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Review

Caspase-11: mediator of pyroptotic and non-pyroptotic host responses

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ABSTRACT

The inflammatory caspases are crucial for innate defenses against invading pathogens. Though the canonical inflammasomes have been extensively studied, recently much attention has been focused on the role caspase-11 plays within the non-canonical inflammasome. Activation of the non-canonical inflammasome promotes caspase-11-dependent pyroptosis, but the downstream signaling pathways have remained obscure. This review will examine critical components required for pyroptosis downstream of caspase-11. In addition, we will discuss novel non-pyroptotic functions of the inflammatory caspases that promote the destruction of microbes. Both of these pathways shed new light into the distinct roles the caspases play in the host response that will contribute to new strategies aimed at enhancing the physiological role of pyroptotic and non-pyroptotic functions during infection and inflammatory diseases.

KEYWORDS: non-canonical inflammasome, caspase-11, caspase-1, pyroptosis, non-pyroptotic function, inflammation

INTRODUCTION

The canonical inflammasome is important for antimicrobial defense as it promotes the secretion of inflammatory cytokines IL-1β & -18 and induces pyroptosis, via the activation of the inflammatory caspase-1 [1, 2]. Recently, several reports have indicated that another inflammatory caspase, caspase-11, also plays a vital role in guarding against bacterial infection. Specifically, these studies detail caspase-11's role in the host inflammatory response against a plethora of Gram-negative pathogens from *Clostridium difficile*, *Citrobacter rodentium*, *Klebsiella pneumoniae*, *Vibrio cholera*, *Salmonella typhimurium*, and *Legionella pneumophila* to *Escherichia coli*, *Hemophilus influenzae*, *Shigella flexneri*, *Enterbacter cloacae*, *Burkholderia thailandensis*, and *Burkholderia pseudomallei* [3-15]. Recently, it has been demonstrated that caspase-11 plays an intimate role in the restriction of *L. pneumophila*, though the mechanism is unclear [4, 9, 10, 12, 14, 16]. However, it is clear that caspase-11 serves as an important factor in the degradation and clearance of *L. pneumophila*. This is accomplished either by elimination of its replicative niche via pyroptosis, phagolysosomal fusion, or by activating neighboring immune cells to bacterial infection through the maturation and release of inflammatory cytokines (IL-1β & -18) or danger signals (IL-1α & HMGB1).

In this review we discuss recent studies that detail unique downstream targets of caspase-11 upon activation of the non-canonical inflammasome and the resulting consequences of this activation for the host cell. We also discuss novel non-pyroptotic functions of caspase-11 and caspase-1 in order to control bacterial infection by *L. pneumophila*. These findings open the door to nascent areas investigating the molecular mechanisms employed by immune cells during infection and inflammatory diseases. Studies discussed in this review provide a basis for

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novel therapies that are directed against pyroptotic and non-pyroptotic targets of the inflammatory caspases.

Caspase-11

Caspase-11 was first discovered 22 years ago in a murine complementary DNA library screen while searching for homologs of caspase-1 [17]. From this, caspase-11 was identified because of its close similarity (46%) to caspase-1 and in *in vitro* studies it was discovered that this new murine caspase plays a direct role in cell death and promotes processing of pro-IL-1β via interaction with and activation of capsase-1 in response to lipopolysaccharide (LPS) but not *L. pneumophila*. It is, however, inefficient in processing IL-1β itself [18, 19]. This was also found to be the case for the human orthologs of caspase-11, caspase-4 and -5 [20-23]. Additionally, it was observed that stimulation with the bacterial pathogen-associated molecular pattern (PAMP) LPS induced expression of caspase-11, as 43 kDa and 38 kDa precursors, in various mouse tissues, especially the macrophage-rich spleen [18]. From this, many studies were carried out to decipher caspase-11's role in a LPS-induced septic shock model, given the finding that *Casp-11*$^{-/-}$ mice were resistant to lethal doses of LPS when compared to wild-type (WT) mice [18, 19]. This finding pointed to a divergent mechanism that regulates caspase-11 expression when compared to caspase-1, which is constitutively expressed in myeloid cells [24]. To date, it has been shown that LPS, type I, and II interferons (i.e. IFN-α, -β, and -γ) are able to promote upregulation of caspase-11 [5, 6, 18, 25]. To understand the mechanism regulating caspase-11 expression, Beyaert's group conducted deletion/ mutation analyses on the caspase-11 promoter along with chromatin immunoprecipitation (ChIP) assays and found that the promoter region contained many putative binding sites for various transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator of transcription-1 (STAT-1), interferon regulatory factor (IRF), nuclear factor of activated T-cells (NFAT), and cAMP response element-binding protein (CREB) [25]. In addition, the activation of p38 mitogen-activated protein kinase (MAPK) in rat glial cells, c-Jun N-terminal kinase (JNK) in mouse embryonic fibroblasts, and C/EBP homologous (CHOP) protein in mice are required for the induction of caspase-11 [26-28].

Because of the various and complex signaling pathways activated by the cell and the multitude of transcription factors that are able to interact with the caspase-11 promoter downstream of these pathways, it is difficult to identify a singular PAMP, inflammatory molecule, or danger signal that activates the main transcription factor responsible for inducing the expression of caspase-11. This supports the hypothesis that caspase-11 is a vital inflammatory mediator of the host response and therefore, the cell has conserved, redundant pathways encoded into its repertoire of tools to ensure caspase-11 expression during times of stress or infection.

The inflammatory caspases: caspase-1 vs. caspase-11

Once expressed and then activated, caspase-11 was thought to activate caspase-1 in the same pathway in a LPS lethal sepsis model, as both *Casp-11*$^{-/-}$ and *Casp-1*$^{-/-}$ were found to be resistant to lethal endotoxic shock [18, 29]. But, a recently published study reports that in fact, the caspases have distinct roles in the inflammatory response to clinically significant bacterial infections [3]. The original *Casp-1*$^{-/-}$ mice were created using embryonic stem (ES) cells from the 129 mouse strain and are inadvertently a double knock out of caspase-1 and -11 (*Casp-1*$^{-/-}$*Casp-11*$^{-/-}$). The authors found that all tested 129 mouse lines contained a 5 bp deletion in the splice acceptor of exon 7 in the caspase-11 genomic DNA. This mutation caused exon 7 to be spliced out, resulting in a frame shift that created a premature stop codon and therefore unstable caspase-11 transcripts. 129 mice do not express this mutant transcript, as it is most likely degraded via nonsense-mediated decay [30]. The inflammatory caspases are situated next to the caspase-1 gene on the same chromosome within the murine genome (chromosome 9) creating what is thought to be an inflammatory gene cluster [31]. Because *Casp-1* and *Casp-11* are in close proximity to each other, as they are only separated by ~1,500 bp (only 0.012 centimorgans), this negates the possibility of segregating the genes by recombination. Consequently, backcrossing to another caspase-11 competent mouse strain (C57BL/6 or NOD/ShiLtJ) would not rescue the caspase-11 deficiency [3]. These revelations highlight the need to revisit previous studies where the original *Casp-1*$^{-/-}$ (*Casp-1*$^{-/-}$*Casp-11*$^{-/-}$) mice were used to decipher caspase-1's contribution to the host's innate immune response during bacterial pathogenesis.

To examine caspase-1's singular role in the inflammatory response to pathogens, single *Casp-1*$^{-/-}$ mice were created by injecting a caspase-11 bacterial artificial chromosome (BAC) transgene in the *Casp-1*$^{-/-}$ *Casp-11*$^{-/-}$ embryos to produce *Casp-1*$^{-/-}$*Casp-11*^{Tg} [3]. Using these mice, researchers are starting to characterize caspase-1 and -11's functions during infections, to determine if they have unique or redundant roles within the host response [10, 16].

In agreement with past studies indicating that caspase-11 is induced upon LPS ligation with toll-like receptor 4 (TLR4) [18, 19], recent studies using Gram-negative pathogens have established that TLR4 is triggered by LPS engagement and is able to recruit the adaptor molecules myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF), thereby activating downstream transcription factors NF-κB, STAT-1, and interferon response factor 3 (IRF3). Once this occurs, many inflammatory genes and host restriction factors are upregulated, such as Nlrc4, Naip5, Nlrp3, pro-caspase-11, pro-IL-1α and -β, and interferon-α and -β [5-7, 9, 10, 13]. This is the first step in the established two-step process of activation thought to be employed to safeguard against unintentional caspase-1 activation. Priming is the first step and includes upregulating gene expression of the many inflammatory factors involved in the clearance of pathogens [32]. Additionally, it has also been shown that these upregulated inflammatory cytokines, specifically interferon-α and -β, acting in an autocrine or paracrine fashion, stimulate the type I interferon receptor (IFNRA) to engage STAT1 and IRF9 that also contribute to caspase-11 expression [5, 6]. But, it is worth noting that there is debate as to whether caspase-11 induction upon infection with Gram-negative bacteria is solely dependent on the type I interferon-IFNRA-TRIF axis [5-7].

Once upregulated, triggering of the inflammasome is the second step in activation and it has been demonstrated that caspase-11 plays a unique role in promoting pyroptosis and the secretion of IL-1β in what is now termed non-canonical inflammasome activation [3]. Using LPS to prime murine bone marrow-derived macrophages (BMDMs), Dixit's group found that capsase-11, not caspase-1, was required for pyroptosis in response to *C. rodentium*, *V. cholera*, and *E. coli*, but at the same time, caspase-11-dependent secretion of IL-1β and -18 required caspase-1, as well as Nlrp3 and apoptosis-associated speck-like protein containing a CARD (ASC). Furthermore, caspase-11, rather than caspase-1, is required for LPS-induced lethality in mice model of endotoxic shock [3]. Moreover, when macrophages are stimulated with factors that engage the canonical inflammasomes such as Nlrp3, Nlrc4/ Naip, Aim2, and Pyrin, caspase-1-dependent responses take over. Therefore, caspase-11-mediated cell death and IL-1β processing is only detected in the absence of canonical activators like LPS and ATP, nigericin, flagellin, T3SS needle or rod, double-stranded DNA and *Francisella tularensis*, and *Clostridium difficile* toxin [3, 8-10, 33]. Together, these were the first studies to describe caspase-11 as a main potentiator of the inflammatory response to pathogens through the activation of the novel non-canonical inflammasome.

Early investigations into non-canonical inflammasome activation

Since the discovery of the non-canonical inflammasome (also known as the caspase-11-dependent inflammasome), many research groups have focused their efforts into elucidating the mechanism of activation and the bacterial PAMP responsible for this activation. There have been many conflicting reports as to the nature of the non-canonical inflammasome that were general attributes of microbes. Gram-negative bacteria elicit a TLR4-TRIF-dependent release of IFN-β that, through autocrine signaling, activates the non-canonical inflammasome. Conversely, Gram-positive and Nlrc4-dependent pathogens mediate immune responses independent of this pathway [5-7]. Others have indicated that reactive oxygen species (ROS) production regulates caspase-11-dependent inflammasome through the JNK pathway [13]. One study has indicated that canonical caspase-1 activation drives the IL-18-IFN-γ axis to promote caspase-11 activation *in vivo* and restriction of the cytosol-invasive bacteria *B. thailandensis* [15]. Other reports have stated that IFN-inducible small GTPase called guanylate binding proteins (Gbp) promote lysis of the pathogen-containing vacuole, allowing bacteria to gain access to the host cytosol where caspase-11 is then activated [11, 12, 34]. Meanwhile, within the *L. pneumophila* field our report has indicated that caspase-11 activation is

flagellin-dependent and associates with the Nlrc4 inflammasome, as caspase-1 and -11 interact to form a catalytically active heterocomplex [3, 4, 19]. But others investigating caspase-11's role during *L. pneumophila* infection have found that in the absence of flagellin, T4SS-dependent caspase-11 activation promoted pyroptosis via the Nlrp3-caspase-1 axis, independent of the classic Nlrc4 inflammasome [35]. Moreover, this T4SS-dependent activation is solely responsible for the release of IL-1α during *L. pneumophila* infection, which is in agreement with Dixit's group's findings in an *in vivo* endotoxic shock model [3, 10].

Altogether, these data helped progressively elucidate caspase-11 activation in the non-canonical inflammasome during bacterial infections, but it wasn't until two independent studies resolved the controversy within the field. These reports discovered that intracellular LPS is an activating factor of the non-canonical inflammasome and that caspase-11 is the sensor of the PAMP [36, 37]. This was demonstrated by using a genetically engineered *E. coli* strain lacking LPS that failed to activate the caspase-11-dependent inflammasome. Moreover, *Francisella novicida* and *Yersinia pestis*, two Gram-negative pathogens that produced non-immunological LPS once cultured, failed to trigger caspase-11 innate sensing [37]. Intracellular LPS detection was furthered characterized when another study established that caspase-11 directly interacts with LPS molecules through its caspase recruitment domain (CARD) and supported previous data that this domain is able to detect mature hexa-acyl lipid A molecules within the cytoplasm of host cells and not precursor tetra-acylated lipid IVa that *Francisella novicida* and *Yersinia pestis* express [38]. Findings from these studies uniquely characterize the inflammatory caspase-11 as an intracellular receptor of bacterial LPS that is able to promote pyroptosis, contributing to the host response during infection.

These reports indicate that intracellular LPS is a potentiator of the non-canonical inflammasome, as caspase-11-dependent cell death bypasses the canonical extracellular sensor of LPS, TLR4. In fact, when stimulating WT and *Tlr4*$^{-/-}$ murine BMDMs with a TLR2 agonist Pam3CSK4, and then transfecting LPS into the intracellular compartment, both strains of macrophages responded similarly and exhibited comparable levels of pyroptosis via the caspase-11 non-canonical inflammasome [36, 37]. In addition, lipid A binding proteins MD-1 and -2 and the LPS-binding protein CD14 were also dispensable for LPS-induced caspase-11 activation [36]. These data further support the claim that caspase-11 is an intracellular, TLR4-independent sensor of LPS that promotes innate immune responses to intracellular infection by Gram-negative pathogens. The existence of multiple pattern recognition receptors (PRRs) that detect the same PAMP is not without precedence. For example, TLR5 and Nlrc4/Naip5 both detect bacterial flagellin, and TLR3 and RIG-I detect RNA to promote anti-pathogen responses [39-43]. Therefore, while TLR4 is vital for the initial detection of extracellular LPS to initiate signaling cascades, caspase-11 exists as a second sensor to detect this PAMP when it contaminates intracellular spaces.

Caspase-11 substrates

Upon detection of LPS within the host cell, the non-canonical caspase-11-dependent inflammasome is activated and promotes pyroptosis [36, 37]. But, the molecular players involved in caspase-11-mediated pyroptosis have been elusive, as it was not precisely understood how caspase-11 promoted inflammatory cell death and non-canonical Nlrp3 inflammasome activation until recently. The Dixit and Shao groups independently have addressed this gap in the caspase-11 substrate repertoire by identifying a key molecule involved in caspase-11-dependent pyroptosis. Shao's group used the gene editing method – genome-wide clustered regularly interspaced palindromic repeat (CRISPR)-Cas9 system and Dixit's group employed a forward screen using *N*-Ethylnitrosourea (ENU) mutagenized mice to find mediators of caspase-11-dependent non-canonical inflammasome activation. Both identified the gene *Gsdmd*, which encodes the protein gasdermin D, a member of the poorly defined gasdermin protein family, as a major substrate downstream of caspase-11 that promotes cell death upon caspase-11 activation [44, 45]. Using several experimental approaches, both groups confirmed that upon detection of intracellular LPS, Gsdmd is activated by caspase-11 via cleavage, producing a catalytically active 30 kDa N-terminal fragment. Cleavage of gasdermin D is necessary and sufficient to promote

pyroptosis of host cells, as a processing mutant of gasdermin (D276A) failed to do so. In addition, both groups found that, in terms of cytoplasmic LPS and infection with *C. rodentium*, *E. coli*, and *S. flexneri*, active Gsdmd was not only essential for lytic cell death, but also the activation of caspase-1 and the maturation and secretion of IL-1β [44, 45]. It is important to note that loss of inflammasome members Nlrp3, ASC, or caspase-1 did not prevent the generation of mature p30 Gsdmd in response to intracellular LPS, indicating that caspase-11-dependent Gsdmd activation is upstream of the canonical inflammasome. Dixit's group also wanted to know whether Gsdmd induces pyroptosis and Nlrp3 inflammasome activation in the same cell or if the events of inflammatory cell death released danger associated molecular patterns (DAMPs) that promote caspase-11-independent Nlrp3 inflammasome activation in neighboring cells. This was elucidated using simple and elegant assays, firstly by co-culturing *Casp-11*$^{-/-}$ and *IL-1β*$^{-/-}$ BMDMs and activating the cells with LPS, which failed to secrete IL-1β, but also by stimulating *Casp-11*$^{-/-}$ macrophages with pyroptotic supernatants of *IL-1β*$^{-/-}$ cells when activated with LPS. The caspase-11-null cells did not secrete IL-1β in response to the supernatants. Together these data indicated that paracrine signaling from a caspase-11-dependent inflammasome-activated cell does not activate the canonical inflammasome in neighboring cells and it is most likely that Gsdmd activation leads to pyroptosis and canonical inflammasome-dependent secretion of IL-1β in a cell intrinsic manner [44]. The exact mechanism of how cleaved Gsdmd promotes canonical Nlrp3 inflammasome activation and pyroptosis is unclear, though it has been theorized that the p30 active Gsdmd contributes to pore formation and cell membrane damage, promoting IL-1β release and perturbation of the intracellular homeostasis that the Nlrp3 inflammasome senses [46, 47] (Figure 1). Altogether, these data designate that gasdermin D is a key mediator of caspase-11-dependent pyroptosis, increasing our conceptual knowledge of the inflammatory caspase effector functions.

In addition to these studies, another report from the Núñez and Liu group shed further light on the downstream events of caspase-11-mediated pyroptosis. This study identified another component downstream of the non-canonical pathway that plays a prominent role in pyroptosis. Yang *et al*. provided evidence that upon delivery of LPS to the cytosol of BMDMs, activation of caspase-11 resulted in the activation of the pannexin-1 channel, a non-selective, large pore channel that releases ATP across the cell membrane to the extracellular space [48, 49]. The authors elucidated that the pannexin-1 channel was activated by caspase-11-dependent cleavage at the C-terminal inhibitory domain. Reconstituting *Casp-11*$^{-/-}$ BMDMs with a catalytically inactive caspase-11 gene did not result in pannexin-1 cleavage upon LPS transfection. In addition, *Panx-1*$^{-/-}$ macrophages complemented back with a pannexin cleavage mutant (D378A) failed to activate the transmembrane protein upon delivery of LPS to the intracellular space [48]. It is this cleavage that opens the channel and promotes the release of ATP [50, 51]. Within the scope of the authors' model, caspase-11-dependent pannexin-1 cleavage resulted in the significant increase in the release of ATP to the extracellular space and therefore cytotoxicity. It has been noted that exogenous ATP activates the purinergic receptor P2X7, opening the ligand-gated channel allowing small ions (K^+ and Na^+) to pass to the extracellular milieu [52, 53]. Perturbation of the cell membrane integrity causes these channels to open, promoting the efflux of K^+ ions out of the cell, leading to a reduction in cytosolic K^+ concentration and thereby providing a trigger for canonical NLRP3 inflammasome activation [54, 55]. In addition to pannexin-1, the P2X7 receptor was required during pyroptosis, and this was found to require caspase-11, indicating that P2X7 is activated downstream of caspase-11 [48] (Figure 1). However, relatively high concentrations of extracellular ATP are required to activate the P2X7 receptor in resting cells [56]. These ATP levels are much higher than those observed to activate the purinergic receptor during caspase-11-mediated pannexin-1 activation, indicating that the amount of ATP released during pyroptosis is incongruous with that which is usually needed. Yang *et al*. deciphered that LPS transfection increases the sensitivity of the P2X7 receptor in responding to ATP to the nanomolar range, thereby essentially lowering the threshold required for opening of the P2X7 channel to levels exhibited during pyroptosis [48]. This is supported by the finding that in the absence of LPS transfection, cells did not respond to nanomolar ATP concentrations and instead required high concentrations (~500 μM)

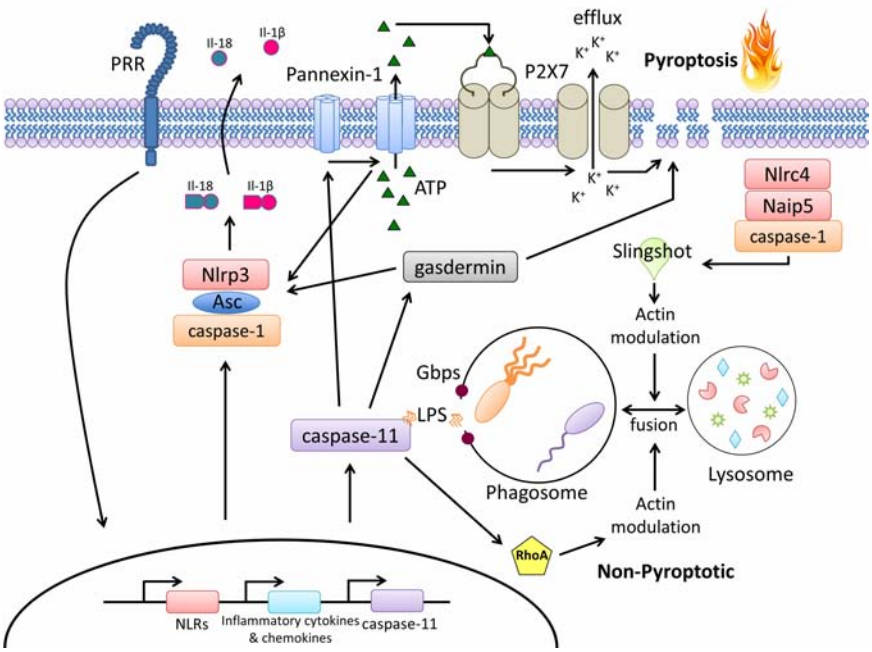

Figure 1. Bimodal consequences of caspase-11 activation. Upon engagement of cell surface pattern recognition receptors (PRRs), innate immune molecules are upregulated, including NOD-like receptors, inflammatory cytokines and chemokines, and caspase-11. Select intracellular pathogens that break free of phagosome compartmentalization, via guanylate-binding proteins (Gbps), access the host cell cytosol. Upon lipopolysaccharide (LPS)-mediated activation, caspase-11 cleaves and activates the novel substrates gasdermin D and Pannexin-1, causing the inflammatory lytic cell death: pyroptosis. Downstream of the non-canonical inflammasome, gasdermin D activates the canonical Nlrp3 inflammasome, promoting caspase-1 activation, leading to maturation of IL-1β and IL-18 and pyroptosis. At the same time, cleavage of Pannexin-1 by caspase-11 promotes the translocation of ATP molecules from their intracellular environment to the extracellular space. In turn, extracellular ATP activates the P2X7 receptor, promoting potassium efflux and Nlrp3 inflammasome activation. This strong inflammasome activation, via gasdermin D and Pannexin-1, not only leads to inflammatory cytokine secretion, but also leads to a loss of membrane integrity and release of cytotoxic material into the extracellular niche. On the other hand, LPS-independent activation of caspase-11 and caspase-1 promotes non-pyroptotic host defenses against *L. pneumophila*. Upon infection, caspase-11 promotes RhoA-GTPase activation while caspase-1 targets the phosphatase Slingshot. Both caspase-11 and caspase-1 work in a balanced fashion to target the actin regulator cofilin, thereby modulating actin. Actin polymerization around phagosomes promotes the efficient deliver and fusion of the phagosome with the lysosome. Both pyroptotic and non-pyroptotic consequences of the inflammatory caspase display the diversity of functions these caspases have in innate host defenses.

of ATP in order to stimulate the P2X7 receptor. This regulation of the purinergic receptor demonstrates that perturbation of the cell membrane is not a ubiquitous characteristic of all forms of cell death and indicates the specificity for LPS/caspase-11-mediated pyroptosis.

Even though the P2X7 receptor promotes K^+ efflux and therefore NLRP3 activation, it was found that, caspase-11 and pannexin-1 were required for caspase-1 activation and IL-1β secretion, while P2X7 was dispensable, suggesting that the pannexin-1/P2X7 pathway diverges during non-canonical inflammasome activation [48]. This is supported by other work that has demonstrated that the P2X7 channel is not required for NLRP3-dependent caspase-1 activation and IL-1β maturation [3]. It is thought that upon delivery of LPS to the cytosol, caspase-11-dependent pannexin-1 activation is sufficient to promote efflux of K^+, driving the assembly of the NLRP3 inflammasome and promoting IL-1β maturation [2]. However, it was demonstrated that P2X7 was needed during ATP

activation of the canonical inflammasome [48, 57]. Still, the study indicates that in some circumstances of pyroptotic cell death, IL-1β release is an active process facilitated by the inflammasome and not a just a by-product of membrane disruption.

Non-pyroptotic functions of caspase-11

Most studies characterizing caspase-11 function have elucidated its role in promoting pyroptosis of host cells in order to restrict bacterial infection. But, our group has demonstrated that caspase-11 has other non-pyroptotic functions in order to promote clearance of microbes. *Casp-11*$^{-/-}$ BMDMs allowed intracellular replication of *L. pneumophila* as a result of significantly diminished fusion of *L. pneumophila*-containing phagosomes with lysosomes [4].

Our report showed that caspase-11 is required for restriction of the bacteria, as complementation of *Casp-11*$^{-/-}$ macrophages with a functional caspase-11 plasmid exhibited significantly less colony forming units (CFUs) when compared to *Casp-11*$^{-/-}$ BMDMs complemented with an empty vector. In addition, complementation with a catalytically inactive caspase-11 completely failed to restrict *L. pneumophila* [4]. Also, caspase-11 promoted bacterial degradation via phagosome-lysosome fusion as immunofluorescent assays showed significantly inhibited colocalization of *L. pneumophila* with Lysotracker stain. Moreover, these fusion events were mediated via actin dynamics as treatment with cytochalasin D after infection abrogated phagolysosomal degradation [4]. Further supporting actin-mediated restriction, it was noted that *L. pneumophila*-containing phagosomes were surrounded by filamentous actin (F-actin) in WT macrophages, while in contrast, *Casp-11*$^{-/-}$ cells exhibited sparse amount of F-actin around phagosomes [4]. This is an interesting finding because it demonstrates that caspase-11 has the ability to modulate actin formation, and also because this finding is supported by reports that establish F-actin formations promote efficient fusion of phagosomes and lysosomes [58]. Further supporting the relationship between the inflammatory caspase and the actin cytoskeletal network, it was found that caspase-11 plays a role in modulating the phosphorylation state of cofilin, an actin regulator, during *L. pneumophila* infection in macrophages [4]. This is in agreement with another report that indicates that caspase-11 is able to interact with actin-interacting protein (Aip1) in order to activate cofilin-mediated actin modulation in a J774 macrophage cell line [59]. Our group demonstrated that caspase-11 status of host cells was able to discriminate between pathogenic and non-pathogenic cargo since caspase-11 is dispensable for trafficking *E. coli*-containing phagosomes to lysosomes [4]. This novel function of caspase-11 indicates that once activated, caspase-11 promotes phagosome maturation through cofilin-regulated actin dynamics in order to restrict intracellular pathogens.

Because the mechanism of how inflammatory caspases control vesicle trafficking during infection has been unclear, our group further characterized this alternative role of caspase-11 and caspase-1 during physiological levels (non-pyroptotic) of *L. pneumophila* infection. Previously, our group established that the absence of caspase-11 allows intracellular replication of the bacteria [4]. This was also thought to be the case with caspase-1-deficient cells, but recently it was discovered that cells derived from caspase-1$^{-/-}$ mice also lacked caspase-11 because of a passenger mutation from the genome of the 129 mouse strain, the line of mice the caspase-1 knockout was constructed in [3]. Since much of what the field knows regarding inflammasome biology was established with the *Casp-1*$^{-/-}$*Casp-11*$^{-/-}$ double knockout mouse, it was unclear if restriction of pathogens that activated the inflammasome and promoted pyroptosis was due to caspase-11 or caspase-1. By using the single caspase-1$^{-/-}$ mouse and its derived macrophages (*Casp-1*$^{-/-}$*Casp-11*^{Tg}), our lab was the first to elucidate that caspase-1 plays a role in the restriction of *L. pneumophila* by promoting proper and efficient fusion of the phagosome and lysosome [60]. In addition, the use of low levels of infection enabled us to discover that caspase-1 did also contribute to cell death during infection. These findings are in line with a recent study from Zamboni's group [16]. Even though pyroptosis via canonical inflammasome activation is a major determinant of bacterial survival, we found increased bacterial replication *in vivo* and *in vitro* in *Casp-11*$^{-/-}$ mice and BMDMs when compared to WT mice and cells, even though both strains exhibited comparable levels of cells death *in vitro*, suggesting that destruction of the host cell is not the main response employed to restricting intracellular pathogens. Both *Casp-11*$^{-/-}$

and *Casp-1*$^{-/-}$*Casp-11*^{Tg} macrophages demonstrated significantly increased CFUs and exhibited blunted phagolysosomal fusion, indicating that proper vesicle trafficking plays a considerable role in the restriction of *L. pneumophila* infection [4, 60].

Transport of intracellular vesicles, like late endosomes and phagosomes, use the actin cytoskeleton as a framework to spatially move towards and fuse with other compartments and lysosomes [58]. In further characterizing the alternative functions of caspases, our group used a molecular approach to elucidate dynamic changes in actin dependent on caspase-1 and -11. WT macrophages displayed increasing ratios of F/G-actin as compared to cells singularly lacking caspase-11 and caspase-1 when infected with low levels of *L. pneumophila*. To understand if the inflammatory caspases played a role in the trafficking of non-pathogens, we tested if *E. coli* elicited changes in the cytoskeletal network. The distribution of actin and also the F/G-actin ratios were unchanged when WT, *Casp-11*$^{-/-}$, or *Casp-1*$^{-/-}$*Casp-11*^{Tg} were infected with *E. coli* [60]. Altogether, these data indicated that capsase-11 and capsase-1 are intimately connected to modulating the actin cytoskeleton during infection with pathogens as opposed to non-pathogenic microbes that are quickly shuttled to lysosomes independently of the caspase status of the host.

To further explore the link between the inflammatory caspases and the actin cytoskeletal network, using a proteomics approach, we found that the actin regulator cofilin is modulated by caspase-11 [4]. In our recent report, we demonstrated that both caspase-11 and caspase-1 converge on cofilin to differentially regulate its activation based upon its phosphorylation state at the serine in the 3rd position [61, 62]. Upon infection with *L. pneumophila*, it was found that WT macrophages dephosphorylated (activated) cofilin, while cofilin in *Casp-11*$^{-/-}$ cells remained unphosphorylated from its basal state, and conversely *Casp-1*$^{-/-}$*Casp-11*^{Tg} BMDMs phosphorylated (inactivated) cofilin after infection [60]. Because caspase-1 is a central protease in the canonical Nlrc4 inflammasome, the requirement of the NLRs Nlrc4 and Naip5 to modulate cofilin activity was assessed. In addition, the Nlrc4 inflammasome detects contaminating molecules of bacterial flagellin within intracellular spaces, therefore the role of flagellin in regulating the phosphorylation of cofilin was also examined. Macrophages lacking either Nlrc4 or Naip5 failed to dephosphorylate when compared to WT counterparts. In addition, WT macrophages infected with a T4SS mutant (*dotA*$^{-}$) or a flagellin mutant (*flaA*$^{-}$) failed to dephosphorylate cofilin in comparison to cells infected with the parental WT *L. pneumophila*. These data, altogether demonstrated the need for canonical Nlrc4 inflammasome activation via detection of flagellin molecules from *L. pneumophila* with an intact T4SS (Dot/Icm) [60]. In addition, this further supports the concept that caspase-11 and caspase-1 differentially modulate cofilin activity, where caspase-11 promotes the phosphorylation of cofilin, while canonical caspase-1 activation promotes it dephosphorylation.

Various upstream regulators promote the phosphorylation/dephosphorylation of cofilin in response to different stimuli [63, 64]. It has been established that the Rho GTPase family (RhoA, Rac, and Cdc42) phosphorylates cofilin while the phosphatase Slingshot promotes its dephosphorylation [65, 66]. Using GTPase ELISA (G-LISA) and Western blot assays, we determined that caspase-11 was required for RhoA GTPase function and caspase-1 was needed for activation of Slingshot [60]. Also, the enzymatic activity of caspase-11 was required in order to promote the modulation of cofilin phosphorylation after infection with *L. pneumophila* [60]. These novel finding suggested that these inflammatory caspases, which are somewhat similar in sequence (~46%), fulfill exclusive functions in different pathways in order to converge on and regulate the activation of an actin-associated factor to modulate dynamics. In acting through different pathways, an intricate balance between phosphorylation and dephosphorylation of cofilin is struck in order for proper modulation of polymerized and unpolymerized actin. This balance is crucial for efficient vesicle trafficking and bacterial restriction via lysosomal degradation (Figure 1).

Human caspase-4 and -5

As opposed to mice, humans do not encode the caspase-11 gene. Instead of caspase-11, the human genome contains orthologs caspase-4 and caspase-5 [18, 19]. Murine caspase-11 exhibits 60% & 55% identity to human capsase-4 and -5, while at the

same time the pro-domains of *Casp-4* and *-5* share 84% and 86% identity, respectively [67]. These caspases are all contained on the same chromosome and with a high degree of similarity between them, which had led to the theory that these multiple caspases came from the amplification of the *Casp-1* gene in early stages of mammalian evolution [31]. Similar to that of *Casp-11*, the *Casp-4* and *-5* expression is inducible upon stimulation with LPS or IFN-γ [24]. In deciphering the functionality of these caspases, *Casp-11*$^{-/-}$ cells were reconstituted with *Casp-4* or *-5* and regained responsiveness to LPS, signifying that either caspase can be functionally exchanged with caspase-11 [68]. The experiment elucidated *Casp-4* and *-5*, like *Casp-11*, as intracellular sensors of LPS and that they themselves can bind the lipid A moiety of LPS via their respective CARD domains with high affinity, leading to oligomerization and activation of the caspases [68]. In addition, all inflammatory caspases (Casp-1/4/5/11) are able to cleave GSDMD to its p30 fragment and induce pyroptosis when stimulated with cytoplasmic LPS, indicating a conserved mechanism of the inflammatory caspases to promote pyroptosis in a substrate-specific manner [44, 45]. In the same vein, targeting *Casp-4* in human cells by RNA interference (RNAi) or CRISPR imparted resistance to *S. flexneri* infection and also LPS-induced pyroptosis [68-70]. But to be clear, it is not known whether Casp-4, -5, or both are the exact functional ortholog of murine Casp-11.

Characterization of caspase-11's role in the inflammatory host defense against Gram-negative pathogens or in response to endotoxic shock has been a major focus of many laboratories as of late. Conversely, the function of caspase-4 and -5 and the consequences of their activation during infection or inflammatory diseases remain in the nascent stage of elucidation. Currently, a few studies have given us insights into the role caspase-4 and -5 play during different models of inflammation and infection. In skin keratinocytes, human caspase-4 was required for maturation of IL-1β in a UV-B induced model of inflammation [71]. The study detailed that caspase-4 was necessary for NLRP3 and AIM2 inflammasome-dependent secretion of IL-1β, noting that caspase-4 physically interacted with the central inflammasome protease caspase-1, forming a heterocomplex, in order to activate caspase-1 and its downstream proteolytic processing of IL-1β [71]. Using a transgenic mouse that expressed *Casp-4*, another group found that mice were sensitive to endotoxin and that just priming macrophages from the mice, with either LPS or Pam3CSK4, caspase-1 was activated in a caspase-4-dependent manner [72]. Furthermore, elucidating the functionality of caspase-4 in an infectious disease model, the Shin group described a role for caspase-4 in promoting pyroptosis and IL-1α release during infection with virulent, Gram-negative bacteria [14].

In addition to their canonical function of pyroptosis, caspase-4 and -5 can promote non-pyroptotic functions. The role the inflammatory caspases play in the modulating the phosphorylation status of cofilin was examined from total lysates of THP-1 monocytes transfected to overexpress plasmids carrying the *Casp-4* and *-5* genes. Human monocytes ectopically expressing *Casp-4* and *-5* dephosphorylated (activated) cofilin upon *L. pneumophila* infection, indicating that not only do the human orthologs have similar sequence and structure to the caspase-11, but also similar pyroptotic and non-pyroptotic functions [4, 44, 45].

Gaps in the field

Because of the ever-evolving elucidation of the caspase-11-dependent pyroptotic pathway, many questions still need to be answered. For example, gasdermin D and pannexin-1 have been identified as downstream targets of the non-canonical caspase-11 inflammasome [44, 45]. Caspase-11 cleaves gasdermin D and pannexin-1 in response to intracellular LPS to promote pyroptosis, but specifically how these players facilitate this remains elusive. Reports indicate activation of these molecules promote caspase-1 activation either by direct cleavage or the activation of the purinergic P2X7 receptor and K^+ efflux that in turn activates caspase-1-dependent pyroptosis [44, 45, 48]. Future studies are needed to determine if either gasdermin D or pannexin-1 act upstream of each other within this newly characterized pathway or they exact their functions cooperatively downstream of caspase-11 to promote P2X7-NLRP3 inflammasome activation. Moreover, both are targets cleaved by caspase-11 to promote pyroptosis, but because caspase-11 and capsase-1 are inflammatory caspases that promote

pyroptosis, it is worth noting if caspase-1 shares target specificity or do these inflammatory caspases rely on distinct substrates to elicit cell death programs? Also, it would be a worthy endeavor to understand the mechanism by which the P2X7 receptor requires a decreased ATP concentration (K_M) during non-canonical inflammasome activation. Yang *et al.* noted that sensitivity of the P2X7 receptor was achieved by LPS transfection in *Casp-11*$^{-/-}$ BMDMs, suggesting this process occurs via the C-terminal intracellular domain of the P2X7 receptor and independently of the non-canonical inflammasome [48]. This raises the prospect that a separate, concurrent pathway, initiated by LPS, is responsible for P2X7 sensitivity to ATP, contributing to pyroptosis. Finally, because IL-1β is a major mediator of inflammation during infection and disease [73], further characterizing the mechanism of IL-1β secretion using *Panx1*$^{-/-}$ and *P2X7*$^{-/-}$ cells would be of value in order to elucidate molecular players crucial for mediating the release of inflammatory cytokines that require processing in order to be released from the cell.

Similarly, it still remains to be elucidated how the inflammatory caspases execute their non-pyroptotic functions. Specifically, how they promote the activation of the upstream regulators Rho-GTPase and Slingshot phosphatase is unclear. Because caspase-11 and caspase-1 are both proteases, it is very likely that these proteins cleave their targets in order to promote their respective non-pyroptotic functions. It remains to be determined if these caspases directly process RhoA and Slingshot proteins in order to activate them by either revealing a putative phosphorylation site or cleaving off inhibitory phosphorylation modifications or if this is achieved indirectly via cleavage of an upstream regulator that is able to promote exchange factor function or dephosphorylation of its downstream target [74-77].

Also, since gasdermin D has recently been elucidated as a downstream target of caspase-11 [44, 45], it would be of note to investigate all of gasdermin D's functions, pyroptotic and non-pyroptotic alike. Therefore, characterizing its potential role downstream of caspase-11 in terms of actin dynamics would greatly add to our knowledge of pathways gasdermin is involved in and which molecular players it interacts with. Determining its involvement with actin-associated factors like cofilin, RhoA, or Slingshot would allow the field to understand if caspase-11 acts directly or indirectly on these molecules. Therefore, it would uniquely add depth to a previously uncharacterized protein and interestingly open up other avenues of investigation to its modus operandi.

The IFN-inducible GTPase guanylate-binding proteins (Gbps) are another set of proteins that have been implicated in their importance in promoting host immunity against pathogens [11, 12, 78-80]. It stands to reason that characterization of the role of these proteins in the activation of the RhoA or Slingshot proteins is needed. Do Gbps allow vacuolar escape of *L. pneumophila* or its components to the cytosol that activate caspase-11 and/or caspase-1 in order to activate the RhoA GTPase and Slingshot proteins? Furthermore, our data suggests that modulation of cofilin activation is flagellin-dependent via a functional T4SS [60]. Therefore, it raises the question if Gbps are needed for access of flagellin to the cytosol or if they selectively allow LPS access to intracellular spaces, or are they dispensable and this process is solely mediated by the T4SS to promote actin rearrangement?

Lastly, it is also worth noting that the Nlrc4 inflammasome is able to detect various PAMPs and is therefore able to sense a variety of other pathogens. The NLRs Nlrc4 and Naip5 have been established as the canonical cytoplasmic detectors of monomeric flagellin [39, 40, 81, 82]. Currently, other Naip proteins have been characterized and thought to act as adaptors for Nlrc4 to add specificity in detecting various other PAMPs [83]. Several reports have nicely elucidated that homologs Naip1, Naip2, and Naip6 are able to detect T3SS needle and rod proteins, and flagellin molecules, respectively [41, 84-86]. This diversity in bacterial PAMPs has enabled the Nlrc4 inflammasome to detect a variegated plethora of pathogens like *L. pneumophila*, *S. typhimurium*, *P. aeruginosa*, *S. flexneri* and *B. pseudomallei* [39, 85, 87-90]. Therefore, it begs the questions if these other pathogens are able to engage this new signaling pathway downstream of the inflammatory caspases in order to promote cofilin-dependent modulation of the actin cytoskeleton. In addition, because our

data suggests flagellin is a major determinant in promoting F-actin polymerization during infection and is sensed by the Nlrc4 inflammasome, it would be interesting to know if other PAMPs detected by the inflammasome (i.e. T3SS needle and rod proteins), via the other Naip homologs, are able to promote similar responses in actin modulation upon cytosolic detection. The actin machinery is an emerging component of the host-mediated immunity [91-93]. Hence, it is vital to understand if other pathogens, specifically those that are known to engage caspase-1 and -11, are intimately involved in activating the upstream regulators of actin dynamics RhoA and/or Slingshot, giving us a fuller picture of the host restriction pathways employed by pathogens during infection.

Concluding remarks

Important studies have recently illuminated many obscure aspects in the field of inflammasome biology. Triggers of canonical inflammasomes and the consequences of their activation have extensively been characterized, but the molecular requirements for the activation of the non-canonical inflammasome are only beginning to be unraveled. Much of the work concerning the restriction of pathogens has centered on elucidating the role of caspase-11 during cell death processes. From Gbps that act as gatekeepers of vacuolar release and intracellular LPS detection to gasdermin D and pannexin-1 cleavage and membrane permeabilization, the field has taken great strides into characterizing the role of caspase-11 and revealing that it is a crucial component within the innate immune system's response to pathogens. Caspase-1 has been shown to have numerous downstream substrates, but only recently have downstream targets of caspase-11 come to light [44, 45, 48, 60, 94]. However, understanding all facets of the inflammatory caspases' functions will greatly help us fully understand the role and contribution of these proteases to infection and inflammatory disease pathology. Therefore, the emerging field of non-pyroptotic functions of caspase-11 and caspase-1 and their substrates is greatly important in understanding their complete role within the immune response. Importantly, unresolved issues still remain regarding the pyroptotic and non-pyroptotic functions of the inflammatory caspases.

Recent studies by our group and others have interestingly pointed to the multi-faceted nature of these caspases and have shed light on not just their canonical inflammatory roles but also their ability to direct cell-intrinsic immunity. Investigating the complete pyroptotic and non-pyroptotic functions of the inflammatory caspases can illuminate molecular partners and pathways that will provide the basis for development of therapeutic interventions that will ameliorate or halt inflammatory, neurodegenerative, cancerous, and/or infectious disease progression.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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