Viewing *Legionella pneumophila* Pathogenesis through an Immunological Lens

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Abstract

Legionella pneumophila is the causative agent of the severe pneumonia Legionnaires' disease. *L. pneumophila* is ubiquitously found in freshwater environments, where it replicates within free-living protozoa. Aerosolization of contaminated water supplies allows the bacteria to be inhaled into the human lung, where *L. pneumophila* can be phagocytosed by alveolar macrophages and replicate intracellularly. The Dot/Icm type IV secretion system (T4SS) is one of the key virulence factors required for intracellular bacterial replication and subsequent disease. The Dot/Icm apparatus translocates more than 300 effector proteins into the host cell cytosol. These effectors interfere with a variety of cellular processes, thus enabling the bacterium to evade phagosome−lysosome fusion and establish an endoplasmic reticulum-derived *Legionella*-containing vacuole, which facilitates bacterial replication. In turn, the immune system has evolved numerous strategies to recognize intracellular bacteria such as *L. pneumophila*, leading to potent inflammatory responses that aid in eliminating infection. This review aims to provide an overview of *L. pneumophila* pathogenesis in the context of the host immune response.

Introduction

In the summer of 1976, over 200 American Legionnaires developed severe pneumonia shortly after they returned home from their state convention in Philadelphia, and 34 individuals died [1]. This mysterious illness was eventually named Legionnaires' disease and was discovered by the Centers for Disease Control and Prevention microbiologist Joseph McDade to be caused by the bacterium *Legionella pneumophila* [2,3]. *L. pneumophila* is now appreciated to be an important cause of community-acquired and nosocomial pneumonia globally [4−6].

L. pneumophila is a gram-negative bacterium found throughout freshwater environments and manmade water systems, such as cooling towers or decorative fountains [7], where it associates with biofilms and normally parasitizes free-living protozoa [8,9]. Strikingly, *L. pneumophila* is able to replicate in a wide range of protozoan hosts [9,10], and the ability to adapt to diverse hosts appears to be due to the acquisition of a large number of genes [11], which has endowed *L. pneumophila* with the ability to also infect humans [12]. Aerosolization of contaminated water supplies allows for inhalation of *L. pneumophila* into the human lung, allowing the bacteria to be phagocytosed by alveolar macrophages, where they can replicate intracellularly. The outcome of infection likely depends on a combination of bacterial virulence factors and host immunity. Most *Legionella* infections are thought to be asymptomatic or lead to a self-limiting and mild respiratory disease called Pontiac fever [13]. Some individuals, particularly the elderly and those with weakened immune systems or chronic lung diseases, are at increased risk of developing severe pneumonia [14,15].

One of the key bacterial virulence factors essential for *L. pneumophila* to cause disease is the type IV secretion system (T4SS), which is a multi-protein machine that delivers more than three hundred bacterial effector proteins into the host cytosol [16,17]. The T4SS is encoded by *dot/icm* genes [18]. Among these Dot/Icm components, several

proteins, including DotA, are essential for the assembly and activity of the T4SS. Dot/Icm T4SS-deficient strains (such as Δ*dotA*) are unable to replicate intracellularly, as they rapidly traffic to the endocytic pathway and fuse with lysosomes, where they are degraded [19–22]. In contrast, bacteria with a functional T4SS manipulate host membrane trafficking, allowing them to rapidly evade phagolysosomal fusion and remodel the *Legionella*-containing vacuole (LCV) into a rough endoplasmic reticulum (ER)-derived organelle that supports bacterial replication [21].

In the lung, alveolar macrophages appear to be the primary cell type that is targeted by T4SS effectors and support intracellular bacterial replication, while neutrophils can be injected by T4SS effectors and harbor live bacteria as well [23,24]. *In vitro*, human-derived primary macrophages and cell lines are permissive for intracellular bacterial replication. In contrast, the majority of inbred mouse strains and macrophages derived from these strains, except for A/J (or A) mice, are non-permissive for *L. pneumophila* replication [25]. This permissiveness is genetically controlled by the neuronal apoptosis inhibitory protein *Naip5* (*Birc1e*) gene, which is located within the Lgn1 locus on murine chromosome 13, and the increased susceptibility of A/J mice or its macrophages is caused by polymorphisms that render the A/J *Naip5* a functional hypomorph [26–29]. As will be further discussed below, NAIP5 is a nucleotide-binding domain, leucine-rich-repeat-containing receptor (NLR) that, upon sensing flagellin, induces potent cell-intrinsic restriction of bacterial infection [30]; thus, the flagellin mutant strain (Δ*flaA*) has the ability to evade NAIP5-dependent restriction in non-permissive mouse strains or macrophages. Therefore, either A/J mice infected with wild-type (WT) bacteria or C57BL/6 mice infected with Δ*flaA* mutant have been widely used as murine models to better understand the host immune response and pathogenesis of Legionnaires' disease.

The main clinical characteristics of *L. pneumophila*-induced pneumonia are acute lung injury and severe hypoxemia [31,32]. Patients present with high levels of inflammatory cytokines in their serum, such as TNFα, IFN-γ, IL-12, IL-6, IL-8, and granulocyte-colony stimulating factor, and an especially prolonged increase in IL-12 levels during the convalescent phase, whereas cytokines such as IL-10 and IL-4 are low or undetectable [33,34]. The intensity of inflammatory cytokine response directly correlates with the severity of the patient's condition [35]. Numerous lines of evidence in mice and humans indicate that proinflammatory cytokines play important roles in immune defense against *L. pneumophila* infection. For example, human patients with autoimmune diseases that are clinically treated with anti-TNFα agents are at significantly increased risk for acquiring Legionnaires' disease [36–39]. Experimental studies support that TNFα is required to control *L. pneumophila* infection in mice and host cells [40–42].

During the acute inflammatory phase, *L. pneumophila* infection induces robust cellular immune responses, which are characterized by a rapid accumulation of immune cells in the lung, including neutrophils, monocytes, and dendritic cells, as well as NK, NKT, B, and T cells [43]. Subsequently there is bacterial clearance, a sharp decline in inflammatory cytokine production, and eventual resolution of inflammatory cellular infiltration in immunocompetent animals [43]. Below, we describe in more detail the molecular and cellular mechanisms underlying *L. pneumophila* pathogenesis, with a primary focus on the immune response to infection.

Type IV Secretion System

T4SSs are multiprotein complexes of diverse structure and function that have been classified into two major subgroups, T4ASS and T4BSS [18,44–46]. The *L. pneumophila* T4BSS shares only very limited similarity with T4ASS [47], but the basic architecture of T4BSS is similar to T4ASS [48], although the *in situ* dimension is slightly different, suggesting that both shared and distinct aspects underlying their mechanism. The T4BSS is assembled by approximately 27 *dot/icm* (defective organelle trafficking/intracellular multiplication) genes encoding 22 structural proteins and five chaperone proteins that interact with effector proteins in the bacterial cytoplasm [18,45,49]. The Dot/Icm T4SS is a key virulence factor essential for *L. pneumophila* pathogenesis and translocates effector proteins into the host cytosol, which allows *L. pneumophila* to manipulate a variety of cellular processes, including membrane trafficking [50,51], protein synthesis [52,53], ubiquitylation [54,55], and autophagy [56,57]. Importantly, the Dot/Icm T4SS is required for bacterial replication in amoebae and macrophages within a compartment termed the LCV [58,59]. Dot/Icm mutants are unable to replicate intracellularly, in part because they cannot evade phagolysosomal fusion [60,61]. Dot/Icm-deficient *L. pneumophila* are also avirulent in animal models [22]. *L. pneumophila* encodes a second Lvh T4ASS that is not required for bacterial replication in macrophages, but seems to play a role in the efficiency of infection when *L. pneumophila* is grown at a lower temperature of 30 °C compared to 37 °C [62].

Type II Secretion System

Type II secretion systems (T2SS) enable bacteria to translocate proteins into the extracellular environment [63,64]. *L. pneumophila* transports more than 25 protein substrates through the T2SS [65]. The

T2SS consists of a homologue of the *Pseudomonas aeruginosa* prepilin peptidase PilD, which is required for type IV pilus formation and functional type II secretion [66–68], as well as 11 structural proteins [68,69]. T2SS mutants exhibit reduced bacterial loads relative to WT bacteria during pulmonary infection [70], suggesting that T2SS substrates are important bacterial virulence factors during *in vivo* infection. The T2SS substrate ChiA, a chitinase, is required for bacterial growth in the lung but not in macrophages *in vitro* [65,71]. *L. pneumophila* mutants lacking a functional T2SS or the T2SS substrate metalloprotease *proA* elicit increased cytokine responses during *in vivo* infection [72], suggesting that T2SS-dependent factors modulate the innate immune response. In partial support of this model, the T2SS dampens Toll-like receptor (TLR) 2 and MyD88 signaling and decreases cytokine production [72,73], although this effect was specific to human macrophages and did not affect murine macrophages during *in vitro* infection [73], indicating that the T2SS differentially modulates innate immune responses in mice and humans. In addition, *L. pneumophila* mutants lacking a functional T2SS exhibit a defect in intracellular replication within amoebae and macrophages [67,68], suggesting that the T2SS is required for intracellular replication. Intriguingly, the T2SS substrates ChiA and ProA appear to be translocated into the cytosol of amoebae and human macrophages and associates with the LCV membrane, leading to a model, whereby T2SS substrates are secreted into the lumen of the LCV and then somehow translocate across the LCV membrane into the macrophage cytosol [73].

Establishment of the LCV to Support Bacterial Replication

Phagocytosis and intimate host cell contact is critical for *L. pneumophila* to begin translocating T4SS effectors into the host cell cytoplasm [74]. Once phagocytosed, *L. pneumophila* rapidly evade the endocytic pathway. Late endosomal markers are not associated with WT bacteria at early stages of infection, although some lysosomal markers such as lysosomal-associated membrane protein 1 (LAMP-1) are observed on the LCV at later stages of infection [19,75]. WT *L. pneumophila* rapidly manipulate host cell membrane trafficking [21], which in turn converts the LCV into an ER-derived compartment that supports bacterial replication [76]. By four hours post-infection, the LCV is decorated with ribosomes and contains ER-resident proteins [77] In contrast, Dot/Icm mutants are trafficked to degradative lysosomes as early as 5 min after uptake. They are found within compartments associated with LAMP-1 and the small GTP-binding protein Rab7 [19].

In aid of this process, the recruitment and maintenance of small guanosine triphosphatases (GTPases), such as Arf1 and Rab1, on the LCV membrane promotes the recruitment of membrane that normally traffics from the ER to the Golgi apparatus [76,78,79]. The recruitment of Arf1 and Rab1 to the LCV is mediated by the effector RalF [80,81] and DrrA/SidM [82], respectively. Subsequently, Rab1 activity is manipulate by additional effectors through a series of post-translational modifications. The effector DrrA/SidM mediates the AMPylation of Rab1, which protects Rab1 from inactivation by GTPase-activating proteins (GAPs) and allows for the accumulation of GTP-bound Rab1 on the LCV membrane [83,84]. The effector SidD is a deAMPylase allowing for the *L. pneumophila* GAP LepB to inactivate Rab1 [85,86]. The effector AnkX directly modifies Rab1 through the covalent attachment of a phosphocholine moiety, whereas the effector Lem3 removes the phosphocholine group [87–89]. Phosphocholination of Rab1 inhibits interactions with GEFs and GAPs, and may possibly stabilize GDP-bound Rab1 on membranes [87,88]. AnkX's phosphocholination activity disrupts host cell endocytic recycling and is critical for inhibiting fusion of the LCV with lysosomes. In addition, the effector SdeA can ubiquitinate Rab1 in a manner that bypasses the need for E1 and E2 enzymes [90]. The effector SidC, which is an E3 ubiquitin ligase, is involved in monoubiquitylation of Rab1 and also plays a role in early LCV maturation and recruitment of Arf1, but the direct ubiquitination of Rab1 by SidC has not yet been demonstrated [91,92]. In addition, the effector SidK is able to inhibit host vacuolar ATPase activity by interacting with VatA, a key component of the proton pump, which in turn prevents acidification of the LCV [93].

Although T4SS translocates >300 effectors into host cells to support creation of the LCV [16,17], loss of individual effectors generally does not affect intracellular replication within macrophages, indicating that many of the effectors possess redundant functions in targeting a given host pathway and that multiple redundant host pathways are manipulated by effectors. To resolve this redundancy, development of a genetic screening strategy termed insertional mutagenesis and depletion, which integrates bacterial mutagenesis and host RNA interference, has allowed for the systematic identification of genetic interactions between bacterial effectors and host pathways based on the observed reduction in bacterial intracellular replication [94]. Thus, *L. pneumophila* has evolved to manipulate a variety of cellular processes that allow for bacterial survival and replication intracellularly. In general, elucidation of the molecular mechanisms underlying *L. pneumophila* effector function and biogenesis of the LCV has provided novel insights into both bacterial and host cell

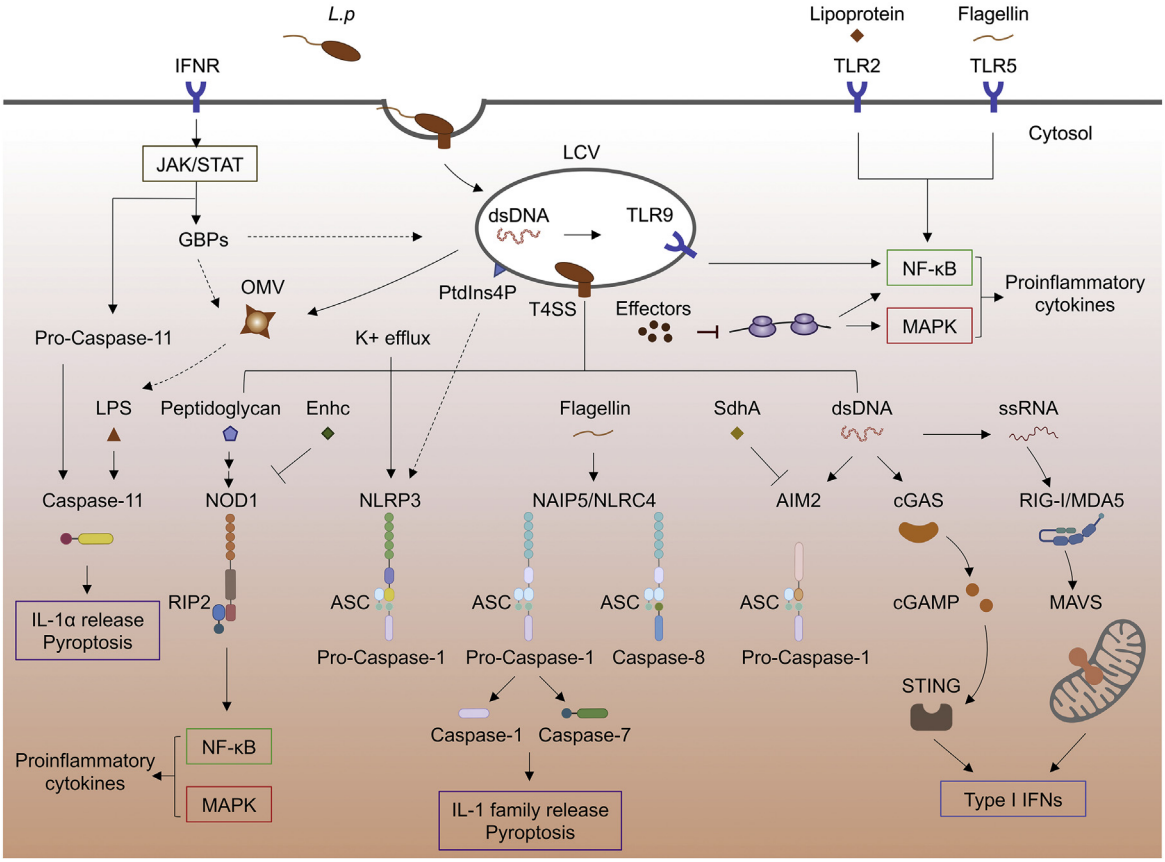

Fig. 1. Innate immune recognition of *L. pneumophila*. Host cells recognize *L. pneumophila* by utilizing multiple PRRs. TLR2, TLR5, and TLR9 sense the bacterial components peptidoglycan-associated lipoprotein (PAL), flagellin, and dsDNA, respectively, which mediate downstream NF-κB activation to induce proinflammatory cytokine production. *L. pneumophila* T4SS translocates numerous bacterial virulence factors into the cytosol. The effector-driven translational block potently induces a unique proinflammatory transcriptional response by means of prolonged NF-κB activation and MAPK signaling activation. Several other factors are recognized individually by distinct cytosolic sensors: (1) NAIP5 recognizes flagellin, resulting in NAIP5/NLRC4/caspase-1 or -8 inflammasome activation and downstream caspase-7 activation, which leads to IL-1 family cytokine release and pyroptosis to restrict bacterial infection. (2) Cytosolic bacterial dsDNA can activate the AIM2/ASC/caspase-1 inflammasome and the cGAS-cGAMP-STING pathway to induce type I interferon production. (3) RIG-I and MDA5 sense ssRNA, also inducing type I interferons. (4) Inflammasome-induced pyroptotic pore formation causes K+ efflux, which activates the NLRP3/ASC/caspase-1 inflammasome. (5) NOD1 recognizes anhydro-disaccharide-tetrapeptide (anhDSTP), a degradation product generated by the bacterial peptidoglycan degrading enzyme SltL, leading to RIPK2-dependent NF-κB and MAPK pathway activation and proinflammatory cytokine production. (6) Caspase-11 senses cytoplasmic LPS, which may be delivered by bacterial OMVs. Interferon signaling upregulates pro-caspase-11 and GBP expression. GBPs may target the LCV and/or OMVs to release LPS into the cytosol. Caspase-11 activation induces IL-1α release and pyroptosis. Meanwhile, several effectors aid in evading cytosolic immune surveillance. For example, SdhA helps maintain LCV integrity, thus evading AIM2 inflammasome activation, while EnhC interferes with SltL to reduce anhDSTP production, thus evading NOD1 sensing.

biology and has been covered more extensively in recent reviews [17,95–98].

Innate Immune Recognition of *L. pneumophila* Infection

The innate immune system is the first line of host defense against invading pathogens. The innate immune system can detect pathogenic bacteria through recognition of pathogen-associated molecular patterns by pattern recognition receptors (PRRs) [99,100]. As further described below, *L. pneumophila* infection activates a diverse array of PRRs, including membrane-bound TLRs, which reside at the cell surface or within endosomal compartments, and a variety of cytosolic immune sensing pathways, including effector-triggered immune responses.

TLR-mediated detection of *L. pneumophila* infection

TLR4 recognizes lipopolysaccharide (LPS) present in gram-negative bacteria, whereas TLR2 recognizes cell wall components found in both gram-negative and gram-positive bacteria, such as lipoteichoic acid, peptidoglycan, and lipoproteins [101–103]. Although TLR4 is important for host defense against many gram-negative bacteria, it appears to be dispensable for immune defense against *L. pneumophila* infection. *L. pneumophila* LPS appears to be poorly recognized by TLR4, as its lipid A consists of unusual long, branched-chain fatty acids [104] and does not interact with CD14 [105]. As a result, TLR4-deficient C3H/HeJ mice showed no differences in bacterial loads and inflammatory infiltration into the lung compared to WT C3H/HeN mice [106]. Similarly, *Tlr4*$^{-/-}$ bone marrow-derived macrophages (BMDMs) and *Tlr4*$^{-/-}$ mice on the C57BL/6 background showed no differences in cytokine production and bacterial loads compared to WT macrophages and mice; in contrast, *Tlr2*$^{-/-}$ BMDMs exhibited defects in IL-12, TNFα and IL-6 production [107], and *Tlr2*$^{-/-}$ mice exhibited higher bacterial loads, as well as defective cytokine and chemokine production in the lung [107–110]. These findings suggest that TLR2, which can recognize *L. pneumophila* peptidoglycan-associated lipoproteins [111], rather than TLR4, is important for host resistance against *L. pneumophila* (Fig. 1). Interestingly, one study showed that individuals heterozygous for two common TLR4 single nucleotide polymorphisms associated with LPS hyporesponsiveness were protected from Legionnaires' disease during an outbreak in the Netherlands [112], suggesting a potential deleterious consequence of TLR4 in inflammatory responses to *L. pneumophila* infection in humans.

Another member of the TLR family, TLR5, detects flagellin from several bacterial pathogens, such as *Listeria monocytogenes* and *Salmonella* Typhimurium [113]. TLR5-deficient alveolar macrophages exhibited defective TNFα responses to WT *L. pneumophila* compared to WT alveolar macrophages and conversely, flagellin-deficient *L. pneumophila* failed to induce TNFα production by WT alveolar macrophages [114], indicating that TLR5 recognizes *L. pneumophila* flagellin. Although WT and *Tlr5*$^{-/-}$ mice did not exhibit any differences in bacterial loads following infection [114,115], *Tlr5*$^{-/-}$ mice exhibited an impaired recruitment of neutrophils into the airway early during infection [114]. Interestingly, *Tlr5*$^{-/-}$ mice exhibited increased production of several cytokines and chemokines, as well as prolonged inflammation in the lung [114], suggesting that TLR5 may play a role in the resolution of pulmonary inflammation. Interestingly, a common stop codon polymorphism in TLR5 was associated with susceptibility to Legionnaires' disease in humans [116], suggesting that TLR5 is important for host defense against *L. pneumophila* in humans.

TLR9 resides within endosomal compartments, where it can recognize bacterial CpG DNA [117–119]. In mice on a mixed C57BL/6/A/J background carrying the A/J *Naip5* gene, which is a partial loss of function (hypomorphic) allele that renders mice more susceptible to *L. pneumophila* [26,28], TLR9 was dispensable for cytokine responses and control of bacterial loads in response to a sublethal dose of *L. pneumophila* [115]. In contrast, in the BALB/c background, mice lacking TLR9 exhibited decreased production of the cytokines IL-12 and IFN-γ, impaired bacterial clearance, and increased mortality [120]. Furthermore, intratracheal administration of the TLR9 agonist CpG oligodeoxynucleotide enhanced bacterial clearance [120], suggesting a critical role for TLR9 in host defense against *L. pneumophila*. The reasons underlying the apparent discrepancy in the two studies is unclear, but may be due to differences in mouse background, intranasal *versus* intratracheal infection, and/or differences in the *L. pneumophila* isolate used.

NAIP/NLRC4 and NLRP3 inflammasomes

The NLR NAIP5 (also known as Birc1e) senses the cytosolic presence of *L. pneumophila* flagellin and mediates restriction of bacterial infection within murine macrophages and during *in vivo* mouse infection [26,28,30,121–123]. Upon binding flagellin, NAIP5 recruits the adaptor protein NLRC4 (also known as IPAF) to assemble a multiprotein complex in the host cytosol termed an inflammasome, which recruits and activates the cysteine protease caspase-1 [124–127]. Purified flagellin, NAIP5, NLRC4, and caspase-1 are sufficient to reconstitute a functional inflammasome in HEK293 cells [124,128]. Active caspase-1 processes IL-1β and IL-18 and also cleaves the host protein gasdermin D (GSDMD) into an active form that inserts into the plasma membrane and forms a pore, resulting in IL-1β and IL-18 secretion and an inflammatory cell death termed pyroptosis [129,130].

The NAIP5/NLRC4 inflammasome restricts *L. pneumophila* through both caspase-1 and caspase-1-independent mechanisms, as NLRC4-deficient macrophages and mice are more susceptible to *L. pneumophila* infection than caspase-1-deficient macrophages and mice [131]. It was recently found that the NAIP inflammasome can activate caspase-8 in an ASC-dependent manner in the absence of caspase-1 or gasdermin D [132]. Subsequent caspase-8 activation can trigger pore formation and cell death in a caspase-1- and gasdermin-D-independent manner and restrict *L. pneumophila* replication [132]. It is unknown whether caspase-8 could be recruited to the NLRC4 inflammasome in other cell types *in vivo* when caspase-1 is still present during bacterial

infection. In addition, it has been shown that the NLRC4 inflammasome can lead to caspase-1-dependent caspase-7 activation, and mice or macrophages lacking caspase-7 exhibited increased bacterial replication compared to their WT counterparts [133], suggesting that NLRC4-dependent activation of caspase-7 is critical for restricting *L. pneumophila* replication. Under conditions where the NLRC4 inflammasome recruits and activates caspase-8, it would be interesting to determine whether there could be subsequent processing and activation of the apoptotic caspases-7 and -3, which could mediate processing of other gasdermin family members, such as gasdermin E (DFNA5), that could carry out pore formation and restriction of bacterial replication [134,135]. These findings collectively suggest that the NAIP5/NLRC4 inflammasome is capable of activating caspase-1, as well as caspases-8 and -7, that are normally thought to be involved in apoptosis, to limit *L. pneumophila* replication.

As a consequence of NAIP5/NLRP4 inflammasome-dependent bacterial restriction, the majority of inbred mouse strains, such as C57BL/6 and BALB/c background mice, are restrictive for *L. pneumophila* growth [25,26]. In contrast, A/J mice carry a hypomorphic *Naip5* allele that causes the mice to be more permissive and allow for *L. pneumophila* replication [25,26,28,136]. Unlike mice, which encode multiple *NAIP* receptors that each have exquisite specificity for individual bacterial type III secretion system (T3SS) or flagellin proteins [126,127], humans encode a single functional *NAIP* gene (*hNAIP*) [137]. A study indicated that hNAIP only recognized the T3SS needle protein [127]. However, hNAIP was subsequently shown to be important for inflammasome responses to bacterial flagellin in both immortalized and primary human monocyte-derived macrophages [138,139]. In contrast to mouse macrophages, human macrophages are more permissive for WT *L. pneumophila* infection, but there is some evidence indicating that hNAIP aids in detection and restriction of bacterial infection. Flagellin-deficient *L. pneumophila* replicate more efficiently than WT bacteria within human immortalized and primary macrophages and lung epithelial cells, and silencing of hNAIP or hNLRC4 expression leads to increased bacterial replication [140]. Interestingly, African populations have a duplication of the *NAIP* gene at a higher frequency than European and Asian populations, and higher NAIP levels correlate with increased cell death during *L. pneumophila* infection, suggesting that increased *NAIP* expression correlates with increased protection against *L. pneumophila* and other bacterial pathogens [141].

The NLRP3 inflammasome is also activated in response to *L. pneumophila* infection [142], which recruits ASC to activate caspase-1, leading to pyroptosis and IL-1 family cytokine release [142–144]. The NLRP3 inflammasome responds to a wide variety of infectious and non-infectious stimuli, and $K+$ efflux appears to be essential for NLRP3 inflammasome activation [145,146]. NLRP3 inflammasome activation can be enhanced by AIM2 inflammasome- and caspase-11-induced plasma membrane damage [147,148]. The precise mechanism underlying the activation of NLRP3 inflammasome in response to *L. pneumophila* or other stimuli is still unclear. Recently, one study revealed that NLRP3 is recruited to the dispersed trans-Golgi network (dTGN) under stimulation of diverse NLRP3 agonists including both $K+$ efflux dependent and independent stimuli through ionic bonding between its conserved polybasic region and negatively charged phosphatidylinositol-4-phosphate (PtdIns4P) on the dTGN [149], and NLRP3 aggregates into multiple puncta on dTGN through PtdIns4P binding, which is essential for NLRP3 inflammasome activation. Notably, the LCV of *L. pneumophila* is enriched with PtdIns4P [150], which is acquired either directly from Golgi apparatus during early LCV maturation [151], or endogenous generation by the dot/Icm effectors LepB and SidF cooperation that LepB kinase domain phosphorylates PtdIns3P to PtdIns(3,4)P2 and subsequently SidF as phosphatase converts PtdIns(3,4)P2 to PtdIns4P [51]. Therefore, it is possible that the LCV-enriched PtdIns4P might serve as NLPR3 agonist to directly activate the inflammasome on dTGN.

Although ASC is required for maximal IL-1 cytokine responses downstream of inflammasome activation in response to *L. pneumophila* infection, ASC may negatively regulate other inflammasome effector functions such as pyroptosis. ASC recruits NLRC4 and caspase-1 to inflammasome puncta during *L. pneumophila* infection, and ASC deficiency results in increased NLRC4-dependent pore formation and cell death [152]. These studies suggest that both NAIP5/NLRC4 and ASC are required for maximal caspase-1 activation and IL-1β and IL-18 secretion during *L. pneumophila* infection, but that ASC also plays a negative feedback role by dampening NLRC4/caspase-1-mediated pyroptosis.

Caspase-11 inflammasome

Caspase-11 is a cytoplasmic sensor of bacterial LPS. Upon binding LPS [153–155], caspase-11 can oligomerize into an inflammasome without the need for upstream NLRs and become proteolytically active, resulting in gasdermin D cleavage, pyroptosis, and release of the alarmins IL-1α and HMGB1 [129,153,155]. Caspase-11-dependent pore formation also triggers $K+$ efflux and subsequent NLRP3 inflammasome activation, resulting in caspase-1-dependent IL-1β and IL-18 processing and secretion [129,153,155]. *Legionella* T4SS activity induces caspase-11-dependent pyroptosis and alarmin release

and subsequent NLRP3 inflammasome activation, thus allowing for IL-1 family cytokine secretion [142,144]. Caspase-11 activation is enhanced in the absence of the T4SS effector SdhA [156], presumably as a consequence of decreased LCV membrane integrity and increased exposure of *L. pneumophila* LPS to the host cell cytosol. In the context of *Escherichia coli* infection, outer-membrane vesicles (OMVs) promote cytosolic entry of LPS and caspase-11 activation [157]. There is evidence that *L. pneumophila* OMVs can deliver bacterial virulence factors into host cells [158]. Perhaps *L. pneumophila* OMVs also deliver LPS into the cytosol to activate caspase-11.

TLR4-dependent TRIF signaling and type I and II IFN signaling enhance caspase-11 responses to *L. pneumophila* and other gram-negative bacteria [142,159–161], in part through upregulating caspase-11 expression. Both inducible and constitutive IFN signaling promote caspase-11 activation in response to infection or bacterial OMVs by inducing expression of the family of guanylate-binding proteins (GBPs) [162–167]. Precisely how GBPs enhance caspase-11 activation is unclear. Several models have been proposed for how GBPs function, including that GBPs recognize and lyse pathogen-containing vacuoles, thus releasing bacterial contents and their associated LPS into the host cell cytosol, or that once bacteria are in the host cell cytosol, GBPs target and lyse the bacterial outer membrane [162,164–167]. In macrophages treated with type I IFN, LCVs were destabilized independently of the GBPs present on mouse chromosome 3, but these GBPs were required for the loss of the rod-shaped morphology of cytosolic bacteria [167], as well as bacterial clearance *in vivo*.

Sensing of bacterial ribosomal protein RpsL

In addition to inflammasome activation, there is some evidence that other forms of cell death can be triggered in macrophages during infection with environmental and clinical *L. pneumophila* isolates. Cytosolic sensing of RpsL appears to induce cathepsin B-dependent lysosomal cell death in murine macrophages that restricts bacterial replication of environmental and clinical strains [168,169]. In contrast, laboratory-adapted *L. pneumophila* strains that are streptomycin resistant, due to a lysine to arginine mutation (K88R) in the bacterial ribosomal protein RpsL [170,171], induced less cell death and are able to replicate within murine macrophages [168,169], indicating that RpsL may be sensed by an as-yet-unknown PRR in mice [172].

NOD1 and NOD2

The NLRs NOD1 and NOD2 are cytosolic sensors of bacterial peptidoglycan [173,174]. NOD1 detects γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), a peptidoglycan motif found in many gram-negative bacteria and some gram-positive bacteria [175–177], whereas NOD2 detects muramyl dipeptide (MDP), a peptidoglycan motif widely found in both gram-positive and gram-negative bacteria [178,179]. NOD1 and NOD2 signal through the adaptor RIPK2 (receptor-interacting protein kinase 2; also known as RIP2) to activate NF-κB and MAPK signaling, resulting in the production of proinflammatory cytokines, chemokines, and antimicrobial effectors [180–182]. During *L. pneumophila* infection, there is NOD1 and NOD2-dependent RIPK2 signaling and proinflammatory cytokine and chemokine production [183–186]. Mice lacking NOD1, NOD2, or RIPK2 exhibited defective CXCL1, IL-6, and G-CSF production, neutrophil recruitment, and bacterial clearance [183]. The *L. pneumophila* periplasmic protein EnhC interferes with soluble lytic transglycosylase (SltL), a bacterial peptidoglycan degrading enzyme, to reduce production of anhydro-disaccharide-tetrapeptide, which can be recognized by NOD1 [187]. EnhC mutants exhibited a defect in intracellular bacterial replication that could be rescued by inhibition of SltL or the absence of NOD1 [187], indicating that EnhC facilitates bacterial evasion from NOD1 detection.

Nucleic acid sensing

AIM2 is a sensor of cytosolic dsDNA that stimulates ASC- and caspase-1-dependent inflammasome activation and subsequent IL-1β and IL-18 secretion and pyroptosis [188–190]. WT *L. pneumophila* infection can trigger AIM2 inflammasome activation in murine macrophages, suggesting that bacterial DNA gains access to the host cytosol, where it is sensed by AIM2. AIM2 activation is enhanced in the absence of the Dot/Icm-translocated effector SdhA, as SdhA helps maintain LCV membrane integrity and prevent exposure of *L. pneumophila* to the host cell cytosol [191,192]. AIM2 inflammasome-induced pore formation then leads to K^+ efflux-mediated NLRP3 inflammasome activation [148], thus amplifying inflammasome responses to infection.

A variety of nucleic acid sensors that lead to potent type I IFN induction are also triggered by *L. pneumophila* infection. The first clues came from studies showing that *L. pneumophila* T4SS activity induced a robust type I IFN response requiring the transcription factor IRF3 [193,194]. The cytosolic RNA sensors RIG-I and MDA5 and the adaptor MAVS were subsequently found to induce type I IFNs during *L. pneumophila* infection [194–196]. Whether *L. pneumophila* RNA gains access to the cytosol and is recognized by RIG-I and MDA5 is unclear. Cyclic GMP-AMP synthase (cGAS), a sensor of cytosolic DNA [197], and STING, a signaling adaptor for cGAS and a sensor of bacterial

cyclic dinucleotides [198–201], also respond to *L. pneumophila* by inducing type I IFNs [202,203]. cGAS- and STING-deficient mice exhibited a defect in inflammatory cytokine production and impaired bacterial clearance [202]. In addition, human macrophages carrying a common HAQ allele of STING were severely impaired in the production of type I IFNs and other inflammatory cytokines in response to *L. pneumophila* [202]. Interestingly, the haplotype frequency of HAQ STING was increased in two cohorts of human Legionnaires' disease patients compared to healthy human controls [202], suggesting that STING is important for host resistance against *L. pneumophila* in humans.

Activation and inhibition of NF-κB signaling

Host transcriptional responses downstream of NF-κB signaling are essential for host cell survival and allowing for intracellular bacterial replication, in part due to Dot/Icm- and NF-κB-dependent anti-apoptotic gene expression [204,205]. *L. pneumophila*-infected host cells exhibit a biphasic pattern of NF-κB activation [204,206]. Within a few hours, there is a robust but transient induction of TLR-dependent and Dot/Icm-independent NF-κB signaling, whereas there is sustained NF-κB activation during later stages of infection that is independent of TLR or NOD1 signaling and requires a functional Dot/Icm system [204,206]. These data indicate that there is Dot/Icm-dependent, effector-triggered NF-κB signaling. The Dot/Icm effectors LnaB and LegK1 potently activate NF-κB [207,208]. LegK1 possesses eukaryotic-like Ser/Thr kinase activity and appears to mimic the host kinase IKK, as it directly phosphorylates IκBα, which leads to canonical NF-κB signaling, and phosphorylates other IκB family members, including p100, which is involved in noncanonical NF-κB signaling [208]. The mechanism by which LnaB promotes NF-κB activation is still unknown. As described in the section below, there are additional effectors that trigger NF-κB signaling due to perturbation of host cell processes. In addition, there are effectors that dampen NF-κB signaling. The Dot/Icm effector MavC (Lpg2147) inhibits NF-κB activation by serving as a transglutaminase that catalyzes monoubiquitination of E2 enzyme UBE2N, which abolishes UBE2N's E2 activity in forming K63-type polyubiquitin chains, thus dampening NF-κB signaling during the initial stages of infection [209]. Furthermore, the T4SS effector RavD is recruited to the LCV [210], where it hydrolyzes linear ubiquitin chains and prevents their accumulation on the LCV, resulting in decreased NF-κB signaling [211].

Effector-triggered immunity

As a consequence of stimulating TLR signaling and perhaps its residence in an ER-derived vacuole, *L. pneumophila* infection induces ER stress and the unfolded protein response (UPR) [212,213]. Induction of UPR involves three different transmembrane receptors, IRE1α, PERK, and ATF6, which sense ER stress and induce downstream signaling events that alleviate the stress and restore cellular homeostasis. A UPR response that fails to be resolved could potentially result in autophagy or apoptosis of infected cells [214]. Thus, activation of UPR can serve as an effector-triggered immune response whereby receptors of the UPR pathway act as guards of ER homeostasis during *L. pneumophila* infection. However, *L. pneumophila* is able to block induction of the UPR. *L. pneumophila* encodes at least 12 effectors that potently inhibit host translational initiation and elongation [53,215–220]. Three of the effectors (Lgt1, Lgt2, and Lgt3), are glycosyltransferases that target the eukaryotic elongation factor eEF1A and block host translation [216], SidI interacts with eEF1A and eEF1Bγ and blocks host translation [215], and SidL blocks host translation through an unknown mechanism [52]. Dot/Icm activity and in particular, a number of T4SS-translocated effectors inhibit mTOR signaling [217,220], thereby contributing to the translational block. In addition, the effector LegK4 phosphorylates HSP70, thereby disrupting its ATPase activity and protein folding capacity and contributing to global translational inhibition [53]. Several of these effectors block the ATF6 and IRE1 branches of the UPR [212,213].

In turn, *L. pneumophila* T4SS activity and the effector-driven translational block potently induce a unique transcriptional program in murine BMDMs, involving the expression of proinflammatory genes such as *Il1a*, *Il1b*, *Il23a*, and *Csf2* [52,186]. The translational block prevents IκB synthesis, which leads to prolonged NF-κB activation [52], and also activates p38 and SAPK/JNK MAPK signaling through an unknown mechanism [221]. Subsequent synthesis of IL-1α and IL-1β by infected macrophages is critical for production of the cytokines TNFα and IL-12 by bystander myeloid cells [222]. Interestingly, inhibition of translation by multiple bacterial toxins or pharmacological inhibitors, in conjunction with TLR signaling, induces expression of an overlapping set of genes as *L. pneumophila* infection [52], suggesting that this transcriptional program represents an immune response to inappropriate disruption of host protein synthesis. Precisely how the infected host cell still synthesizes a subset of proteins despite the translational block is still unclear, but the super-induction of mRNAs is required [223,224]. Collectively, these findings reveal that although *Legionella* employs effectors that interfere with the induction of the UPR by inhibiting host translation, the translational block triggers an effector-triggered immunity response that activates NF-κB and MAPK signaling and super-induction of immune genes that promote bystander cytokine responses. There is emerging evidence that

additional T4SS effectors induce effector-triggered immunity. A transposon insertion screen identified the effector LegC4 as being detrimental to *L. pneumophila* infection, as *legC4* mutants exhibited increased bacterial loads compared to WT bacteria in the lungs of infected mice [225]. Interestingly, BMDMs infected with the *legC4* mutant produced significantly lower levels of IL-12 compared to BMDMs infected with WT *L. pneumophila,* suggesting that LegC4 triggers increased proinflammatory cytokine production [225]. Currently, LegC4's function within host cells and how it leads to cytokine production is unknown.

Inflammatory Cytokine Responses during *L. pneumophila* Infection

Once inhaled into the lung, *L. pneumophila* infects and replicates primarily within alveolar macrophages. Severe pneumonia can develop in immunocompromised individuals, especially in patients with chronic pulmonary diseases who receive immunosuppressants [226–228]. The main clinical characteristics of *L. pneumophila*-induced pneumonia are ALI and severe hypoxemia [31,32]. Patients present with high levels of serum cytokines that likely contribute to both host defense and lung injury during *L. pneumophila* infection. Studies in animal and *in vitro* models have revealed a critical role for several immune cell types and cytokines in host defense against *L. pneumophila*.

IL-1 family cytokines

Following infection of macrophages, *L. pneumophila* blocks host protein synthesis and impairs production of TNFα, IL-6, and IL-12. However, infected macrophages activate a variety of inflammasome-dependent and independent pathways that lead to robust secretion of IL-1α and IL-1β, as described in the previous sections. IL-1α and IL-1β both bind the IL-1R, which is critical for control of *L. pneumophila* infection. IL-1R signaling is required for production of proinflammatory cytokines, such as TNFα and IL-12, by uninfected bystander immune cells, such as alveolar macrophages, neutrophils, monocytes, and dendritic cells [222]. In addition, IL-1R signaling to non-hematopoietic cells, including alveolar epithelial cells, promotes the production of chemokines, such as CXCL1 and CXCL2, which mediate neutrophil recruitment to the lung [144,219,229,230]. Although they signal through the same receptor, IL-1α seems to be more critical than IL-1β in mediating neutrophil recruitment and bacterial clearance in both permissive and nonpermissive mouse models of infection [144,219]. IL-18 is also released in an inflammasome-dependent manner in response to *L. pneumophila* infection [143]. IL-18 is required for production of IFN-γ by NK cells and optimal bacterial clearance in both pulmonary and systemic mouse models of infection [230–232].

Recently, the IL-1 family member IL-36 was found to be critical in host defense against *L. pneumophila* [233]. The IL-36 cytokine family consists of IL-36α, IL-36β, and IL-36γ, which are expressed by a wide variety of cell types, including myeloid cells. In a nonpermissive mouse model, mice lacking the IL-36 receptor exhibited impaired bacterial clearance, increased mortality, as well as reduced inflammatory cell accumulation and decreased expression of proinflammatory cytokines [233]. Furthermore, IL-36α and IL-36γ played redundant and overlapping roles in host defense [233]. It is not clear how infection triggers IL-36 cytokine secretion and which cell types are the major IL-36-producing cells. Overall, these findings provide insight into the roles of different IL-1 family cytokine members in immune defense. There is yet more to learn about the roles of these cytokines in the context of *L. pneumophila* infection.

TNFα

Clinical treatment with anti-TNFα agents (etanercept, infliximab, and adalimumab) is widely used in patients with chronic inflammatory conditions, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, and asthma [234–236]. However, patients on TNFα blockade have an increased risk for developing Legionnaires' disease [36–39]. Similarly, in rodent models, antibody-mediated blockade of TNFα or TNFα receptor deficiency causes a defect in controlling pulmonary *L. pneumophila* infection [42,237,238]. TNFα can signal through two receptors, TNFR1 and TNFR2, that appear to play distinct roles during *L. pneumophila* infection. In a non-permissive mouse model, C57BL/6 mice lacking either TNFR1 or TNFR2 exhibited increased mortality following WT *L. pneumophila* infection [238]. TNFR1-deficient mice showed a slight defect in neutrophil recruitment, IL-12 production, and are defective for bacterial clearance [42,238]. Mixed bone-marrow chimera experiments revealed that cell-intrinsic TNFR1 signaling was required to restrict *L. pneumophila* within alveolar macrophages [42]. In contrast, TNFR2-deficient mice are able to control bacterial loads to the same extent as WT mice [42,238], but exhibit excess neutrophil infiltration [238], suggesting that TNFR2 signaling is not required for controlling *L. pneumophila* infection and instead restrains excessive inflammation. *In vitro*, TNFα suppresses *L. pneumophila* replication within a variety of cell types, including mouse BMDMs, rat alveolar macrophages, and human airway epithelial cells [40–42,239]. Interestingly, TNFα restricts replication of flagellated *L. pneumophila* in either mice or macrophages with a functional NAIP5 inflammasome [42,238,239], suggesting that TNFα signaling somehow contributes to NAIP5-dependent restriction of bacterial replication. The precise mechanisms

underlying how TNFα signaling restricts *L. pneumophila* replication are not fully understood. In macrophages during *in vitro* infection, TNFR1-mediated restriction of intracellular bacterial replication is independent of NLRC4, caspases-1 and 11, and ROS [42]. Instead, restriction within host cells relies on NF-κB signaling [239], as well as lysosome acidification and the activity of unknown caspases [41,42].

IL-12 and type I and II IFNs

IL-12 is produced by Ly6C^{hi} monocytes, dendritic cells, and neutrophils during pulmonary *L. pneumophila* infection [240,241]. IL-12 plays a critical role in controlling *L. pneumophila* infection *in vivo* [242], as IL-12 drives production of type II IFN (IFN-γ) by NK cells, NKT cells, γδ T cells, and memory αβ T cells [231,240]. IFN-γ and the IFN-γ receptor are critical for controlling bacterial infection *in vivo* [115,243,244]. IFN-γ, in concert with type I IFNs, contributes to restricting *L. pneumophila* infection as mice lacking both type I IFN receptor (IFNAR) and type II IFN receptor (IFNGR) have a greater defect in controlling *L. pneumophila* infection than mice lacking either receptor alone [202,203]. *In vivo,* IFN-γ appears to be required for the optimal restriction of *L. pneumophila* within monocytes, but not neutrophils or alveolar macrophages [241]. *In vitro,* exogenous addition of IFN-γ inhibits *L. pneumophila* replication within a variety of host cells, including human alveolar macrophages, human monocytes, and mouse macrophages [245–247]. Similarly, type I IFN signaling restricts *L. pneumophila* replication in macrophages and lung epithelial cells [194,248–250].

Precisely how IFN signaling restricts *L. pneumophila* replication within host cells is not yet fully understood and may involve multiple mechanisms. IFN-γ-activated macrophages infected with *L. pneumophila* robustly produced nitric oxide (NO) [251,252], and pharmacological inhibition of NO synthesis partially impaired IFN-γ-mediated restriction of *L. pneumophila* in the murine RAW264.7 macrophage cell line [252] and rat alveolar macrophages [40]. In contrast, other studies suggest that IFN-γ-mediated restriction of *L. pneumophila* replication is NO-independent in murine macrophages and human monocytes [251,253–255], although inhibition of NO activity in A/J mice affected bacterial clearance *in vivo* [256]. IFN-γ-activated monocytes and macrophages exhibit decreased intracellular iron levels due to downregulation of the transferrin receptor, thus limiting the iron available for *L. pneumophila* replication [252,257]. IFN-γ-mediated bacterial restriction in human monocytes could be reversed by addition of iron-lactoferrin [253,258]. These findings provide a nutrition-dependent mechanism for how IFN-γ restricts *L. pneumophila* replication. In addition, a recent study suggested that IFN-γ and type I IFNs induce remodeling of the LCV and stimulate expression of the immune-responsive gene (IRG)1 in mitochondria, which led to production of itaconic acid [259], which is a metabolite that is bactericidal against *L. pneumophila* [259], as well as the pathogens *Salmonella enterica* and *Mycobacterium tuberculosis,* by inhibiting isocitrate lyase, a key enzyme in the glyoxylate shunt pathway [260]. The findings thus far warrant further exploration of the molecular mechanisms underlying IFN-mediated inhibition of bacterial replication. Given that adenovirus-mediated delivery of IFN-γ robustly promoted *L. pneumophila* clearance in a mouse model [261], and a recombinant form of IFN-γ (Actimmune, IFN-γ-1b) is approved by FDA for reducing the frequency and severity of infections in patients with chronic granulomatous disease [262,263], perhaps recombinant IFN-γ could be a treatment for Legionnaires' disease.

Type 2 cytokines

Th2 cytokines have some effect on host responses to *L. pneumophila* infection. Nonpermissive BALB/c mice lacking IL-4 succumbed to infection due to uncontrolled TNFα production, indicating a role for IL-4 in negatively regulating type 1 cytokine responses during infection [264]. Whether IL-10 participates in the host response to *L. pneumophila* infection *in vivo* is unknown, although it was found that IL-10 could reverse IFN-γ-mediated inhibition of *L. pneumophila* replication in human monocytes and murine BMDMs [265,266]. It would be interesting to examine further whether crosstalk between Th1 and Th2 cytokine responses influences the outcome of *L. pneumophila* infection.

IL-17

In several pulmonary infections, the IL-17 family of cytokines plays critical roles in inflammation and host defense [267]. IL-17A and IL-17F are robustly induced during *L. pneumophila* infection [268,269]. In nonpermissive mouse models, IL-17A/F-deficient mice exhibited a delay in bacterial clearance and succumbed more readily to a lethal dose of *L. pneumophila*, with a more critical role for IL-17A than IL-17F [268]. IL-17A was also important for bacterial clearance and survival in the permissive A/J mouse model [269]. IL-17 was required for maximal production of the cytokines IL-6 and TNFα, neutrophil-attracting chemokines, and subsequent neutrophil recruitment [268,269]. IL-17 was made by both T cells and neutrophils, although neutrophil-derived IL-17 appeared to be more critical for bacterial clearance, and appeared to involve the induction of IFN-γ [269]. A retrospective study of Legionnaires' disease patients detected IL-17A in sera from 4 out of 31 patients and found that all of the IL-17A-positive patients survived, whereas 8 of 27 IL-17A-negative patients died [270], suggesting an association between IL-17 and positive patient outcome. Additional

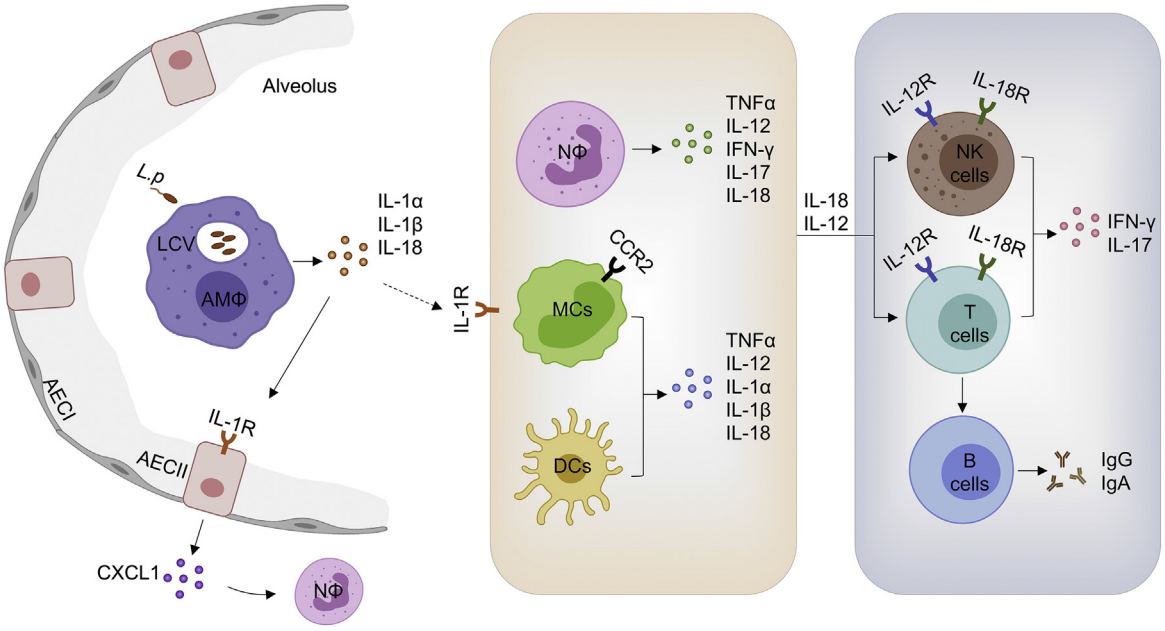

Fig. 2. Cellular immune responses during *L. pneumophila* infection. *L. pneumophila* evades phagosome−lysosome fusion to enable replication in an LCV within permissive alveolar macrophages (AMΦ). The infected AMΦ experience a global block in protein synthesis due to the activity of T4SS effectors and cannot produce TNFα and IL-12, but can still translate and secrete IL-1 family cytokines. IL-1-mediated signaling instructs: (1) type II alveolar epithelial cells to produce chemokines such as CXCL1 to mediate neutrophil (NΦ) recruitment into the lung; (2) bystander uninfected myeloid cells, including NΦs, monocytes (MCs), and dendritic cells (DCs), to produce inflammatory cytokines. NΦs have been shown to produce TNFα, IL-12, type II interferon (IFN-γ), IL-17 and IL-18. MCs and DCs have been shown to produce TNFα, IL-12, and IL-1 family cytokines. These cytokines then instruct NK cells, NKT cells, and memory T cells to produce IFN-γ and IL-17. TNFα and IFN-γ promote the bacterial restriction. In addition, CD4+ T lymphocytes regulate antigen-specific B cell production of IgG and IgA antibodies.

studies are needed to determine whether IL-17A indeed correlates with successful control of *L. pneumophila* infection in humans.

Cellular Responses during *L. pneumophila* Infection *in Vivo*

In murine models, *L. pneumophila* infection induces potent cellular responses (Fig. 2), which are characterized by robust recruitment of inflammatory myeloid cells, including neutrophils, monocytes, and dendritic cells, and NK, NKT, CD4+, and CD8+ T cells to the lung [241]. Cytokine and chemokine responses, including the production of IL-1, CXCL1, and MCP-1, are involved in the recruitment of immune cells, and these cells are critical for producing inflammatory mediators that contribute to control of infection. Below, we describe the contribution of different immune cell types to *L. pneumophila* infection.

Alveolar macrophages

As described above, alveolar macrophages are the primary cell type that are infected by *L. pneumophila* and that support intracellular bacterial replication. Shortly after infection, the number of alveolar macrophages in the lung airway decreases for unclear reasons. Bacterial replication and subsequent lysis and egress from infected alveolar macrophages, as well as inflammasome-dependent pyroptosis, may in part account for the decreased numbers of alveolar macrophages. As alveolar macrophages are able to rapidly transport other pathogens, such as *Streptococcus pneumoniae*, to the lung-draining lymph nodes (dLN) [271], it is possible that alveolar macrophages also transport *L. pneumophila* to dLN following infection for induction of adaptive immune responses. A recent study observed that during *M. tuberculosis* infection, infected alveolar macrophages disseminated from the alveoli to the lung interstitium [272]. This relocalization required IL-1R signaling on non-hematopoietic cells [272]. These studies raise the question of whether *L. pneumophila*-infected alveolar macrophages also relocate into the lung interstitium or dLNs, providing a mechanism to disseminate infection or initiate adaptive immune responses.

Neutrophils

Following *L. pneumophila* infection, neutrophils are recruited to the lung in response to IL-1R signaling via

a non-hematopoietic cell type, likely type II alveolar epithelial cells, that in turn produce neutrophil-attracting chemokines [229]. Neutrophils are important for controlling infection, as inhibiting neutrophil recruitment using anti-CXCR2 blocking antibodies or selectively depleting neutrophils resulted in decreased cytokine levels, increased bacterial loads, and decreased survival [231,240,273]. Neutrophils produce a variety of proinflammatory cytokines, including IL-1, TNFα, IL-12, and IFN-γ, in response to infection [42,240,273]. Although *L. pneumophila* is able to infect and inject T4SS effectors into neutrophils [23], neutrophils possess direct antimicrobial activity and kill *L. pneumophila* through cell-intrinsic ROS production [42]. Interestingly, ROS production and the bactericidal activity of neutrophils do not require either TNFR1 or IFN-γ signaling [42,241], indicating either that these pathways are redundant [42,240,273] or that neutrophils rely on other signaling pathways to become bactericidal against *L pneumophila*.

Monocytes

Ly6C^{hi} inflammatory monocytes are robustly recruited to the lung during pulmonary *L. pneumophila* infection [222,240,241]. Mice lacking the chemokine receptor CCR2, which is required for monocyte egress from the bone marrow [274], have a defect in monocyte recruitment to the lungs and increased bacterial loads during infection [240,241]. Monocytes serve as a critical source of proinflammatory cytokines, including TNFα and IL-12 [222], and are required for downstream IFN-γ production by innate and adaptive lymphocytes [42,240,241,273]. CCR2-deficient animals have impaired recruitment of monocyte-derived dendritic cells (moDCs) into the lung during *L. pneumophila* infection [240,241], as well as in response to non-infectious stimuli [275], indicating that recruited monocytes differentiate into moDCs once in the lung. During pulmonary infection, a large percentage of monocytes seem to phagocytose *L. pneumophila* bacteria or become associated with bacterial-derived material [241]. In addition, monocytes appear to possess direct bactericidal activity in response to IFN-γ signaling [42,240,241,273], although the molecular mechanisms underlying how monocytes restrict *L. pneumophila* infection are not well understood.

Following the acute phase of *L. pneumophila* infection, there is a decline in inflammatory cytokine production that precedes the resolution of inflammatory cellular infiltration [43], suggesting that recruited immune cells not only produce inflammatory cytokines and clear bacterial infection but also aid in dampening inflammation. Programmed cell death of myeloid cells appears to play a role in host resilience to infection, as overexpression of pro-survival B-cell lymphoma-2 protein (BCL-2) specifically in myeloid cells resulted in increased inflammation after infection [276]. This finding indicates that programmed cell death of myeloid cells is important for restoring immune homeostasis during *L. pneumophila* infection.

Dendritic cells

DCs in the lung play a central role in the integration of innate and adaptive immunity in a variety of infectious and non-infectious settings [277]. During pulmonary *L. pneumophila* infection, there is robust recruitment of moDCs into the lung [240,241]. *In vivo*, DCs do not appear to be productively infected by *L. pneumophila* [23], but they produce a variety of proinflammatory cytokines, including IL-12, in response to infection [222,240,241]. Plasmacytoid dendritic cells (pDCs) are also recruited into the lung during *L. pneumophila* infection, and pDCs appear to promote bacterial clearance [278]. Whether other populations of DCs, such as resident DCs, are required for immune defense against *L. pneumophila* infection is not yet known.

During *in vitro* infection, in contrast to macrophages, bone marrow-derived DCs (BMDCs) or splenic DCs from permissive A/J mice restrict *L. pneumophila* replication, although the LCV is able to evade endocytic maturation [279]. Within several hours post-infection, DCs undergo rapid caspase-3-dependent apoptosis specifically in response to *L. pneumophila* Dot/Icm T4SS activity [280]. Eliminating the pro-apoptotic proteins BAX and BAK or overexpressing the anti-apoptotic protein Bcl-2 allowed *L. pneumophila* to replicate within DCs [280]. A screen identified five effectors (Lpg0716, Lpg0898, Lpg1625, Lpg2178, and Lpg2831) that induced caspase-3 activation following transient overexpression in 293T cells, and a mutant bacterial strain lacking these five effectors induced less apoptosis in DCs [281]. Several of these effectors appear to be toxic to cells, and some of the effectors localize to the mitochondria [281]. These studies suggest that innate immune sensing of T4SS-translocated effectors and/or other bacterial products triggers this apoptotic response, although the underlying mechanisms and why this pathway functions in DCs but not in macrophages are unknown. In addition, these findings may provide an explanation for why productive infection of DCs or translocation of T4SS effectors into DCs is not normally observed *in vivo* [23].

Although *L. pneumophila* is able to avoid endocytic maturation and establish an ER-derived vacuole within BMDCs, BMDCs are still able to present *Legionella* antigens on MHC class II molecules and activate CD4+ T cells from *L. pneumophila*-immunized mice [279]; however, it is not yet known which bacterial antigens are presented on MHC class II. Interestingly, WT *L. pneumophila*-infected DCs are better at activating CD4+ T cells than DCs infected

with Dot/Icm mutants, which are transported to lysosomes [279]. *De novo* bacterial protein synthesis is required for Dot/Icm-enhanced antigen presentation to T cells, although bacterial replication is not required [279]. BMDMs are also capable of presenting *Legionella* antigens to CD4+ T cells from *L. pneumophila*-immunized mice [282]. CD4+ T-cell responses were higher in response to BMDMs infected with WT *L. pneumophila* than to Dot/Icm mutant-infected BMDMs, whereas BMDMs infected with IcmR, IcmS, or IcmW mutants induced intermediate levels of CD4+ T-cell activation [282]. This Dot/Icm-enhanced response did not require bacterial replication. These findings suggest that within both BMDCs and BMDMs, *L. pneumophila* traffic to a cellular compartment that evades lysosomal fusion and permits bacterial protein synthesis, subsequently allowing for processing and presentation of a unique subset of bacterial antigens on MHC class II. Furthermore, WT *L. pneumophila*-infected BMDCs and BMDMs produce more IL-12 than Dot/Icm mutant-infected cells [279], which would be expected to enhance IFN-γ production by CD4+ T cells.

Innate lymphocyte responses

During the first several days of *L. pneumophila* infection, NK cells, NKT cells, γδ T cells, and non-cognate memory αβ T cells all serve as early sources of IFN-γ [115,231,240,247,261,283]. These cells produce IFN-γ in response to IL-12 produced by monocytes and IL-18 from infected cells [115,231,240,247]. In permissive mouse models of pulmonary or intravenous infection, depletion of NK cells led to significantly decreased IFN-γ levels, but mice were still able to control bacterial infection, suggesting that other lymphocyte populations serve as redundant sources of IFN-γ [115,247]. Innate-like mucosal-associated invariant T (MAIT) cells are also activated and expand during *L. pneumophila* infection [284]. MAIT cells express a semi-invariant αβ T-cell receptor that recognizes small antigens presented by the MHC class I-related molecule MR1 [285,286]. These antigens are derivatives of the riboflavin biosynthetic pathway found in many bacteria [287–289], including *L. pneumophila*. *In vitro*, *L. pneumophila*-derived antigens are capable of activating MAIT cells [284]. During infection with the related *Legionella* species *L. longbeachae, Mr1*$^{-/-}$ mice, which are MAIT deficient, showed impaired bacterial clearance [284]. Whether MAIT cells also contribute to control of *L. pneumophila* infection is unknown.

Adaptive T-cell responses

During primary infection, CD4+ and CD8+ T cells are critical for immune control, as depletion of CD4+ and/or CD8+ T cells resulted in increased bacterial loads and mortality [43]. However, it is unclear whether control of primary infection is due to an antigen-specific T-cell response, as non-cognate memory αβ T cells are an early source of IFN-γ [241]. Patients that have recovered from Legionnaires' disease develop *L. pneumophila*-specific adaptive immune responses [290]. Infected animals or animals vaccinated with live attenuated strains or *L. pneumophila* antigens, such as major secretory protease (Msp), HSP60, or OmpS, also generate *L. pneumophila*-specific immune responses, which are protective against subsequent infection [279,291–295]. The generation of protective immunity requires both T cells and B cells [296].

During intranasal infection, *L. pneumophila*-specific CD4+ αβ T cells are primed and proliferate in the mediastinal lymph nodes (MLNs) [295]. They then differentiate into Th1 and Th17 cells once they arrive in the lung [295]. Interestingly, initial CD4+ T-cell activation and proliferation in the MLN is equivalent in response to WT or Dot/Icm mutant bacteria, but generation of a Th1/Th17 response requires Dot/Icm activity [295], suggesting that innate immune sensing of Dot/Icm-translocated substrates is important for sustained CD4+ T-cell proliferation, survival, and/or migration from the MLN to the lung. In support of this model, NLRC4 inflammasome-dependent detection of flagellin and IL-1R signaling contribute to the generation of a Th17 response against *L. pneumophila* [295].

Adaptive B-cell responses

Antibody responses are also important for host defense against *L. pneumophila*. Either active vaccination with bacteria or passive immunization with hyperimmune serum leads to a strong reduction of the bacterial burden in the lung [297]. DCs pulsed with *L. pneumophila* and transferred into mice induce an antibody response that requires MHC class II antigen presentation and are protective against lethal respiratory challenge [298]. During intranasal infection, WT *L. pneumophila,* but not Dot/Icm mutants, induce a specific antibody response, with IgG antibodies found systemically and IgA antibodies located in the lung [297]. CD4+ T cells are not required for IgM production by B cells but are required for antibody isotype switching to IgG and IgA [297]. These findings suggest that T follicular helper (Tfh) cells must be involved in promoting antibody responses to *L. pneumophila,* although the Tfh response has not yet been examined.

Both IgG and IgA subclasses are protective against *L. pneumophila* infection [299]. Of the IgG subclasses, although the IgG2c and IgG3 subclasses were the most prevalent in *L. pneumophila*-infected mice, IgG1, IgG2c, IgG2b, and IgG3 were all found to be highly efficacious at reducing bacterial loads following transfer of purified

IgG subclasses into naïve mice and subsequent *L. pneumophila* challenge [300]. Antibodies are protective through complement-independent mechanisms [299]. Within the first hour of infection, opsonizing antibodies provided nearly 10-fold protection in an antibody Fc-dependent, but FcR-independent manner [301]. By 2 days post-infection, antibodies promoted efficient opsonization of *L. pneumophila* and bacterial clearance involved subsequent FcR-dependent targeting of the bacteria to a degradative phagolysosomal compartment, Syk kinase activity in alveolar macrophages, and the induction of ROS [299,301]. Collectively, these findings indicate an important role for antibody responses in host defense and suggest that purified anti-*L. pneumophila* antibodies could be an effective treatment for infection.

Conclusions

L. pneumophila utilizes a variety of strategies to replicate within host cells. Although *L. pneumophila* causes the severe pneumonia Legionnaires' disease in humans, its primary hosts are environmental protozoa. *L. pneumophila* is therefore considered to be an accidental pathogen of humans, and it is thought that *L. pneumophila* has not evolved to evade mammalian-specific immune mechanisms. Thus, the study of the immune response to *L. pneumophila* has been invaluable, as it has provided unique and new insight into how the immune system is able to detect intracellular bacterial pathogens through the use of germline-encoded PRRs and antigen receptors generated as a result of somatic recombination, as well as alternative and emerging modes of immune recognition, such as effector-triggered immunity. Furthermore, the investigation of the immune response to *L. pneumophila* in both *in vitro* and *in vivo* models, as well as the identification of immune gene polymorphisms associated with human susceptibility to Legionnaires' disease, provides insight into the significance of these immune pathways in host defense in humans.

Although *L. pneumophila* subverts numerous host cell functions to replicate within macrophages, it still triggers potent inflammatory cytokine and cellular responses that enable the host to eventually clear infection. In addition to identifying innate immune pathways that are activated within infected murine and human cells, the roles of individual downstream cytokines and immune cells in controlling infection have been investigated in a variety of rodent models. The interplay between inflammatory cytokines and various immune and non-immune cells is beginning to emerge, and there is much to discover moving forward. Although some cell-intrinsic factors, such as the NAIP5 inflammasome, ROS, GBPs, and itaconic acid, are known to contribute to the restriction of *L. pneumophila* within macrophages, it is unclear what the precise roles are of these or additional cell-intrinsic factors in promoting bacterial killing in macrophages and other myeloid cell types. In addition, it is poorly understood how the immune system and other host factors influence host resilience and tolerance mechanisms during *L. pneumophila* infection. This information may provide a better understanding of the diversity of clinical phenotypes observed in individual patients, which range from asymptomatic infection to Pontiac fever to severe pneumonia. Finally, the mechanisms underlying the resolution of inflammation and restoration of lung homeostasis following clearance of *L. pneumophila* infection are poorly understood. A better understanding of the host response to *L. pneumophila* will not only shed light on fundamental principles of host cell biology, immunology, and organismal physiology, but could also provide new insight into the development of improved therapeutics for bacterial infections.

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Keywords:
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Abbreviations used:
T4SS, type IV secretion system; LCV, *Legionella*-containing vacuole; ER, endoplasmic reticulum; WT, wild-type; T2SS, type II secretion system; GAP, GTPase-activating protein; PRR, pattern recognition receptor; TLR, Toll-like receptor; LPS, lipopolysaccharide; BMDM, bone

marrow-derived macrophage; OMV, outer-membrane vesicle; GBP, guanylate-binding protein; NO, nitric oxide; moDC, monocyte-derived dendritic cell; MLN, mediastinal lymph node.

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