at 300 x g for 5 min, room temperature, after cell plating and infection, to ensure homogeneous adherence of cells and infection synchronization, respectively. Incubation of non-infected and infected cells was done at 37°C, 5% CO₂.

Bacteria culture and preparation
Legionella pneumophila strains used were JR32 and isogenic mutants for flaA and fliI as previously described [12,22]. For some experiments, L. pneumophila strains stably expressing the Photorhabdus luminescens luxCDABE operon were used. Bacteria were cultured in Charcoal-Yeast Extract Agar (CYE, 10 g/L 4-morpholinepropanesulfonic acid [MOPS], 10 g/L Yeast extract, 15 g/L technical agar, 2 g/L activated charcoal, supplemented with 0.4 g/L L-cysteine and 0.135 g/L Fe(NO₃)₃) at 35–37°C, for 4 days from frozen stocks. Single colonies were streaked on fresh plates and allowed to grow for another 2 days. For in vitro infections, bacteria grown on solid plates were resuspended in autoclaved distilled water and diluted on RPMI as indicated. For in vivo infections, bacteria grown on solid plates were resuspended in autoclaved distilled water and diluted in Phosphate-buffered saline (PBS) as needed.

Bacterial replication in macrophages
Experiments to quantify bacterial CFU in macrophages were made in 24-well plates. A total of 2 x 10⁵ macrophages were plated per well in R10 and incubated overnight. The medium was replaced with the bacterial suspension in R10 with the indicated multiplicities of infection (MOIs) for 1hr before being replaced again by fresh R10 media. At the indicated time points, the supernatants were collected, cells were lysed with autoclaved distilled water and the lysate was added to the supernatants. Dilutions were plated on CYE and incubated for 4 days for counting of colony-forming units (CFU). Experiments to measure bacterial replication using a luminescence-based replication assays were made as previously described [33]. Briefly, 10⁵ macrophages/well were plated on white 96-well plates and incubated overnight. The medium was replaced with the bacterial suspension in R10 with an MOI of 0.01 or 10. At the indicated time points, luminescence emission was measured at 470 nm with a Spectra-L plate reader (Molecular Devices, California, USA).
Cell toxicity assay by LDH release

5 x 10^5 macrophages/well were plated on 24-well plates in R10 and incubated overnight. The medium was replaced with the bacterial suspension (estimated to reach an MOI of 10) in RPMI without Phenol Red (3.5 g/L HEPES, 2 g/L NaHCO₃, 10.4 g/L RPMI without Phenol Red, 1% glutamine, pH 7.2) and incubated for 7h. The supernatant was collected and LDH release was measured using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Wisconsin, USA) following the manufacturer’s instructions.

Pore formation assay

For estimation of pore formation, 10^5 macrophages/well were plated on black, clear bottom 96-well plates in R10 and incubated overnight. The medium was replaced with the bacterial suspension (estimated to reach an MOI of 10) in RPMI without Phenol Red and low on NaHCO₃ (2 g/L HEPES, 0.38 g/L NaHCO₃, 10.4 g/L RPMI without Phenol Red, 1% glutamine, 2% FBS, pH 7.2), 1:1000 rabbit anti-Legionella antibody and 6 μL/mL propidium iodide (PI). PI was excited at 538 nm and emission was measured at 617 nm with a SpectraMax plate reader (Molecular Devices, California, USA). Triton-X100 1.3% was used as a positive control and for normalization.

Caspase-8 activation assay

A total of 10^5 cells/well were plated on white 96-well plates in R10 and incubated overnight. The medium was replaced with the bacterial suspension in RPMI without Phenol Red and low on NaHCO₃ (2 g/L HEPES, 0.38 g/L NaHCO₃, 10.4 g/L RPMI without Phenol Red, 1% glutamine, 2% FBS, pH 7.2) with an MOI of 10 and the plate was incubated for 8h. The supernatants were collected and Caspase-8 activation was measured using Caspase-Glo 8 Assay (Promega, Wisconsin, USA) following the manufacturer’s instructions.

Western blotting

To measure caspase and GSDMD cleavage by western blot, 10^6 cells/well were plated on 24-well plates (for CASP7 and CASP3) or 48-well plates (for CASP8 and GSDMD) in R10 and incubated overnight. The medium was replaced with the bacterial suspension in R10 with an MOI of 10 and the plate was incubated for the indicated times. The supernatants were discarded (for CASP7 and CASP3) or collected (for CASP8 and GSDMD) and cells were lysed with 50 μL of RIPA supplemented with protease inhibitor (Complete Protease Inhibitor Cocktail, Roche, Basel, Switzerland). For CASP8 and GSDMD, lysates were added to the supernatants. Samples were immediately sonicated for 10 min and frozen at -80°C until analysed. A total of 50 μg of protein from each sample were run on a 15% acrylamide gel, transferred onto a nitrocellulose membrane and the membranes were incubated overnight, at 4°C under mild agitation with Anti-cleaved CASP7 antibody (rabbit) (Cell Signaling Technologies, Massachusetts, USA) diluted 1:1000 in 5% BSA in TBS 1X with 0.01% Tween; or Caspase-8 (D35G2) Rabbit mAb (Cell Signaling Technologies, Massachusetts, USA) diluted 1:1000 in 5% non-fat dry milk in TBS 1X with 0.01% Tween; Caspase-3 antibody (#9662, Cell Signaling Technologies, Massachusetts, USA) diluted 1:1000 in 5% non-fat dry milk in TBS 1X with 0.01% Tween; or a rat monoclonal antibody against GSDMD (G20-13, Genentech) diluted 1:1000 in 5% non-fat dry milk in TBS 1X with 0.01% Tween. Actin was stained with rabbit anti-α-actin (#A2066, Sigma-Aldrich, Missouri, USA) diluted 1:5000 in 5% non-fat dry milk in TBS 1X with 0.01% Tween. The membranes were incubated for 1h with goat anti-rabbit or anti-rat secondary antibodies (Sigma-Aldrich, Missouri, USA) and analyzed using ECL™ Prime.
Western Blotting System (GE Healthcare, Illinois, EUA) and an Amersham Imager 600 (GE Healthcare, Illinois, EUA). Bands were quantified using ImageJ.

**In vivo replication assays**

All mice were matched by sex and age (all were at least 8 weeks old at the time of infection) and were in a C57BL/6 mouse genetic background. For the in vivo experiments, approximately 5–7 mice per group were used, as indicated in the figures. Mice were infected intranasally with $10^5$ bacteria contained in 40 $\mu$L of PBS. The animals were anesthetized with ketamine (50mg/kg) and xylazine (10mg/kg) intraperitoneally and infected. At the indicated time points, the lungs were harvested and macerated for 30 seconds in 5 mL of autoclaved distilled water using a tissue homogenizer (Power Gen 125; Thermo Scientific). Dilutions were plated on CYE + 10 $\mu$g/mL of streptomycin and plates were incubated for 4 days at 37˚C for CFU counting.

**Statistical analysis**

The data were plotted and analyzed using GraphPad Prism 5.0 software. The statistical significance was calculated using the Student’s t-test or analysis of variance (ANOVA). Differences were considered statistically significant when $P$ was $<0.05$, as indicated by an asterisk in the figures.

**Supporting information**

S1 Fig. CASP8 is activated in the absence of CASP1/11 and it is important for restriction of *L. pneumophila* replication in macrophages. Macrophages were infected with Lp02 WT *L. pneumophila* (A), Lp02 flaA mutants (B) or Lp02 dotA^−^ mutants (C) expressing luciferase at an MOI of 0.015 and bacterial replication was estimated by measuring the luminescence (RLU) of each well over 4 days of infection. Statistical significance was calculated using Student’s t test. *, $P<0.05$: compared to C57BL/6. ##, $P<0.05$: compared to Casp1/11/Ripk3^−/−^. (TIF)

S2 Fig. CASP7 is activated downstream of CASP8 in the NAIP5/NLRC4 inflammasome in immortalized macrophages. Immortalized macrophages from Casp1/11^−/−^, Asc/Casp1/11^−/−^, Casp8/1/11/Ripk3^−/−^ and Casp7/1/11/Ripk^−/−^ mice were left uninfected or infected with wild type *L. pneumophila* (WT Lp, grey bars) or flaA mutants (black bars) at an MOI of 10 for 8 hours. Caspase-8 activation was measured by western blot using anti-Casp8 p18 antibody. Bands were quantified using ImageJ. (TIF)

S3 Fig. CASP7 is important for restriction of *L. pneumophila* replication in vivo in the absence of CASP1/11. Bone marrow-derived macrophages from C57BL/6, Casp7^−/−^, Casp1/11^−/−^, Casp7/1/11/Ripk3^−/−^ and Nlr4^−/−^ mice were infected with wild type *L. pneumophila* or flaA mutants at an MOI of 0.015 and bacterial replication was assessed by measurement of luminescence (RLU) emitted by luciferase-expressing bacteria. Statistical significance was calculated using Student’s t test. *, $P<0.05$. (TIF)

S4 Fig. GSDMD cleavage in response to *L. pneumophila* infection for 6 h requires flagellin, NLRC4 and CASP1/11. Macrophages from C57BL/6, Casp1/11^−/−^, Nlr4^−/−^, Asc^−/−^ and Gsdmd^−/−^ mice were left uninfected (NI) or infected with wild type *L. pneumophila* (WT Lp) or flaA mutants (flaA) at an MOI of 10 for 6 hs. GSDMD cleavage in the supernatants plus cell
lysates were measured by western blot using the anti-GSDMD antibody.

(TIF)

Acknowledgments
We are grateful to Roberto Chavez, Maira Nakamura, Livia Yamashiro, Daisy Ji, Alexandre L. N. Silva and Peter Dietzen for their technical assistance. We thank Richard Flavell (Yale University) and Vishva Dixit (Genentech) for providing us with mice strains used in this study. We also thank members of the Vance and Zamboni Labs for discussions.

Author Contributions

Conceptualization: Augusto V. Gonçalves, Shally R. Margolis, Danielle P. A. Mascarenhas, Isabella Rauch, Eduard Ansaldo, Mary F. Fontana, Russell E. Vance, Dario S. Zamboni.

Data curation: Augusto V. Gonçalves, Shally R. Margolis, Gustavo F. S. Quirino, Danielle P. A. Mascarenhas, Isabella Rauch, Russell E. Vance, Dario S. Zamboni.

Formal analysis: Augusto V. Gonçalves, Shally R. Margolis, Gustavo F. S. Quirino, Danielle P. A. Mascarenhas, Dario S. Zamboni.

Funding acquisition: Russell E. Vance, Dario S. Zamboni.

Investigation: Augusto V. Gonçalves, Shally R. Margolis, Gustavo F. S. Quirino, Danielle P. A. Mascarenhas, Isabella Rauch, Randilea D. Nichols, Russell E. Vance, Dario S. Zamboni.

Methodology: Augusto V. Gonçalves, Shally R. Margolis, Gustavo F. S. Quirino, Danielle P. A. Mascarenhas, Isabella Rauch, Randilea D. Nichols.

Project administration: Russell E. Vance, Dario S. Zamboni.

Resources: Russell E. Vance, Dario S. Zamboni.

Supervision: Russell E. Vance, Dario S. Zamboni.

Validation: Augusto V. Gonçalves, Shally R. Margolis, Gustavo F. S. Quirino, Danielle P. A. Mascarenhas.

Visualization: Augusto V. Gonçalves, Russell E. Vance, Dario S. Zamboni.

Writing – original draft: Augusto V. Gonçalves, Russell E. Vance, Dario S. Zamboni.

Writing – review & editing: Augusto V. Gonçalves, Shally R. Margolis, Gustavo F. S. Quirino, Danielle P. A. Mascarenhas, Isabella Rauch, Russell E. Vance, Dario S. Zamboni.

References

1. Broz P, Dixit VM (2016) Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol 16: 407–420. https://doi.org/10.1038/nri.2016.58 PMID: 27291964

2. Schroder K, Tschopp J (2010) The inflammasomes. Cell 140: 821–832. https://doi.org/10.1016/j.cell.2010.01.040 PMID: 20303873

3. von Moltke J, Ayres JS, Kofod EM, Chavarria-Smith J, Vance RE (2013) Recognition of bacteria by inflammasomes. Annu Rev Immunol 31: 73–106. https://doi.org/10.1146/annurev-immunol-032712-095944 PMID: 23215645

4. Mascarenhas DPA, Cerqueira DM, Pereira MSF, Castanheira FVS, Fernandes TD, et al. (2017) Inhibition of caspase-1 or gasdermin-D enable caspase-8 activation in the Naip5/Nlrc4 inflammasome. PLoS Pathog 13: e1006502. https://doi.org/10.1371/journal.ppat.1006502 PMID: 28771586

5. Rauch I, Deets KA, Ji DX, von Moltke J, Tenthorey JL, et al. (2017) Naip-Nlrc4 Inflammasomes Coordinate Intestinal Epithelial Cell Expulsion with Eicosanoid and Il-18 Release via Activation of Caspase-1 and -8. Immunity 46: 649–659. https://doi.org/10.1016/j.immuni.2017.03.016 PMID: 28410991
6. Diez E, Lee SH, Gauthier S, Yaraghi Z, Tremblay M, et al. (2003) Birc1e is the gene within the Lgn1 locus associated with resistance to Legionella pneumophila. Nat Genet 33: 55–60. https://doi.org/10.1038/ng1065 PMID: 12483212

7. Wright EK, Goodart SA, Grownney JD, Hadinoto V, Endrizzi MG, et al. (2003) Naip5 affects host susceptibility to the intracellular pathogen Legionella pneumophila. Curr Biol 13: 27–36. PMID: 12526741

8. Kofoed EM, Vance RE (2011) Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. Nature 477: 592–595. https://doi.org/10.1038/nature10394 PMID: 21874021

9. Tenthorey JL, Haloupek N, Lopez-Blanc o JR, Grob P, Adamson E, et al. (2017) The structural basis of flagellin detection by NAIP5: A strategy to limit pathogen immune evasion. Science 358: 888–893. https://doi.org/10.1126/science.aao1140 PMID: 20173201

10. Zhao Y, Yang J, Shi J, Gong YN, Lu Q, et al. (2011) The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature 477: 596–600. https://doi.org/10.1038/nature10510 PMID: 21918512

11. Molofsky AB, Byrne BG, Whitfield NN, Madigan CA, Fuse ET, et al. (2006) Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection. J Exp Med 203: 1093–1104. https://doi.org/10.1084/jem.20051659 PMID: 16606669

12. Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE (2006) Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog 2: e18. https://doi.org/10.1371/journal.ppat.0020018 PMID: 16552444

13. Amer A, Franchi L, Kanneganti TD, Body-Malapel M, Ozoren N, et al. (2006) Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. J Biol Chem 281: 35217–35223. https://doi.org/10.1074/jbc.M604933200 PMID: 16984919

14. Lightfield KL, Persson J, Brubaker SW, Witte CE, von Moltke J, et al. (2008) Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. Nat Immunol 9: 1171–1178. https://doi.org/10.1038/ni.1644 PMID: 18724372

15. Zamboni DS, Kobayashi KS, Kohlsdorf T, Ogura Y, Long EM, et al. (2006) The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of Legionella pneumophila infection. Nat Immunol 7: 318–325. https://doi.org/10.1038/ni1305 PMID: 16444259

16. Agard NJ, Maltby D, Wells JA (2010) Inflammatory stimuli regulate caspase substrate profiles. Mol Cell Proteomics 9: 880–893. https://doi.org/10.1074/mcp.M900528-MCP200 PMID: 20173201

17. Lamkanfi M, Kanneganti TD, Van Damme P, Vanden Berghe T, Vanoverberghe I, et al. (2008) Targeted peptidomimetic proteomics reveals caspase-7 as a substrate of the caspase-1 inflammasomes. Mol Cell Proteomics 7: 2350–2363. https://doi.org/10.1074/mcp.M800132-MCP200 PMID: 18667412

18. Shao W, Yeretsian G, Doiron K, Hussain SN, Saleh M (2007) The caspase-1 digestome identifies the glycolysis pathway as a target during infection and septic shock. J Biol Chem 282: 36321–36329. https://doi.org/10.1074/jbc.M708182200 PMID: 17959595

19. Kovacs SB, Miao EA (2017) Gasdermins: Effectors of Pyroptosis. Trends Cell Biol 27: 673–684. https://doi.org/10.1016/j.tcb.2017.05.005 PMID: 28619472

20. Kayagaki N, Stowe IB, Lee BL, O’Rourke K, Anderson K, et al. (2015) Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature 526: 660–665. https://doi.org/10.1038/nature15514 PMID: 26375003

21. Shi J, Zhao Y, Wang K, Shi X, Wang Y, et al. (2015) Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature 526: 660–665. https://doi.org/10.1038/nature15514 PMID: 26375259

22. Pereira MS, Morgantetti GF, Massis LM, Horta CV, Hori JL, et al. (2011) Activation of NLRC4 by flagellated bacteria triggers caspase-1-dependent and -independent responses to restrict Legionella pneumophila replication in macrophages and in vivo. J Immunol 187: 6447–6455. https://doi.org/10.4049/jimmunol.1003784 PMID: 22079982

23. Aachoui Y, Leaf IA, Hagar JA, Fontana MF, Campos CG, et al. (2013) Caspase-11 protects against bacteria that escape the vacuole. Science 339: 975–978. https://doi.org/10.1126/science.1230751 PMID: 23348507

24. Akhter A, Caution K, Abu Khweek A, Tazi M, Abdulrahman BA, et al. (2012) Caspase-11 promotes the fusion of phagosome harboring pathogenic bacteria with lysosomes by modulating actin polymerization. Immunity 37: 35–47. https://doi.org/10.1016/j.immuni.2012.05.001 PMID: 22658523

25. Case CL, Kohler LJ, Lima JB, Strowig T, de Zoete MR, et al. (2013) Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to Legionella pneumophila. Proc Natl Acad Sci U S A 110: 1851–1856. https://doi.org/10.1073/pnas.1211521110 PMID: 23307811
GSDMD and CASP7 are the key CASP1 and CASP8 substrates downstream of the NAIP5/NLRC4 inflammasome.

26. Casson CN, Copenhaver AM, Zwack EE, Nguyen HT, Strowig T, et al. (2013) Caspase-11 activation in response to bacterial secretion systems that access the host cytosol. PLoS Pathog 9: e1003400. https://doi.org/10.1371/journal.ppat.1003400 PMID: 23762026

27. Cerqueira DM, Pereira MS, Silva AL, Cunha LD, Zamboni DS (2015) Caspase-1 but Not Caspase-11 Is Required for NLRC4-Mediated Pyroptosis and Restriction of Infection by Flagellated Legionella Species in Mouse Macrophages and In Vivo. J Immunol 195: 2303–2311. https://doi.org/10.4049/jimmunol.1501223 PMID: 26232428

28. Akhter A, Gavrilin MA, Frantz L, Washington S, Ditty C, et al. (2009) Caspase-7 activation by the Nlrc4/Ipaf inflammasome restricts Legionella pneumophila infection. PLoS Pathog 5: e1000361. https://doi.org/10.1371/journal.ppat.1000361 PMID: 19343209

29. Man SM, Tourlonoumis P, Hopkins L, Monie TP, Fitzgerald KA, et al. (2013) Salmonella infection induces recruitment of Caspase-8 to the inflammasome to modulate IL-1beta production. J Immunol 191: 5239–5246. https://doi.org/10.4049/jimmunol.1301581 PMID: 24123685

30. Hirata H, Takahashi A, Kobayashi S, Yonehara S, Sawai H, et al. (1998) Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. J Exp Med 187: 587–600. https://doi.org/10.1084/jem.187.4.587 PMID: 9463409

31. Schneider KS, Gross CJ, Dreier RF, Saller BS, Mishra R, et al. (2017) The Inflammasome Drives GSDMD-Independent Secondary Pyroptosis and IL-1 Release in the Absence of Caspase-1 Protease Activity. Cell Rep 21: 3846–3859. https://doi.org/10.1016/j.celrep.2017.12.018 PMID: 29281832

32. Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, et al. (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471: 363–367. https://doi.org/10.1038/nature09852 PMID: 21366763

33. Coers J, Vance RE, Fontana MF, Dietrich WF (2007) Restriction of Legionella pneumophila growth in macrophages requires the concerted action of cytokine and Naip5/Ipaf signalling pathways. Cell Microbiol 9: 2344–2357. https://doi.org/10.1111/j.1462-5822.2007.00963.x PMID: 17506816

34. Denault JB, Salvesen GS (2003) Human caspase-7 activity and regulation by its N-terminal peptide. J Biol Chem 278: 34042–34050. https://doi.org/10.1074/jbc.M305110200 PMID: 12824163

35. Sarhan J, Liu BC, Muendlein HI, Li P, Nilson R, et al. (2018) Pathogen blockade of TAK1 triggers caspase-8-dependent cleavage of gasdermin D and cell death. Science. 191: 5239–5246. https://doi.org/10.1371/journal.ppat.1003400 PMID: 24123685

36. Pereira MS, Marques GG, Dellama JE, Zamboni DS (2011) The Nlrc4 Inflammasome Contributes to Restriction of Pulmonary Infection by Flagellated Legionella spp. that Trigger Pyroptosis. Front Microbiol 2: 33. https://doi.org/10.3389/fmicb.2011.00033 PMID: 21687424

37. von Moltke J, Trinidad NJ, Moayeri M, Kintzer AF, Wang SB, et al. (2012) Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. Nature 490: 1043–1413. https://doi.org/10.1128/IAI.00905-09 PMID: 20048047

38. Orning P, Weng D, Starheim K, Ratner D, Best Z, et al. (2018) Pathogen blockade of TAK1 triggers caspase-8-dependent cleavage of gasdermin D and cell death. Science.

39. Sarhan J, Liu BC, Muendlein HI, Li P, Nilson R, et al. (2018) Caspase-8 induces cleavage of gasdermin D to elicit pyroptosis during Yersinia infection. Proc Natl Acad Sci U S A 115: E10888–E10897. https://doi.org/10.1073/pnas.1809548115 PMID: 30381458

40. Chen KW, Demarco B, Heilig R, Shkarina K, Boettcher A, et al. (2019) Extrinsic and intrinsic apoptosis activate pannexin-1 to drive NLRP3 inflammasome assembly. EMBO J.

41. Jorgensen I, Zhang Y, Krantz BA, Miao EA (2016) Pyroptosis triggers pore-induced intracellular traps (PITs) that capture bacteria and lead to their clearance by efferocytosis. J Exp Med 213: 2113–2128. https://doi.org/10.1084/jem.20151613 PMID: 27573815

42. Abdelaziz DH, Gavrilin MA, Akhter A, Caution K, Kotrange S, et al. (2011) Asc-dependent and independent mechanisms contribute to restriction of legionella pneumophila infection in murine macrophages. Front Microbiol 2: 18. https://doi.org/10.3389/fmicb.2011.00018 PMID: 21713115

43. Lara-Tejero M, Sutterwala FS, Ogura Y, Grant EP, Bertin J, et al. (2006) Role of the caspase-1 inflammasome in Salmonella typhimurium pathogenesis. J Exp Med 203: 1407–1412. https://doi.org/10.1084/jem.20060206 PMID: 16717117

44. Li P, Allen H, Banerjee S, Franklin S, Herzog L, et al. (1995) Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell 80: 401–411. PMID: 7859282

45. Sutterwala FS, Ogura Y, Szczepanik M, Lara-Tejero M, Lichtenberger GS, et al. (2006) Critical role for NALP3/CIA51/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity 24: 317–327. https://doi.org/10.1016/j.immuni.2006.02.004 PMID: 16546100
46. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, et al. (2011) Non-canonical inflammasome activation targets caspase-11. Nature 479: 117–121. https://doi.org/10.1038/nature10558 PMID: 22002608

47. He S, Wang L, Miao L, Wang T, Du F, et al. (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137: 1100–1111. https://doi.org/10.1016/j.cell.2009.05.021 PMID: 19524512

48. Marim FM, Silveira TN, Lima DS Jr., Zamboni DS (2010) A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. PLoS One 5: e15263. https://doi.org/10.1371/journal.pone.0015263 PMID: 21179419