



# Salmonella and Caspase-1: a complex interplay of detection and evasion

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Salmonellae are intracellular pathogens that replicate within epithelial cells and macrophages, and are a significant public health threat in both developed and developing countries. The innate immune system detects microbes through pattern recognition receptors, which are compartmentalized on the subcellular level to detect either extracellular (e.g., TLRs) or cytosolic (e.g., NLRs) perturbations. *Salmonella* infection is detected by the NLRC4 and NLRP3 inflammasomes, which activate Caspase-1, resulting in reduced bacterial burdens during infection. NLRC4 responds to the SPI1 type III secretion system via detection of inadvertently translocated flagellin and rod protein. The signals for NLRP3 detection during *Salmonella* infection remain undefined. *Salmonella* have evolved evasion strategies to attenuate Caspase-1 responses. We review recent findings describing the interplay between detection and evasion of *S. typhimurium* infection by the inflammasome. We discuss how the interplay between detection and evasion affects Caspase-1 effector functions mediated by IL-1 $\beta$  secretion, IL-18 secretion, and pyroptosis.

**Keywords: Salmonella, Caspase-1, inflammasome, IL-1 $\beta$ , pyroptosis**

## INTRODUCTION

*Salmonella typhi* and *S. paratyphi* cause Typhoid fever, a continuing source of morbidity and mortality in developing countries. With 21 million cases and 200–600,000 deaths annually, antibiotic resistance is a growing concern (Parry et al., 2002; Bhutta and Threlfall, 2009). *S. typhimurium* and other non-typhoidal serotypes are food borne pathogens that cause self-limited gastroenteritis with periodic outbreaks from contaminated centralized food processing sites. The estimated burden of non-typhoidal Salmonellosis in the United States is 1.4 million cases annually (Voetsch et al., 2004). Invasive infections with non-typhoidal *Salmonella* have begun to emerge as the leading cause of community acquired bacteremia in sub-Saharan Africa and are associated with 21–47% mortality despite appropriate antibiotic therapy (Gordon et al., 2008; Kingsley et al., 2009). These isolates are predominantly *S. typhimurium* strains and are typically resistant to multiple antibiotics.

## TYPE III SECRETION

Salmonellae manipulate host cellular physiology during infections by using type III secretion systems (T3SS). T3SS are very common virulence factors used by a wide array of Gram-negative pathogens, which function by facilitating the translocation of effector proteins into the cytosol of host cells (Hueck, 1998). Changing the complement of effectors that are translocated allows different bacteria to exert control over distinct host signaling pathways. For example, Salmonellae encode two virulence-associated T3SS within two distinct *Salmonella* pathogenicity islands (SPI), each resulting in the transfer of a distinct set of effectors (Ibarra and Steele-Mortimer, 2009). The SPI1 T3SS is expressed primarily in the gut lumen, and promotes epithelial cell invasion. It is critical for the induction of gastroenteritis. The SPI2 T3SS is expressed by

bacteria within the vacuolar compartment of macrophages and epithelial cells. It is important for intracellular replication and causing systemic disease.

## INNATE IMMUNE SENSORS

Innate immune system sensors can be classified by the cellular site of detection. Extracellular detectors, such as the Toll-like receptors (TLR) and C type Lectin receptors (CLR), respond to microbe associated molecular patterns (MAMPs) in the extracellular or vacuolar space. They tend to respond to molecules that are broadly conserved, such as LPS (TLR4) or flagellin (TLR5) and induce pro-inflammatory gene expression. Cytosolic detectors such as the Nod-like receptors (NLR), Rig-I-like receptors (RLR), and Aim2-like receptors (ALR) detect MAMPs in the cytosolic compartment or cytosolic perturbations caused by extracellular agonists. They induce either transcriptional or post-translational responses. Some of these sensors can respond to virulence properties of pathogens either directly or indirectly. For example, NLRC4 detects T3SS activity by sensing the inadvertent translocation of both flagellin and the T3SS rod protein into the cytosol of host cells (Miao et al., 2007; Miao and Warren, 2010). Interestingly, while flagellin in the extracellular space is a marker for any flagellated bacterium present within host tissues, in the cytosol it is instead a marker of a flagellated bacterium that possesses virulence factors that allow access to the cytosolic compartment. From the host's perspective, the threat posed by these two bacteria is quite different.

Toll-like receptors induce the expression of multiple cytokines and chemokines. While most of these factors are secreted upon stimulation, pro-IL-1 $\beta$  and pro-IL-18 are held in reserve, awaiting a second signal. Some cytosolic receptors in the NLR and ALR families form inflammasomes, multi-protein complexes which

serve as platforms for Caspase-1 activation. Activated Caspase-1 cleaves pro-IL-1 $\beta$  and pro-IL-18 to their mature secreted forms. Caspase-1 activation also induces a form of programmed cell death called pyroptosis (Bergsbaken et al., 2009).

### INNATE IMMUNE SENSORS DETECTING *S. TYPHIMURIUM*

Several sensors can detect *S. typhimurium* infection. Both TLR4 and TLR5 play a role in the host response to *S. typhimurium* (Figure 1). *Tlr4*<sup>-/-</sup> mice show increased susceptibility to *S. typhimurium* infection, and *Tlr4-Tlr5*<sup>DKO</sup> mice show an even more severe phenotype (Feuillet et al., 2006). *S. typhimurium* is also detected by NLRs, specifically NLRC4 (previously called Ipaf) and NLRP3 (previously called Nalp3 or cryopyrin), which also contribute to a reduced bacterial burden in the mouse model (Broz et al., 2010).

NLRC4 and NLRP3 both form inflammasomes and activate Caspase-1. Mice deficient in Caspase-1 have increased bacterial loads and succumb to *S. typhimurium* infection earlier than WT mice (Lara-Tejero et al., 2006; Raupach et al., 2006). *Nlrp3-Nlrc4*<sup>DKO</sup> mice have a phenotype similar to *Casp1*<sup>-/-</sup> mice (Broz et al., 2010; Figure 1).

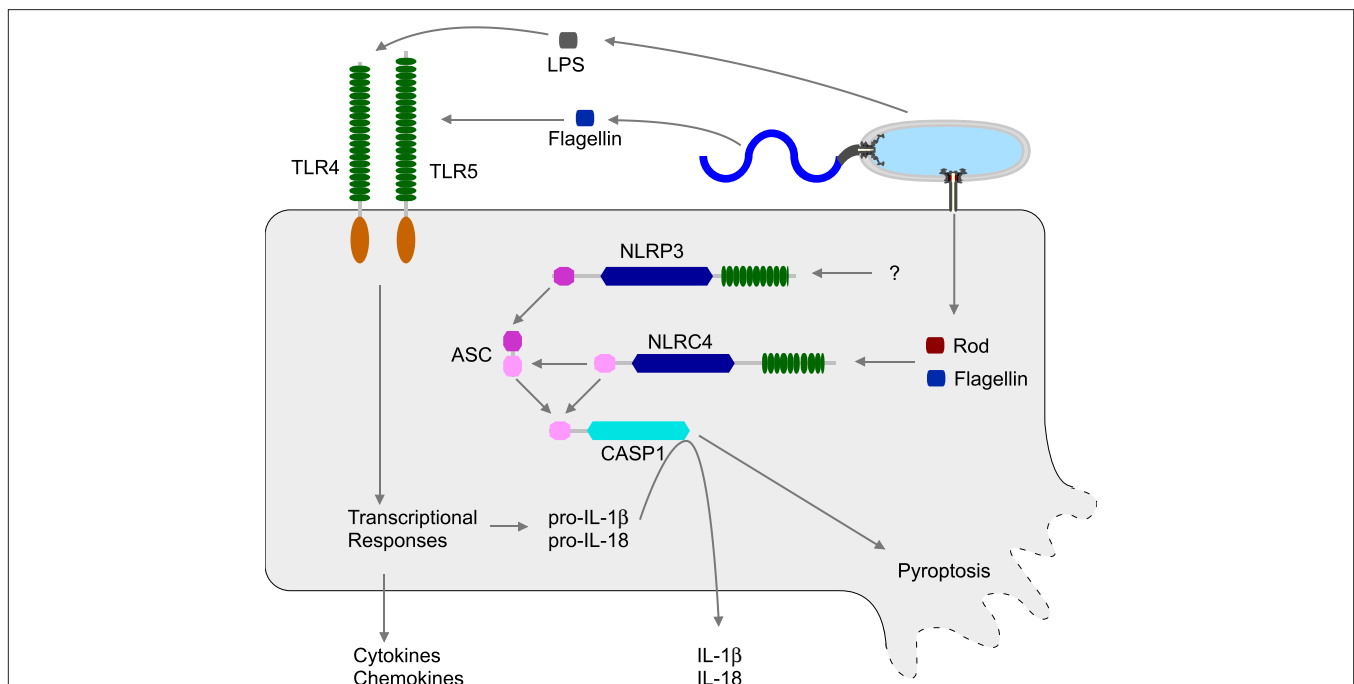
Caspase-1 reduces bacterial loads after *S. typhimurium* infection largely via the activities of IL-18. *Il18*<sup>-/-</sup> and *Casp1*<sup>-/-</sup> mice show similar susceptibilities to *S. typhimurium* infection while IL-1 $\beta$ <sup>-/-</sup> mice have a much more subtle phenotype (Raupach et al., 2006). IL-18 has numerous effects on the immune response, including the transcriptional induction of pro-inflammatory genes and the

potentiation of IFN- $\gamma$  secretion by T-cells and NK cells, both of which play significant roles in host defense against *S. typhimurium* (Eckmann and Kagnoff, 2001).

### NLRP3 DETECTION

NLRP3 detects *S. typhimurium* within macrophages at late time-points. It responds to a diverse set of agonists, including ATP, pore forming toxins, crystals, viruses, bacteria, and fungi (Schroder and Tschopp, 2010; Rajan et al., 2011). These agonists are thought to trigger convergent signaling events that involve lysosomal destabilization, electrolyte imbalances, or mitochondrial dysfunction via ROS production (Hornung and Latz, 2010; Schroder and Tschopp, 2010; Zhou et al., 2011). Whether these pathways converge on a single terminal signal or not is not known. The kinetics of detection of different NLRP3 agonists is variable: ATP and nigericin are detected within 1 h, crystals typically within 4 h, while bacteria, viruses, and fungi are detected only after 6 or more hours. These differences likely reflect the length of time required to trigger a cytosolic event that is sensed by NLRP3.

Several bacteria can trigger NLRP3 activation *in vitro*, including *Listeria monocytogenes* (Mariathasan et al., 2006; Warren et al., 2008; Kim et al., 2010; Wu et al., 2010), *Staphylococcus aureus* (Mariathasan et al., 2006; Craven et al., 2009; Munoz-Planillo et al., 2009; Shimada et al., 2010), *Klebsiella pneumoniae* (Willingham et al., 2009), *Porphyromonas gingivalis* (Huang et al., 2009), *Shigella flexneri* (Willingham et al., 2007), *Chlamydomphila pneumoniae* (He et al., 2010), *Neisseria gonorrhoeae* (Duncan et al., 2009), *Mycobacterium*



**FIGURE 1 | Innate immune detection of *S. typhimurium*.** *S. typhimurium* can be detected by both extracellular (TLR) and cytosolic (NLR) sensors. TLR4 responds to lipopolysaccharide (LPS), while TLR5 responds to extracellular flagellin monomers. TLRs induce transcriptional responses, including the synthesis and secretion of multiple cytokines and chemokines. TLRs also induce the synthesis of pro-IL-1 $\beta$  and pro-IL-18,

but these are reserved in the cytosol awaiting processing by Caspase-1. Caspase-1 activity is controlled by inflammasomes. *S. typhimurium* is detected by two inflammasomes, NLRC4 and NLRP3. NLRC4 responds to cytosolic flagellin monomers or T3SS rod protein monomers, both delivered by the activity of a virulence-associated T3SS. NLRP3 detects *S. typhimurium* via unknown mechanisms.

*tuberculosis* (Koo et al., 2008), *Yersinia pseudotuberculosis* (Brodsky et al., 2010), and *S. typhimurium* (Broz et al., 2010). Although the terminal signaling events triggered by these pathogens are likely the same as those triggered by other NLRP3 agonists, the initial mechanisms are almost certainly divergent. For example *Y. pseudotuberculosis* triggers NLRP3 via the activity of T3SS effectors (Brodsky et al., 2010), but detection of *S. typhimurium* by NLRP3 does not require SPI1 or SPI2 T3SS (Broz et al., 2010). In contrast *L. monocytogenes* detection by NLRP3 requires lysis of the phagosome by listeriolysin O (Warren et al., 2008). *S. aureus* is also detected by NLRP3. Toxin-producing strains of *S. aureus* are detected via the pore forming activities of their toxins (Craven et al., 2009; Munoz-Planillo et al., 2009) while non-toxin-producing strains are not detected, apparently evading NLRP3 detection by producing degradation resistant peptidoglycan structures. *S. aureus oatA* mutants that are defective in these cell wall modifications are detected via NLRP3 by a mechanism that involves peptidoglycan degradation in the phagosome (Shimada et al., 2010). Thus, even within a bacterial species different strains may be detected or may evade NLRP3.

## NLRC4 DETECTION

NLRC4 detects the activity of T3SS and T4SS. It does so in part by responding to flagellin that is inadvertently translocated into the cytosolic compartment by T3/4SS (Figure 2; Molofsky et al., 2005, 2006; Amer et al., 2006; Franchi et al., 2006; Miao et al., 2006; Ren et al., 2006). T3SS efficiently select translocation substrates from the bacterial cytosol for injection into host cells. Because flagellin is targeted to the flagellar T3SS apparatus for secretion to form the flagellar filament (Minamino et al., 2008), it has features that likely predispose mistargeting to the virulence-associated T3SS. Specifically, it binds a secretion chaperone and has the physical properties required for export in an unfolded state through a T3SS secretion apparatus. The chaperone is required for efficient delivery and subsequent detection by NLRC4 (unpublished data). The mechanisms underlying accidental transfer by T4SS are less clear. As a cytosolic flagellin sensor, NLRC4 also detects flagellated bacteria that escape from the vacuolar compartment into the cytosol, such as *Listeria monocytogenes* (Warren et al., 2008).

NLRC4 can also detect T3SS activity in non-flagellated bacteria (Miao et al., 2006; Sutterwala et al., 2007; Suzuki et al., 2007), responding to inadvertent translocation of the T3SS rod protein (Miao and Warren, 2010; Miao et al., 2010b; Figure 2). The rod component of the T3SS apparatus forms the inner channel that spans the bacterial periplasm. Similar to flagellin, it is believed to polymerize into a hollow tube that permits the passage of secreted proteins (Marlovits et al., 2004), and there is sequence conservation at the carboxy-terminus of flagellin and the rod protein (Miao et al., 2010b). If rod proteins are inadvertently translocated into the macrophage cytosol, they are detected by NLRC4 (Miao et al., 2010b). This may occur if the T3SS exports excess rod monomers from the bacterial cytosol, or if rod monomers slough into the interior channel of the needle apparatus, resulting in translocation. In their correct location within the T3SS apparatus, rod proteins are not detected.

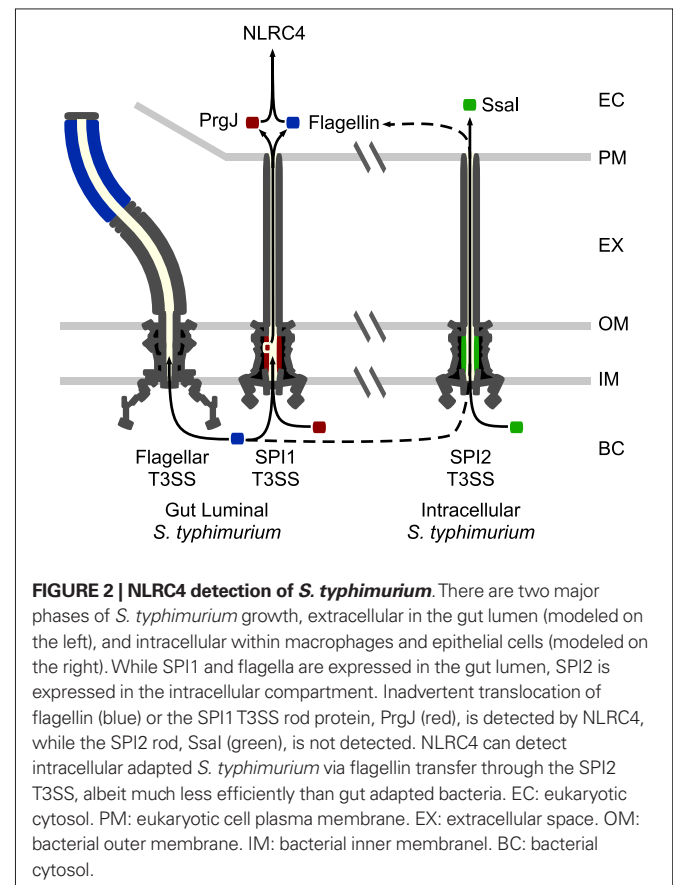
Macrophages detect *S. typhimurium* SPI1 T3SS via inadvertent translocation of flagellin and the SPI1 rod protein PrgJ. Because flagellin is one of the most highly produced proteins in the bacteria

during growth in LB, which is thought to mimic the gut lumen, it is the dominant NLRC4 signal *in vitro*. Both flagellin and SPI1 are highly induced in the late logarithmic phase of growth in LB, and these bacteria are readily detected within 1 h by macrophages *in vitro*. However, if bacteria are grown to stationary phase, they no longer express SPI1 and NLRC4 does not detect *S. typhimurium* within the first several hours (Miao et al., 2010b). Thus the transcriptional profile of the bacterium is critical for detection by NLRC4.

## EVASION OF NLRC4

*Salmonella typhimurium* have developed evasion strategies to prevent NLRC4 detection during intracellular replication in macrophages. Flagellin is repressed in the intracellular environment when SPI2 T3SS is active. In addition, the SPI2 T3SS rod protein, SsaI, has amino acid changes that prevent its detection by NLRC4 (Miao et al., 2010b; Figure 2). Elimination of these two evasion strategies by expressing PrgJ or flagellin from a SPI2 co-regulated promoter results in strong and persistent detection via NLRC4 and complete clearance of the bacteria *in vivo*, demonstrating that evasion of NLRC4 is essential for *S. typhimurium* virulence (Miao et al., 2010a,b).

These evasion strategies appear to be efficient, but not perfect. NLRC4 can detect *S. typhimurium* that are grown under SPI1/flagellin non-expressing conditions (stationary phase LB), but only at very late times post-infection (Broz et al., 2010). After infection of macrophages *in vitro*, NLRC4 does not detect the bacteria



**FIGURE 2 | NLRC4 detection of *S. typhimurium*.** There are two major phases of *S. typhimurium* growth, extracellular in the gut lumen (modeled on the left), and intracellular within macrophages and epithelial cells (modeled on the right). While SPI1 and flagella are expressed in the gut lumen, SPI2 is expressed in the intracellular compartment. Inadvertent translocation of flagellin (blue) or the SPI1 T3SS rod protein, PrgJ (red), is detected by NLRC4, while the SPI2 rod, SsaI (green), is not detected. NLRC4 can detect intracellular adapted *S. typhimurium* via flagellin transfer through the SPI2 T3SS, albeit much less efficiently than gut adapted bacteria. EC: eukaryotic cytosol. PM: eukaryotic cell plasma membrane. EX: extracellular space. OM: bacterial outer membrane. IM: bacterial inner membrane. BC: bacterial cytosol.

during the early hours after infection. However, between 8- and 17-h post-infection, NLRC4 does detect *S. typhimurium* apparently via SPI2 T3SS translocation of flagellin (Broz et al., 2010; we have verified these results). Whether the detected flagellin arises from transcriptional re-induction at late time-points after infection of macrophages, or is due to residual protein left over from the prior growth in LB remains to be determined. In either case, this detection is sufficient to provide a benefit to the host *in vivo*, as NLRC4 dependent Caspase-1 activation results in reduced bacterial burden in infected mice, and a delay in the time of death (Broz et al., 2010). SPI2 detection is significantly delayed compared to SPI1 detection, and the amount of IL-1 $\beta$  secreted is considerably lower.

Thus, there is a fine interplay between detection and evasion of NLRC4 during *S. typhimurium* infection. NLRC4 does detect *S. typhimurium in vivo*, providing a benefit to the host. *S. typhimurium* expresses flagellin in the gut lumen, and represses it in the intracellular environment. We propose that NLRC4 only detects *S. typhimurium* which have recently emigrated from the gut to deeper tissues because these bacteria have residual flagellin present within their cytosol. Indeed, GFP expressed under the control of the *fliC* promoter is detectable in bacteria recovered from the Peyer's patches, but not from the draining lymph nodes or spleen (Cummings et al., 2006). Once the bacteria reprogram their gene expression for the intracellular environment, perhaps after one or more rounds of replication within host cells, we hypothesize that they subsequently evade NLRC4 completely by degrading residual flagellin in the bacterial cytosol and repressing its transcription.

### PYROPTOSIS IS AN EFFECTIVE INNATE IMMUNE EFFECTOR MECHANISM *IN VIVO*

Caspase-1 activation results in both cytokine processing, and a form of cell death called pyroptosis (Bergsbaken et al., 2009), which is associated with stronger Caspase-1 activation than is required for cytokine processing. Like apoptosis, pyroptosis is a form of programmed cell death (Labbe and Saleh, 2008; Duprez et al., 2009). Both pyroptosis and apoptosis require the activation of specific signaling pathways triggered downstream of different caspase family proteases. This requirement contrasts with necrotic cell death (also called oncosis), which is an accidental form of cell death that does not have an absolute requirement for cellular signaling pathways. Pyroptosis, like necrosis, results in the lysis of the affected cell, releasing cytosolic contents after disruption of the plasma membrane, an inherently inflammatory event. In contrast, apoptotic blebs are cleared in an orderly fashion and are non-inflammatory manner (Labbe and Saleh, 2008; Duprez et al., 2009). Pyroptosis occurs rapidly, within 30–60 min *in vitro* and to date has only been characterized in macrophages and dendritic cells.

Pyroptosis was first observed in 1992 *in vitro* (Zychlinsky et al., 1992). Although there was some evidence that it occurred *in vivo* (Bergsbaken et al., 2009), the relevance of pyroptosis *in vivo* was largely undefined until recently. As mentioned above, we generated a *S. typhimurium* strain that persistently expresses flagellin (FliC<sup>ON</sup>; Miao et al., 2010a). This strain was effectively detected and cleared by NLRC4 dependent Caspase-1 activation. Given that IL-18 secretion is the effector mechanism by which Caspase-1 clears WT *S. typhimurium* (Raupach et al., 2006), we expected that IL-18 would be required to clear FliC<sup>ON</sup> *S. typhimurium*. However, mice

deficient for both IL-1 $\beta$  and IL-18 retained their ability to clear these bacteria. Instead, pyroptosis released FliC<sup>ON</sup> *S. typhimurium* into the extracellular space, where they are phagocytosed and killed by neutrophils (Miao et al., 2010a). We obtained similar results for *L. monocytogenes* strains engineered to persistently express and secrete flagellin or the *S. typhimurium* PrgJ rod protein; clearance of these strains could not be attributed to IL-1 $\beta$  and IL-18 secretion (unpublished data). Furthermore, after IP infection with *Legionella pneumophila* or *Burkholderia thailandensis*, Casp1<sup>-/-</sup> mice had increased bacterial burdens in the draining lymph nodes that could not be completely attributed to IL-1 $\beta$  or IL-18 secretion (Miao et al., 2010a). These results provide strong evidence that pyroptosis is an important innate immune effector mechanism against intracellular bacteria, including FliC<sup>ON</sup> *S. typhimurium*.

### SALMONELLA TYPHIMURIUM EFFECTIVELY EVADE PYROPTOSIS, BUT FAIL TO EVADE IL-18

Our work presents an apparent conundrum: both WT *S. typhimurium* and FliC<sup>ON</sup> *S. typhimurium* are detected by NLRC4, but the former is affected by IL-18 while the latter is cleared through pyroptosis. We propose that this difference is attributable to two variables between these strains: the time to detection in a single infected cell, and the persistence of detection over the course of days.

At the single cell level under conditions that mimic systemic infection *S. typhimurium* does not trigger NLRC4 and NLRP3 until 17-h post-infection (under SPI1 repressing growth conditions; for example stationary phase LB cultures; Broz et al., 2010). In contrast, if FliC<sup>ON</sup> *S. typhimurium* trigger NLRC4 within 6 h (Miao et al., 2010a). This is a significant difference. Under both conditions, Caspase-1 mediates cytokine secretion and pyroptosis. In the case of FliC<sup>ON</sup> *S. typhimurium*, relatively early pyroptosis occurs before the bacteria can replicate in the macrophage, essentially short-circuiting the replicative cycle. Thus, FliC<sup>ON</sup> are released and exposed to neutrophil killing before replication occurs. Some FliC<sup>ON</sup> bacteria will also be taken up by macrophages, where they are again predicted to induce early pyroptosis before replication, exposing them to neutrophils before they can undergo a replicative cycle within the macrophage. This mechanism of clearance is so effective that any contribution of IL-1 $\beta$  and IL-18 is obscured in the mouse model. In contrast, WT *S. typhimurium* do not trigger NLRC4/NLRP3 until late times after infection of a single cell. At this time, pyroptosis should still occur, but only after the bacteria have replicated. Released bacteria are predicted to be exposed to neutrophils, but many will also infect new macrophages and continue the replicative cycle. Thus, we propose that WT *S. typhimurium* do not prevent pyroptosis from occurring, rather they void its effectiveness by delaying its onset.

Since *S. typhimurium* prevents the effectiveness of pyroptosis, one might be tempted to conclude that the role of pyroptosis in host defense against real world pathogens is negligible. However, we have evidence that during systemic infection with *Legionella pneumophila* or *Burkholderia thailandensis*, Caspase-1 reduces bacterial burdens independent of IL-1 $\beta$  and IL-18 (Miao et al., 2010a). These models require further research to accurately define the role of pyroptosis. Nevertheless, the fact that *S. typhimurium* actively constrains the effectiveness of pyroptosis attests to its potency in clearing intracellular pathogens that target macrophages or dendritic cells.



Unlike pyroptosis the effectiveness of IL-18 secretion is not avoided by WT *S. typhimurium*. In part, this may relate to the fact that pyroptosis is a cell intrinsic effector mechanism while IL-18 exerts its effects in a paracrine manner, inducing IFN- $\gamma$  expression in NK cells and T cells. Therefore, although the effects of IL-18 can be delayed for several hours by delaying NLRC4/NLRP3 detection, it is likely that they cannot be circumvented, as is the case with pyroptosis.

## COMPLEX INTERPLAY OF DETECTION AND EVASION

WT C57BL/6 mice succumb to *S. typhimurium* infection despite detection by TLR4, TLR5, NLRC4, and NLRP3, raising the question of the utility of these sensors to the host. However, it is crucial to note that this strain of mice is highly susceptible to *S. typhimurium* infection, at least in part due to a defect in *Nramp1* (also called *Slc11A1*), a metal ion transporter that depletes the phagosome of Fe<sup>2+</sup> and Mn<sup>2+</sup> (Gruenheid et al., 1997; Cellier et al., 2007). Even so, infection in C57BL/6 mice remains a powerful model to study innate immunity and bacterial pathogenesis because of the availability of knockout mice. The extension of results in this mouse strain to human disease should be interpreted appropriately. In humans, it is likely that detection by TLR4, TLR5, NLRC4, and NLRP3 will result in reduced bacterial

burdens during both gastroenteritis and systemic disease, and we would predict that this will result in decreased symptom severity and earlier resolution of the infection. Further, IL-1 $\beta$  and IL-18 are expected to promote protective adaptive responses to reinfection (Sims and Smith, 2010).

## FUTURE DIRECTIONS

*Salmonella typhimurium* is an important pathogen, and understanding how it interacts with the immune system during infection has implications for human disease caused by it and many other pathogens. In addition to the importance of studying *S. typhimurium* in its own right, it remains a particularly useful tool for dissecting the innate immune system because of the powerful genetic tools available. Studies using *S. typhimurium* have revealed the mechanisms of NLRC4 activation and the utility of pyroptosis. One of the crucial paradigms to emerge from the study of *S. typhimurium* detection is the complex interplay between detection and evasion, wherein innate immune detectors are able to respond to pathogens, but pathogens have evolved strategies that prevent maximal responses. Future studies examining the intricacies of detection and evasion of NLRC4 and NLRP3 will undoubtedly lead to novel insights into the mechanisms by which these sensors respond to *S. typhimurium* as well as many other bacterial pathogens.

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