Induction and Consequences of the Type I IFN Response to *Listeria monocytogenes*

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Introduction

Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium that causes foodborne illnesses in animals and humans. *L. monocytogenes* is the causative agent of listeriosis, a life-threatening systemic infection that primarily affects aged or immune compromised individuals and pregnant women. Clinical features of *L. monocytogenes* infection range in severity from gastroenteritis to septicemia and meningitis. When infecting pregnant individuals, *L. monocytogenes* also causes abortions, still births, and neonatal meningitis. The incidence of listeriosis is low, but the mortality rate is high. Hence, *L. monocytogenes* remains a leading cause of death from foodborne illness within the USA. For example, in 2011 a *L. monocytogenes* outbreak associated with cantaloupes infected 147 individuals with 33 deaths, for a mortality rate of 22 % [1].

L. monocytogenes gains entry into a wide variety of mammalian cells, both hematopoietic and non-hematopoietic, by phagocytosis or clathrin mediated uptake [2–4]. The bacterium usually does not replicate within phagosomes or vacuolar compartments but instead escapes these compartments to grow in the cell cytosol. A major bacterial virulence factor required for phagosomal escape is the pore-forming toxin listeriolysin O (LLO), encoded by the *hly* gene. LLO is secreted and active preferentially under acidic conditions found in maturing phagosomes, where

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it destroys the phagosomal membrane with additional contributions by two bacterial phospholipases [5]. The exact mechanism of phagosomal escape is still under debate. However, *L. monocytogenes* strains with mutation of *hly* or otherwise deficient in LLO are attenuated and fail to escape acidified phagosomes [6]. *L. monocytogenes* strains that invade into the cytosol trigger CD8+ T cell responses and long-lasting protective immunity, while LLO-deficient strains are poor at eliciting CD8+ T cell responses and protective immunity [7].

Following systemic infection of mice, *L. monocytogenes* primarily localizes to the liver and spleen. The bacteria are rapidly phagocytosed by resident macrophages and dendritic cells (DC) within these tissues. Some of the phagocytosed bacteria escape phagosomes and replicate within these cells. In response to *L. monocytogenes*, phagocytes produce pro-inflammatory cytokines such as TNFα and type I interferons (IFN). Type I IFNs have long been associated with effective anti-viral immunity, but their role during bacterial infections is less clear. During infections by *L. monocytogenes*, *Mycobacterium tuberculosis*, and several other bacteria type I IFN are detrimental to the host. A better understanding of how type I IFN responses are regulated during *L. monocytogenes* infection thus has potential impact for treatment of bacterial infections. Though much has been learned in this regard, the detailed mechanisms for induction of these cytokines (abbreviated IFN-α/β) are still being unraveled. The goal of this chapter is to summarize the current state of research in this area. We outline the pattern recognition receptors (PRRs) and signaling pathways involved in the production of type I IFNs during a *L. monocytogenes* infection and the biological effects their production has on the host. Pathways known to be important for induction of type I IFN within *L. monocytogenes*-infected phagocytes are diagrammed in Fig. 1.

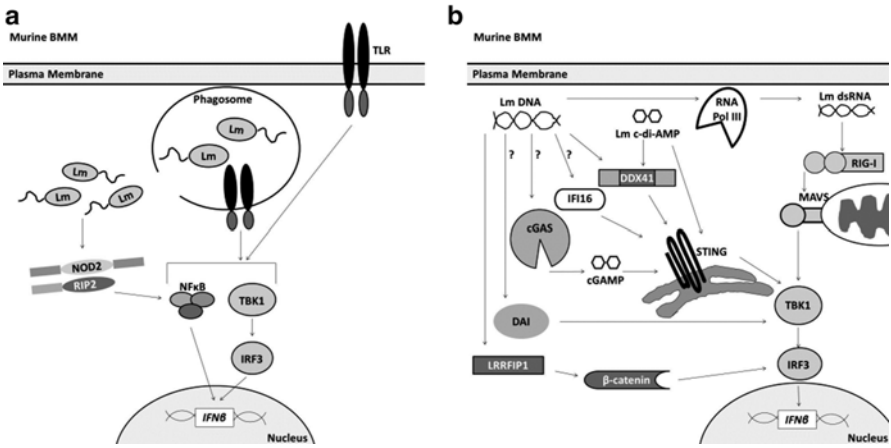

Fig. 1 Mammalian sensing of *L. monocytogenes* microbial components leading to induction of type I IFNs. (**a**) TLR and NOD pathways commonly recognize *L. monocytogenes* cell wall and envelope moieties. These pathways have not been shown to be required, but may augment IFN-β production. (**b**) Nucleic acid sensing pathways are known to induce type I IFNs by *L. monocytogenes* secretion of DNA, RNA, and cyclic di-nucleotides. While many of these pathways have been verified by direct recognition of *L. monocytogenes* nucleic acids, question marks (?) indicate potential but unconfirmed *L. monocytogenes* DNA sensors

IFN Regulatory Factor 3 Is Crucial for Type I IFN Responses During *L. monocytogenes* Infection

Members of the IFN regulatory factor (IRF) family of transcription factors regulate type I IFN production during viral infections and in response to other inflammatory stimuli. IRF3 in particular acts as an early factor regulating the type I IFN response. In resting cells, IRF3 is found in an inactivated state within the cytoplasm [8]. Phosphorylation on serine residues near the C-terminus of IRF3 enables it to dimerize and form complexes with CBP/p300, and to translocate to the nucleus where it can bind promoter regions of *Ifnb* and other genes. IRF3 thus helps initiate *Ifnb* transcription and subsequent secretion of IFN-β [8]. Once produced, IFN-β mediates autocrine and paracrine signaling through the IFN-α/β receptor (IFNAR). Such signaling activates transcriptional complexes involving STAT1, STAT2, IRF7, and IRF9. These complexes bind promoters to regulate expression of diverse interferon regulated genes (IRGs), including those encoding other type I IFNs (e.g. IFNα proteins). Thus, IRF3 activation directly or indirectly triggers production of multiple type I IFN proteins.

IRF3 is involved in IFN-β production during *L. monocytogenes* infection of macrophages. Specifically, infected murine bone marrow derived macrophages (BMDMs) showed significant nuclear localization of IRF3 at 4 h after infection [9]. Unlike wild-type BMMs, BMMs derived from IRF3-deficient mice also failed to induce expression of IFN-β upon infection by *L. monocytogenes* [9]. Studies with C57Bl/6ByJ mice also indicated an important role for IRF3 in the response to *L. monocytogenes*. BMDMs from this inbred sub strain of C57Bl/6 mice transcribed ~100-fold lower *Ifnb* mRNA upon *L. monocytogenes* infection [10]. Consistent with the reduced type I IFN response, these mice also showed significantly increased resistance to challenge with a lethal infection dose. The defect in type I IFN production mapped to a single A-T mutation found to be important for efficient splicing of *Irf3*. This mutation resulted in reduced IRF3 protein levels that correlated with the reduced type I IFN synthesis [10]. Subsequent studies from several other groups have independently confirmed the importance of IRF3 in the induction of type I IFNs by *L. monocytogenes* [9, 11–14].

TNFR-Associated NF-κB Kinase- Binding Kinase 1 (TBK1) is Crucial for Type I IFN Responses During *L. monocytogenes* Infection

The phosphorylation of IRF3 and stimulation of IFN-β production during viral infections or stimulation of cells with dsRNA requires two serine kinases, TNFR-associated NF-κB kinase (TANK)-binding kinase 1 (TBK1) and I-κB kinase ε (IKKε) [15–18]. TBK1 is an ubiquitously expressed member of the IKK protein kinase family that can associate with IKKε and TANK to regulate NF-κB activation

and expression of several proinflammatory cytokines [19]. Knockdown of either IKKε or TBK1 abolishes the production of IFN-β in response to dsRNA stimulation, suggesting a non-redundant role for these two kinases [16]. Evidence that TBK1 plays a role in IRF3 activation during a *L. monocytogenes* infection comes from experiments with infected murine embryonic fibroblasts (MEF) from TBK1 sufficient and deficient littermates. Unlike control MEFs, *Tbk1*$^{-/-}$ MEF showed no nuclear translocation of IRF3 and no production of IFN-β [11]. In contrast, infection-induced nuclear localization of the p65 NFκB subunit was not affected by TBK1 deficiency, suggesting a specific requirement for TBK1 in IRF3 activation [11]. Additional evidence that TBK1 promotes IRF3 nuclear translocation and type I IFN synthesis during an infection with *L. monocytogenes* comes from studies with BMDMs lacking both TBK1 and TNFR1. The double knockout cells were used as TBK1 deletion causes embryonic lethality in TNF-responsive mice. IFN-β production by the *Tbk1*$^{-/-}$*Tnfr1*$^{-/-}$ BMDMs was drastically, but not completely, reduced [11]. These results demonstrate that TBK1 is important but also argue there may be some functional overlap between TBK1 and IKKε in IRF3 activation during *L. monocytogenes* challenge [11, 16].

Toll-Like Receptors Recognize *L. monocytogenes* and in Some Situations May Contribute to Type I IFN Production

The Toll-like receptor (TLR) family of transmembrane receptors recognize molecular patterns associated with bacteria and viruses (PAMPs). Ligation of various TLRs by microbial products initiates signaling pathways involving NFκB, MAPK, and in several cases IRFs [20]. Thus, stimulation of TLRs can result in the production of proinflammatory cytokines and in some cases type I IFNs. The extracellular regions of TLRs contain leucine rich repeats (LRR) that mediate ligand binding, while their cytosolic regions contain Toll/IL-1 receptor (TIR) domains that interacts with other TIR containing adaptor proteins. Notably, TIR domains in TLRs recruit signaling adapters myeloid differentiation primary response gene 88 (MyD88) and/or TIR domain containing adapter inducing IFN-β (TRIF) [20]. This latter factor associates with TBK1 to ultimately stimulate IRF3 activation and IFN-β production.

Work with mouse cells has shown that several TLRs are capable of detecting *L. monocytogenes* products. In some cases, such recognition might conceivably contribute to the induction of type I IFNs. For example, TLRs 2, 3, and 4 have been shown to recruit TRIF to activate TBK1, IRF3, and production of IFN-α/β [18, 21]. TLR4 is best known as the receptor for lipopolysaccharide (LPS), which is produced exclusively by Gram-negative bacteria. However, TLR4 can also reportedly recognize lipoteichoic acids present in the cell envelope of *L. monocytogenes* and other Gram-positive bacteria [22]. Nevertheless, TLR4 expression was not required for nuclear translocation of IRF3 or type I IFN production by *L. monocytogenes-infected* BMMs [11]. TLR4 deficiency also failed to reduce IFN-β production by

L. monocytogenes-infected peritoneal macrophages [13]. TLR3 stimuli are well known to elicit type I IFN production. However, this TLR recognizes double-stranded RNA present in certain viral particles or produced during viral infections [23]. Thus, ligands for TLR3 are presumably rare during bacterial infections. Nonetheless, a study by Aubry et al. [13] reported that peritoneal macrophages lacking TLR3 produced significantly less IFN-β than wild-type cells when infected with *L. monocytogenes*. The nature of the *L. monocytogenes* ligand(s) recognized by TLR3 in this setting is unclear. One possibility is that TLR3 is activated due to an association with TLR2 [13]. TLR2 recognizes lipoproteins/lipopeptides commonly found in the peptidoglycan and lipoteichoic acid of bacterial cell walls and appears to be important for recognition of *L. monocytogenes* during in vivo infections, since mice lacking TLR2 or MyD88 show impaired resistance to *L. monocytogenes* [24–27]. Furthermore, signaling from internalized TLR2 has been shown to induce type I IFN production [28, 29]. One group reported detecting type I IFN production that was dependent on IRF1 and IRF7 (but independent of IRF3) in BMDMs stimulated with the synthetic TLR2 ligand diacylated lipopeptide Pam3CSK4 [29]. Conversely, Barbalat et al. [28] reported that stimulation of TLR2 in inflammatory monocytes induced type I IFNs in response to viral but not bacterial components. Consistent with this latter report, IRF3 nuclear localization and IFN-β production were not reduced in *Tlr2*$^{-/-}$ BMMs infected with *L. monocytogenes* [11]. The lack of a role for TLR2 in type I IFN production by *L. monocytogenes*-infected BMDMs was confirmed in the study by Aubry et al. [13]. Yet, these authors also reported that TLR2 deficiency significantly reduced type I IFN production by *L. monocytogenes* infected peritoneal macrophages. Resident peritoneal macrophages are more bactericidal than BMDMs. Thus, these studies suggest TLR2 signaling may augment type I IFN production by cell types that are capable of delaying phagosomal escape of and/or digesting phagocytosed *L. monocytogenes*. Consistent with a requirement for bacterial internalization, peritoneal macrophages pre-treated with Cytochalasin D to inhibit actin mobilization before *L. monocytogenes* infection produced very little type I IFNs [13]. However, preventing internalization of *L. monocytogenes* also prevents bacterial access to the host cell cytosol and subsequent replication and stimulation of cytosolic PRRs.

Evidence for Involvement of Cytosolic PRRs

In addition to cell surface and vacuolar TLRs, macrophages and other cells can recognize microbial products using cytosolic PRRs. Recognition of microbes by different PRRs may also elicit distinct cellular responses. In the context of *L. monocytogenes* infection, it was demonstrated that a gene expression profile observed during the "early phase" (1–2 h) of BMDMs infection by virulent wild-type *L. monocytogenes* strains was also seen upon treatment of the cells with killed bacteria or Δ*hly L. monocytogenes* mutants unable to escape from vacuole compartments into the host cell cytosol [14, 30]. Several upregulated "early phase" genes

(for example *Il1b*, *Tnfa*, and several chemokines) are known to be induced by TLR and NF-κB signaling pathways [14, 30], and were no longer or not as strongly induced upon infection of *MyD88*$^{-/-}$ macrophages [14]. These findings are consistent with involvement of TLR mediated pathways in the "early phase" of the macrophage response to killed or live bacteria. A distinct, "late-phase," response was also observed at 4–8 h after the infection with wild-type bacteria [14, 30]. However, this "late phase" gene expression profile was not observed after infection by killed or Δ*hly L. monocytogenes* strains [14, 30]. Hence, the late response appears to be indicative of infections where bacteria can access the cytosol and replicate within the macrophages. The "late phase" BMDMs genes included *Ifnb,* multiple subtypes of *Ifna*, and several additional IFN dependent genes [14, 30], and was almost entirely dependent on IRF3 activation [14]. These findings support the notion that the type I IFN response is elicited by cytosolic PRRs that are stimulated upon escape of phagocytosed wild-type *L. monocytogenes* from vacuolar compartments.

Nucleotide-Binding Oligomerization Domain-Containing (NOD) Proteins May Augment Type I IFN Responses to *L. monocytogenes*

The nucleotide-binding domain, LRR protein family referred to as NLRs includes several cytosolic and nuclear proteins. The NLR protein family has three distinct domain structures; a caspase recruitment domain (CARD) thought to regulate homotypic and heterotypic binding; a nucleotide binding domain (NBD) thought to be involved to self-oligomerization; and the LRR domain that is also thought to function in ligand binding [31]. Some LRR proteins have been shown to act as innate sensors in the detection of microbial products. For example, nucleotide-binding oligomerization domain-containing protein (NOD) 1 and NOD2 detect distinct muropeptide fragments derived from the cell wall of Gram-positive and/or Gram-negative bacteria [32]. Recognition of these fragments by NOD1 and NOD2 activates a serine/threonine kinase receptor interacting protein (RIP) 2 that is required for initiating downstream signaling and activation of NF-κB [33]. The *L. monocytogenes* cell wall contains moieties that are capable of recognition by both NOD1 and NOD2, and infection of BMDMs with *L. monocytogenes* elicits RIP2-dependent production of multiple pro-inflammatory cytokines [33–35]. However, deficiencies in NOD1, NOD2, or RIP2 do not completely ablate the cytokine response to *L. monocytogenes* indicating that this is not an essential recognition pathway [33]. Moreover, studies with RIP2 null or NOD2 null BMMs failed to reveal an essential role for these factors in mediating type I IFNs synthesis in response to *L. monocytogenes* [11, 12]. Thus, these NOD proteins do not appear to be essential for the type I IFN response elicited by replicating cytosolic *L. monocytogenes*.

However, there is some evidence that NOD proteins may, like TLRs, augment type I IFN production by *L. monocytogenes*-infected BMDMs. Specifically, while stimulation of BMDMs with synthetic MDP (the agonist for NOD2) alone elicited very little IFN-β production MDP treatment did increase IFN-β production in BMDMs transfected with *L. monocytogenes* genomic DNA by approximately twofold. The IFN-β produced in response to the DNA required expression of TBK1 and the enhancement by MDP required RIP2 [14]. To further evaluate the necessity for NOD2 in this response, BMMs were first tolerized by treatment with the TLR2 agonist, Pam3CSK4, then infected [14]. At 4 h post infection, tolerized NOD2-deficient BMMs had a twofold reduction in IFN-β synthesis compared to tolerized wild-type BMMs [14]. These findings suggest that NFκB signaling downstream of RIP2 enhances type I IFN production in *L. monocytogenes*-infected BMDMs.

Possible Contributions of RNA Helicases to the *L. monocytogenes*-Induced Type I IFN Response

During viral infections two cytosolic RNA helicases, retinoic acid inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), detect viral "patterned" RNA to initiate the interferon response [36]. Both RIG-I and MDA5 contain two CARD domains required for dimerization and adaptor protein association, plus a DExD/H-box RNA helicase domain that allow for dsRNA recognition [36, 37]. Once dsRNA is detected, RIG-I or MDA5 molecules dimerize and are recruited to the mitochondria where they encounter their adaptor protein, mitochondrial antiviral signaling (MAVS) [37]. MAVS links RIG-I and MDA5 signaling to TBK1, IRF3 phosphorylation, and IFN-β synthesis [37, 38]. RIG-I is required for the type I IFN response to several ssRNA viruses while MDA5 is required for detection of another viral group, usually involving longer pieces of dsRNA [36]. Additionally, RIG-I is able to induce IFN-β production in response to cytosolic DNA when it is transcribed into a dsRNA species within the cytosol by RNA polymerase III [39].

Soon after MAVS was found to be important for viral detection, investigators asked if this adapter protein might also be involved in the type I IFN response to cytosolic *L. monocytogenes*. Studies with BMDMs from knockout mice showed that MAVS was not required to produce wild-type amounts of IFN-β in response to *L. monocytogenes* [38]. Similar conclusions were reached in studies using siRNA knockdown of MAVS in the RAW 264.7 macrophage-like cell line [40]. These findings thus argued against an essential role for MDA5 or RIG-I in the interferon response to *L. monocytogenes*. However, when Abdullah et al. [41] more directly evaluated the effects of RIG-I and MDA5 during *L. monocytogenes* challenges they found that both reacted to cytosolic *L. monocytogenes*. They reported that IFN-β production was significantly reduced in the *RigI*$^{-/-}$ BMMs and modestly reduced in *Mda5*$^{-/-}$ BMMs. However, RIG-I deletion did not completely ablate IFN-β production [41]. Additional evidence suggested that *L. monocytogenes* may actively secrete

RNA [41]. Such secreted RNA (seRNA) may also interact with RIG-I differently than RNA isolated from *L. monocytogenes* lysates [41], as seRNA induced a stronger IFN-β response when transfected into macrophages [41]. Along with secreting RNA, *L. monocytogenes* was also reported to secrete DNA, which enhanced IFN-β production through an RNA polymerase III and RIG-I dependent mechanism. These studies also included experiments using a *L. monocytogenes* SecA2 mutant (ΔSecA2) *L. monocytogenes* strain. SecA2 is a key component of an auxiliary secretory system originally identified as a protein secretion system that contributes to bacterial pathogenesis [42]. Mutants lacking SecA2 still access the cytosol of infected BMMs but do not induce the same level of IFN-β production as wild-type *L. monocytogenes*, thus the authors concluded that the SecA2 secretion system may contribute to release of nucleotides involved in activating RNA helicase pathways [41]. However, the original studies with SecA2 showed that deficiency alters bacterial morphology, impairs bacterial cell–cell spread, and impairs secretion of several *L. monocytogenes* proteins, some with demonstrated roles in pathogenicity. Thus, it is possible that one or more of these other factors contributed to the observed reduction in type I IFNs. Recent work by Hagmann et al. [43] suggests that RIG-I may play a larger role in activating type I IFN production in non-immune cell types, but additional work is needed to confirm this.

Cytosolic DNA Sensors in the Interferon Response to *L. monocytogenes* Infection

Stetson and Medzhitov [44] were first to show that IFN-β production could be induced in BMMs by a DNAse-sensitive component of *L. monocytogenes* lysates. Upon further analysis, this recognition was independent of CpG motifs in the DNA that are required for TLR9 stimulation as well as MyD88 and RIP2 [44]. Rather, the response required the sugar-phosphate DNA backbone and IRF3. These results suggested the existence of a receptor capable of sequence-independent recognition of *L. monocytogenes* DNA. This spurred a hunt for cytosolic DNA sensors that activate TBK1/IRF3 to trigger type I IFN production.

DNA-dependent activator of IFN-regulatory factors (DAI) was discovered as a potential DNA sensor through a screen for IFN inducible genes that also contained DNA binding domains [45, 46]. DAI is localized to the cytoplasm and when overexpressed in cell lines can enhance type I IFN responses to DNA. Conversely, knockdown of DAI using RNAi inhibits IFN-β induction by DNA [45]. DAI was shown to directly bind dsDNA and promote association of TBK1 and IRF3 [45]. However, siRNA knockdown of DAI had no effect on IFN-β production by human cell lines infected with *L. monocytogenes* [47]. These findings argue that DAI is not essential for the type I IFN response to *L. monocytogenes* infection, though additional studies are needed to fully understand the role this protein plays in innate DNA sensing.

LRRFIP1 is a LRR domain containing protein originally discovered for its interaction with the mammalian homolog of the gelsolin family member, *Drosophila flightless I* [48, 49]. LRRFIP1 is localized to the cytoplasm of most cells and is also known to bind dsRNA and G-C rich dsDNA [48–50]. LRRFIP1 was identified in a screen for siRNAs that reduced IFN-β production by *L. monocytogenes* infected primary peritoneal macrophages. Knockdown of LRRFIP1 reduced IFN-β secretion from infected mouse peritoneal cells by greater than 50 %, while stable knockdown in RAW 264.7 cells suppressed *L. monocytogenes* induced *Ifnb* transcripts by almost 80 % [49]. LRRFIP1 appears to act as a co-stimulator of *Ifnb* transcription. The protein was shown to interact with β-catenin to enhance its ability to bind IRF3 and recruit p300 for acetylation of histones at the *Ifnb* promoter [49]. Type I IFN production in response to *L. monocytogenes* infection was also shown to be significantly reduced in primary peritoneal macrophages deficient for β-catenin [49]. These data suggest a mechanism by which *L. monocytogenes* nucleic acids can activate LRRFIP1 to enhance *Ifnb* transcription. However, depletion of both LRRFIP1 and β-catenin failed to completely impair the type I IFN response [49].

Absent in melanoma 2 (AIM2) is another cytosolic DNA sensor. DNA binding to AIM2 causes formation of a complex called the AIM2 inflammasome, which activates caspase 1 to cleave and activate inflammatory cytokines including IL-18 and IL-1β. *L. monocytogenes* infection activates the AIM2 inflammasome, but AIM2 stimulation has not been shown to impact production of type I interferons [51–53]. In contrast, the IFI16 protein both interacts with cytosolic viral DNA and regulates production of IFN-β in both macrophages and MEFs [54, 55]. Binding of *L. monocytogenes* DNA to IFI16 has not been shown to occur, nor is it yet published whether IFI16 impacts type I responses during *L. monocytogenes* infection.

STING-Dependent Sensing of DNA or Cyclic Dinucleotides Regulates the Interferon Response to *L. monocytogenes* Infection

Stimulator of interferon genes (STING), also called MITA, MPYS, or ERIS, is an evolutionarily conserved protein that contains five transmembrane regions and is localized in the endoplasmic reticulum [56–59]. The involvement of STING in type I IFN responses was first discovered in a screen where full length cDNA expression vectors were transfected into 293T cells containing a luciferase construct driven by the IFN-β promoter [56, 57]. Over-expression of STING increased IRF3 activation and IFN-β production in response to viral challenges [56, 57, 59]. RNAi knockdown or a direct knockout of STING resulted in a decreased activation of IRF3 and decreased IFN-β production, ultimately leading to increased viral susceptibility [56, 57, 59]. In fact, STING expression levels correlated with the degree of inhibited viral replication [57]. Upon viral infection, STING dimerizes and directly interacts with TBK1 in immunoprecipitation experiments [56, 57, 59]. STING also enhances

interaction of TBK1 and IRF3 and both of these factors are required for STING-induced type I IFN production [56, 57, 59]. To identify stimuli leading to STING-dependent induction of IFN-β, MEFs derived from wild-type and STING$^{-/-}$ mice were transfected with various DNA ligands. STING expression enhanced IFN-β synthesis in response to cytosolic delivery of both viral and bacterial DNA, as well as synthetic non-CpG dsDNA, but not dsRNA [60]. Macrophages and dendritic cells isolated from *Sting*$^{-/-}$ mice also demonstrated significantly reduced or undetectable levels of IFN-I when transfected with synthetic DNA or infected with *L. monocytogenes* [60–62].

STING does not appear to be a direct sensor of DNA. Rather, cyclic di-nucleotides—which act as second messengers in a number of bacterial species—are able to induce type I IFN production in a STING-dependent manner [61–63]. STING binds radiolabeled cyclic diguanylate monophosphate (c-di-GMP) in a manner competed by unlabeled cyclic dinucleotides but not other nucleic acids such as dsDNA [63]. Another study found that biotinylated c-di-GMP and c-di-AMP also bound to the DEAD-box helicase, DDX41, with a higher affinity than to STING [64]. Unlike STING, DDX41 also bound cytosolic DNA. Mouse or human cells deficient for DDX41 also showed decreased IFN-β responses to *L. monocytogenes* infection or cytosolic delivery of c-di-AMP and c-di-GMP [64]. Yet, STING was still required for type I interferon production to these stimuli as well as synthetic dsDNA and DNA viruses [65]. Since DDX41 also binds to STING, it may act as a co-factor to regulate STING-dependent type I IFN responses [64, 65].

Evidence suggests that cyclic di-nucleotides are actively released from replicating *L. monocytogenes* [66]. The release of c-di-AMP from *L. monocytogenes* appears to be mediated by a family of multidrug efflux transporters (MDRs) [66]. *L. monocytogenes* strains containing increased or reduced expression of MDRs such as MdrM show corresponding increases and reductions in their ability to elicit IFN-β production by infected BMDMs [66]. *L. monocytogenes* production of c-di-AMP requires a diadenylate cyclase (DacA), which is required for establishment and optimal growth within mammalian cells, as well as the overall stability of its bacterial cell wall [67]. Strains deficient in DacA are significantly attenuated during infections of mice, yet still induce type I IFN production [67]. The residual activation of type I IFNs could reflect the release of c-di-GMP other cyclic di-nucleotides that activate STING, or the release of DNA or RNA. Knockdown of STING in RAW 264.7 cells and BMMs derived from a *Sting*$^{-/-}$ mouse significantly decreased IRF3 activation and IFN-β production in response to *L. monocytogenes* infection or cytosolic delivery of c-di-AMP and c-di-GMP [61]. During systemic *L. monocytogenes* infection in mice, STING deficiency also impacted early production of type I interferons as *Sting*$^{-/-}$ mice had significantly reduced IFN-β in the sera 8 h post infection [61]. Similar results were independently observed using an *N*-Ethyl-*N*-Nitrosourea (ENU) generated mouse with a loss of function mutation in STING [62]. These data indicate the importance of STING in the initial type I interferon response to cytosolic *L. monocytogenes* and suggest this could be due to bacterial release of cyclic di-nucleotides. It is also possible that bacterial DNA released into the cytosol could contribute to this STING-dependent response. It was recently shown that cytosolic

or viral DNA can be processed into a "non-canonical" 2′–5′ linked cyclic dinucleotide, cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) by an enzyme named cGAMP synthase (cGAS) [68, 69]. This contrasts with the canonical 3′–5′ linkage seen in the cyclic di-nucleotides produced by bacteria. Like bacterial cyclic di-nucleotides, cGAMP binds STING and does so in a manner competed by high concentrations of unlabeled c-di-GMP, c-di-AMP, and cGAMP, but not by DNA [68]. Furthermore, over-expression of cGAS induced IFN-β production that was dependent on STING expression and knockdown of cGAS significantly reduced IRF3 activation and *Ifnb* transcription in response to DNA [69]. Whether cGAS might also play a role in the type I interferon response by macrophages or other cell types infected with *L. monocytogenes* is not yet known.

Biological Consequences of Type I IFN Production

Type I interferons bind a common cell surface receptor to alter gene expression in a manner that induces an antiviral state that increases cell intrinsic resistance to viral replication. Thus, production and response to these interferons increases host resistance to numerous viral infections. The opposite occurs during infections by *L. monocytogenes* and several other bacteria, where responsiveness to type I IFNs is actually detrimental to the host [9, 70–72]. Mice deficient in IFNAR and IRF3 are also significantly more resistant to *L. monocytogenes* challenge [9, 70]. In wild-type mice, treatment with the type I interferon-inducing synthetic dsRNA agonist poly:IC also significantly increased *L. monocytogenes* titres in both the livers and spleens [9]. These results indicate that type I IFN production and responsiveness exacerbate *L. monocytogenes* pathogenicity. However, although STING-deficient mice have reduced production of IFN-β early after *L. monocytogenes* infection, they were not more resistant to *L. monocytogenes* and showed similar bacterial burdens in the both the livers and spleens compared to STING sufficient mice [61]. These results suggest that the lack of IFN production very early after infection is not sufficient to increase host resistance and also that redundancy exists in the pathways required for *L. monocytogenes* induced type I IFN during systemic infection.

Multiple mechanisms have been proposed to account for the deleterious effects of type I IFNs during *L. monocytogenes* challenge. O'Connell et al. [9] observed that type I IFN signaling increased the expression of several pro-apoptotic genes such as TRAIL, PML, and Daxx. Additionally, more macrophages and inflammatory monocytes were found in the spleens of *L. monocytogenes* infected *Ifnar*$^{-/-}$ mice compared to wild type [9]. These results suggested to the authors that type I IFNs may be deleterious because they induce apoptosis of monocytes within the spleens. Another group observed decreased terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining in the spleens of *Ifnar*$^{-/-}$ mice after 2–3 days of *L. monocytogenes* infection [73]. These authors concluded that the apoptotic cells were lymphocytes and not monocytes, and thus that IFN-induced apoptosis of lymphocytes was deleterious to the host [73]. In contrast, Auerbuch

et al. [70] reported increased numbers of splenic CD11b$^+$ cells secreting the pro-inflammatory cytokine, TNFα, within the spleens of *L. monocytogenes* infected *Ifnar*$^{-/-}$ mice, leading them to suggest type I IFN signaling suppresses accumulation of TNFα producing monocytes that might protect against *L. monocytogenes* infection [70].

In contrast to type I IFNs, the type II IFN or IFNγ is critical for the pro-inflammatory activation of macrophages. IFNγ enhances macrophage ability to kill bacteria, increases their secretion of pro-inflammatory cytokines such as TNFα and IL-12, and increases expression of MHC class II and co-stimulatory molecules [74]. IFNγ signals through a heterodimeric receptor IFN gamma receptor (IFNGR). During a *L. monocytogenes* infection, it was observed that the IFNGR was selectively down regulated from the surface of myeloid cells, but not T cells [71, 75]. This phenomenon was observed both in vivo and in vitro upon challenge with *L. monocytogenes* and was mediated by type I IFNs [71, 75]. BMDMs derived from wild-type mice also decrease surface expression of IFNGR upon stimulation with IFN-β [71, 75]. The suppression of the IFNGR receptor decreased the responsiveness of the myeloid cells to IFNγ, potentially suppressing pro-inflammatory activation of macrophages and decreasing their ability to clear bacterial infections [71]. This thus represents an additional potential mechanism to account for the ability of type I IFNs to increase host susceptibility to bacterial infections.

Mechanistically, down regulation of the IFNGR involves transcriptional silencing by type I IFNs [71, 75]. Kearney et al. [75] demonstrated that IFN-β stimulation silences new transcription at the *ifngr* locus in macrophages, as indicated by loss of activated RNA polymerase II at the transcriptional start site as well as epigenetic marks indicative of condensed chromatin. Additionally, recruitment of early growth response factor 3 (Egr3) to the *ifngr* promoter was observed shortly after IFN-β treatment [75]. Egr3 can act as a activator or repressor of transcription [76–79]. Association of Egr proteins with the NGFI-A binding protein, Nab1, causes transcriptional silencing and Nab1 was recruited to the *ifngr* promoter shortly after Egr3 [75]. Knockdown of Nab1 in mouse RAW 264.7 macrophages prevented IFNGR down regulation in response to type I IFN stimulation [75]. These data provide evidence of a direct antagonistic effect between type I and type II IFNs in myeloid cells and suggest this antagonism lowers myeloid cell responsiveness to IFNγ and thus host resistance. However, there is not yet direct evidence to support whether one of these possible mechanisms is responsible for the increased bacterial burdens in response to type I IFNs.

Conclusions

Sensing of microbial products is important for host defense against pathogens. Yet, sensing of *L. monocytogenes* and other bacterial pathogens appears to be deleterious to the host when this leads to the production of type I IFNs. *L. monocytogenes* may thus promote such sensing as there is evidence it actively secretes RNA, DNA, and

cyclic di-nucleotides that are recognized by cytosolic PRRs including RIG-I, STING, DDX41, IFI16, and cGAS. STING expression is most critical for the induction of IFN-I in cultured macrophages, but whether this is through a direct interaction with *L. monocytogenes* c-di-AMP is uncertain. However, mice lacking STING still produce type I IFNs in response to *L. monocytogenes* infection, highlighting the redundancy in these pathways mediating detection of pathogen-derived molecules and triggering of IFN-β production. The creation of double and triple knockout mice would provide a valuable tool to further dissect which sensing pathways are most crucial for *L. monocytogenes* sensing in vivo. Further understanding of how type I IFNs are triggered, and the effects they have on host biology, is essential for improving our knowledge of and ability to improve host resistance to bacterial infections.

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