Listeria monocytogenes and the Inflammasome: From Cytosolic Bacteriolysis to Tumor Immunotherapy

Erin Theisen and John-Demian Sauer

Abstract Inflammasomes are cytosolic innate immune surveillance systems that recognize a variety of danger signals, including those from pathogens. *Listeria monocytogenes* is a Gram-positive intracellular bacterium evolved to live within the harsh environment of the host cytosol. Further, *L. monocytogenes* can activate a robust cell-mediated immune response that is being harnessed as an immunotherapeutic platform. Access to the cytosol is critical for both causing disease and inducing a protective immune response, and it is hypothesized that the cytosolic innate immune system, including the inflammasome, is critical for both host protection and induction of long-term immunity. *L. monocytogenes* can activate a variety of inflammasomes via its pore-forming toxin listeriolysin-O, flagellin, or DNA released through bacteriolysis; however, inflammasome activation attenuates *L. monocytogenes*, and as such, *L. monocytogenes* has evolved a variety of ways to limit inflammasome activation. Surprisingly, inflammasome activation also impairs the host cell-mediated immune response. Thus, understanding how *L. monocytogenes* activates or avoids detection by the inflammasome is critical to understand the pathogenesis of *L. monocytogenes* and improve the cell-mediated immune response generated to *L. monocytogenes* for more effective immunotherapies.

Contents

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1 Introduction

1.1 Overview

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen that has evolved to survive in a variety of severe environments and infect a wide range of hosts. These features combine to make *L. monocytogenes* an important foodborne pathogen most frequently associated with unpasteurized dairy products, deli meats, and more recently, fresh produce (Ferreira et al. 2014). Infection *with L. monocytogenes* can result in a noninvasive gastroenteritis that is likely severely underreported (Swaminathan and Gerner-Smidt 2007). More importantly, systemic listeriosis poses a severe risk to the immunocompromised, including the very young and old, as well as pregnant women. Complications of listeriosis include septicemia, meningitis, encephalitis, abortion, and still-borne births, resulting in a strikingly high mortality rate of 20–30 % even with proper antibiotic therapy (Swaminathan and Gerner-Smidt 2007).

While *L. monocytogenes* remains an important foodborne pathogen, there is an increasing interest in harnessing the robust cell-mediated immune response induced upon *L. monocytogenes* infection for use as a potential immunotherapy. Due to its almost exclusive intracellular lifecycle, described more in detail below, *L. monocytogenes* stimulates a robust $CD8^+$ T-cell response. Additionally, *L. monocytogenes* is genetically tractable and is able to break self-tolerance, key factors in making *L. monocytogenes* an extremely promising cancer immunotherapeutic platform (Le et al. 2012). As access to the cytosol is an essential prerequisite for both the pathogenesis and the induction of immunity following *L. monocytogenes* infection, understanding how bacteria are sensed by the innate immune system in

this environment has been a focal point of *L. monocytogenes* research for the past decade. Particularly, activation and avoidance of the inflammasome, a critical innate immune signaling pathway that all cytosolic pathogens must deal with, has become a recent focus.

1.2 Life Cycle

Listeria monocytogenes can infect a variety of cell types either through phagocytosis by myeloid cells or through active invasion of epithelial cells or hepatocytes with its virulence factors, internalin A or internalin B, respectively (Mengaud et al. 1996; Shen et al. 2000). Upon entry into the host cell, *L. monocytogenes* is initially contained within a phagocytic vacuole. Using its cholesterol-dependent pore-forming toxin listeriolysin-O (LLO, encoded by the gene *hly*) and a pair of phospholipases (PlcA and PlcB), *L. monocytogenes* escapes from the phagosome and enters into the cytosol (Portnoy et al. 1988; Mengaud et al. 1991; Camilli et al. 1991; Vazquez-Boland et al. 1992; Hamon et al. 2012).

Unlike most bacterial pathogens, *L. monocytogenes* is able to not only survive but also flourish and replicate within the host cytosol. This is a property unique to *L. monocytogenes* and other cytosol-adapted pathogens as intracellular pathogens that mislocalize to the cytosol or non-intracellular pathogens that are placed within the cytosol are unable to survive or replicate (Beuzón et al. 2000; Goetz et al. 2001; Slaghuis et al. 2004; Creasey and Isberg 2012). These studies suggest that *L. monocytogenes* has evolved specific adaptations to deal with cytosolic stresses, cell autonomous defense mechanisms, and innate immune detection in the cytosol, including the inflammasome. For example, *L. monocytogenes* modifies its peptidoglycan through N-deacetylation and O-acetylation to avoid killing by lysozyme and detection by the innate immune system (Boneca et al. 2007; Rae et al. 2011). *L. monocytogenes* can also modulate the production of the innate signaling cytokine IL-6 through its virulence factor InlH (Personnic et al. 2010); misregulation of these factors results in the attenuation of *L. monocytogenes*, demonstrating the importance of *L. monocytogenes* carefully controlling its detection by the host (Boneca et al. 2007; Personnic et al. 2010; Rae et al. 2011).

Once in the cytosol, *L. monocytogenes* avoid exposure to the extracellular milieu and the host defenses found there by utilizing the virulence factor ActA to hijack host actin and propel itself through membrane protrusions into neighboring cells (Kocks et al. 1992). Both *Δhly* and *ΔactA L. monocytogenes* mutants are attenuated; however, of these, only *ΔactA L. monocytogenes* are able to mount a protective cell-mediated immune response (Portnoy et al. 1988; Goossens and Milon 1992; Bahjat et al. 2009), further highlighting the importance of the cytosol and cytosolic innate immune recognition in stimulating robust $CD8^+$ T cell-mediated immunity.

1.3 Innate Immune Response

Once *L. monocytogenes* accesses the cytosol, it is rarely exposed to extracellular host defenses; as such, detection by cytosolic innate immune sensors is likely critical for combating infection. Likewise, as *L. monocytogenes* must access the cytosol to cause disease, avoidance or direct inhibition of the cytosolic innate immune system is likely critical for *L. monocytogenes'* virulence. Indeed, multiple cytosolic innate immune pathways have been demonstrated to recognize *L. monocytogenes* infection (reviewed in Witte et al. 2012). Briefly, peptidoglycan fragments of *L. monocytogenes* can activate nucleotide binding oligomerization domain (NOD) proteins leading to the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and transcription factor NF-κB activation, further leading to inflammatory cytokine production (O'Riordan et al. 2002; Opitz et al. 2006). Further, NOD signaling can synergize with interferon (IFN) signaling to enhance the host innate response (Leber et al. 2008), although it should be noted that type I IFN induction has been shown to inversely correlate with host protection (O'Connell et al. 2004; Auerbuch et al. 2004; Carrero et al. 2006). More recently, cyclic dinucleotides secreted by *L. monocytogenes* have been shown to be recognized by the STING/IRF3 pathway resulting in the production of type I IFNs (Woodward et al. 2010; Sauer et al. 2011b). Additionally, as will be highlighted in this chapter, *L. monocytogenes* has been reported to activate the inflammasome through a variety of receptors, ultimately resulting in the activation of caspase-1 (Fig. 1). Caspase-1 activation leads to an inflammatory cell death, pyroptosis, release of the pro-inflammatory cytokines IL-1β and IL-18, and the production and release of lipid mediators known as eicosanoids (Fig. 2). The engagement of these innate pathways resulting in the production of pro-inflammatory cytokines and the recruitment of innate immune cells is hypothesized to be critical in the development of adaptive immunity.

In this chapter, we will focus on how *L. monocytogenes* activates different inflammasomes. We will also discuss the downstream consequences of inflammasome activation on the pathogenesis of *L. monocytogenes*. Finally, we will discuss how engagement of the inflammasome influences the innate and adaptive immune responses to *L. monocytogenes*, with a focus on its impact on the development of *L. monocytogenes* as an immunotherapeutic platform.

2 Activation of Different Inflammasomes

2.1 NLRP3 Activation

NLRP3 belongs to the Nod-like receptor family of pattern recognition receptors and recognizes a variety of danger signals including ATP, uric acid crystals, and toxins (Mariathasan et al. 2006). *L. monocytogenes* was first observed to activate the NLRP3

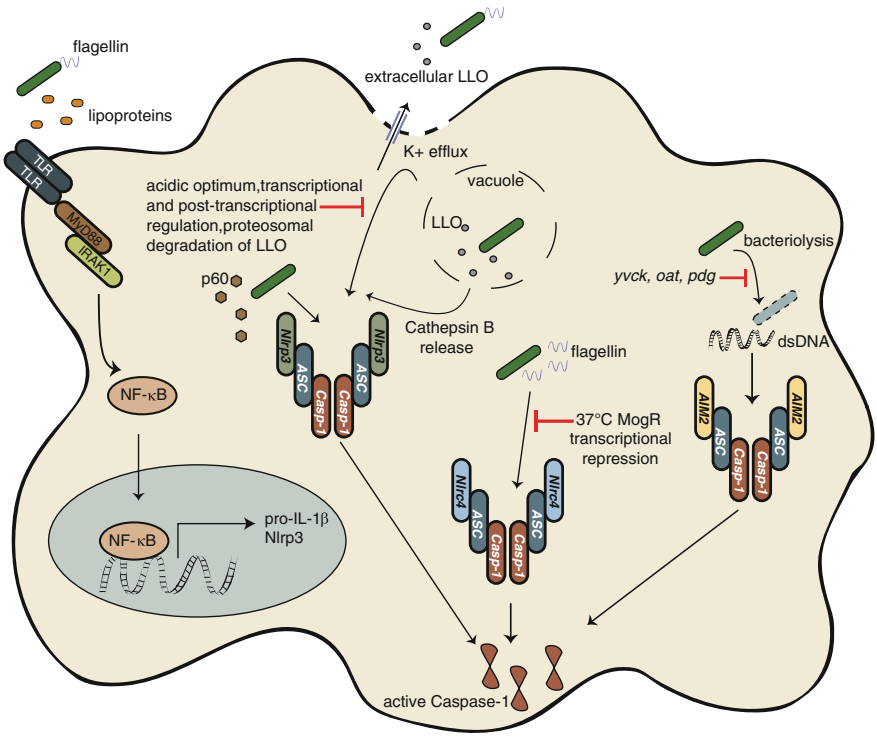

Fig. 1 In vitro mechanisms of activation and avoidance of the inflammasome by *L. monocytogenes*. *L. monocytogenes* lipoproteins and flagellin can signal through toll-like receptors (TLRs) via MyD88 and IRAK1 to activate the transcription factor NF-κB and prime cells for inflammasome activation by up-regulating IL-1β and NLRP3 transcripts. *L. monocytogenes* can then activate the NLRP3 inflammasome via a K^+ efflux secondary to extracellular or intracellular LLO-induced pores. Vacuole rupture by LLO can result in cathepsin B release that along with p60 can also activate the NLRP3 inflammasome. Flagellin can activate the NLRC4 inflammasome, while bacteriolysis and the subsequent DNA release can activate the AIM2 inflammasome. *L. monocytogenes* limits the activation of any of these inflammasomes via the tight control of LLO or flagellin, or by unknown mechanisms of combating cytosolic stress and limiting bacteriolysis

inflammasome in the seminal paper by Mariathasan et al. (2006) who demonstrated that cytosolic *L. monocytogenes* can activate the NLRP3 inflammasome, while vacuole-contained *L. monocytogenes* fail to activate the inflammasome, suggesting that the cytosolic recognition is critical. Concurrently, Kannengati et al. (2006) observed that *L. monocytogenes* total RNA could stimulate the NLRP3 inflammasome, further suggesting that *L. monocytogenes* could activate the NLRP3 inflammasome through a variety of mechanisms. More recently, activation of the Nrlp3 inflammasome specifically in unprimed bone marrow-derived macrophages infected with *L. monocytogenes* has been reported (Wu et al. 2010; Fernandes-Alnemri et al. 2013; Lin et al. 2014). However, the importance of NLRP3 in *L. monocytogenes* infection has also been questioned, as Franchi et al. (2007) failed to find any

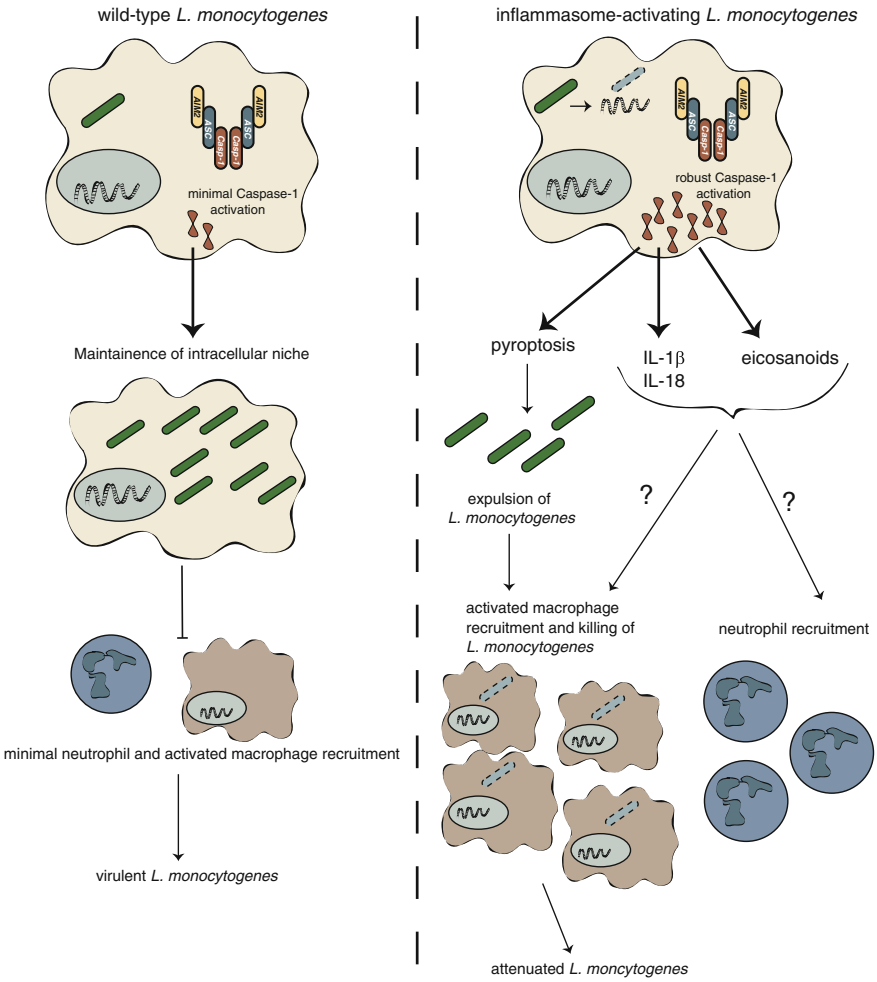

Fig. 2 Activation of the inflammasome attenuates *L. monocytogenes* through macrophage recruitment. In wild-type *L. monocytogenes* infection, *L. monocytogenes* minimally activates caspase-1 and maintains its intracellular niche, ultimately promoting a pathogenic infection. In contrast, inflammasome-activating *L. monocytogenes* results in robust caspase-1 activation and pyroptosis, release of IL-1β and IL-18, and eicosanoids. These effects result in expulsion of *L. monocytogenes,* increased activated macrophage recruitment, and potentially an influx of neutrophils. Increased macrophage recruitment restricts *L. monocytogenes* infection

difference in caspase-1 processing and IL-1β release in Nlrp3$^{-/-}$ cells following *L. monocytogenes* infection. Similarly, Sauer et al. (2010) failed to find any significant role for the NLRP3 inflammasome during *L. monocytogenes* infection.

These differing observations beg the questions: "Which components of *L. monocytogenes* stimulate the NLRP3 inflammasome and what role, if any, does it

play under physiologic conditions?" Pore-forming toxins have been demonstrated to activate the NLRP3 inflammasome, and Mariathasan and colleagues hypothesized that LLO could act like many other NLRP3 agonists and result in intracellular K^+ disruptions to activate the NLRP3 inflammasome (Mariathasan et al. 2006). While their original interpretation was that the importance of LLO was to facilitate the access of *L. monocytogenes* to the cytosol, it is also possible that pore formation, and not cytosolic access, was the critical function of LLO in NLRP3 activation during their studies, particularly as these studies used a high MOI of 50. Additionally, Meixenberger et al. (2010) suggest that cathepsin B release from LLO-damaged phagosomes can stimulate the NLRP3 inflammasome. While some data have directly suggested that cytosolic access of intact bacteria is required for *L. monocytogenes* NLRP3 activation, more data are consistent with a role of extracellular LLO in NLRP3 activation (Meixenberger et al. 2010; Hamon and Cossart 2011; Sakhon et al. 2013). Extracellular LLO can induce caspase-1 processing and IL-1β release depending on NLRP3 (Meixenberger et al. 2010) by stimulating a K^+ efflux (Hamon and Cossart 2011). Blocking bacterial uptake with cytochalasin D still results in the activation of caspase-1, suggesting that low amounts of LLO from extracellular *L. monocytogenes* can activate the inflammasome (Hamon and Cossart 2011). Further, purified LLO that maintains pore-forming ability, but not hemolytically inactive LLO (LLO^{W492A}), can activate caspase-1 (Sakhon et al. 2013), and these results are independent of the cholesterol-binding activity (Hara et al. 2008; Hamon and Cossart 2011). Taken together, these results suggest that pore formation from extracellular LLO can trigger K^+ efflux resulting in NLRP3 inflammasome activation. However, LLO expression, activity, and stability are tightly regulated during *L. monocytogenes* infection (Glomski et al. 2002; Schnupf et al. 2006a, b), thus limiting the likelihood of high extracellular concentrations of LLO during infection in vivo and potentially suggesting that NLRP3 activation by LLO is an in vitro artifact.

While LLO is believed to be the primary molecule triggering the NLRP3 inflammasome, both bacterial RNA (Kanneganti et al. 2006) and the secreted virulence factor, p60 encoded by the gene invasion-associated secreted endopeptidase (*iap*), from *L. monocytogenes* have been shown to activate the NLRP3 inflammasome (Schmidt and Lenz 2012). The N-terminal LysM and SH3 domain region (L1S) of p60 stimulates IL-1β and IL-18 release, independent of the act of pyroptotic cell death, in bone marrow-derived dendritic cells (Schmidt and Lenz 2012). Given the recent reports about the importance of gasdermin D-dependent host cell death in cytokine secretion (Shi et al. 2015; Kayagaki et al. 2015), it is unclear how IL-1β and IL-18 are being secreted in this scenario; however, Schmidt and Lenz found that ROS inhibition with the inhibitor DPI impaired IL-1β secretion, while IL-18 secretion remained intact. Further, in BMDCs from mice on a 129S6 background that lack caspase-11, IL-1β secretion was impaired, while IL-18 was unaffected (Schmidt and Lenz 2012). These observations suggest different licensing mechanisms for IL-1β and IL-18, potentially allowing either the host cell or the invading pathogen to fine-tune the downstream consequences of inflammasome activation.

Most work examining *L. monocytogenes* and the NLRP3 inflammasome has been done with murine cells. However, human peripheral blood mononuclear cells (PBMCs) infected with *L. monocytogenes* can undergo LLO-induced NLRP3-dependent inflammasome activation that depends on phagosomal acidification and cathepsin B release (Meixenberger et al. 2010). These results suggest that the observed differences for the role of the NLRP3 inflammasome may also stem from the cell types used. More importantly, the state of the cells used in experiments matters. Multiple groups were able to find a role for the NLRP3 inflammasome in the first hour of *L. monocytogenes* infection when using unprimed cells (Wu et al. 2010; Fernandes-Alnemri et al. 2013; Lin et al. 2014). Interestingly, for *L. monocytogenes* to induce a rapid NLRP3-dependent inflammasome activation, intact toll-like receptor (TLR) signaling must be present as MyD88$^{-/-}$ cells fail to activate the inflammasome (Fernandes-Alnemri et al. 2013; Lin et al. 2014). Further, this response is dependent on the MyD88 downstream signaling molecule IL-1 receptor-associated kinase (IRAK1) as IRAK1$^{-/-}$ cells also fail to activate the inflammasome and secrete IL-1β and IL-18 (Thomas et al. 1999; Fernandes-Alnemri et al. 2013; Lin et al. 2014). In the presence of priming, however, IRAK1 is dispensable for inflammasome activation (Lin et al. 2014); additionally, a prolonged infection results in NLRP3-independent inflammasome activation (Lin et al. 2014). These results suggest that *L. monocytogenes* can rapidly activate the NLRP3 inflammasome early in infection, as long as an intact TLR pathway is present. Many groups do not use unprimed cells, as priming is generally required for inflammasome activation in vitro (Latz et al. 2013), potentially explaining the observed discrepancies in the importance of NLRP3.

2.2 NLRC4 Activation

The NLRC4 inflammasome recognizes flagellin and type III secretion system components (Miao et al. 2010b). As *L. monocytogenes* is a Gram-positive pathogen and lacks type III secretion components, its flagellin is likely the key activator of the NLRC4 inflammasome. In line with this, *L. monocytogenes* engineered to hyper-express flagellin can activate the NLRC4 inflammasome (Sauer et al. 2011a; Warren et al. 2011). Additionally, *ΔflgK L. monocytogenes* lack the adaptor molecule between the flagellar hook and flagellin monomers, and infection with this strain results in excess flagellin secretion and hyperactivation of the NLRC4 inflammasome (Warren et al. 2008). However, *L. monocytogenes* likely avoids the activation of the NLRC4 inflammasome by shutting off flagellin expression in mammalian hosts (Peel et al. 1988; Shen and Higgins 2006). As such, *L. monocytogenes* inflammasome activation was shown to be independent of NLRC4 (Franchi et al. 2007; Sauer et al. 2010). Similarly, infection with *L. monocytogenes* deficient in flagellin (*ΔflaA*) resulted in similar caspase-1 processing and IL-1β release (Warren et al. 2008) as infection with wild-type *L. monocytogenes*. Taken together, these results suggest that although *L. monocytogenes* flagellin can activate

the NLRC4 inflammasome, likely in a manner similar to other bacterial flagellin being dependent on the adaptor molecule Naip5 (Kofoed and Vance 2011; Zhao et al. 2011), *L. monocytogenes* largely avoids the activation of this inflammasome through the control of its flagellin in vivo.

2.3 AIM2 Activation

As opposed to the NLR family members described above, AIM2 (absent in melanoma) is a member of the interferon-inducible class of genes (DeYoung et al. 1997). The AIM2 inflammasome through its HIN200 domain recognizes double-stranded DNA, rich in adenine and thymine that is mislocalized to the cytosol (Fernandes-Alnemri et al. 2009; Hornung et al. 2009). Initial evidence for the involvement of AIM2 in *L. monocytogenes* infection came from observations that *ΔflaA* mutant *L. monocytogenes* could still induce caspase-1 activation in the absence of NLRP3 (Warren et al. 2008; Wu et al. 2010). However, caspase-1 activation was abrogated in $Aim2^{-/-}$ cells, suggesting that the AIM2 inflammasome is responsible for the remaining caspase-1 activation (Wu et al. 2010; Kim et al. 2010). Additionally, Warren et al. (2010) observed that a component of lysed *L. monocytogenes* could activate the AIM2 inflammasome. Similarly, transfected *L. monocytogenes*' genomic DNA could also activate the AIM2 inflammasome (Warren et al. 2010). Using Hoechst-labeled *L. monocytogenes*, Wu et al. (2010) were able to observe colocalization of *L. monocytogenes* with GFP-labeled AIM2, further suggesting that *L. monocytogenes* DNA is able to activate the AIM2 inflammasome.

Finally, Sauer et al. (2010) demonstrated that bacteriolysis within the cytosol resulted in the activation of the AIM2 inflammasome, identifying a likely mechanism for how bacterial DNA accesses the cytosol to be recognized by AIM2. These experiments demonstrated that wild-type *L. monocytogenes* lyses at low levels throughout the course of infection resulting in the recognition of cytosolic DNA by AIM2. Treatment of cytosolically replicating *L. monocytogenes* with the bactericidal β-lactam antibiotic, ampicillin, but not the bacteriostatic antibiotic chloramphenicol, triggered the increased bacteriolysis and subsequently increased inflammasome activation (Sauer et al. 2010). Additionally, a *L. monocytogenes* mutant was identified that was defective for cytosolic survival leading to increased cytosolic bacteriolysis and ultimately increased AIM2-dependent inflammasome activation. Finally, *L. monocytogenes* engineered to lyse specifically within the host cytosol through the expression of the bacteriophage proteins holin and lysin resulted in a significant bacteriolysis and subsequent cell death (Sauer et al. 2010). Importantly, these data implicated cytosolic survival and avoidance of cell autonomous defenses as important mechanisms of avoiding detection by the AIM2 inflammasome. Further, a similar mechanism of cytosolic bacteriolysis was subsequently found to be the mechanism of AIM2 activation following *Francisella tularensis* infection using a similar reporter system (Peng et al. 2011).

2.4 Other NLR Engagement

The inflammasomes mentioned above are the traditionally described inflammasomes as well as the primary inflammasomes that *L. monocytogenes* has been demonstrated to engage. Beyond the receptors described above, other NLRs including NLRC5 and NLRP6 have been studied in regard to *L. monocytogenes* infection. The NLRC5 inflammasome largely regulates MHC class I-associated gene expression (Meissner et al. 2010). Nlrc5$^{-/-}$ mice infected with *L. monocytogenes* have impaired CD8^{+} T-cell responses, both in the number and in the ability to produce the effector cytokine, IFNγ. CD4^{+} T-cell responses are not impaired in these mice, consistent with the role of NLRC5 in regulating MHC I-associated gene expression (Yao et al. 2012; Biswas et al. 2012). NLRC5 has also been shown to associate with NLRP3 and contribute to NLRP3 inflammasome activation (Davis et al. 2011; Yao et al. 2012). As discussed above, *L. monocytogenes* has been shown, in some cases, to activate the NLRP3 inflammasome, although the specific contribution of NLRC5 to this activation will require additional studies. That said, infection of Nlrc5$^{-/-}$ mice with *L. monocytogenes* resulted in partial impairments in caspase-1 processing and IL-1β secretion, suggesting a role for this protein in a more traditional inflammasome-dependent response (Yao et al. 2012). Finally, Nlrc5$^{-/-}$ mice infected with *L. monocytogenes* have higher bacterial burdens (Yao et al. 2012; Biswas et al. 2012), although it is unclear whether this is due to deficient CD8^{+} T-cell responses or to a more canonical inflammasome-dependent infection control mechanism.

NLRP6 has been shown to have roles in altering the gut microbiota to promote gut inflammation and ultimately tumorigenesis (Elinav et al. 2011; Normand et al. 2011; Chen et al. 2011). Interestingly, Nlrp6$^{-/-}$ mice infected orally with *L. monocytogenes* are largely protected from lethal *L. monocytogenes* infection, with 75 % surviving lethal challenge. Further, Nlrp6$^{-/-}$ mice harbor lower bacterial burdens in their spleens and livers and fail to lose weight following infection (Anand et al. 2012). These effects appear to be independent of the gut microbiota composition, but instead depend on increases in circulating monocytes and neutrophils in Nlrp6$^{-/-}$ mice after *L. monocytogenes* infection both systemically in the blood and locally in the peritoneal cavity (Anand et al. 2012). Though NLRP6 seems to have important roles in *L. monocytogenes* infection, this appears to occur independent of canonical inflammasome activation as caspase-1 activation and IL-1β processing are not impaired following *L. monocytogenes* infection in Nlrp6$^{-/-}$ macrophages (Anand et al. 2012). Similarly, in human PBMCs, NLRP6 is dispensable for canonical inflammasome activation by *L. monocytogenes* (Meixenberger et al. 2010). Taken together, these results suggest that though NLRP6 has a role in *L. monocytogenes* pathogenesis, it is likely independent of the effects from inflammasome signaling.

Finally, among the remaining NLRs, a few have been tested for *L. monocytogenes*-dependent phenotypes. HEK293 cells reconstituted with NLRP2 or NLRP12 result in increases in IL-1β production when infected with *L. monocytogenes*,

suggesting that *L. monocytogenes* can also engage these inflammasomes, although which components of *L. monocytogenes* are recognized by these receptors remains unknown (Tsuchiya et al. 2010). Conversely, in human PBMCs, NLRP1 and NLRP12 are dispensable for inflammasome activation by *L. monocytogenes* (Meixenberger et al. 2010). These results suggest that *L. monocytogenes* can potentially engage other NLRs; however, the effects of this engagement are unclear and the mechanisms by which they are activated require more in-depth future studies.

In conclusion, although under in vitro conditions *L. monocytogenes* has been found to activate the NLRP3, NLRC4, and AIM2 inflammasomes (Fig. 1), the in vivo relevance of each of inflammasomes is unclear. As discussed in more detail below and as shown in Fig. 1, molecules that engage the NLRP3 and NLRC4 inflammasomes are under the tight control in physiologic settings, thus limiting their ability to be sensed by their respective inflammasomes. Although AIM2 is the most likely relevant inflammasome in vivo, *L. monocytogenes* also has yet undefined ways of avoiding cytosolic bacteriolysis, suggesting that overall, *L. monocytogenes* limits inflammasome activation.

3 Avoidance of Inflammasome Activation

Avoidance of cell death is critical for the virulence of *L. monocytogenes* as mutants that misregulate LLO activity in the cytosol cause necrosis and are highly attenuated in vivo (Glomski et al. 2003). Similarly, *L. monocytogenes* that hyperactivate the inflammasome or induce apoptosis are also attenuated (Sauer et al. 2011a; Warren et al. 2011; Theisen and Sauer, unpublished results). Thus, although *L. monocytogenes* can activate multiple inflammasomes during infection in vitro, it is likely that it largely avoids doing so during infection in vivo to protect its intracellular niche. As a pathogen that utilizes a replication niche rich in inflammasome receptors, it is critical that *L. monocytogenes* has evolved mechanisms to avoid detection or actively inhibit the inflammasome.

3.1 Avoidance of NLRP3

As described above, LLO, through its pore-forming activity, is capable of activating the NLRP3 inflammasome, likely when found in high concentrations extracellularly. However, expression, stability, and activity of LLO are tightly regulated to prevent host cell toxicity, either directly through pore formation and necrosis or potentially through pore-induced NLRP3 activation. LLO expression is both transcriptionally and post-transcriptionally regulated through the activity of the PrfA transcription factor and the 5′ UTR, respectively (Shen and Higgins 2005; Schnupf et al. 2006a). Additionally, LLO in the host cytosol is ubiquitinated and targeted for

proteosomal degradation (Schnupf et al. 2006b). Finally, and perhaps most importantly, LLO toxicity is limited at the level of pore-forming activity such that LLO is maximally active only at an acidic pH, a characteristic unique to LLO among the cholesterol-dependent cytolysins (Glomski et al. 2002). Each of these levels of regulation contributes to minimizing the toxicity of LLO to maximize virulence as misregulated LLO can lead to cytotoxicity and result in severe attenuation of virulence (Glomski et al. 2003). Taken together, these studies demonstrate that *L. monocytogenes* carefully regulates the expression of its pore-forming toxin to limit host cell damage and maintain its virulence, in the process likely avoiding a robust activation of the NLRP3 inflammasome under physiologic conditions.

3.2 Avoidance of NLRC4

Similar to the tight regulation of LLO, *L. monocytogenes* has developed exquisite control over expression of its flagellin, regulating the expression through temperature sensing as well as yet to be identified signals found in the host cell cytosol. At the physiologic temperature of 37 °C, *L. monocytogenes* limits the expression of its flagellin through the transcriptional repressor, MogR. However, at temperatures below 37 °C, such as those found in the external environment, *L. monocytogenes* must express flagellin (Peel et al. 1988), and MogR repression must be removed.

Repression by MogR is relieved by the anti-repressor GmaR (Shen et al. 2006). Thus, the balance between these two factors is important in the expression of flagellin. As an added measure to ensure that flagellin is not expressed at 37 °C, there is post-transcriptional regulation of GmaR so that MogR is the dominant component regulating flagellin expression (Kamp and Higgins 2009). Therefore, similar to what is observed for LLO, although flagellin can activate the NLRC4 inflammasome, *L. monocytogenes* tightly limits its expression in the presence of inflammasome components. Whether this level of regulation evolved to facilitate the avoidance of TLR5 detection or inflammasome activation is unclear; however given that the majority of *L. monocytogenes'* lifecycle in the host is in the cytosol, it is enticing to imagine a strong selective pressure exerted by inflammasome detection leading to the tight control of flagellin expression. Indeed, recent evidence suggests that, contrary to previous reports, primary human macrophages utilize a specific splice variant of NLR family, apoptosis inhibiting protein (NAIP) to facilitate the recognition of flagellin through the NLRC4 inflammasome (Kortmann et al. 2015).

3.3 Avoidance of AIM2

In addition to regulating LLO and flagellin to avoid detection by the inflammasome, as a pathogen that makes its life in the cytosol, fidelity during replication and

evasion of host defense mechanisms is essential for *L. monocytogenes* to avoid detection by the AIM2 inflammasome. Work by Werner Goebel's laboratory suggests that cytosolic pathogens have developed unique defenses to survive the harsh environment, that is, the host cytosol. Non-cytosolic pathogens that are placed into the cytosol by microinjection fail to replicate and in some cases are killed, whereas cytosol-adapted pathogens survive and thrive (Goetz et al. 2001; Slaghuis et al. 2004). Further, additional studies demonstrated that even intracellular pathogens that mislocalize to the cytosol, such as *ΔsifA* mutant *Salmonella enterica* subsp. Typhimurium, are unable to survive (Beuzón et al. 2000). Finally, *Legionella pneumophila ΔsdhA* mutants mislocalize to the cytosol, subsequently lyse, and activate the inflammasome (Creasey and Isberg 2012; Ge et al. 2012). Taken together, these data suggest that bacteria such as *L. monocytogenes* that are able to survive and replicate within the harsh environment of the cytosol must have evolved mechanisms to deal with these stresses and/or active host defenses. Recent evidence suggests that the loss of these survival adaptations ultimately results in the lysis of *L. monocytogenes* in the cytosol, leading to AIM2 activation and virulence attenuation, further highlighting the importance of inflammasome avoidance in promoting *L. monocytogenes* virulence (Sauer et al. 2010, Daniel Pensinger, Grischa Chen and JD Sauer unpublished data).

The specific adaptations that allow *L. monocytogenes* to survive within the cytosol of cells and avoid AIM2 inflammasome activation are largely unknown. *L monocytogenes Δyvck* mutants lyse in the macrophage cytosol, resulting in hyperactivation of the AIM2 inflammasome and virulence attenuation (Sauer et al. 2010). The function of YvcK, a conserved protein of unknown function, in maintaining cell wall integrity specifically in the cytosol remains unknown, though the loss of *yvck* sensitizes *L. monocytogenes* to lysozyme and numerous cell wall-acting antibiotics in vitro, suggesting that *yvck* plays an integral role in dealing with cell wall stress (Burke et al. 2014, Daniel Pensinger and JD Sauer unpublished data). Similarly, *L. monocytogenes* deficient in either the N-deacetylation (*Δpgd*) or O-acetylation (*Δoat*) of peptidoglycan are sensitive to lysozyme and lyse within the host cytosol (Rae et al. 2011). Finally, Witte et al. (2012) reported a number of *L. monocytogenes* mutants that hyperinduce host cell death and undergo bacteriolysis in the host cell cytosol, though these mutants were not fully characterized as activating the AIM2 inflammasome. However, it is likely given their lysis phenotypes correlating with increased host cell death that these genes are required for the avoidance of host cytosolic defense mechanisms to promote the avoidance of the AIM2 inflammasome. Importantly, maintaining bacterial integrity in the cytosol as a mechanism of avoiding AIM2 detection is a conserved virulence mechanism as Peng et al. (2011) recently demonstrated that a series of *Francisella tularensis* mutants that hyperactivate the inflammasome do so due to cytosolic bacteriolysis. Taken together, these data suggest that maintenance of the cell wall is a critical determinant of *L. monocytogenes* virulence, in part due to the necessity of avoiding AIM2 activation.

3.4 Active Inhibition of the Inflammasome

Although pathogens such as *Yersinia* spp. and *Pseudomonas aeruginosa* express molecules that actively inhibit inflammasome activation (Sutterwala et al. 2007; Brodsky et al. 2010), there is little evidence of *L. monocytogenes* actively inhibiting the inflammasome. Instead, it is presumed that the lack of robust inflammasome activation observed during *L. monocytogenes* infection is due to avoiding detection. These data are supported by the studies demonstrating that *L. monocytogenes* engineered to activate the inflammasome through ectopic expression of flagellin are able to potently activate the inflammasome (Sauer et al. 2011a; Warren et al. 2011). Likewise, *L. monocytogenes*-infected macrophages are still capable of robustly responding to transfected DNA, suggesting that *L. monocytogenes* is not actively inhibiting AIM2 inflammasome activation (JD Sauer unpublished observations). While it is possible, and maybe even likely given its importance in host defense, that *L. monocytogenes* actively inhibits pyroptosis under certain conditions or for the activation of specific inflammasomes, studies to date have not identified such a mechanism. The lone potential example is during in vivo infection where *L. monocytogenes* can activate cholesterol 25-hydroxylase (Ch25h) downstream of liver X receptors (LXRs) through type I INF production (Zou et al. 2011). Ch25h results in the expression of Cd5l, a pro-survival gene that limits the activation of both caspase-3 and caspase-1, ultimately limiting host cell inflammasome activation and pyroptosis. Further, this response seems to be conserved among other downstream targets of LXRs (Zou et al. 2011), suggesting that *L. monocytogenes* may activate this pathway to limit caspase-1 activation and preserve its niche.

4 Role in Pathogenesis

4.1 Role of Caspase-1/11

As described above, *L. monocytogenes* can interact with many different inflammasomes in vitro, culminating in the activation of caspase-1. Initial studies of murine caspase-1 were actually studies of both caspase-1 and caspase-11 double deletions (Kayagaki et al. 2011). Caspase-11 has largely been implicated in Gram-negative infections recognizing the LPS moiety of many of these pathogens (Wang et al. 1998; Kayagaki et al. 2011). *L. monocytogenes* has been found to induce caspase-11 expression upon infection; however, genetic deletion of caspase-11 still allows caspase-1 to function normally (Akhter et al. 2012), suggesting that caspase-11 is dispensable in *L. monocytogenes* infection.

Initial reports suggested that mice deficient in caspase-1/11 had impaired clearance of *L. monocytogenes*, resulting in an increased death rate, particularly at late time points in infection (Tsuji et al. 2004). Mice were rescued with exogenous IL-18 or IFNγ, suggesting that the production of these critically important cytokines

is impaired in caspase-1/11$^{-/-}$ mice (Tsuji et al. 2004). While these initial experiments suggest a critical role for caspase-1/11 in controlling *L. monocytogenes* infection, further experiments have failed to find a similar role. Both Sauer et al. (2011a) and Tsuchiya et al. (2014) observed no increased susceptibility to *L. monocytogenes* in caspase-1/11$^{-/-}$ mice at 2 days post infection. Similarly, there was no increased susceptibility to *L. monocytogenes* in zebrafish lacking caspase-1 (Vincent et al. 2015). These studies are consistent with the tight levels of regulation observed with potential inflammasome agonists as well as the critical nature of host cell survival that has previously been reported to promote *L. monocytogenes* virulence. The differences between the initial characterization of caspase-1 and more recent studies likely stem from the time points examined, the background of mice used in the initial studies (C57Bl/6 J × 129SV/J), as well as the differences in the strains of *L. monocytogenes* used for each of the studies. Taken together, the more recent studies suggest that caspase-1 is dispensable for the control of *L. monocytogenes* early during infection, likely due to *L. monocytogenes* avoiding the activation of the inflammasome and maintaining its intracellular niche. However, it is possible that later in infection when bacterial burdens are high, the inflammasome plays a role in host protection. Importantly, as briefly discussed above and in more detail below, under conditions in which *L. monocytogenes* does activate the inflammasome, due to ectopic flagellin expression or mutations that result in detection by AIM2, caspase-1 activation and the subsequent pyroptosis severely attenuate virulence, further highlighting the importance of inflammasome evasion for *L. monocytogenes* pathogenesis.

4.2 Role of ASC

ASC is an adaptor molecule required for cytokine secretion and pyroptosis downstream of both the NLRP3 and AIM2 inflammasomes (Broz et al. 2010). In the case of CARD-containing inflammasomes, such as the NLRC4 inflammasome, ASC is only required for cytokine secretion and is dispensable for pyroptosis (Broz et al. 2010). Thus, as discussed above, the majority of inflammasome activation by *L. monocytogenes* requires signaling through ASC for both pyroptosis and cytokine secretion (Fig. 1). As such, ASC$^{-/-}$ peritoneal macrophages fail to activate caspase-1 or secrete IL-1β and IL-18 (Ozören et al. 2006). Consistent with observations that the loss of caspase-1 has no effect on *L. monocytogenes* virulence in vivo (Sauer et al. 2011a; Tsuchiya et al. 2014), the loss of ASC results in similar susceptibility to *L. monocytogenes* (Sauer et al. 2011a). Interestingly, recent work by Tsuchiya suggests that ASC deficiency may in some cases result in enhanced host protection during *L. monocytogenes* infection (Tsuchiya et al. 2014). ASC$^{-/-}$ mice are able to withstand a bacterial load of 1×10^6 colony forming units (CFU) of *L. monocytogenes*, a log higher than the LD_{50} for wild-type C57Bl/6 mice, and harbor lower bacteria burdens in their livers. The investigators report that ASC promotes the production of the anti-inflammatory cytokine IL-10 and that

protection in ASC-deficient mice is likely due to reduced IL-10 production (Tsuchiya et al. 2014). Taken together, these results suggest that similar to the loss of caspase-1, the loss of ASC does not promote the virulence of *L. monocytogenes*, and in fact may result in enhanced protection, although this phenotype may be independent of ASC's role in caspase-1 activation (Tsuchiya et al. 2014).

4.3 Role of IL-1β and IL-18

IL-1β and IL-18 have been examined in the context of *L. monocytogenes* infection well before these cytokines were known to be downstream of the inflammasome. Initial reports of IL-1 receptor-deficient (IL-1R1$^{-/-}$) mice that lack signaling of IL-1α and IL-1β suggested that these mice are more susceptible to *L. monocytogenes* (Labow et al. 1997); however, this was done in a mixed background (B6/129) and is likely confounded by the fact that 129 mice are more susceptible to *L. monocytogenes* at baseline (Gahan and Collins 1995). Further examination on a clean C57Bl/6 background suggests that there is no alteration in acute virulence in the absence of IL-1 signaling (Glaccum et al. 1997). Similarly, mice deficient just in IL-1β show no differences in susceptibility (Zheng et al. 1995), suggesting that IL-1 signaling is dispensable during initial infection.

Priming of cells via signal one, largely through NF-κB signaling, is critical in up-regulating IL-1β transcripts (Latz et al. 2013). In TLR2$^{-/-}$ macrophages, IL-1β secretion is impaired following *L. monocytogenes* infection likely through transcriptional impairment (Ozören et al. 2006). Further, the loss of tumor progression locus 2 (TPL2), a factor critical in orchestrating cytokine responses downstream of TLR signaling, impairs IL-1β secretion following *L. monocytogenes* infection through impairments of IL-1β transcript up-regulation (Mielke et al. 2009). Interestingly, the loss of TPL2 increases the susceptibility of mice to *L. monocytogenes*, though the regulation of other key innate cytokines such as TNF-α and IFN-γ is similar regardless of whether TPL2 is present or not (Mielke et al. 2009). Even though TPL2 influences the levels of IL-1β, the increased susceptibility of TPL2$^{-/-}$ mice does not recapitulate the phenotype of IL-1R1$^{-/-}$ mice; thus, the increased susceptibility of TPL2$^{-/-}$ mice is not likely due to the changes in IL-1β but instead to some other undescribed effect of loss of TPL2.

Characterization of the role of IL-18, particularly in acute infection, has also resulted in conflicting reports. Initial studies suggested that IL-18 is critical in acute infection, and the lack of IL-18 leads to an increased susceptibility (Neighbors et al. 2001). IL-18 is critical in stimulating IFN-γ, a crucial cytokine for controlling *L. monocytogenes* infection, and thus, decreases in IL-18 production result in less IFN-γ production, leading mice to succumb to infection. While these observations were made in initial reports, the authors also observed decreases in TNF-α production, another critical cytokine in innate susceptibility, and suggest that the corresponding lack of TNF-α from IL-18-deprived mice is largely what is responsible for susceptibility to infection. More recent studies in both IL-18$^{-/-}$ and

IL-18Rα$^{-/-}$ contradict these earlier results and suggest that IL-18$^{-/-}$ mice are actually more resistant to *L. monocytogenes*, particularly at high doses (Lochner et al. 2008; Tsuchiya et al. 2014). These results are in line with reports that suggest that ASC$^{-/-}$ mice, which also lack IL-18 secretion, are less susceptible to *L. monocytogenes* (Tsuchiya et al. 2014). The reason for this decreased susceptibility could stem from an increased influx of leukocytes in the initial 72 h postinfection that are resistant to apoptosis (Lochner et al. 2008).

While most IL-1β and IL-18 signaling is believed to occur downstream of the inflammasome, there have been caspase-1-independent observations of IL-1β and IL-18 secretion (Uchiyama et al. 2013; Tsuchiya et al. 2014). One such mechanism relies on Fas (CD95/Apo-1) signaling. Peritoneal exudate cells (PECs) deficient in Fas secreted less IL-1β and IL-18 after *L. monocytogenes* infection (Uchiyama et al. 2013), suggesting that Fas is required for cytokine secretion. Interestingly, this mechanism is caspase-1, caspase-11, NLRP3, and NLRC4 independent, while it is dependent on ASC (Uchiyama et al. 2013). In line with the reports that suggest IL-1β maturation can occur through caspase-8 activation (Gringhuis et al. 2012; Bossaller et al. 2012), *L. monocytogenes* can activate caspase-8 in a Fas-dependent manner, suggesting that in this instance, IL-1β and IL-18 could be processed through caspase-8 and not caspase-1. Additionally, IL-18 can be processed to its mature form through neutrophil serine proteases (Sugawara et al. 2001). In caspase-1/11$^{-/-}$ mice infected with lethal doses of *L. monocytogenes*, elevated neutrophil serine protease activity was observed that correlated with the increased IL-18 production (Tsuchiya et al. 2014). Taken together, while not all studies are consistent, and differences in mouse backgrounds, *L. monocytogenes* strains, doses, and infection kinetics could account for discrepancies, the preponderance of data suggests that the inflammatory cytokines downstream of inflammasome activation are dispensable for host defense during *L. monocytogenes* infection.

4.4 Innate Immune Cell Infiltrate

During murine *L. monocytogenes* infection, there is a robust, initial infiltrate of myelomonocytic cells (MMCs) consisting of neutrophils and Ly6C^{hi} monocytes into the splenic white pulp (Waite et al. 2011; Williams et al. 2013). Because *L. monocytogenes* largely avoids activating the inflammasome, much of the work examining the host response to pyroptosis has used an engineered strain of *L. monocytogenes* that ectopically expresses flagellin resulting in hyperactivation of the inflammasome and attenuation (Sauer et al. 2011a; Warren et al. 2011; Williams et al. 2013; Vincent et al. 2015). Infection with inflammasome-activating *L. monocytogenes* resulted in an earlier MMC infiltrate that penetrates into the deep T-cell zones of the spleen; however, this infiltrate is less robust and clears earlier than with wild-type infection, corresponding to a drop in burden (Williams et al. 2013). Similar early innate immune cell infiltrates consisting of predominantly macrophages were observed in zebrafish infected with inflammasome-activating

L. monocytogenes ultimately resulting in attenuation (Fig. 2) (Vincent et al. 2015). Previous work with *Salmonella enterica* subsp. Typhimurium similarly engineered to robustly activate the inflammasome suggests that pyroptosis expels bacteria for uptake by bactericidal neutrophils ultimately resulting in phagocyte oxidase-dependent attenuation (Miao et al. 2010a). However, neutrophil depletion does not specifically rescue virulence defects following the activation of pyroptosis by *L. monocytogenes* (Sauer et al. 2011a; Vincent et al. 2015). Instead, depletion of macrophages rescues the virulence defect of inflammasome-activating *L. monocytogenes* (Vincent et al. 2015). Why the attenuation of *L. monocytogenes* is independent of neutrophils while *S. typhimurium* is dependent on neutrophils is unclear, although it is important to note that the depletion of neutrophils was not directly assessed in the *Salmonella* studies; rather, the loss of phagocyte oxidase implicated the role for neutrophils (Miao et al. 2010a). In wild-type *L. monocytogenes* infection, neutrophils are dispensable for defense, while inflammatory monocytes are critical (Shi et al. 2011), suggesting that attenuation following inflammasome activation likely depends on the cell type critical in clearing the pathogen normally.

5 Adaptive Immune Response to *L. monocytogenes*

The specific mechanisms by which *L. monocytogenes* infection triggers robust $CD8^+$ T cell-dependent cell-mediated immune responses are largely unknown. Generally, the initial innate response is posited to inform the type and robustness of the adaptive immune response (Janeway 1989). It was hypothesized that activation of the inflammasome and its resulting robust innate inflammatory response would contribute to mounting a better adaptive immune response. As discussed below, surprising results suggest that activation of the inflammasome is detrimental for the activation of *L. monocytogenes*-triggered adaptive immunity (Sauer et al. 2011a). Importantly, understanding how $CD8^+$ T-cell responses form following *L. monocytogenes* infection and how we can improve them is critical in the development of *L. monocytogenes* as a cancer immunotherapeutic platform.

5.1 Protective Immunity

To understand how the inflammasome impacts the generation of adaptive immunity, multiple groups independently generated strains of *L. monocytogenes* that hyperactivate the inflammasome (Sauer et al. 2011a; Warren et al. 2011). Both groups utilized expression systems whereby flagellin expression was activated exclusively in the cytosol of infected macrophages, limiting flagellin detection to the NAIP5/NLRC4 inflammasome while limiting the potential impact of TLR5-mediated flagellin recognition. Warren et al. (2011) observed that mice immunized with flagellin-expressing *L. monocytogenes* could survive a subsequent

lethal challenge, suggesting that the activation of the inflammasome allows a protective immune response to form. While all mice immunized with a high dose of inflammasome-activating bacteria survived the subsequent lethal dose challenge, no wild-type control mice were analyzed as a comparison, and neither T-cell responses nor bacterial burdens after lethal challenge were examined to determine whether the response was better or worse than that activated by non-inflammasome-activating *L. monocytogenes*.

In contrast, similar immunization studies with inflammasome-activating *L. monocytogenes* found that adaptive immune responses were impaired. While immunization of mice with high doses of attenuated inflammasome-activating or nonactivating *L. monocytogenes* both conferred some degree of protection from the subsequent lethal challenge, mice were significantly less protected when immunized with strains that activated the inflammasome. More strikingly, when immunized at low doses, inflammasome-activating strains were unable to confer any protection above mock-immunized mice, whereas non-inflammasome-activating immunizations led to a significant protection from the subsequent lethal challenge (Sauer et al. 2011a). Similarly, immunization with inflammasome-activating *L. monocytogenes* resulted in an impaired antigen-specific primary $CD8^+$ T-cell response, with about half as many antigen-specific $CD8^+$ T cells forming compared to immunization with non-inflammasome-activating strains (Sauer et al. 2011a). Further, these $CD8^+$ T-cell deficits persist into the memory stage (Sauer et al. 2011a), and levels of the recall $CD8^+$ T cells at 2 days after challenge are also impaired (Theisen and Sauer, unpublished results). Interestingly, the recall response examined at 5 days post challenge results in similar levels of expanded antigen-specific $CD8^+$ T cells (Sauer et al. 2011a), suggesting that the rate of expansion upon recall is slower and likely contributes to the differences seen in bacterial burdens upon challenge. Finally, caspase-1 mice immunized with *L. monocytogenes* mount more robust protective immune responses compared to the wild-type (Sauer et al. 2011a, JD Sauer unpublished data). Taken together, these data suggest that at high immunizing doses, some protection can be conferred from *L. monocytogenes* strains that activate the inflammasome, but that this response is impaired compared to strains that avoid inflammasome activation, and at lower immunizing doses, T-cell activation is insufficient to confer the protection from a subsequent lethal challenge (Fig. 3).

How inflammasome activation inhibits *L. monocytogenes*-stimulated immunity remains unclear. Inflammasome-activating strains of *L. monocytogenes* are attenuated in vivo (Sauer et al. 2011a; Warren et al. 2011; Vincent et al. 2015); however, some of the adaptive immunity studies have been done with *Δact L. monocytogenes* to normalize the differences in bacterial burden (Sauer et al. 2011a; Williams et al. 2013). Further, the depletion of MMCs, which partially rescues inflammasome-activating *L. monocytogenes* virulence defects, does not affect the defects in the development of $CD8^+$ T cells or protective immunity (Fig. 3) (Williams et al. 2013), suggesting that deficits in adaptive immunity are independent of virulence defects and are consistent with evidence that immunity is independent of bacterial burden (Mercado et al. 2000). Interestingly, inflammasome activation is required

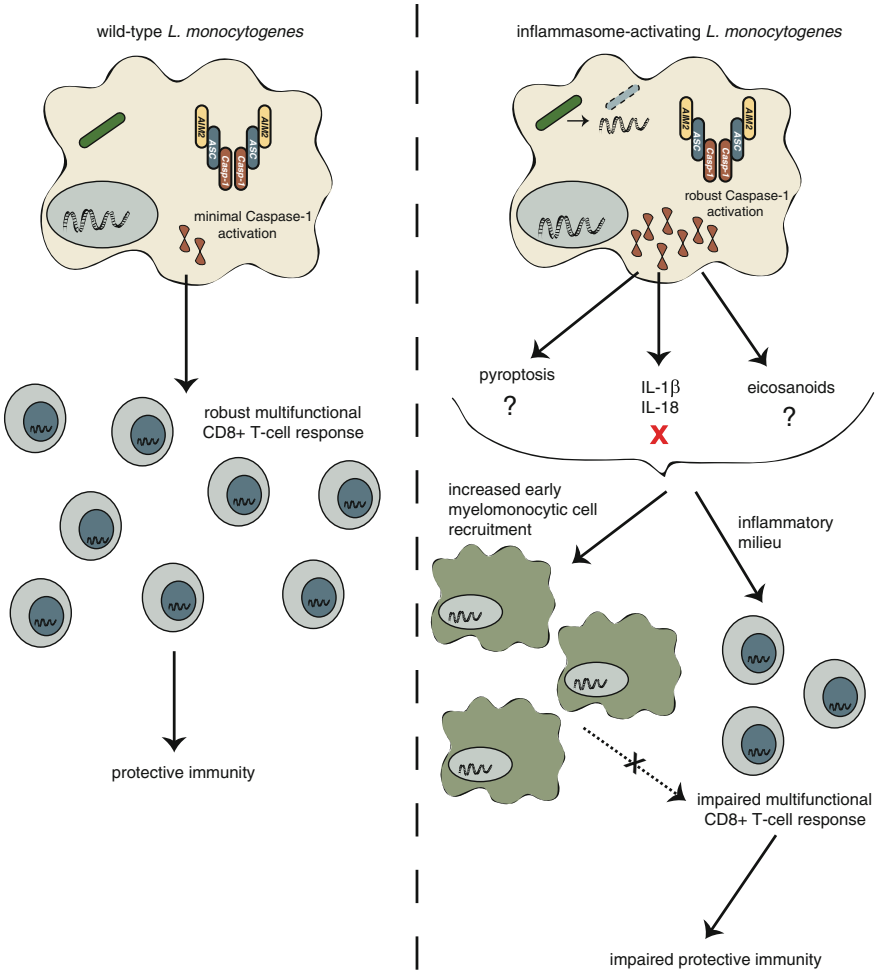

Fig. 3 Inflammasome-associated inflammation impairs host cell-mediated immune responses to *L. monocytogenes*. In wild-type *L. monocytogenes* infection, *L. monocytogenes* minimally activates caspase-1 and, through a not fully understood mechanism, induces a robust multifunctional $CD8^+$ T-cell response. In contrast, activation of the inflammasome by *L. monocytogenes* results in robust caspase-1 activation and impairment of multifunctional $CD8^+$ T-cell generation through the inflammasome-associated inflammatory milieu, independent of IL-1β and IL-18. Inflammasome activation also results in a robust, early recruitment of myelomonocytic cells that while attenuating *L. monocytogenes* do not impair $CD8^+$ T-cell generation

for T-cell responses to a variety of other pathogens, including *Candida albicans, Schistosoma mansoni*, and *Bordetella pertussis* (Dunne et al. 2010; Ritter et al. 2010; van de Veerdonk et al. 2011). However, these responses largely require CD4+ T cell-dependent Th1 or Th17 responses, not the $CD8^+$ T-cell response that

L. monocytogenes induces, suggesting that inflammasome activation may have different roles in regulating the adaptive immune response pending the type of response required for pathogen control.

5.2 *Influence of Cytokines*

Preliminary data suggest that inflammasome-dependent inhibition of immunity is largely mediated by the inflammatory milieu and not deficits in antigen presentation or costimulation. However, the specific inflammatory mediators that inhibit the generation of robust cell-mediated immunity remain unknown (Erin Theisen and JD Sauer, unpublished data). We and others have examined how deficiency of either IL-1β or IL-18 influences the adaptive immune response. In our hands, the loss of IL-1β, IL-18, or both still results in impairments in the protective immune response following the immunization with inflammasome-activating *L. monocytogenes*, suggesting that these cytokines do not negatively influence the development of adaptive immunity. Further, IL-18$^{-/-}$ or IL-18Rα$^{-/-}$ mice do not have impairments in generating antigen-specific CD8^{+} T cells that have effector functions (Lochner et al. 2008; Haring and Harty 2009). Similarly, the loss of IL-18Rα does not impact the rate of contraction or formation or maintenance of memory T cells (Haring and Harty 2009). Interestingly, it seems that the loss of IL-18 does result in fewer CD4^{+} T cells; however, this ultimately does not impact the generation of protective immunity as IL-18$^{-/-}$ or IL-18Rα$^{-/-}$ mice harbor similar burdens of *L. monocytogenes* following the lethal challenge (Lochner et al. 2008; Haring and Harty 2009).

Taken together, these results suggest that inflammasome activation is detrimental to the influence of adaptive immunity; however, the reason for this inhibition remains largely unknown. We hypothesize that an undescribed inflammatory component is responsible for these defects.

6 Concluding Remarks

In this chapter, we have discussed the mechanisms by which *L. monocytogenes* can activate the inflammasome. However, in vivo data suggest that under physiologic conditions, *L. monocytogenes* largely avoids activating the inflammasome as evidenced by multiple studies that demonstrate no change in virulence in caspase-1-deficient mice and attenuation when *L. monocytogenes* is engineered to robustly activate the inflammasome (Sauer et al. 2011a; Warren et al. 2011; Vincent et al. 2015).

Attenuated *L. monocytogenes* is currently being developed as a cancer immunotherapeutic platform, and early returns in multiple phase I and II clinical trials have been exceptionally positive (Le et al. 2015). How *L. monocytogenes* is

able to mount a $CD8^+$ T-cell response that is able to break self-tolerance remains unknown. It was initially hypothesized that inflammasome activation and its associated inflammation would positively influence cell-mediated immunity; however, inflammasome activation ultimately impairs the adaptive immune response generated to *L. monocytogenes* (Sauer et al. 2011a; Williams et al. 2013). It is possible that although the inflammasome is detrimental in the context of strong non-self-antigens, it could be beneficial in the context of less robust or self-antigens. Studies to assess this possibility are currently underway.

While *L. monocytogenes* largely avoids inflammasome activation, immunization of caspase-1/11$^{-/-}$ mice results in better protective immune responses than wild-type mice, suggesting that even low-level inflammasome activation is detrimental to the development of adaptive immunity (Sauer et al. 2011a). Additionally, current platforms of *L. monocytogenes* immunotherapy are limited in the complexity of antigens they can produce, and for the production of more complex antigens, the host must be able to transcribe and translate the antigens—similar to the idea of immunization with plasmid DNA. Platforms of *L. monocytogenes* designed to release plasmid DNA encoding complex tumor antigens through engineered bacteriolysis or antibiotic-mediated lysis (van Pijkeren et al. 2010; Sauer et al. 2010) are severely inhibited due to inflammasome activation. Thus, understanding and modulating inflammasome activation by *L. monocytogenes* is critical for its success as an immunotherapeutic platform.

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