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Canonical inflammasomes drive IFN-γ to prime caspase-11 in defense against a cytosol-invasive bacterium

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SUMMARY

The inflammatory caspases-1 and -11 are activated in response to different agonists and act independently to induce pyroptosis. In the context of IL-1β/IL-18 secretion however, in vitro studies indicate that caspase-11 acts upstream of NLRP3 and caspase-1. By contrast, studying infection in vivo by the cytosol-invasive bacterium *Burkholderia thailandensis*, we find that caspase-1 activity is required upstream of caspase-11 to control infection. Caspase-1 activated IL-18 induces IFN-γ to prime caspase-11 and rapidly clear *B. thailandensis* infection. In the absence of IL-18, bacterial burdens persist, eventually triggering other signals that induce IFN-γ. Whereas IFN-γ was essential, endogenous type I interferons were insufficient to prime caspase-11. Although mice transgenic for caspase-4, the human orthologue of caspase-11, cleared *B. thailandensis* in vivo, they did not strictly require IFN-γ priming. Thus, caspase-1 provides priming signals upstream of caspase-11 but not caspase-4 during murine defense against a cytosol-invasive bacterium.

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AUTHOR CONTRIBUTIONS

Y.A. and E.A.M led the project and wrote the manuscript. Y.A., Y.K., I.A.L., J.P-Y.T, J.C., A.A., J.D.B. and E.A.M. designed the study, and analyzed results. Y.A., Y.K., I.A.L., and D. M. performed the experiments.

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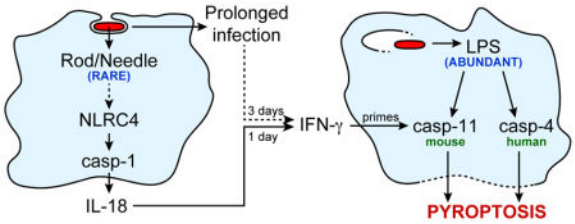
Graphical Abstract



INTRODUCTION

Inflammatory caspases, including caspase-1 and -11, are able to distinguish virulent from avirulent bacteria (Aachoui et al., 2013b). When activated, caspase-1 processes pro-IL-1β and pro-IL-18 to their mature and secreted forms; it additionally triggers a lytic form of programmed cell death called pyroptosis (Aachoui et al., 2013b). In contrast, caspase-11 induces pyroptosis, but cannot process pro-IL-1β or IL-18 alone (Kayagaki et al., 2011). During infection, IL-1β and IL-18 promote host defense via pleiotropic mechanisms. IL-1β is best known to promote local inflammation and to recruit neutrophils to site of infection. IL-18 is best known to induce interferon-γ (IFN-γ) secretion by natural killer cells and cytotoxic T lymphocytes (Dinarello, 1999). Pyroptosis restricts intracellular bacterial growth by lysing infected cell, thereby eliminating the intracellular replication niche, and exposing the bacteria to neutrophils (Aachoui et al., 2013b; Miao et al., 2010a). When considering any particular infection, IL-1β, IL-18, and pyroptosis could work coordinately to clear the infection, or one of these three effector mechanisms may be dominant.

Caspase-1 is activated by the canonical inflammasomes, including several NOD-like receptors (NLRs; including NLRP3 and NLRC4), certain AIM2-like receptors, and Pyrin, which respond to contamination of the cytosol, or perturbation of cellular physiology. NLRP3 senses cytosolic perturbation that originates from diverse stimuli, although, the precise signal that NLRP3 detects remains controversial (Franchi et al., 2012). NLRC4 detects the activity of type III secretion system (T3SS) and T4SS (Kofoed and Vance, 2011; Miao et al., 2010b; Zhao et al., 2011). Unlike other inflammasomes, NLRC4 is activated in conjunction with a helper NLR in the NAIP family, forming mixed NLRC4-NAIP oligomeric inflammasome platforms. C57BL/6 mice encode four NAIPs; NAIP1 binds the T3SS needle, NAIP2 binds the T3SS rod, and NAIP5 and NAIP6 bind flagellins. By contrast, humans have a single NAIP that detects the T3SS needle (Kofoed and Vance, 2011; Rayamajhi et al., 2013a; Yang et al., 2013; Zhao et al., 2011).

Caspase-11 differentiates Gram-negative bacteria that invade the cytosol from those that remain extracellular or confined to the vacuole by detecting the presence of LPS in the cytosol (Aachoui et al., 2013a; Case et al., 2013; Hagar et al., 2013; Kayagaki et al., 2013). In a unique mechanism of activation among caspases, caspases-11 directly binds LPS leading to its activation without an upstream sensor (Shi et al., 2014). In cultured macrophages, caspase-11 activation requires prior priming by type I or II interferons (Aachoui et al., 2013a; Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012). Priming stimuli induce transcription of *Casp11*, however, it is

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unclear whether this is sufficient to account for the priming effects. Poly(I:C) is sufficient to prime caspase-11 during endotoxic shock models in vivo(Hagar et al., 2013; Kayagaki et al., 2013). However, nothing is known about the importance of caspase-11 priming during defense against cytosol invasive bacteria in vivo. Caspase-11 is duplicated in human as caspase-4 and -5, which both bind LPS directly and induce pyroptosis (Shi et al., 2014). Whether caspase-4 and/or -5 serve to defend against cytosol invasive bacterial infection in vivo remains unstudied.

Although caspase-1 and -11 respond to different agonists, they both induce pyroptosis. In this regard, caspase-1 and -11 act independently and in parallel to each other. In contrast, caspase-11 can only trigger IL-1β and IL-18 secretion in conjunction with NLRP3, ASC, and caspase-1, although the molecular mechanism underlying this pathway remains to be fully elucidated. One possible mechanism to explain this link is that NLRP3 can be considered to be a sensor of cellular catastrophe, and thus would be expected to respond to the process of pyroptosis, which is indeed a catastrophic cellular event. In this regard, caspase-11 would be upstream of caspase-1. In this study we perform a detailed analysis of the role of caspase-1 and caspase-11 in vivo. We provide evidence that, in contrast to the two above paradigms, caspase-1 provides priming signals upstream of caspase-11 during defense against the cytosol invasive bacterium *Burkholderia thailandensis*.

RESULTS

Both caspase-1 and -11 confer resistance to *Burkholderia thailandensis*

B. thailandensis is a Gram-negative bacterium that uses a T3SS to escape the phagosome and replicate in the cytosol (Wiersinga et al., 2012). In our previous studies, we found that *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice are susceptible to *B. thailandensis* infection. However, mortality was asynchronous, occurring anywhere from 4 to 30 days after infection (Figure 1A and (Aachoui et al., 2013a)). Mice appeared healthy until one day before succumbing, at which point they rapidly progressed to a moribund state. Thus, at day 14 post infection symptomatic mice had significant bacterial burdens, whereas littermates infected at the same time that had not progressed to clinically apparent symptoms had undetectable bacterial loads (data not shown). We therefore hypothesized that *B. thailandensis* may persist in a non-pathogenic state until a reactivation event occurs, likely a mutation. Strikingly, after mouse passage in a *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mouse, *B. thailandensis* showed rapid and synchronized virulence; all animals died within 1–3 days of infection (Figure 1A). We previously showed that caspase-1 was dispensable for resistance to unpassaged *B. thailandensis* (Aachoui et al., 2013a). However, because mouse-passaged *B. thailandensis* is phenotypically distinct from, and more experimentally robust than, unpassaged *B. thailandensis*, we revisited the role of caspase-1 and -11 in defense (*Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice serve as a proxy for *Casp1*$^{-/-}$ mice). In contrast to unpassaged *B. thailandensis* (Aachoui et al., 2013a), both caspase-1 and caspase-11 were required for resistance to high dose infection by mouse-passaged *B. thailandensis* (Figure 1B). While wild type mice were resistant and had undetectable bacterial loads, *Nlrc4*$^{-/-}$*Asc*$^{-/-}$, *Casp11*$^{-/-}$, and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice all had similarly elevated bacterial burdens (Figure 1C). Therefore, our data indicate that after mouse passage, *B. thailandensis* stably mutated into a higher

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virulence state. Nevertheless, WT mice remained completely resistant to mouse-passaged *B. thailandensis* through the combined action of caspase-1 and -11.

Unpassaged and mouse-passaged *B. thailandensis* were similarly detected by caspase-1 and -11 in vitro (Figure S1A–D). Thus, the effects of mouse passage are observable only in vivo. The specific mutation in mouse-passaged *B. thailandensis* and the mechanism whereby it confers enhanced virulence in vivo is the subject of ongoing investigation.

We next evaluated the degree of susceptibility of the *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice to mouse-passaged *B. thailandensis*. While WT mice resist 2×10^7 cfu, *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice rapidly succumb to as few as 100 cfu (Figure 1D–E). This result is truly remarkable, and is, by many orders of magnitude, the strongest role for inflammasomes in defense against any infectious agent. To our knowledge, the only other pathogen that shows a similar phenotype is another environmental microbe, *Chromobacterium violaceum*, where WT mice survive a 10^6 cfu challenge while *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice again succumb to 100 cfu (unpublished data). Hereafter, we continue to study the mouse-passaged *B. thailandensis*, which is a potent probe to examine the functions of caspase-1 and -11 in defense against cytosol-invasive bacteria.

Differential contribution of caspase-1 and -11 in defense against *B. thailandensis*

We chose three representative doses for further study: 2×10^7 cfu (high dose), 10^4 cfu (medium dose) and 10^2 (low dose). *Casp11*$^{-/-}$ mice succumbed even to 10^2 cfu, similar to *Casp1*$^{-/-}$*Casp11*$^{-/-}$ animals, albeit with slightly slower kinetics (Figure 2A–B). In contrast, whereas *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice succumbed high doses, they survived medium and low dose challenges (Figure 1B, 2A–B). The role for caspase-1 in defense against *B. thailandensis* was also detectable during medium and low dose infection as *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ had significant bacterial loads that were fully cleared by day 5 after low dose infection (Figure 2C–E). Therefore, during high dose infection, both caspase-1 and -11 detection are required concomitantly for survival, whereas, at low infectious doses non-canonical inflammasome caspase-11 plays a dominant role.

We next considered whether *Nramp1*-deficiency is involved in the extreme susceptibility of C57BL/6 background *Casp11*$^{-/-}$ mice. Conveniently, *Nramp1*-sufficient 129/SvEv mice are naturally defective for *Casp11* (Kayagaki et al., 2011); 129/SvEv mice were also extremely susceptible to *B. thailandensis* (Figure 2F), consistent with a dominant role for caspase-11 in defense against cytosol invasive bacterial infection. In contrast, Nramp1-defective *Casp11*$^{+/+}$ BALB/c mice remained resistant (Figure S2). It should be noted, however, that these mouse strains have many genetic differences besides *Nramp1* and *Casp11*.

Several NLRs are dispensable for caspase-11 activation

As this work was underway, most investigators hypothesized that an upstream sensor activated caspase-11. Less studied NLRs were candidates for this noncanonical inflammasome. We found that *Nlrc3*$^{-/-}$, *Nlrc5*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Nlrx1*$^{-/-}$ mice survived high dose *B. thailandensis* infection (Figure 3A). Further, *Nod1*$^{-/-}$*Nod2*$^{-/-}$, *Nod2*$^{-/-}$, and *Myd88*$^{-/-}$*Nod1*$^{-/-}$*Nod2*$^{-/-}$, *Card6*$^{-/-}$, *Nlrc3*$^{-/-}$, and *Nlrc5*$^{-/-}$ BMMs were fully responsive to

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cholera toxin B delivered cytosolic LPS (Figure 3B–C). These results ruled out many candidates as obligatory LPS sensors upstream of caspase-11, consistent with the recent finding that caspase-11 itself directly binds LPS through its CARD domain (Shi et al., 2014).

NLRC4 and NLRP3 both contribute to defense against *B. thailandensis*

Next we examined the canonical inflammasome pathways responsible for caspase-1 activation in response to *B. thailandensis* during high dose infection. *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice lack inflammasome signaling through all PYD-containing inflammasomes and the CARD-containing NLRC4. *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$ mice were susceptible to high, but not medium dose *B. thailandensis* (Figure 3D–E), indicating that other inflammasomes such as AIM2 and Pyrin are dispensable. In contrast, *Nlrp3*$^{-/-}$ or *Nlrc4*$^{-/-}$ mice survived, suggesting a level of functional redundancy. Interestingly, analysis of bacterial burdens revealed that *Nlrc4*$^{-/-}$ mice carried significantly higher burdens compared to *Nlrp3*$^{-/-}$ mice (Figure 3F). Thus, NLRC4 is more efficient at responding to *B. thailandensis* than is NLRP3, but NLRP3 can serve as a failsafe canonical inflammasome when NLRC4 is absent.

IL-18 is required for defense against *B. thailandensis*

Caspase-1 promotes IL-1β and IL-18 secretion, and triggers pyroptosis. To investigate these three effector mechanisms, we first examined *Il1b*$^{-/-}$ and *Il18*$^{-/-}$ mice, since the pyroptosis-inducing target of caspase-1 is unknown. While *Il1b*$^{-/-}$*Il18*$^{-/-}$ mice lack the two cytokines but are pyroptosis competent, *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice are deficient for both. *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice succumbed to high dose *B. thailandensis* infection faster than *Il1b*$^{-/-}$*Il18*$^{-/-}$ mice, and had higher bacterial burdens (Fig 4A–C), suggesting a partially protective role for caspase-1-dependent pyroptosis. Because *Il1b*$^{-/-}$*Il18*$^{-/-}$ mice had increased susceptibility and higher bacterial burdens in comparison to WT mice (Figure 4A–D), we also conclude that one or both of these cytokines play a role in defense. *Il18*$^{-/-}$ mice phenocopied *Il1b*$^{-/-}$*Il18*$^{-/-}$ mice, while *Il1b*$^{-/-}$ mice survived high dose infection and exhibited only a moderate increase in bacterial burdens (Figure 4A–D). It should be noted that we have not examined IL-1α in these studies. *Il18*$^{-/-}$ and *Il1b*$^{-/-}$*Il18*$^{-/-}$ mice survived medium dose infection (Figure 4B) in agreement with the resistance of *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice to this dose (Figure 2B and 4B).

IL-18 secretion could be fully attributed to the combined effects of NLRC4 and NLRP3 (Figure 4E–F). The high serum IL-18 levels seen in *Nlrc4*$^{-/-}$ mice is likely a consequence of their high bacterial burden. *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice had slightly higher bacterial burdens than *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$ mice, suggesting a third inflammasome, however this third inflammasome was not activated strongly enough to achieve detectable serum IL-18 levels, even at very high bacterial burdens (Figure 4F). *Casp11*$^{-/-}$ mice remained susceptible (Figure 2) despite having high levels of serum IL-18 (Figure 4E), suggesting that IL-18 works upstream of caspase-11.

IFN-γ is critical to defend against *B. thailandensis*

Although IL-18 is a pleiotropic cytokine, one of its primary functions is to induce production of IFN-γ (Souza-Fonseca-Guimaraes et al., 2012). We therefore hypothesized

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that *Il18*$^{-/-}$ mice are susceptible due to a deficiency in IFN-γ production. Indeed, like *Il18*$^{-/-}$ mice, *Ifng*$^{-/-}$ mice also succumbed to high dose infection. Surprisingly, unlike *Il18*$^{-/-}$ mice, *Ifng*$^{-/-}$ mice also succumbed to the medium and low dose infection (Figure 5A–C); in this regard, they are more similar to *Casp11*$^{-/-}$ mice. Bacterial burdens were similarly elevated in both *Ifng*$^{-/-}$ and *Casp11*$^{-/-}$ mice (Figure 5D). Serum IFN-γ concentrations correlated with the resistance of WT and *Il1b*$^{-/-}$ mice to infection, whereas IFN-γ was absent in *Il18*$^{-/-}$ mice (Figure 5E), consistent with its role as an inducer of IFN-γ. *Casp11*$^{-/-}$ mice had high serum IFN-γ (Figure 5E), yet this failed to provide protection, as was seen with serum IL-18 (Figure 4F). This suggested that both IL-18 and IFN-γ act upstream of caspase-11.

We next considered why *Ifng*$^{-/-}$ mice are more susceptible than *Il18*$^{-/-}$ mice. We hypothesized that we would observe a delayed IFN-γ response in canonical inflammasome deficient mice (*Nlrc4*$^{-/-}$*Asc*$^{-/-}$) or *Il1b*$^{-/-}$*Il18*$^{-/-}$ mice that correlated with bacterial clearance. While WT mice had a robust IFN-γ response at day 1 post infection with 10^4 CFU that correlated with bacterial clearance, *Il1b*$^{-/-}$*Il18*$^{-/-}$ or *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice had significant bacterial burdens 1 day post infection, and insignificant levels of serum IFN-γ (Figure 5F–H). However, at day three, a small amount of serum IFN-γ was detected in the serum of these mice (Figure 5G–H), and thereafter no bacterial burdens were detected (Figure 5H) and the mice survive (Figure 4B). Finally, we could bypass the need for IL-18 by providing exogenous IFN-γ early; this reduced bacterial loads in *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice (Figure S3A). This suggests that efficient detection of *B. thailandensis* by the canonical inflammasomes provides a burst of IL-18, which drives a rapid and protective IFN-γ response. Interestingly, after high dose infection (2×10^7 CFU) bacterial burdens quickly rose, and the delayed IFN-γ seen at day 3 post infection (Figure 5I–J) is presumably too late to rescue the mice from morbidity (Figure 4A). Therefore, in the absence of IL-18, prolonged bacterial burdens eventually provide weak IFN-γ through other signaling pathways that is sufficient to clear a medium dose infection, but too late to clear a high dose infection.

IFN-γ primes caspase-11 in vivo to protect against *B. thailandensis*

Previous studies with cultured macrophages showed that caspase-11 requires priming by either IFN-β or IFN-γ (Aachoui et al., 2013a; Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012). Indeed, IFN-β and IFN-γ were equally efficient at priming caspase-11 responses in vitro (Figure 6A). However, unlike the susceptibility of IFN-γ receptor deficient mice *Ifngr1*$^{-/-}$, type I interferon receptor deficient mice *Ifnar1*$^{-/-}$ survived high dose *B. thailandensis* infection (Figure 6B). TLR4 drives type I IFN responses to LPS, and concomitantly, *Tlr4*$^{-/-}$ mice also survived to high dose *B. thailandensis* (Figure 6B). Thus, the presence of the type I interferon pathway does not rescue mice that are deficient in IFN-γ, suggesting that endogenous type I IFN responses during *B. thailandensis* infection are insufficient to prime caspase-11.

To further substantiate our hypothesis that IFN-γ acts to prime caspase-11, we sought to bypass this priming in vivo. During endotoxic shock, priming is required for caspase-11 activation; this can be achieved by the TLR4 agonist LPS, or the TLR3 agonist poly(I:C) (Hagar et al., 2013; Kayagaki et al., 2013). Indeed, poly(I:C) primed caspase-11 responses in

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vitro similar to IFN-γ (Figure 6A). Further, poly(I:C) can induce caspase-11 expression in vivo independently of IFN-γ (Figure 6C). Poly(I:C) priming decreased bacterial burdens in *Ifng*$^{-/-}$ mice, but had no effect upon *Casp11*$^{-/-}$ mice (Figure 6D, S3B). Poly(I:C) should thus drive a synchronized priming event, resulting in caspase-11 activation and pyroptosis. During pyroptosis cytosolic contents, including HMGB1, are released to the extracellular space (Kayagaki et al., 2011). Therefore, as a correlative measure of pyroptosis, we assessed the release of HMGB1. Poly(I:C) treatment caused a marked increase in serum HMGB1 in *Ifng*$^{-/-}$ mice, but not *Casp11*$^{-/-}$ mice (Figure 6E). The genetic programming of HMGB1 release by caspase-11 is consistent with this lytic event being potentially pyroptosis. WT mice had minimal serum HMGB1 consistent with an early clearance of the bacteria prior to 24h post infection. These results are again consistent with IFN-γ acting upstream of caspase-11.

IFN-γ priming does not require NOS2 or GBPs encoded on chromosome 3

The complete mechanism by which interferons prime caspase-11 remains incompletely described. While transcriptional induction of caspase-11 is one aspect of priming, it is unclear whether this explains the full effect of priming. Guanylate-binding proteins (GBPs) are interferon inducible GTPases that play key roles in immunity to infections(Kim et al., 2012). Two recent studies demonstrated that GBPs on mouse chromosome 3, specifically GBP2 and 5, enhance detection of LPS or non-cytosol-invasive Gram-negative bacteria by the caspase-11 (Meunier et al., 2014; Pilla et al., 2014). It was proposed that this effect occurs as GBPs localize to pathogen-containing vacuoles and promote their lysis, which in turn causes the bacteria to be expelled to the cytosolic compartment where they are detected by caspase-11. We observed that *Gbp*$^{chr3-/-}$ mice clear *B. thailandensis* in similar fashion as WT mice (Figure 6F). Our observation is consistent with the fact that *B. thailandensis* efficiently enters the cytosol, thus making GBP-mediated vacuolar instability dispensable for caspase-11 activation.

Another major antibacterial interferon stimulated gene is the inducible nitric oxide synthase (Utaisincharoen et al., 2003). However, *Nos2*$^{-/-}$ mice survived high dose *B. thailandensis* infection (Figure 6G). This is consistent with IFN-γ acting upstream of caspase-11.

Caspase-4-transgenic mice do not require IFN-γ priming in vivo

The murine caspase-11 has been duplicated in the human genome as caspase-4 and -5. We recently generated *CASP4* transgene (*CASP4*^{Tg}) mice in order to study the role of human caspase-4 in vivo, and found that *CASP4*^{Tg} complements *Casp11*$^{-/-}$ mice for the detection of CTB-delivered cytosolic LPS in vitro and endotoxic shock in vivo (Kajiwara et al., 2014). We now use these mice to study the role of caspase-4 in defense against cytosol-invasive bacteria. Indeed, addition of the *CASP4*^{Tg} complemented the inability of *Casp1*$^{-/-}$*Casp11*$^{-/-}$ macrophages to detect *B. thailandensis* (Figure 7A). Further, the *CASP4*^{Tg} restored the resistance of *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice to *B. thailandensis* (Figure 7B). Concomitantly, while *Casp11*$^{-/-}$ mice had high bacterial burdens, *Casp11*$^{-/-}$ *CASP4*^{Tg} mice fully cleared *B. thailandensis* (Figure 7C). Altogether, these results indicate that caspase-4 and -11 both confer resistance to cytosol-invasive *B. thailandensis* infection.

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Casp11 and *CASP5* are weakly expressed in macrophages, and both are transcriptionally induced by IFN-γ (Lin et al., 2000; Schauvliege et al., 2002). In contrast, human caspase-4 expression is high in many unstimulated immunologic and epithelial cells, and has minimal responsiveness to priming (Lin et al., 2000) (Figure S4). Similarly, *CASP4*^{Tg} mice showed minimal alteration in caspase-4 expression in vivo after IFN-γ priming (Figure 7D). We therefore considered whether IFN-γ is as important for caspase-4-mediated defense as it is for caspase-11. IFN-γ blockade caused WT mice to become highly susceptible to *B. thailandensis* (Figure 7E), in agreement with the phenotype of *Ifng*$^{-/-}$ mice (Figure 6). Although a small increase in bacterial burdens after IFN-γ blockade was observed in the *CASP4*^{Tg} mice, the resistance conferred by the *CASP4*^{Tg} was largely unaffected by IFN-γ blockade (Figure 7E). This suggests that unlike caspase-11, caspase-4 may not strictly dependent upon IFN-γ priming to protect against *B. thailandensis* infection in vivo. This result also further solidifies our conclusion that IFN-γ acts upstream of caspase-11.

DISCUSSION

We previously showed that caspase-11 defends against infection by cytosol invasive *B. pseudomallei* and *B. thailandensis* (Aachoui et al., 2013a). *B. pseudomallei* is a highly virulent pathogen, uniformly killing mice at infectious doses as low as 200 bacteria (Ceballos-Olvera et al., 2011). *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice succumb to even lower dose of 25 cfu (Ceballos-Olvera et al., 2011). This roughly eight-fold shift in the lethal dose is typical for the in vivo phenotypes seen during infection of inflammasome-deficient mice. In contrast, *Casp11*$^{-/-}$ mice have over a two hundred thousand-fold shift in the lethal dose of *B. thailandensis*. These two phenotypes are the strongest in vivo role inflammasomes play against any infectious agent – be it bacterial, fungal, viral, or parasites (Allen et al., 2009; Fernandes-Alnemri et al., 2010; Gross et al., 2009; Lara-Tejero et al., 2006; LaRock and Cookson, 2012; Mayer-Barber et al., 2010; Raupach et al., 2006; Thomas et al., 2009; Tsuji et al., 2004). *B. thailandensis* and *C. violaceum* are therefore exquisite tools with which to study the roles of caspase-1 and caspase-11 in defense against infection.

While much attention has focused on individual impact that each caspase has on host defense, their interactive and collaborative contribution is less studied. Caspase-1 is fully competent to induce pyroptosis and maturation of IL-1β and IL-18. In contrast caspase-11 is fully competent only to trigger pyroptosis. In order to drive IL-1β and IL-18 maturation, caspase-11 must partner with the NLRP3-ASC-caspase-1. Thus, the current conceptual model suggests that after caspase-11 binds LPS, caspase-1 can become activated as a downstream event. In contrast to this paradigm, we propose that caspase-1 functions upstream of caspase-11 at the priming step during an actual infection in vivo. We propose that the NLRP3 and NLRC4 canonical inflammasomes detect virulence traits, such as the *B. thailandensis* T3SS, and activate caspase-1, resulting in subsequent IL-18 secretion. This IL-18 then promotes the production of IFN-γ, likely from a mixture of NK, NKT, T cells, and innate lymphoid cells, which primes caspase-11. Without IL-18, IFN-γ production is delayed and extremely weak, but nonetheless this weak response is sufficient to prime caspase-11 during medium or low dose infection to rescue the mice from lethality. The ancillary stimuli for this IFN-γ may arise from prolonged bacterial burdens, leading to IL-12

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and IL-15 secretion, or to direct TLR stimulation of IFN-γ producing cells. Once primed, caspase-11 then triggers pyroptosis to eliminate the intracellular replication niche.

From previous studies showing that pyroptosis is an efficient defense mechanism against intracellular bacterial infection (Ceballos-Olvera et al., 2011; Miao et al., 2010a), one would expect that the function of pyroptosis would be the same regardless of whether it is initiated by caspase-1 or -11. Although, in vitro *B. thailandensis* efficiently triggers both caspase-1 and -11 dependent pyroptosis (Aachoui et al., 2013a; Ceballos-Olvera et al., 2011), in vivo caspase-1-dependent pyroptosis plays only a minor role, and does not fully substitute for caspase-11-dependent pyroptosis. While it is tempting to conclude that caspase-1 and -11 dependent pyroptosis are not equivalent, we instead propose that caspase-1-dependent pyroptosis fails in this instance because the NLRC4 agonists are in much lesser abundance than the caspase-11 agonist. NLRC4 responds to three agonists delivered to the cytosol by T3SS: flagellin, T3SS rod, and T3SS needle. Flagellin may be highly expressed; however *B. thailandensis* flagellin is not detected (Zhao et al., 2011). In contrast, T3SS rod and needle proteins are expressed in vanishingly small quantities (Kimbrough and Miller, 2000). Thus, during *B. thailandensis* infection, NLRC4 may provide relatively slow and inefficient activation of caspase-1. In contrast, when we engineered *S. typhimurium* to efficiently and robustly express flagellin in vivo, it was rapidly and efficiently cleared by caspase-1 dependent pyroptosis (Miao et al., 2010a). Therefore, ligand concentration could explain the disparity between caspase-1-dependent and caspase-11-dependent pyroptosis in defense against *B. thailandensis*.

If NLRC4 is inefficient during *B. thailandensis* infection, NLRP3 is even less efficient. In this case, it appears that NLRP3 activates only when NLRC4 fails to detect the bacteria (as occurs *Nlrc4*$^{-/-}$ mice). Thus, NLRP3 could be considered a fail-safe mechanism that activates as a last resort; this is sufficient to rescue *Nlrc4*$^{-/-}$ mice during high dose *B. thailandensis* infection. How is NLRP3 activated during *B. thailandensis* infection? NLRP3 can be activated by caspase-11, thus conceptually, NLRP3 driven IL-18 could form a positive feedback loop to ensure caspase-11 priming in surrounding cells. However, given the multitude of mechanisms by which NLRP3 activates, it is impossible to attribute the protective effect of NLRP3 solely to the NLRP3-caspase-11 loop. Further, since the *Nlrp3*$^{-/-}$ mice are highly resistant while *Casp11*$^{-/-}$ are extremely sensitive, we can conclude that the pathway whereby caspase-11 activates NLRP3 has minimal physiologic relevance during *B. thailandensis* infection.

Caspase-11 must be primed by type I IFN or IFN-γ to enable it to detect cytosolic LPS. In vitro, these stimuli are routinely supplied before activating caspase-11, however, whether one or both of these priming pathways is operational during infection in vivo had not been studied. Prior studies hint at type I interferons playing a more prominent role in caspase-11 priming (Broz et al., 2012; Case et al., 2013; Hagar et al., 2013; Kayagaki et al., 2011; Rathinam et al., 2012). This makes some sense because the innate immune system should first encounter extracellular LPS, triggering TLR4, a known inducer of type I IFNs. However, our results show the converse, that IFN-γ is required to prime caspase-11 during *B. thailandensis* infection. In this case, IFN-β was likely not induced to sufficient levels during in vivo infection, perhaps because *B. thailandensis* LPS is a somewhat weak TLR4

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agonist (Novem et al., 2009), or because *B. thailandensis* escapes the vacuole before vacuolar TLR4 can activate TRIF (Kagan et al., 2008). Nevertheless, the role of IFN-γ during *B. thailandensis* infection can be bypassed by providing poly(I:C), a known inducer of IFN-β(Wu et al., 2014). During any particular infection there may be a stronger IFN-γ or IFN-β response. Which of these two cytokines is most likely to precede a cytosolic invasion event? While IFN-γ-deficient mice are susceptible to a wide array of intracellular bacterial pathogens, type I IFN-deficient mice are most susceptible to viral infection. Thus, it may be that IFN-γ is the more physiologically relevant pathway to prime caspase-11.

We next assessed the importance of priming to the human cytosolic LPS response pathway. Murine caspase-11 is duplicated in human as caspase-4 and -5. Similar to caspase-11, both caspase-4 and -5 have been shown to detect cytosolic LPS (Shi et al., 2014). Nevertheless, it remains unclear whether both are functional orthologue of caspase-11 in vivo. While the function of caspase-5 remains to be studied in vivo, we show here that caspase-11 and caspase-4 are fully competent to defend against *B. thailandensis* infection. However, unlike caspase-11, caspase-4-transgenic mice do not strictly require IFN-γ priming to defend against *B. thailandensis* infection. While one must keep in mind the caveats of using transgenic mice, our data is consistent with the observations that caspase-4 is constitutively expressed in vitro by human cells (Lin et al., 2000). This difference may have significant relevance to human disease. We would predict that humans are therefore more resistant to infection by cytosol-invasive Gram-negative bacteria, but would simultaneously be more susceptible to aberrant translocation of LPS that could occur during sepsis. Indeed, the *CASP4*^{Tg} mice have an increased susceptibility to LPS challenge (Kajiwara et al., 2014). Therefore, whereas WT mice are highly resistant to LPS challenge, the constitutive expression of caspase-4 in humans could render them more sensitive to the effects of LPS during sepsis. It will be important to consider the differences between caspase-4 and -11 priming in mind when considering the translation impact of murine studies to human sepsis.

EXPERIMENTAL PROCEDURES

Mice

Wild-type C57BL/6 (Jackson Laboratory), *Nlrp3*$^{-/-}$ (Mariathasan et al., 2006), *Nlrc4*$^{-/-}$ (Mariathasan et al., 2004), *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$, *Nlrc4*$^{-/-}$*Asc*$^{-/-}$, *Nlrc3*$^{-/-}$ (Schneider et al., 2012), *Nlrc5*$^{-/-}$ (Robbins et al., 2012), *Nlrx1*$^{-/-}$ (Allen et al., 2011), *Il1b*$^{-/-}$, *ll18*$^{-/-}$ (Takeda et al., 1998), *Il1b*$^{-/-}$ *Il18*$^{-/-}$ (Shornick et al., 1996), *Casp11*$^{-/-}$ (Kayagaki et al., 2011), *Casp1*$^{-/-}$*Casp11*$^{129mut/129mut}$ referred to as *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Tlr4*$^{lps\text{-}del/lps\text{-}del}$ referred to as *Tlr4*$^{-/-}$ (Jackson # 007227), *Ifnar1*$^{-/-}$ (Jackson # 032045), *Ifng*$^{-/-}$ (Jackson # 002287), *Ifngr1*$^{-/-}$ (Jackson # 0022216), *Nos2*$^{-/-}$ (Jackson # 002609), *Gbp*$^{chr3-/-}$, *Casp11*$^{-/-}$/*CASP4*^{Tg} (Kajiwara et al., 2014), *Casp1*$^{-/-}$*Casp11*$^{-/-}$/*CASP4*^{Tg} (Kajiwara et al., 2014) and 129/SvEv (Taconic# 129/SvEv Tac) mice were used in this study. Bones from *Nod2*$^{-/-}$, *Nod1*$^{-/-}$*Nod2*$^{-/-}$, *Myd88*$^{-/-}$*Nod1*$^{-/-}$*Nod2*$^{-/-}$, and *Card6*$^{-/-}$, to prepare bone marrow derived macrophages (BMMs) were a Kind gift from Russell Vance and Thirumala Kanneganti Laboratories. All mice were 8–12 weeks old, male or female, and house under specific pathogen free condition–free facility. All protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill, the

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Institute for Systems Biology, Seattle Biomedical Research Institute, or The Icahn medical institute at Mount Sinai and met guidelines of the US National Institutes of Health for the humane care of animals.

In vivo infections

For all experiment, Burkholderia strains were grown in Luria-Bertani medium (LB) overnight at 37°C. Bacteria were pelleted from 1 mL of culture and washed in PBS before inoculation to mice. Bacteria titer was determined by OD measurement at 600 nm and plating serial dilution on LB agar plate. In this study, we used in addition to unpassaged *B. thailandensis* strain (u.p. *Bth*, Strain E264), a *B. thailandensis* strain that was passaged through *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice (m.p. *Bth*, strain E264-1); this strain displays more synchronized infection kinetics than the parental E264. For lethal challenge, mice were inoculated via the intraperitoneal (i.p.) route with the dose specified in the text. For bacterial burden measurements, spleen, liver, and mediastinal lymph nodes (MLN) were collected on day 1, 3, or 5 post infections when dose of infection used were 2×10^7 cfu, 10^4 cfu, or 10^2 cfu respectively. All organ harvested were homogenized in sterile PBS. Viable cfu in homogenates were enumerated by plating serial dilutions on agar plates. At the same time of organ harvest, serum was collected from blood drawn by cardiac puncture for cytokines measurement. Alternatively, in figure 6 WT, *Ifng*$^{-/-}$, or *Casp11*$^{-/-}$ mice were infected i.p. with 10^4 cfu of *Bth*, 24 hrs post infection they were injected with PBS or poly(I:C) (10mg/kg), and 48 hrs after PBS or poly(I:C) priming, bacterial burdens in organs homogenates were determined as described above. In Figure 6b, Mice were infected with 10^4 cfu of *Bth*, 48 hrs post infection they were injected with PBS or poly(I:C) (10mg/kg), and 9 hrs after PBS or poly(I:C) priming, serum was collected as described above and HMGB1 levels were assessed by ELISA. In Figure 3S, Mice were infected i.p. with 2×10^7 cfu of *Bth*, 6 hrs post infection they were injected with PBS, or IFN-γ (4ug/mice), or poly(I:C) (10mg/kg), and 18 hrs after PBS, IFN-γ, or poly(I:C) priming, bacterial burdens in organs homogenates were determined as described above.

In vitro infection and analysis of inflammasome activation

For in vitro infections, Burkholderia strains grown as described above were pelleted from 1mL of culture were opsonized with 50μL of mouse sera for 30 min at 37°C and then suspend ed in 1 ml of DMEM. BMMs were prepared as described (Miao et al., 2010b). For infections, macrophages were seeded into 96-well tissue culture treated plates at a density of 5×10^4 cells/well. When indicated, macrophages were primed with lipopolysaccharide (50 pg/ml) or IFN-γ (8 ng/ml) overnight. Bacteria were added to BMMs at MOI 50, centrifuged for 5 min at 200 ×g, and then incubated at 37°C for 1hr. After 1 hour, extracellu lar bacterial growth was stopped by addition of 300 μg/ml kanamycin and supernatant samples were collected at the indicated time points. Alternatively, for caspase-11 activation using cytoplasmic LPS, BMM were primed as indicated in the text, and then media was replaced with OptiMEM containing combinations of CTB (20ug/ml) plus LPS (10ug/ml) or *L. monocytogenes* (strain 10403s) (MOI 7.5) plus LPS (10ug/ml). Supernatant samples were collected at the indicated time points in figures. Cytotoxicity was defined as the percentage of total lactate dehydrogenase released into the supernatant and was determined as described

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(Rayamajhi et al., 2013b). IL-1β secretion was determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

Cytokine Measurements in mouse sera

Mouse IL-1β, IL-18 and IFN-γ in serum were measured with an ELISA assay (R&D Systems). Protein level of HMGB-1 in serum was quantitatively measured using ELISA (antibodies-online) the according to the guidelines of the manufacturer.

IFN-γ depletion

The indicated mice were infected with 10^4 cfu of *Bth*. IFN-γ was depleted using two doses (0.5 mg of protein in PBS) anti-IFN-γ antibodies or isotype controls 24 hrs prior and 24 hrs post infection with 10^4 cfu of m.p. *Bth*. IFN-γ depletion was confirmed by measuring IFN-γ serum level 2 day post infection at time of organ harvest for bacterial burden measurement. Rat Anti-IFN-γ Monoclonal Antibody, Unconjugated, Clone XMG1.2 from BioXCell was used to deplete IFN-γ in vivo. Control animals were injected with matching isotype control antibody obtained from BioXcell.

Western Blots

Spleen from WT, *Casp11*$^{-/-}$, or *Ifng*$^{-/-}$ mice were harvested 6h after PBS or poly(I:C) priming and lysate were prepared. Total protein from lysates was determined using BCA assay and analyzed by Western blot. Caspase-11 or Caspase-4 expression was analyzed using anti-caspase-11 antibody (17D9, Novus) diluted 1:1000, caspase-4 antibody (4B9, MBL international) diluted 1:1000 respectively. Blots were stripped and equivalent loading of protein was ensured by Western blot using anti-β-actin HRP antibody (Cat. # 20272, AbCam) diluted 1:20,000.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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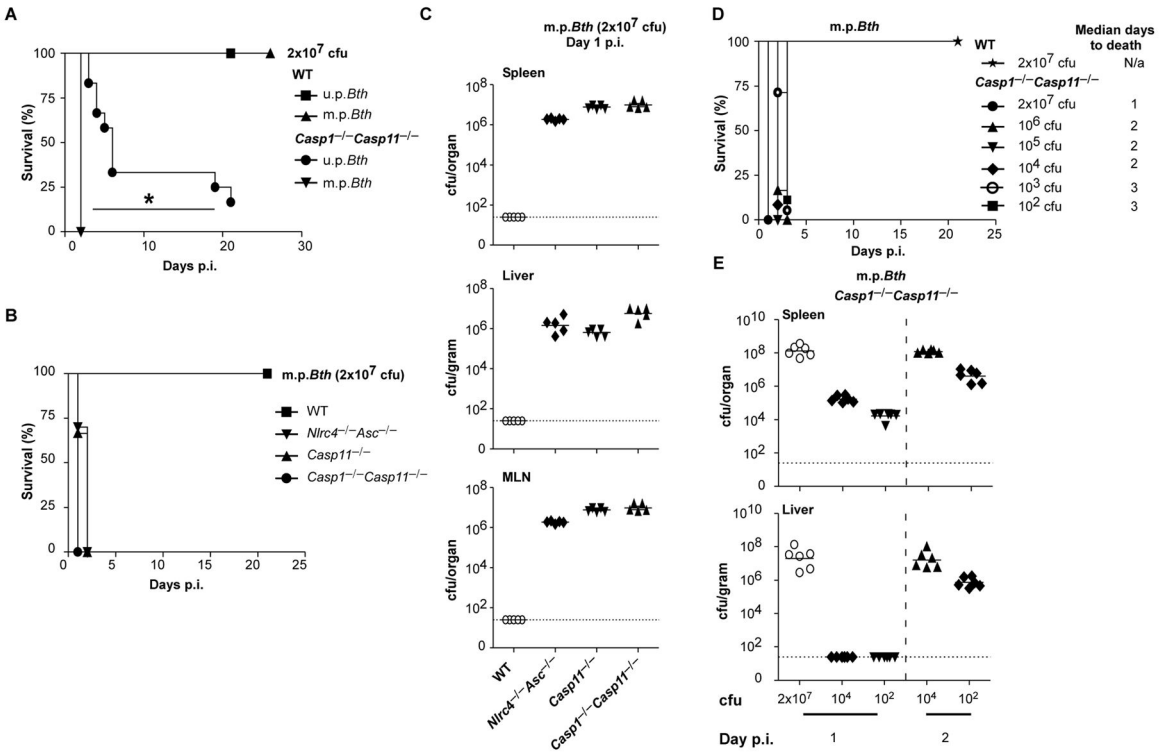

Figure 1. Both caspase-1 and -11 confer resistance *to Burkholderia thailandensis*

(A, B) Mice were infected with 2×10^7 cfu of either unpassaged (u.p.) or mouse-passaged (m.p.) *B. thailandensis* (*Bth*) and survival was monitored. Data are representative of two (A) or pooled from at least three experiments (B).

(C) Mice were infected with 2×10^7 cfu of m.p. *Bth*, and bacterial burdens were measured in spleen, liver, or mediastinal lymph nodes 24 hours later. Data are representative of three independent experiments. Dashed line: limit of detection.

(D, E) Mice were infected with the indicated dose of m.p. *Bth* and survival monitored or bacterial burdens assessed at the indicated time. Data are representative of two (D) or pooled from two experiments.

* $p \leq 0.05$; log rank test (a). For number of mice in each panel see Table S1. See also Figure S1.

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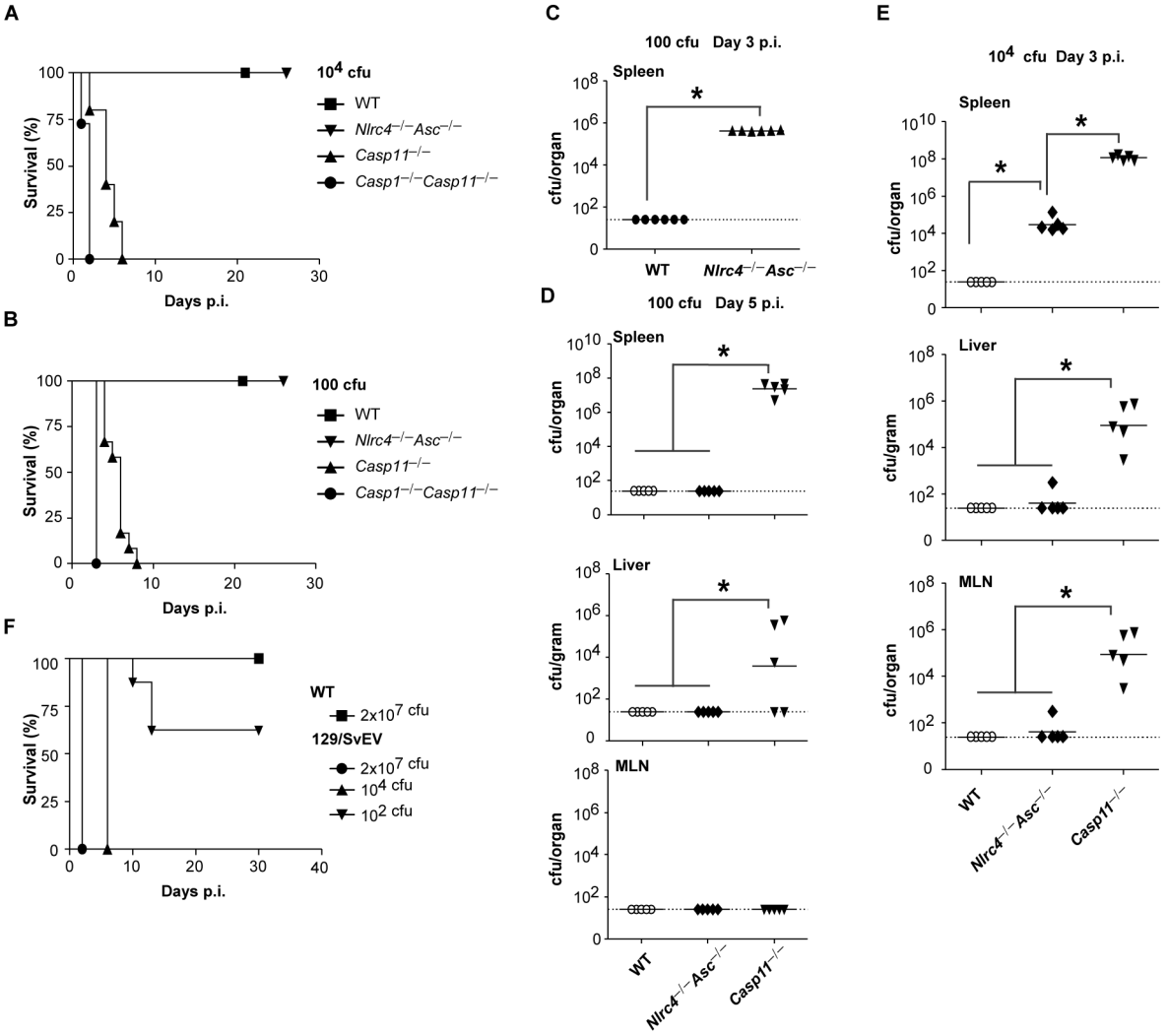

Figure 2. Differential contribution of caspase-1 and -11 to defense against *B. thailandensis*

(A, B, and F) Mice were infected with *Bth* at the indicated dose and survival was monitored. Data are pooled from at least two (E) or three experiments (A and B).

(C, D, and E) Bacterial burdens were determined in spleen, liver, or mediastinal lymph nodes at the indicated time post-infection. Data are representative of two (C) or three (D, and E) independent experiments.

*Student's *t* test (C, D, and E). For number of mice in each panel see Table S1. See also Figure S2

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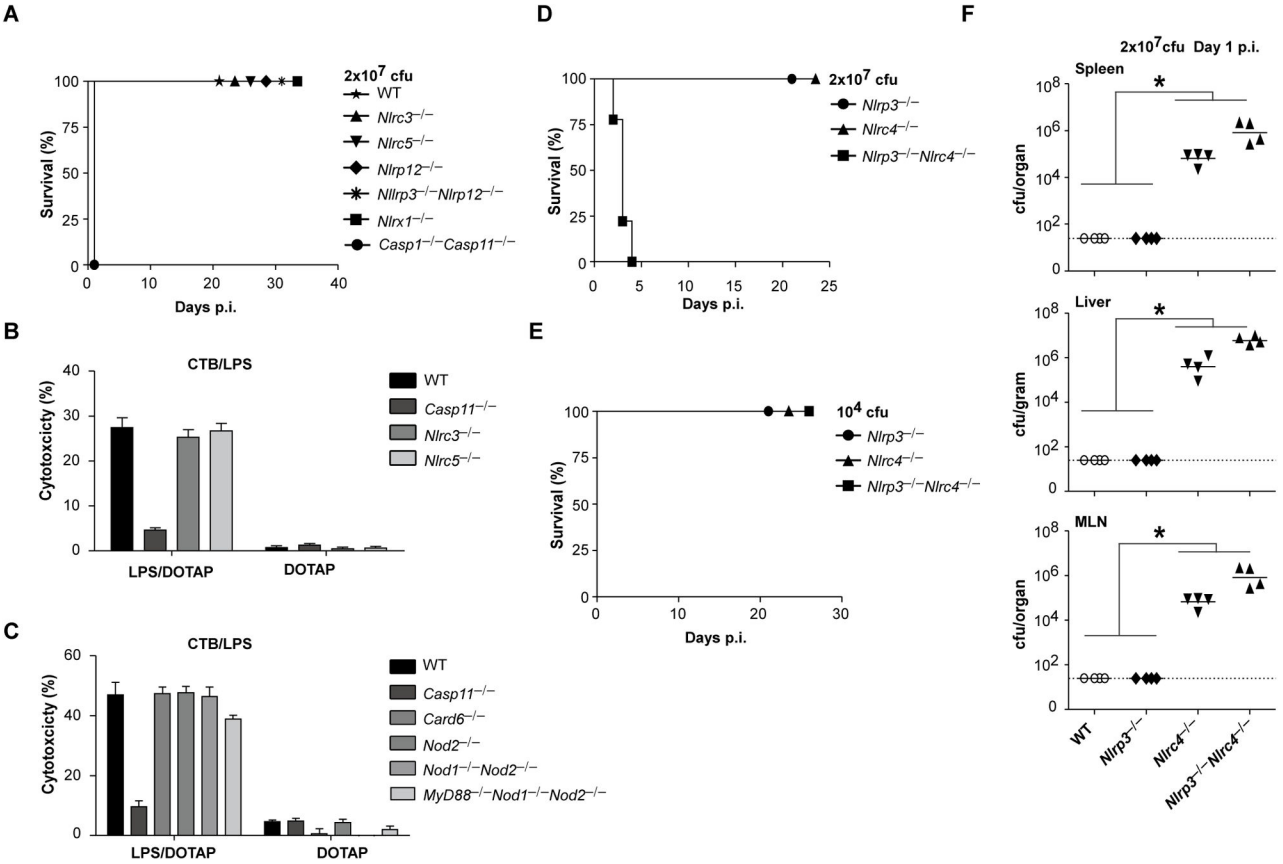

Figure 3. NLRC4 and NLRP3 both contribute to defense against *B. thailandensis*

(A) Mice were infected i.p with 2×10^7 CFU of *Bth* and survival was monitored. Data are pooled from at least two experiments.

(B and C) IFN-γ primed BMMs were stimulated with CTB/LPS for 4h and cytotoxicity was determined by LDH release assay. Histogram represents mean ± SEM of at least two independent experiments.

(D and E) Mice were infected i.p. with the indicated doses of *Bth* and survival was monitored. Data are pooled from 3 experiments.

(F) Mice were sacrificed 24 hrs post-infection with 2×10^7 CFU and the bacterial burdens in organs were assessed. Data are representative of three independent experiments.

* Student's *t* test (F). For number of mice in each panel see Table S1.

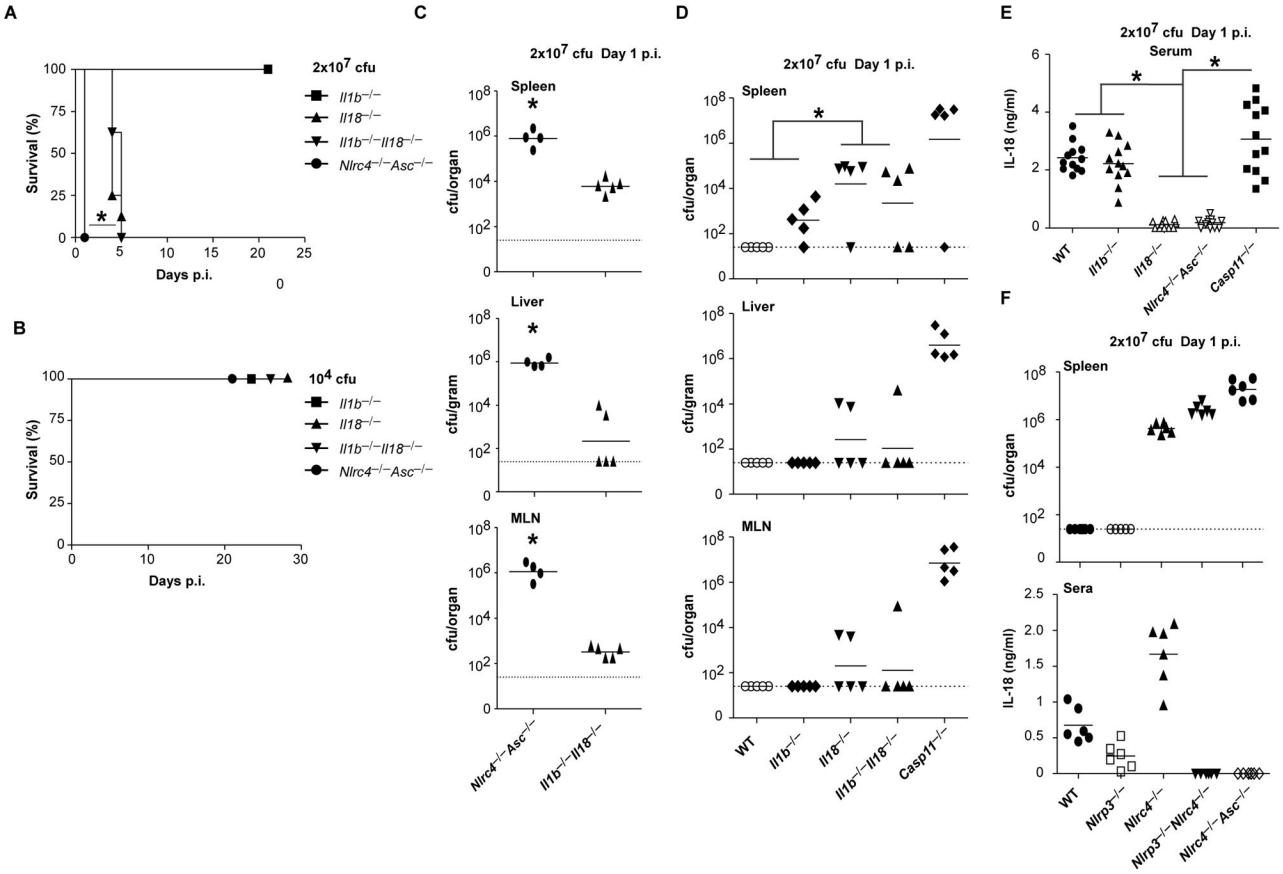

Figure 4. IL-18 is required for defense against *B. thailandensis*

(A and B) Mice were infected i.p. with the indicated dose of and survival was monitored. Data are pooled from at least three experiments.

(C and D) Mice were infected with 2×10^7 cfu of *Bth* and bacterial burdens were assessed. Data are representative of three independent experiments.

(E–F) Serum IL-18 (E–F) or bacterial burdens (F) were determined. Data are pooled from three (E) or two (F) experiments.

* $p \leq 0.05$; log rank test (A) and Student's *t* test (C, D, and E).

For number of mice in each panel see Table S1.

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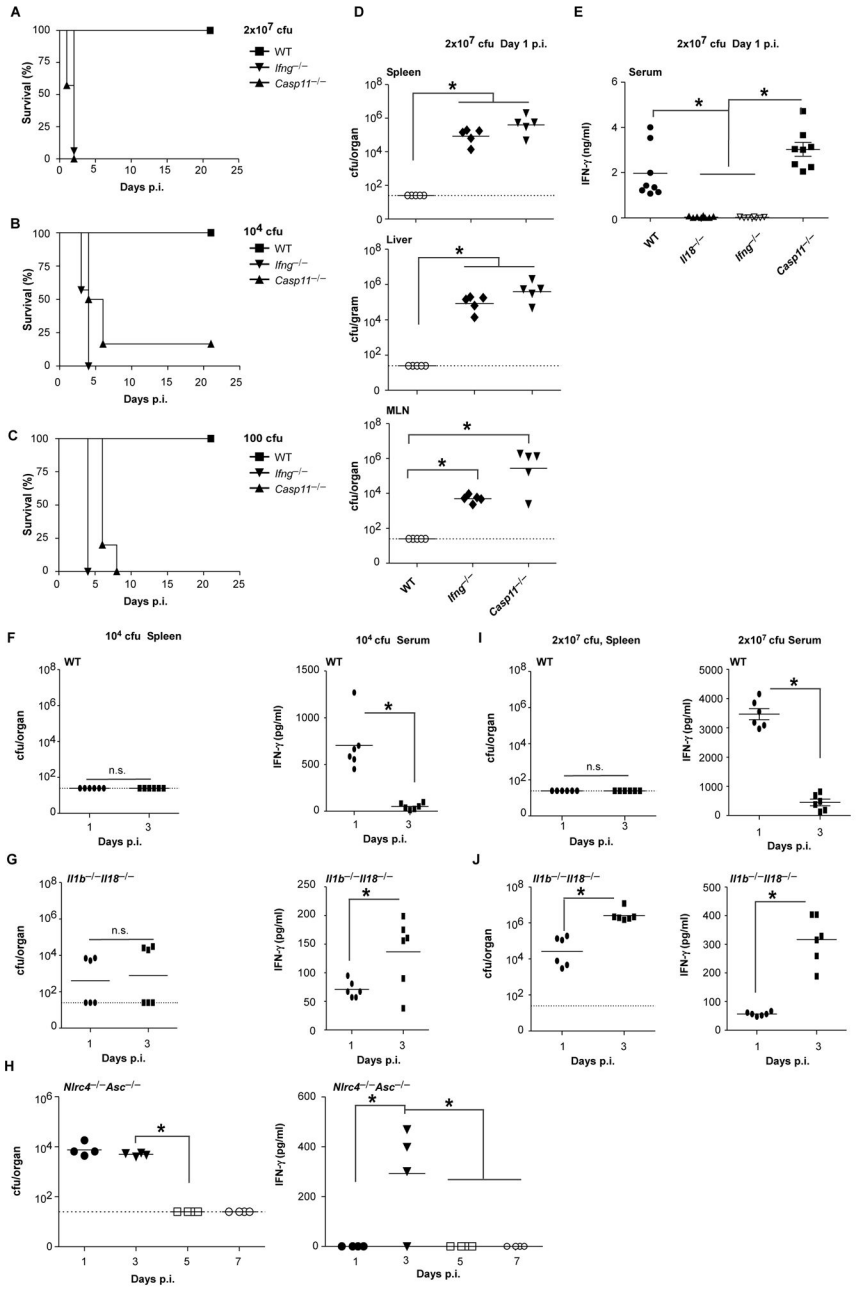

Figure 5. IFN-γ is critical to defend against *B. thailandensis*

(A, B, and C) Mice were infected i.p. with *Bth* at the indicated dose, and survival was monitored. Data are pooled from at least experiments.

(D and E) Bacterial burdens (D) and serum IFN-γ levels (E) were assessed 24 h after infection at the indicated dose. Data are representative of 3 independent experiments (D), or pooled from 3 experiments (E).

(F–J) Mice were infected i.p. with 10^4 or 2×10^7 cfu *Bth* and splenic bacterial burdens and IFN-γ levels were determined at the indicated time. Data are representative of two (F, G, I, J) or three (H) independent experiments.

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* Student's *t* test (D–J). For number of mice in each panel see Table S1. See also Figure S3A.

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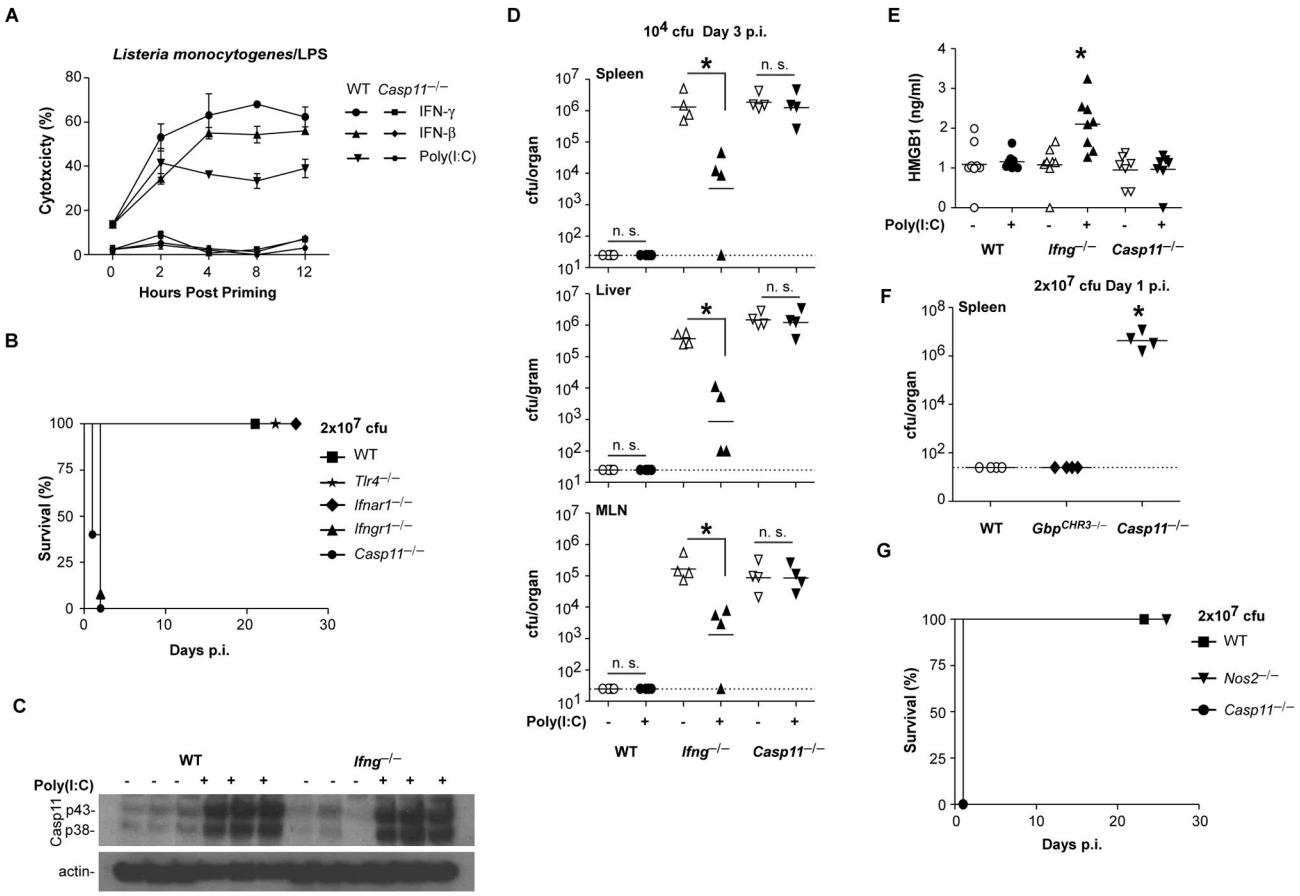

Figure 6. IFN-γ primes caspase-11 in vivo to protect against *B. thailandensis*

(A) IFN-γ, IFN-β, or poly(I:C) primed BMMs were infected with *L. monocytogenes* (*Lm*) plus LPS for 4hrs. Cytotoxicity was determined by LDH release assay. Line represents mean ± SEM of at least two independent experiments.

(B) Mice were infected with *Bth* at the indicated doses and survival was monitored. Data are pooled from at least two experiments.

(C) Western blot for caspase-11 in splenic lysates from WTor *Ifng*$^{-/-}$ mice 6h after PBS or poly (I:C) priming. Representative of three experiments.

(D) Mice were infected with 10^4 cfu of *Bth*. 24 hrs post infection they were injected with PBS or poly (I:C). 48 hrs after PBS or poly (I:C) priming, bacterial burdens in organs homogenates were determined. Data are representative of three independent experiments.

(E) Mice were infected with 10^4 cfu of *Bth*. 48 hrs post infection they were injected with PBS or poly(I:C). 9 hrs after PBS or poly(I:C) priming, serum HMGB1 levels were assessed by ELISA. Data are pooled from three experiments.

(F) Splenic bacterial burdens in WT, *Casp11*$^{-/-}$, and *Gbp*$^{\text{chr3}-/-}$ 3 days post infection with 10^4 cfu *Bth*. Data are representative of two experiments. (G) Survival of WT, *Casp11*$^{-/-}$, and *Nos2*$^{-/-}$ mice after infection with 2×10^7 cfu of *Bth*. Data are pooled from at least three experiments.

* Student's *t* test (D and E). For number of mice in each panel see Table S1. See also Figure S3B

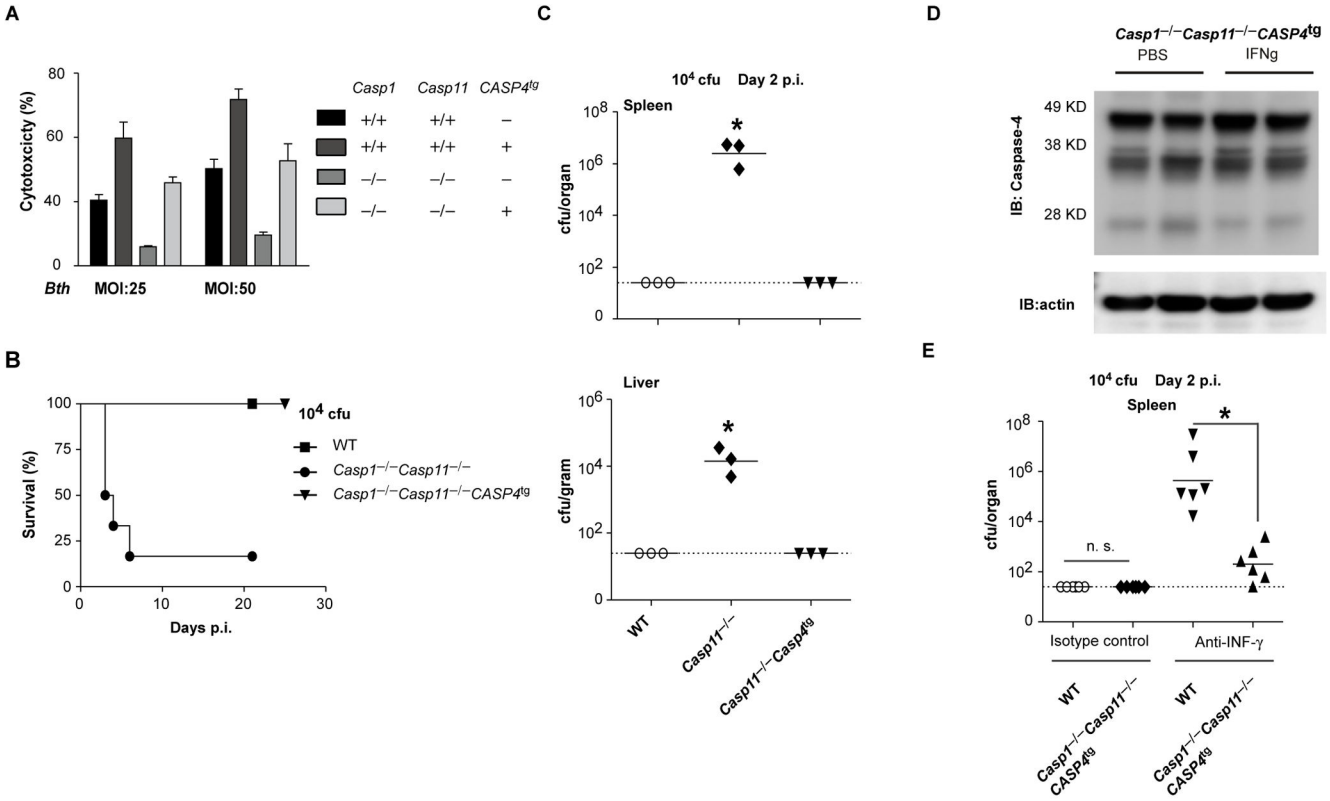

Figure 7. Human caspase-4 does not require IFN-γ priming in vivo

(A) LPS primed BMM were infected with *Bth* for 4hrs and cytotoxicity was determined by LDH release. Histogram represents mean ± SEM of at least two independent experiments.

(B and C) Mice were infected with 10^4 cfu *Bth*, and survival (B) or bacterial burden in spleen and liver were determined 48 hrs post infection (C). Data are representative of two independent experiments.

(D) *Casp1*$^{-/-}$*Casp11*$^{-/-}$*CASP4*^{Tg} mice were injected with PBS or 5μg IFN-γ. 6h later spleens were harvested and caspase-4 and actin levels determined by immunoblot.

(E) Mice were infected with 10^4 cfu of *Bth*. IFN-γ was depleted using two doses of anti-IFN-γ antibodies or isotype controls 24 hrs prior and 24 hrs post infection. Splenic bacterial burdens were determined 72hrs post infection. Data are representative of two independent experiments.

* Student's *t* test (C and D). For number of mice in each panel see Table S1. See also Figure S4

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