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Detection of cytosolic bacteria by inflammatory caspases

Jon A. Hagar and **Edward A. Miao**

Department of Microbiology and Immunology and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

Abstract

The sanctity of the cytosolic compartment is rigorously maintained by a number of innate immune mechanisms. Inflammasomes detect signatures of microbial infection and trigger caspase-1 or caspase-11 activation, culminating in cytokine secretion and obliteration of the replicative niche via pyroptosis. Recent studies have examined inflammatory caspase responses to cytosolic bacteria, including *Burkholderia*, *Shigella*, *Listeria*, *Francisella*, and *Mycobacterium* species. For example, caspase-11 responds to LPS introduced into the cytosol after Gram-negative bacteria escape the vacuole. Not surprisingly, bacteria antagonize these responses; for example, *Shigella* delivers OspC3 to inhibit caspase-4. These findings underscore bacterial coevolution with the innate immune system, which has resulted in few, but highly specialized cytosolic pathogens.

INTRODUCTION

The immune defenses of the extracellular environment are severe, as are those of the phagolysosome. The prospect of refuge from these insults therefore makes the cytosolic compartment a theoretically attractive refuge for potential bacterial pathogens. However, the fact that bona fide cytosolic bacteria can be counted on one's fingers (see Table 1 for a summary of these pathogens, their cell tropisms, and their mechanisms for invading the cytosol) highlights the successful immune defenses employed to maintain the sterility of the cytosolic niche. A number of cytosolic sensors detect signatures of infection, initiating potent inflammatory responses and/or host cell death. The importance of inflammatory caspases in this regard is underscored by the extreme susceptibility of mice deficient in these enzymes to infection by cytosolic pathogens. Interestingly, the few cytosolic specialist pathogens are among the most virulent known. Herein, we discuss the role of inflammatory caspases in the innate immune response to cytosolic bacteria, focusing on recent advances in our understanding of how cells detect intruders and trigger caspase activation, and how caspases mediate containment of the infection.

THE INFLAMMATORY CASPASES

Caspases are ancient and evolutionarily conserved proteases that are integral to development, homeostasis, and immunity. Some caspases are involved in apoptosis, an immunologically silent form of programmed cell death. In contrast, the inflammatory caspases, caspase-11 (or the presumed human homologs caspase-4 and caspase-5) and

Correspondence to: Edward A. Miao, emiao@med.unc.edu, 609 Mary Ellen Jones Building, 116 Manning Drive, Chapel Hill, NC 27599-7290, USA, Campus Box 7290, (919) 966-6773.

caspase-1, initiate a form of lytic cell death termed pyroptosis following their activation, which releases inflammatory mediators, removes the replicative niche of cytosolic bacteria, and exposes intruders to extracellular defenses and neutrophils [1] (reviewed in [2]). In addition, caspase-1 mediates the maturation and secretion of pro-IL-1β and pro-IL-18, two pleiotropic inflammatory cytokines best known for inducing fever and interferon (IFN)-γ secretion, respectively [3].

THE INFLAMMASOMES

The inflammatory caspases are expressed as inactive zymogens. The canonical inflammasomes, a class of cytosolic pattern recognition receptors (PRR), activate caspase-1 in response to specific signatures of infection. A theorized non-canonical inflammasome(s) is proposed to activate caspase-11 [4]. Relevant inflammasomes and their agonists are detailed in Table 2; for in-depth review, see [2] and [3].

Burkholderia

B. pseudomallei and *B. thailandensis* have served as models for studying the interaction of inflammatory caspases and cytosolic bacteria. These Gram-negative bacteria exist ubiquitously in the soil of southeast Asia and sporadically elsewhere [5]. Although closely related, only *B. pseudomallei* causes severe human and murine disease; however, *B. thailandensis* can infect macrophages and epithelial cells both in vitro and in vivo. *B. pseudomallei* and *B. thailandensis* rapidly escape the vacuole via their type III secretion system (T3SS) [6][7]. NLRC4 is positioned to detect signatures of T3SS activity, alerting the immune system to pathogens that reprogram and parasitize host cells. Not surprisingly, we and others found that macrophage infection triggers NLRC4 activation [8][9]. Mediating this activation, we showed that the T3SS rod protein BsaK is detected through NLRC4 [10], and Zhao and colleagues demonstrated that NAIP2 is the sensor upstream of NLRC4 [11]. Later the T3SS needle protein BsaL, as well as needle proteins from a variety of other bacteria, was found to be detected by murine NAIP1 and human NAIP, both signaling through NLRC4 downstream [11][12][13]. By an ill-defined mechanism, *Burkholderia* species also activate NLRP3 [8][9]. Together, NLRC4 and NLRP3 are critical for mice to resist intranasal *B. pseudomallei* challenge [8]. In this model, IL-18 is central to this resistance, coordinating bacterial clearance, whereas IL-1β secretion mediates immune pathology driven by neutrophil recruitment.

Recently, we determined that caspase-11 is critical for mice to resist infection by both virulent *B. pseudomallei* as well as avirulent *B. thailandensis* [9]. Caspase-11 functions independently of all known inflammasomes, instead working in parallel with caspase-1 to mediate protection against ubiquitous environmental bacteria. We discovered that caspase-11 responds specifically to Gram-negative cytosolic bacteria, where normally vacuolar bacteria such as *Legionella pneumophila* and *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) rapidly induce caspase-11 dependent pyroptosis only after aberrant translocation to the cytosol. In complementary studies, we and Kayagaki and colleagues determined that cytoplasmic translocation of penta- and hexa-acylated LPS, but not tetra-acylated LPS, triggers caspase-11 activation [14][15]. Although enhanced by TLR4 signaling, this pathway can proceed independently of extracellular LPS signaling. Thus, $Tlr4^{-/-}$ mice primed with a TLR3 agonist succumb to secondary LPS challenge in a model of endotoxic shock. Previous studies indicate that during prolonged infections, caspase-11 activates in response to all Gram-negative bacteria [4][16][17][18]. We speculate that such activation may reflect vacuole leakage events that accumulate over 16h, which may have relevance in the setting of Gram-negative septic shock. In contrast, caspase-11 rapidly responds to *L. pneumophila* infection in pre-activated macrophages [19][20]; whether vacuolar integrity is compromised under these conditions remains to be examined. The

physiologic role of caspase-11 during infection is to combat cytosolic bacteria. The upstream sensor that detects cytosolic LPS remains unknown.

Shigella

Members of the Gram-negative *Shigella* genus are exquisitely adapted to cause human gastrointestinal disease. *S. flexneri* infects a variety of cell types, such as intestinal epithelial cells and macrophages. Following phagocytosis by macrophages or T3SS-mediated uptake by epithelial cells, *S. flexneri* rapidly escapes the phagosome. In vitro, *S. flexneri* is robustly detected by caspase-1 via NLRC4 [21] and, under some conditions NLRP3 [22]. As an aflagellate bacterium, *S. flexneri* does not expressed flagellin. We showed that the MxiI rod protein is detected via NLRC4 [10], and Zhao showed this was via NAIP2 [11]. The *S. flexneri* needle component MxiH is also detected by murine NAIP1 and human NAIP [12]. As with *Burkholderia*, NLRC4 is positioned to detect *Shigella* before cytosolic invasion, and thus does not differentiate it from vacuolar T3SS utilizing bacteria such as *S. typhimurium*. Whether inflammasome pathways more tailored to detecting cytosolic bacteria (AIM2 or caspase-11) function in resistance to *Shigella* infection remains to be determined; however, we have found that both *S. flexneri* infection and transfection of *S. flexneri* lysates into macrophages activate caspase-11 in vitro (our unpublished observations), indicating that *S. flexneri* lipid A can be detected by the caspase-11 pathway.

Recently, work employing a Guinea pig model of *Shigella* infection, which more faithfully models human infection than mouse models, has implicated caspase-4 in host resistance to *S. flexneri* [23]. Kobayashi and colleagues found that caspase-4 mediates epithelial cell death in response to several enteric pathogens, and that *S. flexneri* secretes an inhibitor of caspase-4 activation, OspC3, to counteract this innate immune response in vitro and in vivo. Remarkably, the authors found that OspC3 is specific in antagonizing caspase-4 and does not associate with caspase-11, highlighting the specificity of *Shigella* species for infecting humans. Future research will determine whether caspase-4 responds to cytoplasmic LPS as does caspase-11, which would situate caspase-4 as key preserver of cytosolic sterility.

Francisella

The causative agent of tularemia, Gram-negative *F. tularensis* is among the most infectious and virulent pathogens; thus, it is classified as a category A bioweapon. *F. tularensis* infects a variety of cell types, with macrophages and neutrophils representing the primary replicative niches during pneumonic infection [24]. *F. novicida* is closely related to *F. tularensis*, but is far less virulent. *F. novicida* lyses in the cytosol of murine macrophages, releasing DNA that triggers AIM2/ASC/caspase-1 [25][26][27][28][29][30]. In vivo, *Aim2*-deficient mice have increased susceptibility to *F. novicida* infection [27][28]. In some experimental systems, *F. novicida* also triggers NLRP3 activation [31]. However, murine infection by *F. tularensis*, unlike by*F. novicida*, results in little detectable caspase-1 activation [32], suggesting virulent strains have evolved to evade AIM2. A better understanding of this difference may have implications for both the treatment of and vaccination against tularemia.

Francisella species express tetra-acylated LPS. Not surprisingly, we have found that macrophages do not activate caspase-11 after infection by *F. novicida* [15]. However, transfection of penta-acylated lipid A from an *lpxF* mutant, but not wild-type tetra-acylated lipid A, triggers caspase-11 dependent pyroptosis. Therefore, *Francisella* species appear to have evolved to evade a major host cytosol surveillance pathway, the non-canonical inflammasome.

Listeria

Listeria monocytogenes is a Gram-positive saprophyte and facultative pathogen that causes self-limited gastroenteritis in immunocompetent individuals. Of particular concern for the immunocompromised, *L. monocytogenes* infections can progress to cause sepsis, encephalitis, and death; in pregnant mothers, it can trigger abortion. *L. monocytogenes* readily escapes into the cytosol of epithelial cells and macrophages using the pore-forming toxin listeriolysin O (LLO).

In vitro, macrophages detect cytosolic *L. monocytogenes* via NLRC4 and AIM2; NLRP3 also detects infection under certain experimental conditions [26][33][34][35][36][37][38], but not others [39][40]. In the absence of infection, the pore-forming activity of purified LLO protein is sufficient to trigger NLRP3 activation [33]. NLRC4 responds to flagellin sloughed from *L. monocytogenes* in the cytosol. In this case, NLRC4 acts as a specific sensor of cytosolic invasion, whereas it does not differentiate between cytosolic or vacuolar T3SS-expressing bacteria. AIM2 responds to DNA released into the cytosol following infrequent lysis of *L. monocytogenes*.

In vivo, *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice may have increased susceptibility to *L. monocytogenes* infection [41]; however, this was not replicated in another publication [40]. Furthermore, the contributions of individual inflammasomes during in vivo infection are not defined. Nevertheless, *L. monocytogenes* appears to have evolved to limit inflammasome detection: LLO activity is optimal in the acidic environment of the phagosome, thus limiting its potential to trigger NLRP3; flagellin expression is repressed during growth at host temperature; and few bacteria lyse in the cytosol, thus limiting cytosolic DNA exposure. The efficiency of these evasive strategies is demonstrated by the rapid clearance of *L. monocytogenes* forced to express flagellin in vivo [40][42].

By virtue of its nature as a Gram-positive bacterium, *L. monocytogenes* does not contain LPS, and thus is not detected by caspase-11 [15][43].

Rickettsia

Members of the genus *Rickettsia* are Gram-negative, obligate intracellular pathogens that invade the cytosol of vascular endothelial cells and macrophages, causing a variety of arthropod-borne diseases. Little research to date has investigated the interactions of inflammatory caspases and *Rickettsia*; however, infected mouse peritoneal macrophages secrete IL-1β [44], suggesting that caspase-1 responds to certain *Rickettsia* species. Interestingly, IFN-γ primed RAW264.7 macrophage-like cells undergo rapid cell death (within 4h) following infection with *R. prowazekii* [45]. It is tempting to speculate that the enhanced bactericidal activity of IFN-γ primed macrophages potentiates AIM2 or caspase-11 detection of *Rickettsia*.

Mycobacterium

Among *Mycobacterium* species, *M. marinum* is distinct in that it rapidly escapes the phagosome to replicate in the cytosol and spread cell-to-cell. Vacuolar escape requires ESAT-6, a secretion product of the ESX-1 type VII secretion system suggested to have membrane pore forming activity [46]. Although *M. tuberculosis* is traditionally considered a vacuolar pathogen of macrophages, recent studies suggest it may exist in the cytosol for at least part of its intracellular life cycle (reviewed in [47]).

A number of studies have investigated the role of inflammatory caspases in immunity to *M. tuberculosis* and *M. marinum*. While the in vivo importance of IL-1α and IL-1β are well accepted, the role of NLRP3, ASC, and caspase-1 remain controversial both in vivo and in

vitro (for a more in-depth review, see [3]). Herein we limit our discussion to the recent studies examining caspase-1 activation in response to cytosolic bacterial exposure. Several studies implicate ESX-1 and ESAT-6 in caspase-1 activation [48][49][50][51][52][53]. Abdallah and colleagues suggest that ESX-1 translocation of mycobacteria to the cytosol potentiates subsequent ESX-5 dependent inflammasome activation [54]. *M. tuberculosis* DNA can access the cytosol in a manner dependent on ESX-1, where it triggers STING-dependent type I interferon production [55]. DNA from *M. tuberculosis* and *M. bovis* also trigger AIM2/ASC/caspase-1 [56][57], and *Aim2*$^{-/-}$ mice appear susceptible to *M. tuberculosis* infection, suggesting a physiologic relevance to the in vitro detection data [56]. A recent contradictory report suggests that virulent *M. tuberculosis* strains actually inhibit AIM2 activation, whereas nonvirulent strains do not [58]; use of different macrophage types in these studies may reconcile their conflicting findings.

CONCLUSIONS

In recent years, our understanding of inflammatory caspase activation has expanded to include several new sensor-stimulus pairs, such as AIM2 and DNA, NAIP1 and the T3SS needle, and LPS and the non-canonical inflammasome. These findings have elucidated how the inflammatory caspases and, more generally, the innate immune system restrict the ability of pathogens to establish cytosolic growth niches. At the same time, they pose a number of questions, such as the identity of the non-canonical inflammasome. Furthermore, several models of cytosolic pathogen interaction with inflammatory caspases remain under-explored, such as *Rickettsia* infection and the emerging paradigm of cytosolic *M. tuberculosis*. Future studies will begin to fill these gaps and, surely, raise a number of new questions.

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HIGHLIGHTS

- Specific NAIPs activate NLRC4 in response to flagellin and the T3SS rod and needle proteins
- Caspase-11 defends against *Burkholderia* species by responding to cytosolic LPS
- OspC3 translocation by *Shigella*is a novel mechanism of caspase-4 antagonism
- *Mycobacterium tuberculosis* may have a cytosolic phase of its lifecycle that exposes it to cytosolic sensors

Table 1

Cell tropism and vacuolar escape determinants of cytosolic bacteria.

Genus	Gram +/−	Cell tropism	Vacuolar escape determinants, bacterial
Burkholderia	−	Mϕ, PMN, epithelial cells	$T3SS_{BSA}$
Shigella	−	Mϕ, DC, intestinal epithelial cells	Mxi-Spa T3SS, IpaB
Francisella	−	Mϕ, PMN, DC, epithelial cells, hepatocytes	IglC, MglA, FTT11103
Listeria	+	Mϕ, intestinal epithelial	LLO, phospholipase C
Rickettsia	−	Vascular endothelial, Mϕ	Phospholipases, hemolysin
Mycobacterium	Acid-fast +	Mϕ	ESX-1 T7SS, ESAT-6

Table 2

Interaction of inflammatory caspases and cytosolic bacteria.

Bacteria	**Caspase-1**			**Caspase-11 or -4**
Stimulus/sensor	**NLRC4**	**NLRP3**	**AIM2**	
Burkholderia	BsaK/NAIP2	Infection		LPS/casp11
Shigella	Needle/NAIP1 and human NAIP Rod/NAIP2	Infection		LPS/casp11 ?/casp4
Francisella		Infection (human)	DNA	
Listeria	Flagellin/NAIP5	Infection, LLO	DNA	
Rickettsia				
Mycobacterium		Infection, ESAT-6	DNA	
Antagonism				
Burkholderia				
Shigella				OspC3 inhibits casp4
Francisella			possible	Tetra-acyl LPS
Listeria	Represses flagellin at host temperatures	pH dependent LLO activity		
Rickettsia				
Mycobacterium		Zmp1 metalloprotease		