



Innate immunity to *Legionella pneumophila*

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Innate immune cells, such as macrophages, are highly adapted to rapidly recognize infections by distinct pathogens, including viruses, bacteria, fungi, and protozoa. This recognition is mediated by pattern recognition receptors (PRRs), which are found in host cell surface membranes and the host cell cytoplasm. PRRs include protein families such as the toll-like receptors, nod-like receptors, RIG-I-like receptors, and sensors of cytosolic DNA. The activation of these PRRs by pathogen-associated molecular patterns leads to transcriptional responses and specific forms of cell death. These processes effectively contribute to host resistance to infection either via cell-autonomous processes that lead to the intracellular restriction of microbial replication and/or by activating pathogen-specific adaptive immune responses. *Legionella pneumophila*, the causative agent of Legionnaires' disease, is a Gram-negative bacterium that triggers responses by multiple PRRs. Here, we review a set of studies that have contributed to our specific understanding of the molecular mechanisms by which innate immune cells recognize and respond to *L. pneumophila* and the importance of these processes to the outcome of infection.

Keywords: *Legionella pneumophila*, innate immunity, pattern recognition receptors, nod-like receptors

INTRODUCTION

Activation of innate immune cells is critical for the initiation of adaptive immune responses. This process relies mostly on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Among the canonical PAMPs are molecules such as lipopolysaccharide, peptidoglycan, bacterial lipoproteins, flagellin, and nucleic acids derived from viruses, bacteria, fungi, and protozoa (Janeway and Medzhitov, 2002; Akira et al., 2006; Gazzinelli and Denkers, 2006). Upon direct or indirect ligand recognition, toll-like receptors (TLRs) dimerize and trigger a signaling cascade leading to the activation of proinflammatory responses (Uematsu and Akira, 2006). TLRs are transmembrane proteins containing an extracellular leucine-rich repeat (LRR) domain that facilitates PAMP recognition and an intracellular domain that mediates intracellular signaling via four different adaptor proteins: TRAM, MAL/TIRAP, MyD88, and TRIF (O'Neill, 2008). Depending on the nature of their specific ligands, TLRs are embedded in either the extracellular membrane (TLR-1, -2, -4, -5, -6, -10, -11) or in the membranes of endocytic vacuoles (TLR-3, -7, -8, -9). In addition to TLRs, other PRR families have already been described; these include DNA/RNA-sensing proteins such as the RIG-I-like receptor (RLR) family and sensors of membrane damage and intracellular PAMPs such as the nod-like receptors (NLRs; Creagh and O'Neill, 2006).

The RNA helicase domain-containing proteins retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) comprise a group of cytoplasmic receptors important for the recognition of viral nucleic acids. PAMP recognition by RIG-I and MDA5 triggers the activation of IRF3 via MAVS (IPS-1), culminating in the production of type I interferons (IFN). Recent studies have demonstrated that these receptors also induce type I IFN production upon recognition of nucleic acids from intracellular bacteria (Cao, 2009). In addition, some studies have

shown that the recognition of DNA by RIG-I/MDA5 is dependent on cytosolic RNA polymerase III (Pol III; Ablasser et al., 2009; Chiu et al., 2009).

The NLRs comprise a PRR family that can be classified into three sub-groups. The first sub-group is composed of receptors that trigger intracellular signaling pathways leading to the activation of transcriptional factors mediating the expression of inflammatory response genes. Both NOD1 and NOD2 are members of this first group, and they signal via RIP2, a kinase that ubiquitinates NEMO to induce the activation of NF- κ B and MAPK (Shaw et al., 2008). The second sub-group comprises NLRs that do not require ASC to trigger caspase-1 activation. Among these proteins are NAIP5 (BIRC1e) and NLRC4 (IPAF), which have been suggested to assemble a unique inflammasome (hereafter referred to as the NLRC4 inflammasome). Activation of this inflammasome triggers a specific form of host cell death called pyroptosis (Lightfield et al., 2008; Case et al., 2009; Broz et al., 2010; Silveira and Zamboni, 2010; Whitfield et al., 2010). The third sub-group of NLRs comprises those that trigger caspase-1 activation via the adaptor protein ASC. These proteins assemble into a multimeric molecular platform known as the "classical" inflammasome. Among the NLRs that trigger the ASC-dependent inflammasome is NALP3, which has been extensively characterized and shown to be important for the recognition of danger-associated molecular patterns (DAMPs) reviewed by Schroder and Tschopp (2010).

In addition to TLRs, NLRs, and RLRs, previous studies have described a class of proteins that recognize cytoplasmic DNA (Ishii and Akira, 2006; Stetson and Medzhitov, 2006). These multiple protein families include DNA-dependent activators of IFN-regulatory factors (DAI; Takaoka et al., 2007), RNA polymerase III, which induces type I IFN production through the RIG-I pathway (Ablasser et al., 2009; Chiu et al., 2009), and the recently described protein absent in melanoma (AIM2), which activates

inflammasomes in an ASC-dependent manner (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).

Legionella pneumophila, a Gram-negative bacterial pathogen that evolved infecting unicellular protozoa in freshwater reservoirs, may not have encountered strong selective pressure to avoid recognition by mammalian PRRs. Consequently, *L. pneumophila* triggers multiple PRR and has been a useful model for understanding the biology of PRRs and the induction of appropriate adaptive immune responses against intracellular pathogens. The successful use of *L. pneumophila* as a tool for studying immunology has been reviewed elsewhere (Vance, 2010). Here, we will review the salient findings that have contributed to our understanding of the molecular mechanisms underlying innate immune cell recognition and response to *L. pneumophila* infection (Figure 1). Furthermore, we will summarize studies that have elucidated the importance of these processes to the outcome of *L. pneumophila* infection.

TOLL-LIKE RECEPTORS

It was originally speculated that TLR4, a general LPS sensor, would recognize *L. pneumophila*. However, work on non-enterobacteriaceae species has indicated that although TLR4/MD2 very efficiently recognizes enterobacterial lipid A, the same is not true for other Gram-negative bacteria. These non-enterobacteriaceae bacterial species often express lipid A containing long fatty acid chains that either fail to trigger TLR4 activation or antagonize the TLR4 receptor (Zamboni et al., 2004). In *L. pneumophila*, studies performed with TLR4-deficient or TLR4 knockout mice have confirmed that this receptor does not effectively participate in the recognition of *L. pneumophila* LPS. Even at high MOIs, there is no difference in *L. pneumophila* infection between wild-type and C3H/HeJ mice, which are defective for TLR4 signaling due to a missense mutation in the *Tlr4* gene resulting in the replacement of a proline with a histidine at position 712 (Poltorak et al., 1998; Lettinga et al., 2002). The initial studies on TLR4 function using C3H/HeJ mice were further corroborated in *tlr4*^{-/-} mouse experiments, which supported the hypothesis that TLR4 deficiency does not influence the outcome of *L. pneumophila* infection (Akamine et al., 2005; Archer and Roy, 2006; Fuse et al., 2007). Studies by Girard et al. (2003) have shown that lipid A of *L. pneumophila* signals via TLR2 to induce the expression of CD14. These findings led to the suggestion that *L. pneumophila* LPS is recognized by TLR2, but the mechanisms underlying the recognition of lipid A by TLR2 have not been completely elucidated; some researchers have speculated that lipid A-mediated TLR2 activation requires either a long chain fatty acid or the presence of a substituent or a branch on the penultimate carbon of a fatty acid chain (Brandenburg et al., 1993). Nonetheless, future studies using a synthetic form of *L. pneumophila* lipid A may be required to unequivocally confirm that *L. pneumophila* LPS is a bona fide agonist of TLR2.

Regardless of the proposed role of TLR2 in LPS recognition, other *L. pneumophila* PAMPs, such as lipopeptides and lipoproteins, are sufficient to activate TLR2. Activation of this receptor is critical to the outcome of *L. pneumophila* infection in mice. This was unequivocally demonstrated by experiments using *tlr2*^{-/-} mice, which show impaired cytokine production and are more susceptible to bacterial multiplication in the lungs (Akamine et al., 2005; Archer and Roy, 2006; Hawn et al., 2006).

In addition to TLR2, other TLRs are also important for the host response to *L. pneumophila*. As a flagellated bacteria, *L. pneumophila* is recognized by TLR5, and a common polymorphism in the ligand-binding domain of TLR5 causes increased susceptibility to Legionnaires' disease in humans (Hawn et al., 2003). These data have been corroborated by studies using *tlr5*^{-/-} mice showing that TLR5 recognition of *L. pneumophila* *in vivo* contributes to the recruitment of leukocytes to the pulmonary cavity (Hawn et al., 2007). However, TLR5 deficiency by itself does not render mice more susceptible to infection as measured by CFU counts and cytokine production (Hawn et al., 2007; Archer et al., 2009).

Another TLR important in *L. pneumophila* infection is TLR9. Mice lacking this receptor exhibit reduced levels of cytokines when challenged with *L. pneumophila* and are therefore more permissive of *L. pneumophila* replication in the lungs (Newton et al., 2007; Archer et al., 2009). This observation was corroborated by experiments involving the *in vivo* administration of CpG oligodeoxynucleotide, a synthetic agonist of TLR9, which protected mice that were pre-infected with *L. pneumophila* (Bhan et al., 2008).

Importantly, these studies using mice deficient for a single TLR indicate that disruption of a single *tlr* gene does not result in a striking susceptibility to *L. pneumophila*; this is possibly due to redundancy in the signaling pathways triggered by these receptors. In contrast, the deletion of the common adaptor protein MyD88, which is important for the signaling of several TLRs, produces mice that are highly susceptible to infection. Mice deficient for MyD88 show impaired cytokine production in response to pulmonary infection with *L. pneumophila*; they also show high numbers of CFUs in the lungs and succumb to *L. pneumophila* infection even at low multiplicities of infection (Neild et al., 2005; Archer and Roy, 2006; Hawn et al., 2006; Sporri et al., 2006; Archer et al., 2009, 2010). The increased susceptibility of *myd88*^{-/-} mice suggests that the deletion of multiple TLR genes will produce mice as susceptible to *L. pneumophila* infection as those lacking *myd88*^{-/-}. To test this hypothesis, Archer et al. (2009) constructed mice deficient for multiple TLRs and showed that mice lacking both TLR5 and TLR9 or deficient for TLR2 and either TLR5 or TLR9 are still able to clear *L. pneumophila* infection. Archer and colleagues elegantly concluded that IL-18 signaling via MyD88 is essential for NK cell production of IFN- γ , a cytokine critical for the restriction of *L. pneumophila* infection *in vivo* (Archer et al., 2009). Interestingly, although the authors showed that NK cells signal via IL-18 to produce IFN- γ , they also demonstrated that mice deficient for the IL-18 receptor are no more susceptible to *L. pneumophila* infection than wild-type animals (Archer et al., 2009). Additional studies will therefore be required to further determine the importance of this pathway *in vivo* and its redundancy with other pathways.

NOD-LIKE RECEPTORS: NOD1 AND NOD2

The first study addressing NOD1 and NOD2 signaling in response to *L. pneumophila* infection was performed by Shin et al. (2008). In this study, the authors evaluated the transcriptional responses of macrophages infected with wild-type and *dotA* mutants of *L. pneumophila* to identify macrophage genes induced in a Dot/Icm-dependent manner. By comparing macrophages deficient for MyD88 and RIP2 kinase, which impairs both NOD1 and NOD2 signaling, or lacking both MyD88 and TRIF, the authors identified

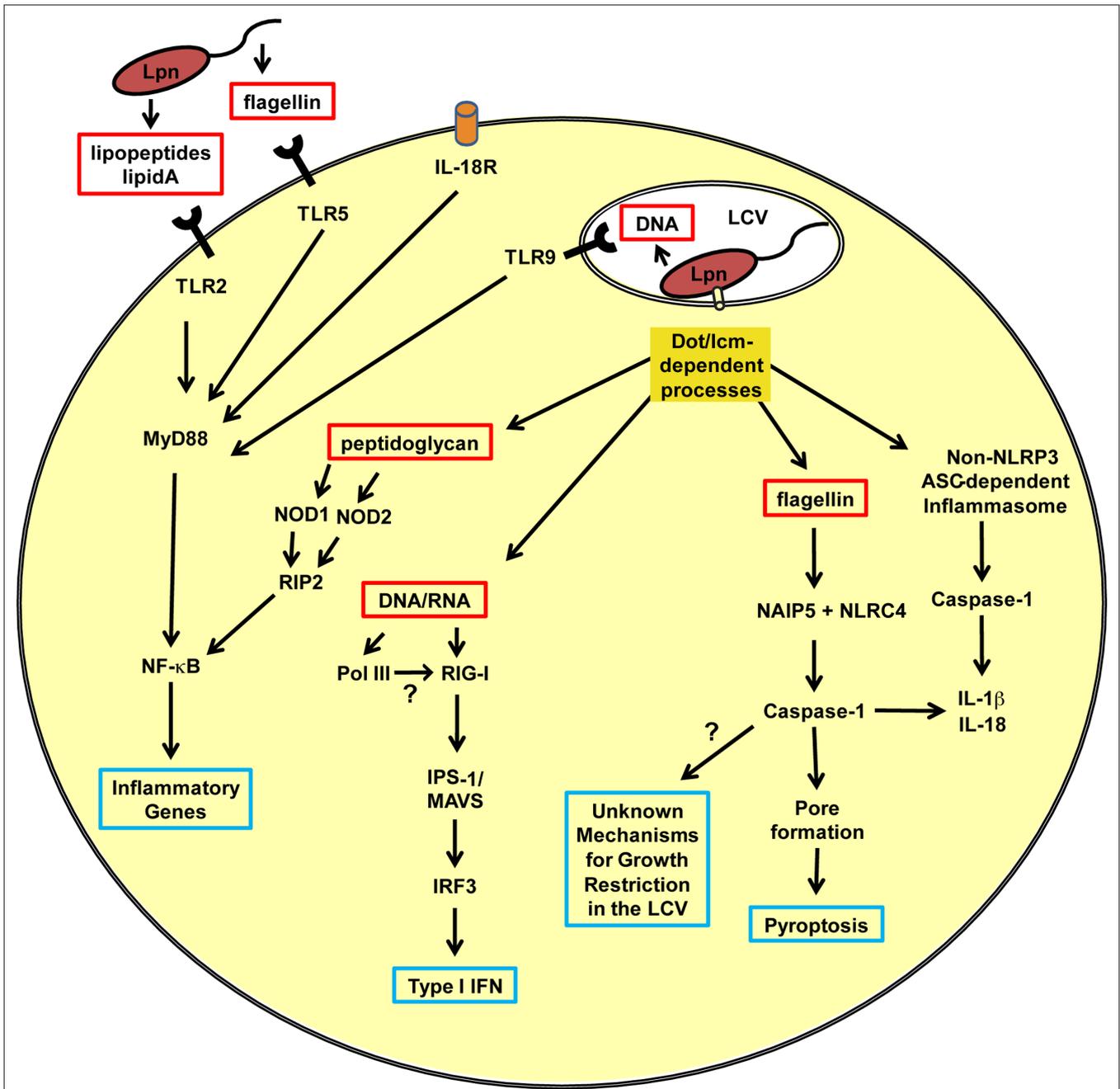


FIGURE 1 | Innate immune responses of a mammalian phagocyte infected with *Legionella pneumophila*. A schematic representation of the pathways activated in a phagocyte after infection with *L. pneumophila*. The red boxes indicate *L. pneumophila*-associated molecular patterns important for the activation of pattern recognition receptors. Blue boxes indicate molecules or processes involved in the cell-autonomous restriction of *L. pneumophila* replication. LCV, *Legionella*-containing vacuole; Lpn, *L. pneumophila*; Dot/Icm, type IVB secretion system; IL,

interleukin; IL-18R, IL18 receptor; TLR, toll-like receptor; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa B; NOD, nucleotide-binding oligomerization domain-containing protein 2; Pol III, RNA polymerase III; RIG-I, retinoic-acid-inducible protein I; IPS-1, IFN-β promoter stimulator 1 (also known as MAVS, mitochondrial antiviral signaling); IRF3, interferon regulatory factor 3; NAIP5, neuronal apoptosis inhibitory protein 5; NLRC4, NLR family CARD domain-containing protein 4.

several genes that were regulated by a RIP2-dependent pathway (Shin et al., 2008). Importantly, this study revealed that multiple responses occur after *L. pneumophila* infection of macrophages; some of these were dependent on MyD88, others on RIP2 and some responses that were induced via unknown sensors were independent

of both MyD88 and RIP2 (Shin et al., 2008). This study was further corroborated by *in vivo* experiments using mice deficient for both RIP2 and MyD88. The *rip2^{-/-}/myd88^{-/-}* mice were significantly more susceptible to *L. pneumophila* than the *myd88^{-/-}* mice, and they succumbed to infection even at low MOIs (Archer et al., 2010).

Importantly, this and another study demonstrated that although a RIP2-dependent response was not critical for restricting *L. pneumophila* infection *in vivo*, a RIP2-dependent response did contribute to the recruitment of phagocytes to the sites of infection (Archer et al., 2010; Frutuoso et al., 2010). Notably, the RIP2-dependent responses that contributed to the recruitment of neutrophils to the lungs of infected mice were at least partially dependent on the NOD1 and NOD2 receptors (Berrington et al., 2010; Frutuoso et al., 2010). These studies confirmed that NOD1 and NOD2 effectively participate in the pulmonary detection of *L. pneumophila* infection, but NOD1 and NOD2 deficiency results only in a minor attenuation of bacterial growth restriction in mouse lungs (Berrington et al., 2010; Frutuoso et al., 2010). Importantly, these studies of *L. pneumophila* infection in mice deficient for TLRs and the NOD/RIP2 pathway demonstrate the substantial redundancy of innate immune receptors in the host response to bacterial infection.

NOD-LIKE RECEPTORS: NLRC4 AND NAIP5

Approximately 30 years ago, it was demonstrated that macrophages from A/J mice fail to restrict the intracellular replication of *L. pneumophila* (Yamamoto et al., 1988). These phenotypic differences between A/J and other mouse strains provided a useful model for investigating the genes responsible for the phenotypic variations. In later years, the genomic region responsible for *L. pneumophila* resistance was mapped to the autosomal recessive locus *Lgn1* on chromosome 13 (Beckers et al., 1995; Dietrich et al., 1995). In early 2003, it was finally revealed that the susceptibility gene within the *Lgn1* locus was NAIP5 (also known as BIRC1e), a member of the NLR proteins family (Diez et al., 2003; Wright et al., 2003). The mechanisms by which NAIP5 contributes to the host control of infection was unraveled a few years later by the discovery that NAIP5 interfered with caspase-1 activation in response to macrophage infection by *L. pneumophila* (Zamboni et al., 2006). This response is dependent on the Dot/Icm system and effectively contributes to the restriction of bacterial replication in macrophages *in vitro* and *in vivo* (Zamboni et al., 2006). The NAIP5-dependent restriction of *L. pneumophila* growth required another NLR protein called NLRC4 (IPAF), which contributes for caspase-1 activation upon *L. pneumophila* infection (Amer et al., 2006; Molofsky et al., 2006; Zamboni et al., 2006). An elegant screening experiment identified the putative agonist of this NAIP5/NLRC4 inflammasome: *L. pneumophila* deficient for the flagellin gene *flaA* bypassed the NLRC4 inflammasome and replicated in macrophages harboring the restrictive *Lgn1* allele (Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). Although the role of NAIP5 in caspase-1 activation was questioned (Lamkanfi et al., 2007), assays with *naip5*^{-/-} mice unequivocally demonstrated the requirement of NAIP5 for caspase-1 activation in response to *L. pneumophila* flagellin (Lightfield et al., 2008). Furthermore, both NLRC4 and NAIP5 were required for the detection of a carboxy-terminal domain of flagellin, a region not required for TLR5 activation (Lightfield et al., 2008). The same group has recently demonstrated that the N-terminus of *L. pneumophila* flagellin relieves the requirement for NAIP5 during activation of the NLRC4 inflammasome, which suggests that NAIP5 regulates the specificity of the NLRC4 inflammasome for certain species of bacteria (Lightfield et al., 2011). These data explain why for some species, such as *L. pneumophila*, the activation

of the NLRC4 inflammasome requires NAIP5, whereas for other species, such as *Salmonella enterica* serovar Typhimurium, NAIP5 is dispensable. Strikingly, activating this NLRC4/NAIP5 inflammasome requires a functional Dot/Icm type IV secretion system. This finding led to the speculation that flagellin may leak from the *Legionella* cell to the macrophage cytoplasm through the Dot/Icm. However, this hypothesis has not yet been experimentally validated.

The mechanisms by which the NLRC4 inflammasome restricts *L. pneumophila* replication remains incompletely understood. Activation of these receptors triggers a caspase-1-dependent pore formation in macrophage membranes and leads to a specific form of cell death called pyroptosis (Derre and Isberg, 2004; Case et al., 2009; Silveira and Zamboni, 2010; Whitfield et al., 2010). However, the activation of NAIP5 and NLRC4 also facilitates a process independent of pyroptosis that culminates with the restriction of *L. pneumophila* multiplication within the replicative vacuole occupied by the bacteria (Swanson and Molofsky, 2005; Amer et al., 2006; Fortier et al., 2007). These processes are possibly dependent on the transcriptional regulation of macrophage genes. This hypothesis has been supported by the demonstration that NAIP5 recognition of *L. pneumophila* triggers IRF1- and IRF8-mediated upregulation of genes important for macrophage resistance to bacterial infection (Fortier et al., 2009). The inducible nitric oxide synthase (NOS2) gene is a possible candidate, as the NAIP5- and caspase-1-dependent induction of NOS2 expression and nitric oxide production has been observed in macrophages transfected with flagellin (Buzzo et al., 2010).

ASC AND THE NLRP3-INDEPENDENT INFLAMMASOME

The adaptor protein called apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC/PYCARD) is a small molecule composed of a PYRIN and a CARD domain. ASC bridges caspase-1 to PYRIN-containing molecules, such as NALP/NLRP family members, via CARD/CARD interactions (Mariathasan et al., 2004). Studies performed with macrophages from ASC-deficient mice have indicated that this molecule is important for the secretion of IL-1 β in response to infection but is not required for controlling *L. pneumophila* replication in C57BL/6 macrophages (Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). This finding led to the proposition that ASC may participate in the NLRC4-dependent activation of caspase-1 in response to *L. pneumophila* (Case et al., 2009; Pedra et al., 2009). However, subsequent studies demonstrated that *L. pneumophila* triggers at least two different inflammasomes: one dependent on NLRC4, NAIP5, and flagellin; and another dependent on ASC and independent of NLRP3 (Sutterwala et al., 2006; Case et al., 2009). Although ASC is dispensable for restricting *L. pneumophila* replication in mice and murine macrophages, a recent report demonstrated that ASC does contribute to the control of *L. pneumophila* infection in human monocytes (Abdelaziz et al., 2010). The proteins participating in this ASC-dependent inflammasome and the molecular signals that trigger its activation have not yet been elucidated.

RIG-I-LIKE RECEPTOR AND INDUCTION OF TYPE I INTERFERON

Several studies have reported the production of type I IFN in response to *L. pneumophila* infection (Opitz et al., 2006; Stetson and Medzhitov, 2006; Lippmann et al., 2008; Chiu et al., 2009;

Monroe et al., 2009), and these experiments have contributed to our understanding of the host cell pathways responsible for type I IFN induction. Although RIG-I and MDA5 were initially reported as sensors of viral infection, recent studies indicate that RLRs are also important for the host cell recognition and response to bacterial infection, and both RIG-I and MDA5 have been implicated in type I IFN production by macrophages in response to *L. pneumophila* infection (Opitz et al., 2006; Stetson and Medzhitov, 2006; Ablasser et al., 2009; Monroe et al., 2009). The production of type I IFN in response to *L. pneumophila* infection was shown to be dependent of MAVS and IRF3 (Opitz et al., 2006; Chiu et al., 2009; Monroe et al., 2009). Furthermore, this response accounted to bacterial growth restriction, as the addition of exogenous type I IFN to macrophages lead to the inhibition of *L. pneumophila* replication in non-permissive macrophages (Schiavoni et al., 2004; Plumlee et al., 2009). Several groups have independently demonstrated that this IRF3-dependent innate immune response does not require the flagellin/NAIP5/NLRC4 axis and occurs in both mice and human cells (Opitz et al., 2006; Coers et al., 2007; Lippmann et al., 2008). Activation of the IRF3 pathway was found to be dependent on a functional bacterial Dot/Icm and to require the presence of bacterial DNA in the host cell cytoplasm (Stetson and Medzhitov, 2006). The current hypothesis is that *L. pneumophila* DNA leaks into the host cell cytoplasm via the Dot/Icm. However, a recent report has demonstrated that *L. pneumophila* RNA, but not DNA, is responsible for RIG-I-dependent response to *L. pneumophila* (Monroe et al., 2009). A subsequent study showed that the host protein RNA polymerase III, which converts poly (dA-dT) DNA into 5'-ppp RNA, is important for IFN β induction through the RIG-I pathway (Chiu et al., 2009). Future studies are required to determine the ligand and receptor responsible for DNA/RNA recognition. Collectively, the investigations of type I IFN in *L. pneumophila* infection indicate that type I IFN signaling effectively restricts *L. pneumophila* replication in phagocytes, thus confirming the importance of this pathway for macrophage resistance to *L. pneumophila*. Conversely, the importance of type I IFN for

murine resistance to *L. pneumophila* infection is less pronounced. Work from independent groups has demonstrated that mice deficient for IFNAR, which impairs the activation by both IFN- α and IFN- β , show no increased susceptibility to *L. pneumophila* infection (Monroe et al., 2009; Ang et al., 2010). Nevertheless, further investigation may be required to determine why type I IFN affects macrophage but not mouse resistance to *L. pneumophila*, and future studies are necessary to identify the ligand and receptors involved in the production of type I IFN in response to *L. pneumophila* infection.

CONCLUDING REMARKS

Different families of PRRs, including TLRs, NLRs, and RLRs, effectively recognize *L. pneumophila*. This recognition leads to several events important for the outcome of infection: (1) phagocytes activate cell-autonomous mechanisms to restrict bacterial replication; (2) phagocytes trigger the expression of hundreds of inflammatory genes, including those for cytokines and chemokines; (3) phagocytes then express stimulatory and co-stimulatory molecules important for antigen presentation; (4) the secreted cytokines and chemokines recruit additional cells to the sites of the infection; (5) antigen presentation will proceed and the immune system may generate specific acquired responses that are highly protective against reinfection. The continued use of *L. pneumophila* to dissect these processes will aid us in understanding how the immune system fights to prevent Legionnaire's disease and will continue to provide important insight into the biological functions of the innate immune responses.

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