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Cell biology and immunology lessons taught by *Legionella pneumophila*

Wenhan Zhu‡ & Zhao-Qing Luo*

Department of Biological Sciences, Purdue University, West Lafayette, IN47907, USA

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Legionella pneumophila is a facultative intracellular pathogen capable of replicating within a broad range of hosts. One unique feature of this pathogen is the cohort of ca. 300 virulence factors (effectors) delivered into host cells via its Dot/Icm type IV secretion system. Study of these proteins has produced novel insights into the mechanisms of host function modulation by pathogens, the regulation of essential processes of eukaryotic cells and of immunosurveillance. In this review, we will briefly discuss the roles of some of these effectors in the creation of a niche permissive for bacterial replication in phagocytes and recent advancements in the dissection of the innate immune detection mechanisms by challenging immune cells with *L. pneumophila*.

Type IV secretion, inflammasome, effectors, posttranslational modification, vesicle transport

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Legionella AND THE DOT/ICM TYPE IV SECRETION SYSTEM

The discovery of *Legionella pneumophila* dates back to 1976, when a mysterious disease affected 221 American veterans attending the American Legion Convention in Philadelphia. The causative agent for this highly concerning outbreak was identified three year later and was designated as *Legionella pneumophila,* a gram-negative bacterium that belongs to the γ-proteobacteria (McDade et al., 1977). Subsequent studies revealed that *L. pneumophila* is an intracellular pathogen capable of replicating in highly diverse hosts, ranging from fresh water amoebae to mammalian alveolar macrophages (Stone et al., 1999). One salient feature associated with *L. pneumophila* infection is that the bacterial phagosome does not undergo the default phagosomal maturation process characterized by fusion with the lysosomal network, particularly in the early phase of infection; rather it develops into an organelle permissive for intracellular bacterial replication termed the *Legionella*-containing vacuole (LCV) (Ensminger and Isberg, 2009; Horwitz, 1983; Tilney et al., 2001) (Figure 1). The capability of this bacterium to construct such a unique niche is mostly attributed to the activity of effectors translocated by the Dot/Icm type IV secretion system (Huang et al., 2011; Isberg et al., 2009; Lifshitz et al., 2013; Luo and Isberg, 2004; Xu and Luo, 2013; Zhu et al., 2011). These effectors function to re-orchestrate various host processes to allow intracellular bacterial growth. The infection biology of *L. pneumophila* provides an excellent example of the sophistication of the interactions between bacterial pathogens and their hosts. Further, investigations of the sensing of the Dot/Icm translocated proteins and/or their activity by immune cells such as macrophages has allowed the identification of novel immunosurveillance mechanisms often not seen in infections with more adapted pathogens.

*Corresponding author (email: luoz@purdue.edu)
‡Current address: Department of Microbiology, UT Southwestern Medical Center, Dallas, TX 75390, USA

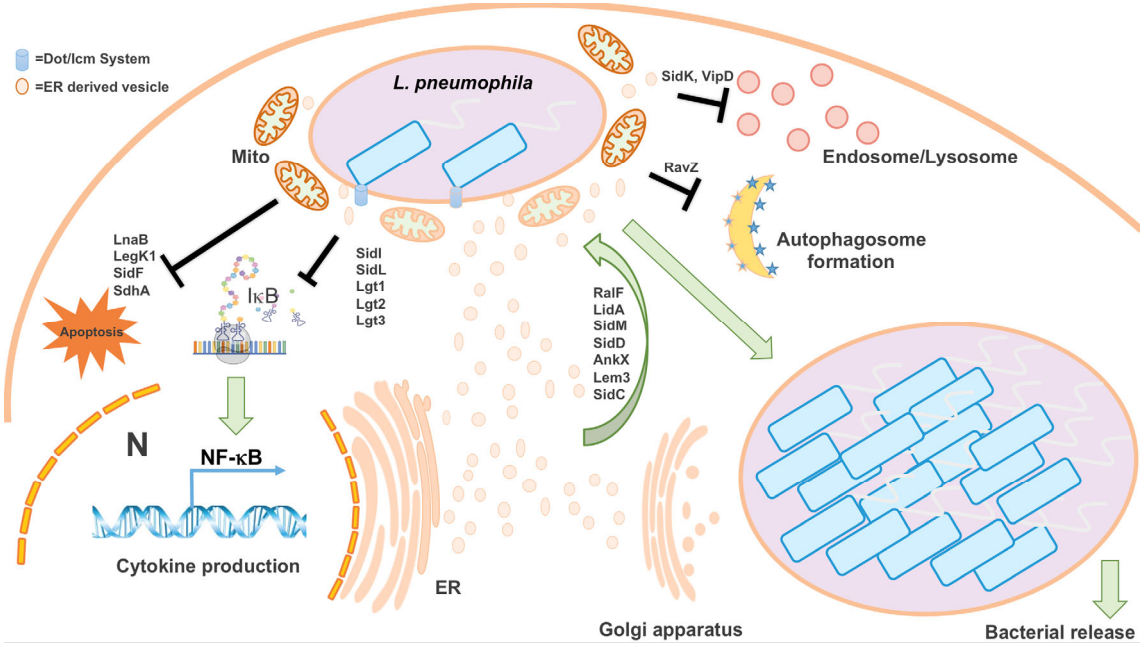

Figure 1 Modulation of multiple host processes by Dot/Icm effectors. The formation of the LCV containing numerous *L. pneumophila* cells in permissive macrophages requires active modulation of multiple host processes by a large cohort of effectors. The events shown in this Figure include cell death, activation of the NF-κB pathway, membrane transport between the ER and the Golgi apparatus, autophagy and the default phagosome maturation pathway that delivers newly formed phagosomes to the lysosomal network. Dot/Icm effectors involved in these processes were listed individually or as groups.

MODULATION OF HOST PROCESSES BY DOT/ICM EFFECTORS

L. pneumophila actively modulates host processes at both transcriptional and posttranslational levels. For example, infection with virulent *L. pneumophila* induces many anti-apoptotic genes via the activation of the NF-κB pathway (Abu-Zant et al., 2007; Losick and Isberg, 2006); effectors such as LegK1 and LnaB are involved in such activation (Ge et al., 2009; Losick et al., 2010). Histone methylation by RomA/LegAS4 causes the repression of innate immune genes and the activation of rDNA transcription, respectively (Li et al., 2013; Rolando et al., 2013), which are important to intracellular replication. Both the inhibition of protein synthesis (Belyi et al., 2006; Fontana et al., 2011) and the induction of cell death (via the activation of caspase 3) are mediated by multiple effectors (Zhu et al., 2013), most likely at the posttranslational level. Similar to many intracellular pathogens, *L. pneumophila* also targets components of the host actin network (Franco et al., 2012; Guo et al., 2014; Michard et al., 2015), likely to facilitate the biogenesis of the LCV by finely modulating the function of the actin cytoskeleton.

The rapid conversion of the bacterial phagosome into an organelle resembling the endoplasmic reticulum (ER) is a unique feature associated with *L. pneumophila* infection; this process involves the function of many effectors, some of which target important signaling hubs of host membrane trafficking. Phosphatidylinositols (PIs) are essential in defining organelle identity and in signaling cascades in vesicle trafficking and actin cytoskeleton structure (Di Paolo and De Camilli, 2006; Odorizzi et al., 2000). *L. pneumophila* harnesses the metabolism and cellular distribution of PIs using specific enzymes. On one hand, this bacterium injects a PI(3)P phosphatase into host cells (Toulabi et al., 2013), which may together with Rab5-activated VipD (Gaspar and Machner, 2014), function to remove PI(3)P from the surface of specific organelles (including the LCV), thus preventing the fusion of the LCV with the lysosomal network. On the other hand, *L. pneumophila* actively enriches PI(4)P on the LCV by employing enzymes of both bacterial and host origins. The PI-3-phosphatase SidF produces PI(4)P from PI(3,4)P2 directly or from PI(3,4,5)P3 together with OCRL, a host 5-phosphatase (Hsu et al., 2014). The host phosphatidylinositol-4-kinase IIIα (PI4KIIIα) also contributes to such enrichment (Hubber et al., 2014). The PI(4)P on the LCV allows the anchoring of PI(4)P-binding effectors such as SidM/DrrA, SidC and SdcA, which may compartmentalize the activity of these effectors to the surface of the LCV (Brombacher et al., 2009; Ragaz et al., 2008; Weber et al., 2006). Enriched PI(4)P may also mimic the lipid composition of the *cis*-Golgi compartment (Di Paolo and De Camilli, 2006; Odorizzi et al., 2000), making the LCV a “natural” recipient compartment for the incoming ER-derived vesicles. *L. pneumophila* also interferes with the biosynthesis of diacylglycerol (DAG) and phosphatidic acid (PA) in host cells (Viner et al., 2012) but the importance of such manipulation is not clear.

Concomitant with its interference with host lipid metabolism, *L. pneumophila* remodels the LCV membrane by hi-

jacking the activity of key regulators of membrane trafficking such as Arf1 (Nagai et al., 2002) and Rab1 (Machner and Isberg, 2006), the small GTPases essential for vesicle transport between the ER and the Golgi apparatus. At least five Dot/Icm effectors completely control the activity of this regulatory protein. Amongst these, SidM/DrrA is a multifunctional protein; it anchors to the LCV surface by binding to PI(4)P and activates Rab1 with its guanine nucleotide exchange factor (GEF) activity (Machner and Isberg, 2006; Murata et al., 2006) and locks the GTPase in its active form with its AMPylating (adenylyl transferase) activity (Muller et al., 2010). Following the removal of the AMPylation modification by SidD (Neunuebel et al., 2011; Tan and Luo, 2011), Rab1 is inactivated by LepB, a bacterial GTPase activation protein (GAP) (Ingmundson et al., 2007). In murine macrophages, these proteins exert a temporal regulation of Rab1 activity on the LCV in the first 4 hours after bacterial uptake (Neunuebel et al., 2011; Tan and Luo, 2011) (Figure 1). The complexity of Rab1 activity regulation is aggregated by the phosphorylcholine transferase AnkX (Mukherjee et al., 2011; Tan et al., 2011) and Lem3, an enzyme that reverses the modification imposed by AnkX (Tan et al., 2011). It is possible that AnkX targets Rab1 at the *cis*-Golgi compartment to block the fusion of ER-derived vesicles, which may facilitate the re-direction of such vesicles to the LCV. In this model, the role of Lem3 is to safeguard (accidental) AnkX-mediated inhibition of Rab1 activity on the LCV. The bacterium also targets the Ran small GTPase to regulate microtubule polymerization, motility of its phagosome and infected cells (Rothmeier et al., 2013; Simon et al., 2014).

Dot/Icm effectors also target other cellular events important for vesicle transport such as autophagy (Choy et al., 2012) and the v-ATPase (Xu et al., 2010), which regulates membrane fusion by controlling the pH and other physiological features of vacuoles. Finally, re-orchestration of the host ubiquitination pathways is emerging as an important branch of the bacterium's strategy. At least eight Dot/Icm effectors appear to possess F-box or U-box domains typical of known E3 ligases (Hubber et al., 2013). Among these, LegU1, LegAU13/AnkB and LubX have been demonstrated to possess E3 ligase activity (Ensminger and Isberg, 2010; Kubori et al., 2008). SidC and SdcA are recently discovered unique E3 ligases important for efficient enrichment of both ubiquitinated species and proteins of ER origin onto the bacterial phagosome (Hsu et al., 2014). Interestingly, Rab1 is monoubiquitinated during *L. pneumophila* infection in a manner that requires SidC/SdcA (Horenkamp et al., 2014), yet these two E3 ligases do not detectably ubiquitinate this small GTPase *in vitro* or in cells transfected to express these proteins (Hsu et al., 2014). Future identification of the protein substrates of these E3 ligases will provide exciting insights into the signaling cascades that potentially link ubiquitination to vesicle transport and other cellular events important for the biogenesis of the LCV.

IMMUNE RECOGNITION IN *L. pneumophila* INFECTION

Extracellular immune recognition

Most individuals susceptible to *L. pneumophila* infection are immunocompromised (Kumpers et al., 2008), suggesting that this bacterium does not effectively evade immune detection. The immune system recognizes *L. pneumophila* by employing an arsenal of germ-line coded Pattern-Recognition-Receptor (PRRs) distributed on different cellular locales to perceive the presence of associated Pathogen-Associated-Molecular-Pattern (PAMPs), or by detecting alterations of the cellular activities imposed by the bacterium (Abt et al., 2012)

The PRRs are categorized based on the subcellular location where the receptor initiates the signaling. Toll-like-receptors (TLRs) and C-type lectins are among the most well-characterized membrane associated receptors that mainly function to recognize extracellular ligands (some TLRs are located in endosomes that recognize intracellular ligands) (Lee and Kim, 2007). These receptors recognize a wide range of ligands. For example, mouse codes for 13 TLRs, and the ligands to these receptors includes bacterial lipopolysaccharides (LPS), peptidoglycans, triacyl lipopeptides, and flagellin, as well as the zymosan of fungi (Broz and Monack, 2013). It has been demonstrated that TLRs are essential to mounting appropriate immune responses to *L. pneumophila*, as mice lacking MyD88, the signaling adaptor shared by all TLRs except TLR3, fail to clear *L. pneumophila* in the lung and eventually succumb to acute pulmonary infection, with bacteria spreading to lymph nodes and the spleen (Archer and Roy, 2006). Among TLRs, TLR2, TLR4 and TLR5 are the major contributors to the recognition of *L. pneumophila* during infection, as the lack of TLR2 enhances bacterial growth in a mouse model, and the polymorphisms of TLR4 and TLR5 are associated with resistance to Legionnaires' disease (Archer et al., 2009; Hawn et al., 2005; Hawn et al., 2003).

Intracellular immune recognition

Extracellular PRRs alone are not sufficient in defending against microbial infections, especially those by intracellular pathogens. It is therefore not surprising that mammals code for a large cohort of cytoplasmic PRRs, including the NOD-like receptors (NLRs), pyrin and HIN domain containing family (PYHIN), RIG-I-like receptors (RLRs) and numerous cytosolic nucleic acid sensors to guard the cytosol from invading microbes (Broz and Monack, 2013). Some NLRs upregulate the transcription of downstream signaling components, such as the NF-κB and the IFN-β production pathways upon engaging their ligands (Strober et al., 2006). Other NLRs, however, drive the assembly of large protein complexes termed inflammasomes, which cause pyroptotic cell death and the secretion of mature inflammatory cytokines, such as IL-1β and IL-1α (Lamkanfi and Dixit, 2014).

The diverse ligand recognition capacity of NLRs makes them one of the most important guardians of the cytoplasm. For example, NLRP1b responds to the protease activity of the *Bacillus anthracis* lethal toxin and to *Toxoplasma gondii* infection (Cirelli et al., 2014), whereas the AIM2 inflammasome responds to cytosolic DNA molecules (Rathinam et al., 2010). Similarly, the NLRC4 inflammasome responds to many bacterial PAMPs, such as the rod and needle proteins of bacterial Type III secretion system as well as flagellin (Zhao et al., 2011). The capability of NLRC4 to recognize such a wide repertoire of PAMPs is attributed to the NAIP proteins (also called baculoviral IAP repeat-containing protein), which serve as adaptors to bridge the interaction between NLRC4 and the PAMP molecules (Zhao et al., 2011). So far, the NLRP3 inflammasome is known to respond to the most diverse PAMPs, which include bacterial, fungal and viral pathogens, as well as pore formation toxins, protein amyloid, extracellular ATP, and inorganic substances such as silica and alum (Lamkanfi and Dixit, 2014).

The discovery of the intracellular sensor for flagellin is largely attributed to the research aiming at understanding the inability of macrophages from most of the inbred mouse strains to support intracellular *L. pneumophila* replication (Yamamoto et al., 1988). For example, BMDMs (Bone Marrow Derived Macrophage) from C57BL/6 mouse effectively clear *L. pneumophila*, and the bacterial clearance is accompanied by extensive pyroptosis (Derre and Isberg, 2004), suggesting the involvement of active host immune responses. However, *L. pneumophila* successfully replicates in macrophages from A/J mice (Yamamoto *et al*., 1988). Taking advantage of the sharply different responses of the two mouse strains to *L. pneumophila*, Dietrich *et al*. mapped the genetic elements responsible for the permissiveness of A/J mice to the Lgn1 locus, which was later pinpointed to the gene *Naip5* (Diez et al., 2003). The very same phenotype also was utilized to identify flagellin as the bacterial factor that engages Naip5 (Molofsky et al., 2006; Ren et al., 2006). Upon engaging flagellin, Naip5 recruits and activates NLRC4, which recruits and activate caspase-1(Kofoed and Vance, 2011; Zhao et al., 2011), leading to the secretion of inflammatory cytokines (IL-18 and IL-1β) and pyroptotic cell death. These events eventually lead to further recruitment of other immune cells and the removal of the replicative niche, explaining the resistance to *L. pneumophila* infection by most mouse lines.

In permissive mouse macrophages, *L. pneumophila* maintains its lifestyle as a vacuolar pathogen. The stability of LCV membrane is affected by the lipase PlaA, which seems to destabilize the vacuole (Creasey and Isberg, 2012). Interestingly, the effects of PlaA are counteracted by the effector SdhA, which maintains the integrity of the LCV, very likely by modulating host membrane trafficking (Creasey and Isberg, 2012). *L. pneumophila* lacking SdhA aberrantly enters the cytosol, leading to the activation of the AIM-2 inflammasome and the noncanonical caspase-11 inflammasome (Aachoui et al., 2013; Ge et al., 2012). These phenomena have facilitated the discovery of the caspase-11 inflammasome, which was later shown to be activated directly by intracellular LPS (Aachoui et al., 2013; Shi et al., 2014). These exciting successes in revealing previously underappreciated immune pathways by studying *L. pneumophila* infection clearly demonstrate the great potential of investigating host responses using less adapted pathogens (Figure 2).

Other cytoplasmic PRRs such as RLRs, including retinoic acid-inducible gene I (RIG-I), LGP2 and the melanoma differentiation-associated protein 5 (MDA5), detect nucleic acid and their derivatives, which are one of most important categories of MAMPs (Broz and Monack, 2013). RIG-I, for example, recognizes cytoplasmic dsRNA to promote the prion conversion of MAVS (Mitochondrial antiviral-signaling protein), which signals to activate the production of IFN-β (Cai et al., 2014). Of particular interest is the long-anticipated revelation of the major cytoplasmic dsDNA sensor, cGAS (Cyclic GMP-AMP synthase) (Sun et al., 2013). This sensor is special as it is an enzyme itself, which catalyzes the formation of cyclic di-GMP-AMP upon recognizing dsDNA. The cyclic di-GMP-AMP is then sensed by STING (STimulator of INterferon Genes), which signals the production of type I IFN (Burdette et al., 2011). *L. pneumophila* can also be recognized by the AIM2 inflammasome, probably by bacterial DNA "leaked" into the host cytosol by the Dot/Icm system (Berrington et al., 2010) (Figure 2). Since *L. pneumophila* infection also induces Type I Interferon production in a STING-(stimulator of interferon genes) dependent manner, it is tempting to postulate that the "leaked" bacterial DNA also engages the cGAS (cyclic GMP-AMP synthase)-c-di-AMP-GMP-STING pathway (Ge et al., 2012; Lippmann et al., 2011; Monroe et al., 2009) (Figure 2). Alternatively, bacterial cyclic di-GMP-AMP may reach host cytosol via the Dot/Icm system or in conjunction with other transporters, akin to that observed in *Listeria monocytogenes* (Crimmins et al., 2008; Woodward et al., 2010).

In addition to pyroptosis, intracellular surveillance of foreign molecules causes permeabilization of the lysosome, an organelle that is emerging as a central signaling hub for various pathological stresses, including stroke, acute pancreatitis, parasitic and infections (Aits and Jaattela, 2013). Upon perceiving these stresses, lysosomal membranes undergoes selective or complete permeabilization, leading to the release into the cytosol, of its contents, including proteases of the cathepsin family (Aits and Jaattela, 2013). Cathepsins are capable of inducing cell death of various forms (Turk and Turk, 2009). A recent discovery reveals that *L. pneumophila* "accidently" delivers RpsL, a conserved bacterial ribosomal protein into the cytosol, where it causes lysosomal membrane permeabilization (LMP), presumably by activating a signaling cascade upon engaging a yet to be identified receptor (Zhu et al., 2015). Cathepsins such ca-

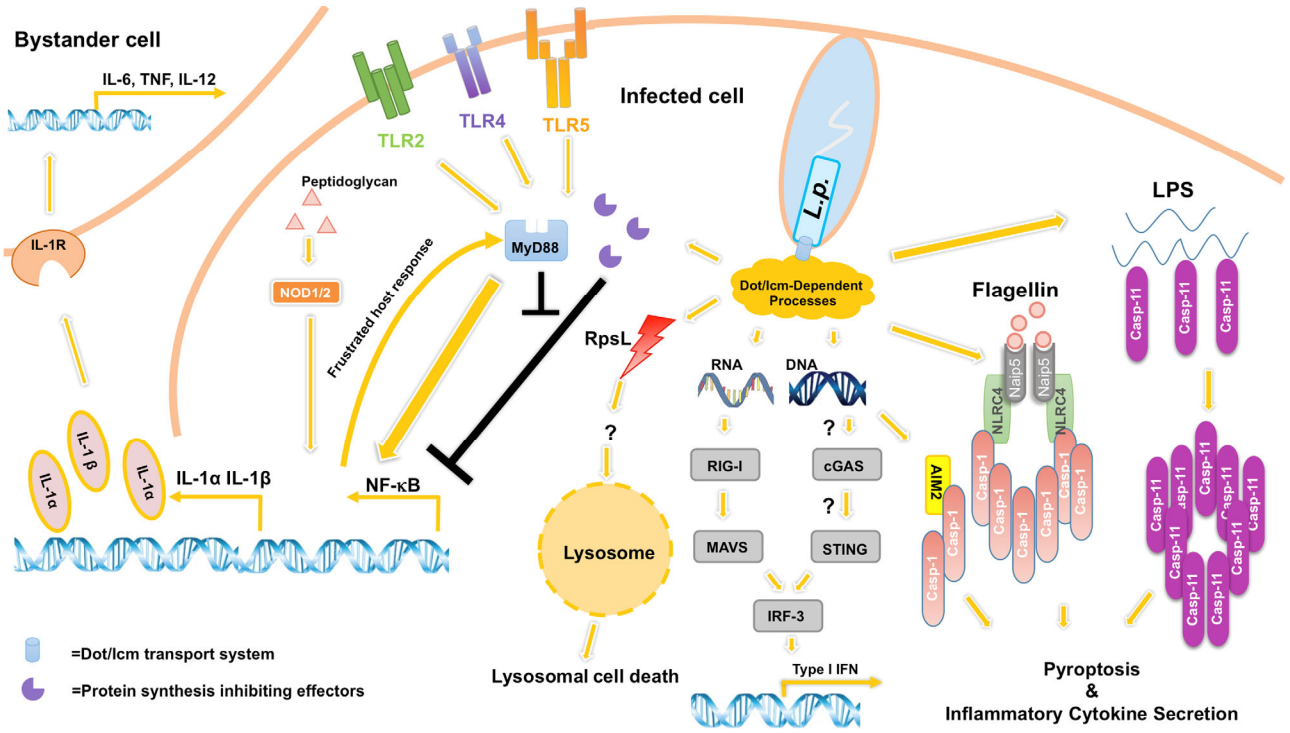

Figure 2 Immune recognition of *L. pneumophila*. Interactions between *L. pneumophila* and macrophages activate immune responses at both transcriptional and posttranslational levels. The most notable events are those that occur in response to the activity of the Dot/Icm transporter, including the production of type I interferon, the activation of various inflammasomes and the induction of lysosomal membrane permeabilization (LMP). AIM2, Absent in melanoma 2; Casp-1, capase-1; Casp-11, caspase-11; cGAS, cyclic di-GMP-AMP synthase; IFN, Interferon; IRF-3, Interferon regulatory factor 3; LPS, lipopolysaccharide; MAVS, mitochondrial antiviral-signaling protein; MyD88, Myeloid differentiation primary response gene 88; Naip5, NLR family, apoptosis inhibitory protein 5; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRC4, NLR family CARD domain-containing protein 4; NOD1/2, Nucleotide-binding oligomerization domain-containing protein 1 and 2; RIG-I, retinoic acid-inducible gene 1; STING, stimulator of interferon genes; TLR2, Toll like receptor 2; TLR5, Toll like receptor 5.

thepsin B and cathepsin D are released in response to infections with *L. pneumophila* expressing wild type RpsL or artificially delivered protein (Zhu et al., 2015). More interestingly, a Lys88Arg mutation that also confers resistance to the antibiotic streptomycin, allows the bacterium to replicate in mouse macrophage (Zhu et al., 2015), suggesting the recognition of the same epitope by antibiotics and the immune system.

IMMUNITY TRIGGERED BY *L. pneumophila* EFFECTORS

PAMPs are not the only microbe-associated features sensed by that the immune systems are sensing. The observation that different cultivars of the same plant respond distinctively to different pathogen races has prompted plant pathologists to postulate the "gene-for-gene" hypothesis (Flor, 1971), which suggests that resistant plants code for the "resistant gene (R gene)" that is required for the recognition of the virulence factor "avirulence gene (*avr* gene)". Since the presence of the a*vr* gene is sufficient to trigger immune responses from resistant plants, it was proposed that the R gene product directly recognizes the gene product of the Avr, which is similar to the PRR-PAMP paradigm in metazoans (Stuart et al., 2013). However, as the interactions between Avr and R genes were rarely demonstrated, and that the Avr genes usually codes for bacterial virulence factors with distinct biochemical activities, Dangl and other researchers hypothesized that R gene products are not recognizing the Avr proteins *per se*, but instead their biochemical consequences (Chisholm et al., 2006). This hypothesis was supported by the observation that Avr proteins lacking their enzymatic activities are not immunogenic (Jones and Dangl, 2006). Thus, the immunity triggered by perceiving the biochemical consequences of bacterial effectors is defined as effector-triggered immunity (ETI) (Jones and Dangl, 2006). Similar hypothesis called "pattern of pathogenesis" was proposed for the mammalian system (Jones and Dangl, 2006). It is now clear that ETI exists in metazoan (Chisholm et al., 2006) as well. For instance, modification of the host protein RAC2 by the *Escherichia coli* CNF (cytotoxic necrotizing factor-1) activates the NF-κB pathway (Boyer et al., 2011; Gurcel et al., 2006). Similarly, the pore formation activity of various bacterial toxins activates the NLRP3 inflammasome (Boyer et al., 2011; Gurcel et al., 2006) and the GAP activity of the *Yersinia* T3SS effector YopE triggers bacterial killing by macrophages (Wang et al., 2014). Finally, a recent study reveals that the Pyrin in-

flammasome is an R gene equivalent in mammals, which senses various effector-mediated modifications on the small GTPase RhoA, including AMPylation, ADP-ribosylation, deamindation and glucosylation (Xu et al., 2014). Thus, ETI is an important branch of innate immune surveillance in metazoans.

ETI also contributes to the immune response to *L. pneumophila* infection. In macrophages, the MAP kinase pathway and the NF-κB pathway are highly induced by infections by wild type *L. pneumophila* but not by its *dot/icm* mutants (Abu-Zant et al., 2007; Losick and Isberg, 2006; Shin et al., 2008). Interestingly, protein synthesis inhibition conferred by at least five Dot/Icm substrates is part of the signals sensed by both the NF-κB and the MAP kinase pathways (Fontana et al., 2011; Fontana et al., 2012). Pro-longed activation of the NF-κB pathway seen in infections with wild type *L. pneumophila* is achieved by delayed re-synthesis of IκB, the labile inhibitory protein of NF-κB (Fontana et al., 2011). The activation of the MAP kinase response by protein synthesis inhibition is multifaceted. First, many genes involved in this pathway are transcribed but not translated. The "frustrated" responses trigger signaling pathways to allow selective translation of at least two inflammatory cytokines, IL-1α and IL-1β, in a MyD88 dependent manner (Asrat et al., 2014). Analyses in a pulmonary infection mouse model suggest that, *in vivo*, bypassing the translational inhibition is mediated by the bystander cells not infected by *L. pneumophila* (Copenhaver et al., 2015).

In sum, the study of *L. pneumophila* pathogenesis in the past decade has greatly advanced our understanding of not only the mechanisms underlying the interactions between this pathogen and its hosts, but also the mechanisms in the biochemistry of posttranslational modifications, signaling and immune detection. Given the fact that the activity has only been assigned to a small fraction of the Dot/Icm substrates, it is anticipated that more excitement will be made in our analysis of the hundreds of effectors and the host response to these proteins in years to come.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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