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Complementary Immune Roles for Infected and Uninfected Cells During Legionella Pneumophila infection

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Complementary Immune Roles for Infected and Uninfected Cells During Legionella Pneumophila infection

Abstract
The innate immune system responds to virulent pathogens, yet many pathogens manipulate host-signaling pathways, which should limit immune activation. The intracellular bacterium Legionella pneumophila is the cause of the severe pneumonia Legionnaire's disease. L. pneumophila encodes a type IV secretion system (T4SS) to translocate bacterial proteins into the cytosol of infected host cells. Several of these bacterial effectors (Lgt1, Lgt2, Lgt3, SidI, SidL, Pkn5, and Lpg1489) inactivate host cell elongation factors involved in protein translation. Despite the ability of L. pneumophila to block host protein translation, inflammatory cytokines are still made during infection both in vivo and in vitro. It is unclear how infected cells can mount a cytokine response when host protein synthesis is blocked. By creating a fluorescence resonance energy transfer-based system to track the activity of the T4SS in infected host cells, this study investigates how innate immune cells produce cytokines during L. pneumophila infection. In vitro, cells targeted by the T4SS of L. pneumophila are poor producers of cytokines critical for control of infection, such as TNF, IL-6, IL-12, and do not express CD86 in response to infection. Instead, uninfected, bystander cells produce these cytokines. Infected host cells do produce IL-1alpha; and IL-1beta; de novo and transcribe many proinflammatory genes. During pulmonary infection, alveolar macrophages and neutrophils are targeted by the T4SS for translocation and contain viable L. pneumophila. These cells provide a niche for bacterial replication during infection, but also secrete IL-1 in response to virulent bacteria. Uninfected alveolar macrophages, neutrophils, as well as inflammatory monocytes and dendritic cells produce TNF during L. pneumophila infection in vivo. Inflammatory monocytes and dendritic cells also increase CD86 expression during infection. Importantly, this bystander TNF production and CD86 expression requires IL-1 signaling, as mice deficient for the IL-1R have diminished levels of TNF and CD86 expression during infection. These data suggest that infected cells have mechanisms to overcome protein synthesis inhibition to produce IL-1 and that uninfected bystander cells are important contributors to the immune response during infection with L. pneumophila. This mechanism of immune activation has broad significance as many other bacterial pathogens manipulate host cell processes, including immune cell signaling.

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Immunology

First Advisor
Sunny Shin

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IL-1, IL-1R, Legionella pneumophila, Type IV secretion system

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COMPLEMENTARY IMMUNE ROLES FOR INFECTED AND UNINFECTED CELLS DURING LEGIONELLA PNEUMOPHILA INFECTION

Alan M. Copenhaver

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2015

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COMPLEMENTARY IMMUNE ROLES FOR INFECTED AND UNINFECTED CELLS
DURING LEGIONELLA PNEUMOPHILA INFECTION

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Alan M. Copenhaver
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ABSTRACT

COMPLEMENTARY IMMUNE ROLES FOR INFECTED AND UNINFECTED CELLS DURING LEGIONELLA PNEUMOPHILA INFECTION

Alan M. Copenhaver
Sunny Shin

The innate immune system responds to virulent pathogens, yet many pathogens manipulate host-signaling pathways, which should limit immune activation. The intracellular bacterium Legionella pneumophila is the cause of the severe pneumonia Legionnaire’s disease. L. pneumophila encodes a type IV secretion system (T4SS) to translocate bacterial proteins into the cytosol of infected host cells. Several of these bacterial effectors (Lgt1, Lgt2, Lgt3, SidI, SidL, Pkn5, and Lpg1489) inactivate host cell elongation factors involved in protein translation. Despite the ability of L. pneumophila to block host protein translation, inflammatory cytokines are still made during infection both in vivo and in vitro. It is unclear how infected cells can mount a cytokine response when host protein synthesis is blocked. By creating a fluorescence resonance energy transfer-based system to track the activity of the T4SS in infected host cells, this study investigates how innate immune cells produce cytokines during L. pneumophila infection. In vitro, cells targeted by the T4SS of L. pneumophila are poor producers of cytokines critical for control of infection, such as TNF, IL-6, IL-12, and do not express CD86 in response to infection. Instead, uninfected, bystander cells produce these cytokines. Infected host cells do produce IL-1α and IL-1β de novo and transcribe many proinflammatory genes. During pulmonary infection, alveolar macrophages and neutrophils are targeted by the T4SS for translocation and contain viable L. pneumophila. These cells provide a niche for bacterial replication during infection, but also secrete IL-1 in response to virulent bacteria. Uninfected alveolar macrophages, neutrophils, as well as inflammatory monocytes and dendritic cells produce TNF during L. pneumophila infection in vivo. Inflammatory monocytes and dendritic cells also increase CD86 expression during infection. Importantly, this bystander TNF production and CD86 expression requires IL-1 signaling, as mice deficient for the IL-1R have diminished levels of TNF and CD86 expression during infection. These data suggest that infected cells have mechanisms to overcome protein synthesis inhibition to produce IL-1 and that uninfected bystander cells are important contributors to the immune response during infection with L. pneumophila. This mechanism of immune activation has broad significance as many other bacterial pathogens manipulate host cell processes, including immune cell signaling.
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<tr>
<td>AEC:</td>
<td>Alveolar epithelial cell</td>
</tr>
<tr>
<td>AMΦ:</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>BFA:</td>
<td>Brefeldin-A</td>
</tr>
<tr>
<td>BlaM:</td>
<td>β-lactamase</td>
</tr>
<tr>
<td>BMDC:</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>BMDM:</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>CFU:</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>cDC:</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>CYE:</td>
<td>Charcoal yeast extract</td>
</tr>
<tr>
<td>DAP:</td>
<td>Diaminopimelic acid</td>
</tr>
<tr>
<td>DC:</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ELISA:</td>
<td>Enzyme-linked immuno assay</td>
</tr>
<tr>
<td>ER:</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EΦ:</td>
<td>Eosinophil</td>
</tr>
<tr>
<td>FAK:</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS:</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FRET:</td>
<td>Fluorescent resonance energy transfer</td>
</tr>
<tr>
<td>GEF:</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>iMC:</td>
<td>Inflammatory monocyte</td>
</tr>
<tr>
<td>JNK:</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LCV:</td>
<td>Legionella containing vacuole</td>
</tr>
<tr>
<td>LPS:</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK:</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDP:</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MKK:</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MyD88:</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NF-κB:</td>
<td>Nuclear factor kappa-light-chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NK:</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLR:</td>
<td>Nod-like receptor or nucleotide-binding oligomerization domain receptor</td>
</tr>
<tr>
<td>NΦ:</td>
<td>Neutrophil</td>
</tr>
<tr>
<td>PAMP:</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS:</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PRR:</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RIP2:</td>
<td>Receptor-interacting protein 2</td>
</tr>
<tr>
<td>TLR:</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>T3SS:</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>T4SS:</td>
<td>Type IV secretion system</td>
</tr>
<tr>
<td>Unfx:</td>
<td>Uninfected</td>
</tr>
<tr>
<td>Untx:</td>
<td>Untreated</td>
</tr>
<tr>
<td>UPR:</td>
<td>Unfolded protein response</td>
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<tr>
<td>WT:</td>
<td>Wild Type</td>
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Mammals provide large and diverse niches for microbial species. To date, every mammalian surface, including the skin, gastrointestinal tract, airways, and oral cavities are known to contain a multitude of living microbes including bacteria, archaea, viruses, and fungi (Bäckhed et al., 2005; Beck et al., 2012; Delwart, 2013; Dewhirst et al., 2010; Fontana et al., 2012; Grice and Segre, 2011; Hoffmann et al., 2013; Somerville, 1969).

The vast majority of known microorganisms do not infect their hosts and induce disease. These organisms are referred to as non-pathogenic organisms or commensals. Some of these non-pathogenic organisms benefit their hosts by aiding digestion and nutrient uptake or by preventing colonization and infection of the host with pathogenic organisms (Casadevall and Pirofski, 2003; Case et al., 2009; Kamada et al., 2013a; 2013b; Kau et al., 2011). Pathogenic organisms are known to cause infection and disease, and thus their presence is detrimental to host organisms (Casadevall and Pirofski, 2003; Kamada et al., 2013a; 2013b; Kau et al., 2011; Mouchtouri et al., 2010; Nguyen et al., 2006). To detect pathogenic organisms, hosts encode and express pattern recognition receptors (PRR) (Janeway and Medzhitov, 2002). These PRRs detect conserved pathogen-associated molecular patterns (PAMP) expressed by pathogens (Vance et al., 2009). These PAMPs include lipopolysaccharide (LPS), flagellin, foreign peptidoglycans, and foreign genetic material. However, commensal organisms express many of these PAMPs (Vance et al., 2009). For instance, LPS is a component of the Gram-negative cell wall, and thus is ubiquitously expressed by all Gram-negative organisms, including non-pathogenic microbes (Rietschel et al., 1994). Similarly, flagellin is a component of the Gram-negative flagellum, which is used by these organisms for locomotion and is not
specifically associated with pathogenic organisms (Bardy et al., 2003; Neville et al., 2013). Thus, PRRs alone cannot distinguish between non-pathogenic and pathogenic organisms.

One distinction between commensal bacteria and their virulent counterparts is that many pathogens access the cytosol of host cells either by using specialized secretion systems to translocate bacterial products across host cell membranes or by using pore-forming proteins to disrupt host cell membranes and gain direct access to the cytosol (Krachler et al., 2011; Roy and Mocarski, 2007; Vance et al., 2009). Pathogens, such as *Yersinia* and *Salmonella spp.*, encode type III secretion systems (T3SS) that form syringe-like structures to translocate bacterial effectors proteins across the plasma or phagosomal membranes (Cornelis, 2006; Viboud and Bliska, 2005). *Burkholderia spp.* also encode a T3SS, but unlike *Yersinia and Salmonella spp.*, these bacteria utilize their T3SS to break out of the phagosome allowing for bacterial replication within the cytosol of host cells (Cornelis, 2006; Galyov et al., 2010). Other bacterial pathogens, such as certain *Vibrio spp.* and *Listeria monocytogenes*, utilize pore-forming proteins to either translocate effectors into the cell or disrupt phagosomal and other membranes allowing for access to the cytosol (De Haan and Hirst, 2004; Hamon et al., 2012). These secretion systems and pore-forming proteins often translocate bacterial effector proteins into the cytosol of host cells to manipulate host cell processes such as cytoskeletal rearrangement, vesicular trafficking, host cell signaling, and protein translation (Kahn et al., 2002; Roy and Mocarski, 2007). These mechanisms benefit the replication, and spread of the pathogens within hosts and are essential for their survival.
A. Pathogenic manipulation of host cells

A variety of bacterial pathogens encode effectors that modulate the activity of host cell kinases, ultimately altering host cell activation (Figure 1-1) (Krachler et al., 2011). Vibrio parahaemolyticus translocates the acetyltransferase VopA/P into host cells, which inactivates a number of MAP kinase kinases (M KK), ultimately inhibiting host cell growth (Selyunin et al., 2011; Trosky et al., 2007). Shigella flexneri encodes two effectors, OspF and OspG that inhibit extracellular signal-regulated kinases (ERK) and nuclear factor kappa-light-chain-enhancer of B cells NF-κB activation respectively, preventing the production of proinflammatory immune responses against the pathogen (Arbibe et al., 2007; Kim et al., 2005; Kramer et al., 2007; Li et al., 2007). Yersinia spp. encode three effectors that alter host cell kinase activity. YopJ and its more pathogenic homolog YopP are acetyltransferases that inactivate both MAPK and NF-κB signaling in host cells via an unknown mechanism (Mukherjee et al., 2006; Palmer et al., 1998; Schesser et al., 1998). Yersinia spp. encodes YopH that inhibits the protein kinases Fyn and focal adhesion kinase (FAK) signaling, preventing phagocytosis of the bacteria (Black and Bliska, 1997; Yuan et al., 2005). Finally, Yersinia spp. encode invasin that inhibits FAK signaling and prevents phagocytosis (Uliczka et al., 2009).

Beyond inhibiting host cell signaling, a handful of pathogenic bacterial species encode toxins or effectors that inhibit protein translation by host cells (Fontana and Vance, 2011). This host protein translation inhibition is thought to limit the production of many proteins involved in immune responses, including cytokines, antimicrobial agents, and lysosomal enzymes. This inability to produce proteins that are important for immune responses promotes infection (Fontana and Vance, 2011). Shigella spp. encode shiga toxin, a protein translation inhibitor that acts by cleaving the 28S RNA of the host ribosome (Sandvig and van Deurs, 1996). Corynebacterium diphtheriae express
diphtheria toxin that ribosylates elongation factor 2 in host cells, thus inhibiting protein translation (Wilson and Collier, 1992). *Pseudomonas aeruginosa* exotoxin ribosylates elongation factor 2 and therefore inhibits protein synthesis in a similar fashion (Wilson and Collier, 1992). *Legionella pneumophila* encodes seven known effectors that inhibit host protein synthesis via inhibition of host elongation factors or via currently unknown mechanisms (Barry et al., 2013; Belyi et al., 2006; 2008; Fontana et al., 2011; Shen et al., 2009).

**B. Immune responses during pathogenic manipulation**

Although bacterial pathogens manipulate host cells processes and therefore limit the immune response against these pathogens, successful immune responses are still mounted against many bacterial pathogens. Importantly, these immune responses are required for protection against infection. Several immune pathways are important for controlling *Yersinia spp.* infections, including the inflammasome, cytokine production, and T cell responses (Bohn and Autenrieth, 1996; Brodsky et al., 2010). *Shigella spp.* activate IFNγ production via IL-12 and IL-18 signaling that are critical for the survival of infected mice (Pore et al., 2012; Sansonetti et al., 2000; Way et al., 1998). *P. aeruginosa* infection induces inflammasome activation and neutrophil recruitment, both of which limit bacterial replication and dissemination (Hirche et al., 2008; Sutterwala et al., 2007). Like *P. aeruginosa* infection, *Legionella pneumophila* infection is controlled by a number of cytokines, the activation of the inflammasome, and neutrophil recruitment (Brieland et al., 1998; Ren et al., 2006; Tateda et al., 2001b).

Thus, many pathogens express toxins, secretion systems, or effector proteins to block and manipulate host cell process, including the immune response, responses are still mounted against these pathogens and are critical for pathogen control and clearance.
Little work, however, has uncovered how a successful immune response is mounted during pathogenic manipulation of host cells. Some pathways have been elucidated. Manipulation of MAPK and NF-κB signaling during Y. pseudotuberculosis infection results in a pro-inflammatory form of cell death known as necroptosis that has been shown to activate the immune response in infected mice (Philip et al., 2014). Likewise, signaling by pattern recognition receptors on host cells during protein translation inhibition induce or increase induction of a number of proinflammatory genes (Dunbar et al., 2012; Fontana and Vance, 2011; Fontana et al., 2012; McEwan et al., 2012). Yet, these phenomena have not been shown to be important in all bacterial infections and other pathways may exist to combat pathogenic manipulation. To study this phenomenon, we have utilized the pathogenic bacterium L. pneumophila.

C. Legionella pneumophila ecology and infection

Legionella pneumophila is a species of Gram-negative bacteria of the genus Legionella, which is found ubiquitously in freshwater environments including ponds, lakes, and streams (Fields, 1996). It parasitizes and replicates within various species of freshwater amoeba, including Hartmannella vermiformis and Acanthamoeba castellani. L. pneumophila can form planktonic biofilms on surfaces in freshwater environments (Piao et al., 2006). In its natural environment, L. pneumophila is generally harmless to mammalian hosts and no documented disease was caused by L. pneumophila until the 20th century. Modern technology has allowed for the collection and aerosolization of vast quantities of fresh water for use in devices such as air conditioners, cooling towers, and misters. If the source of water used for aerosolization is contaminated with L. pneumophila, the bacteria become aerosolized where mammalian hosts can inhale them. This can lead to a severe form of pneumonia, known as Legionnaires’ disease, or cause a milder, flu-like illness known as Pontiac fever (Chandler et al., 1977; Fraser et
al., 1977; McDade et al., 1977; Winn et al., 1978). *L. pneumophila* was first discovered in Philadelphia in 1976 when 200 people attending the convention of the American Legion fell ill with a mysterious pneumonia that killed 34 individuals (Fraser et al., 1977; McDade et al., 1977). The source of infection was determined to be a contaminated air conditioner at the Bellevue hotel where many of the convention-goers were housed. Since then, many efforts have been made to prevent contamination of aerosolizing machines, although *L. pneumophila* infection still occurs. Cooling towers have been implicated as having the greatest ability to spread infection, as contaminated aerosols can travel up to 6 kilometers and infect individuals over a wide radius (Mouchtouri et al., 2010; Nguyen et al., 2006; Vance et al., 2009). *L. pneumophila* is one of the leading causes of community-acquired pneumonia (Musher and Thorner, 2014; Rietschel et al., 1994). There is no immunization for *L. pneumophila*; however, treatment with antibiotics resolves most infections (Bardy et al., 2003; Garau et al., 2010; Neville et al., 2013; Yu et al., 2004).

Once in the lung of an individual, *L. pneumophila* encounters specialized macrophages of the airway space, known as alveolar macrophages (Chandler et al., 1977; Krachler et al., 2011; Roy and Mocarski, 2007; Vance et al., 2009). Infection of a single cell by *L. pneumophila* lasts approximately 16-24 hours, allowing for approximately five rounds of intracellular replication (Abu Kwaik et al., 1993; Cornelis, 2006; Horwitz, 1983; Horwitz and Silverstein, 1980; Viboud and Bliska, 2005). After this time, *L. pneumophila* is released from host cells via an unknown mechanism, resulting in death of the host cell. The bacteria are then able to disseminate to new cells. It is thought that some individuals have been exposed to *L. pneumophila* multiple times, as evidenced by anti-*L. pneumophila* serum titers of antibodies in the human population (Borella et al., 2008; Boshuizen et al., 2006; Cornelis, 2006; Galyov et al., 2010; Nagelkerke et al., 2003).
This indicates that most people control infection without subsequent disease. In those that cannot control infection, however, *L. pneumophila* disseminates throughout the body and bacteria can be found in the kidneys and heart (De Haan and Hirst, 2004; Hamon et al., 2012; Lowry and Tompkins, 1993). Patients who succumb to *L. pneumophila* infection often die of multi-organ failure (Fraser et al., 1977; Kahn et al., 2002; Roy and Mocarski, 2007). Smokers, individuals with chronic lung disease, recent transplant recipients, and immunocompromised individuals are at the greatest risk of acquiring *L. pneumophila* (Boer et al., 2006; Kool et al., 1998; Singh et al., 1993; Yu et al., 2004).

The risk of *L. pneumophila* infection increases with age, but infection can occur in both children and young adults (Centers for Disease Control and Prevention (CDC), 2011). In 2009, there were 3,522 reported cases of legionellosis in the US. Etiological studies estimate that only a third of all infections with *Legionella spp.* are reported and estimate that there are approximately 8,000 to 18,000 cases of legionellosis per year (Centers for Disease Control and Prevention (CDC), 2011). The prevalence of legionellosis has increased over the past decade, highlighting a need for better preventative measures, including proper water treatment (Parr et al., 2014).

**D. Legionella pneumophila type IV secretion system**

*Legionella pneumophila* expresses a specialized type IVb secretion system (T4SS) that is encoded by *dot/icm* genes and is essential for virulence as well as survival of *L. pneumophila* inside of host cells (Berger and Isberg, 1993; Hubber and Roy, 2010; Marra et al., 1992). This secretion system is similar, but genetically distinct from the type IVa secretion system expressed by *P. aeruginosa* and other Gram-negative bacteria (Vincent et al., 2006). Many of the components of this T4SS share genetic sequence and protein structure homology with the type IVa/VirB secretion system of such bacteria as *Brucella spp.* and *Agrobacterium tumefaciens* (Juhas et al., 2008). Despite this
homology, many aspects of the secretion system are unique and distinct from the type IVa secretion system (Nagai and Kubori, 2011). *L. pneumophila* lacking key components of the T4SS is still phagocytosed by amoebae and macrophages, but once inside host cells are unable to establish a replicative niche and are quickly shuttled to a degradative lysosome (Berger and Isberg, 1993; Marra et al., 1992). About 30 genes encode the T4SS of *L. pneumophila* (Vincent et al., 2006). Deletion of some of these genes, such as dotA, dotB, icmC, and icmD render the T4SS completely inactive, whereas mutations in other genes such as icmS, dotF, icmF, and dotU allow for some T4SS activity to still occur, but for intracellular growth to be partially impaired (Nagai and Kubori, 2011; Vogel et al., 1996). Beyond replication and translocation, the *dot/icm* protein, IcmT, has a demonstrated role in allowing for *L. pneumophila* egress from host cells at the end of infection, but the exact mechanism for this escape is unknown (Molmeret et al., 2002).

Research has resolved some of the base structure of the T4SS, mainly those components that are embedded in bacterial membranes or are localized to the bacterial cytosol (Sutherland et al., 2013). Unlike the type III secretion system and the type IVa secretion system, no syringe-like structure has been visualized for the T4SS of *L. pneumophila* and no pore-formation into host-derived membranes has been directly assessed (Nagai and Kubori, 2011). Therefore, the exact mechanism of T4SS-mediated translocation into the cytosol of host cells has not been described.

For pathogenesis, *L. pneumophila* uses its T4SS to translocate bacterial effector proteins from the cytosol of the bacterium, through the phagosomal membrane into the cytosol of the host cell (Ensminger and Isberg, 2009; Ninio and Roy, 2007). To date, over 300 different effector proteins have been identified via screening, but the vast majority of these effectors have not been characterized (Burstein et al., 2009). The overall activity of the T4SS on host cells, however, has been described (Roy and
Mocarski, 2007). Translocation by the T4SS occurs in as little as five minutes post phagocytosis and translocation of effectors is thought to occur throughout the infectious cycle (Nagai et al., 2005; Ninio and Roy, 2007). Not all effectors are translocated at all times, however, and many effector proteins have opposing activities, suggesting that translocation is temporally orchestrated (Bardill et al., 2005; Kubori et al., 2010). Unlike with other secretion system, the cues for this temporal structure as well as the overall signal to induce translocation by the T4SS of *L. pneumophila* are not known (Qiu and Luo, 2013). Likewise, no definitive motif that targets bacterial proteins to the T4SS to be translocated has been identified, although many effectors have “disorganized” C-termini (Nagai et al., 2005). Other effectors, however, do not contain this disordered C-terminus and attachment of the disordered C-terminus alone to reporter constructs does not confer translocation (Nagai et al., 2005). Many of the T4SS effectors contain eukaryotic protein domains, such as ankyrin repeats and serine/threonine kinase domains (Ge et al., 2009; Pan et al., 2008). Likewise, the genes encoding these effectors have a lower GC content than other *L. pneumophila* genes on the bacterial chromosome, indicating that many effectors have been acquired via horizontal gene transfer, although this has not been directly demonstrated (Juhas et al., 2008).

**E. Type IV secretion system effectors**

The T4SS and many of the effectors are involved in preventing phagosomal maturation, as evidenced by a lack of maturation markers on the *Legionella* containing vacuole (LCV) (Roy et al., 1998). The T4SS promotes the assembly of the LCV, which is comprised of ER-derived vesicles and resembles an ER-like compartment by electron and fluorescent microscopy (Nash et al., 1984; Tilney et al., 2001). Much of vesicular transport in eukaryotic cells is controlled by the activity of small, Ras-like GTPases such as Arf1 (Goody and Itzen, 2013). Interestingly, many of the characterized T4SS effectors
alter the activity of GTPases, often by mimicking the activity of guanine nucleotide exchange factors (GEF) or GTPase activating proteins (GAP) and therefore can recruit or exclude various Rabs to the LCV (Goody and Itzen, 2013). RalF, one of best-characterized effectors, recruits the host GTPase, Arf1, to the LCV, although deletion of RalF does not alter infection in mammalian macrophages (Amor, 2004; Nagai et al., 2005). The effectors DrrA, SidD, LepB, LidA, AnkX, Lem3, and VipD have been demonstrated to interact with various host Rabs that are crucial regulators of ER-Golgi trafficking (Goody and Itzen, 2013). Similar to RalF, deletion of a single effector does not greatly alter infection in host macrophages, although minor effects on the LCV are evidenced. In fact, deletion of over 70 of the known effectors in combination does not alter *L. pneumophila* growth in murine macrophages, suggesting a large amount of redundancy in the activity of various effectors (O’Connor et al., 2011). Indeed, many effectors have overlapping activities or similar outcomes to pathogenesis. This redundancy appears to be required for a large host range, as deletion of various groups of effectors alters *L. pneumophila* replication in certain species of amoeba, but not in others (Ensminger et al., 2012; O’Connor et al., 2011). Currently, only one T4SS effector has a demonstrated, non-redundant role: SdhA (Creasey and Isberg, 2012). This effector is involved in preventing PlaA-induced lysis of the LCV during infection, and deletion of this effector releases *L. pneumophila* into the cytosol where it is degraded by autophagosomes, thus limiting growth of SdhA-deficient *L. pneumophila in vitro* (Creasey and Isberg, 2012).

Although many of the effectors described have roles in preventing degradation of *L. pneumophila* and the creation and maintenance of the LCV, other T4SS effectors have different activities. The effector RomA is a methyltransferase that enters the nucleus of host cells and directly trimethylates histone H3 at the K14 residue, thereby globally
altering the chromatin landscape of infected host cells (Rolando et al., 2013). Autophagy of \textit{L. pneumophila} can be blocked by the activity of the effector RavZ, which inactivates the host protein Atg8 (Choy et al., 2012). The effector LegK1 directly phosphorylates \textit{IkB\alpha} on the serine 32 and 36 residues, ultimately leading the activation of NF-\textit{\kappa}B (Ge et al., 2009). Like the effectors that alter ER-Golgi trafficking, however, deletion of these effectors either singly or in combination does not alter bacterial replication in murine macrophages.

Many groups have observed a greater than 90\% reduction in host protein synthesis during \textit{L. pneumophila} infection that depends on the presence of a functional T4SS (Barry et al., 2013; Belyi et al., 2006; McCusker et al., 1991). Currently, seven effectors have been described to inhibit host protein translation (Figure 1-2). The effectors Lgt1, Lgt2, and Lgt3 are all glycosyltransferases that modify the host factor eEF1A, allowing for translational initiation but blocking elongation (Belyi et al., 2006; 2008). SidI binds and inactivates eEF1A, but can also bind the host factor eEF1B\gamma (Shen et al., 2009). SidL, Pkn5, and Lpg1489 block host protein translation via unknown mechanisms (Barry et al., 2013; Belyi et al., 2013). Of note, these effectors are toxic to mammalian cells as demonstrated in ectopic expression assays (Fontana et al., 2011). Deletion of five or all seven of the known effectors, however, does not restore translational activity in infected cells and does not alter bacterial replication \textit{in vitro} (Barry et al., 2013; Fontana et al., 2011). These data suggest that other effectors may block host protein translation.

Alternatively, macrophages treated with rapamycin and infected with avirulent \textit{L. pneumophila} translate less protein than control-infected cells, and cells infected with virulent \textit{L. pneumophila} exhibit suppressed mTOR signaling, suggesting that host mTOR signaling may also diminish translational activity in infected cells (Ivanov and Roy, 2013).
F. The immune response to *Legionella pneumophila*

Despite the ability of the T4SS of *L. pneumophila* to alter many of the aspects of host cell physiology, including chromatin accessibility, ER-Golgi trafficking, autophagy, and protein translation, mammalian host cells still mount an immune response to virulent *L. pneumophila* (Friedman et al., 2002; Spörri et al., 2008). This immune response includes the production of many proinflammatory cytokines, the recruitment of innate and adaptive immune cells to the site of infection, and even the induction of a form of proinflammatory cell death (Casson and Shin, 2013; Neild and Roy, 2004; Tateda et al., 2001a). The immune response to *L. pneumophila* is critical both *in vitro* and *in vivo* in the control and eventual clearance of bacteria from the host, as deletion or manipulation of many key immune pathways leads to subsequent increases in bacterial burden, increased bacterial growth in host phagocytes, and, in severe cases, the death of infected animals (Archer and Roy, 2006; Brieland et al., 1998; 1995; Derré and Isberg, 2004; Hawn et al., 2006; Spörri et al., 2006).

Many of the downstream signaling pathways activated by *L. pneumophila* in host cells have been characterized during *in vitro* and *in vivo* infection. The production of the majority of cytokines *in vitro* depends heavily on the activity of the adaptor protein myeloid differentiation primary response 88 (MyD88), and macrophages deficient for MyD88 have markedly reduced or absent production of many proinflammatory cytokines (Archer and Roy, 2006; Shin et al., 2008). MyD88-deficient mice are the most susceptible to *L. pneumophila* infection *in vivo* and die around 14 days post infection (Archer et al., 2010). The adaptor protein TIR-domain-containing adaptor-inducing interferon-beta (TRIF) plays a minor role *in vitro* in the induction of type I interferon and other cytokines, though the role of TRIF *in vivo* has never been investigated (Shin et al., 2008; Stetson and Medzhitov, 2006). The kinase receptor-interacting serine/threonine-
protein kinase 2 (Rip2) contributes mildly to IL-6 production in vitro and helps clear bacterial CFUs as well as induce production of IL-6, KC, and G-CSF in vivo (Archer et al., 2010; Frutuoso et al., 2010; Shin et al., 2008). IRF3 is induced by L. pneumophila infection and leads to the production of type I interferon (Lippmann et al., 2011; Stetson and Medzhitov, 2006). In vivo, this signaling pathway has little to no effect on bacterial burden.

Many of the aforementioned signaling pathways converge upon classical immune signaling pathways. MyD88, TRIF, and Rip2 all lead to the induction of NF-κB signaling as measured by IκB degradation (Losick and Isberg, 2006; Shin et al., 2008). Interestingly, in the absence of these signaling adaptors, IκB degradation still occurs during WT L. pneumophila infection, but not T4SS-deficient bacteria, suggesting that additional signaling pathways lead to NF-κB induction (Shin et al., 2008). MyD88, TRIF, and Rip2 lead to the induction of MAPK signaling, including ERK1/2 and the kinase c-Jun N-terminal kinase (JNK), which like NF-κB are also activated by other, unknown T4SS-dependent signals as well as in response to T4SS-dependent protein translation inhibition (Fontana et al., 2012; Shin et al., 2008).

Toll-like receptor (TLR) signaling during L. pneumophila infection has been extensively studied both in vivo and in vitro. Although TLR4 is known to recognize bacterial LPS, LPS isolated from L. pneumophila is not a strong activator of TLR4 signaling (Archer and Roy, 2006; Lettinga et al., 2002). TLR2 is activated by outer wall components of L. pneumophila and TLR2 signaling leads to the production of many of the cytokines seen in response to L. pneumophila in vitro (Archer and Roy, 2006; Hawn et al., 2006; Shin et al., 2008). During L. pneumophila infection in vitro, TLR9 is stimulated upon recognition of CpG DNA motifs enriched in bacterial genomes (Archer et al., 2010; Newton et al.,
Although *L. pneumophila* expresses flagellin, determining a role for TLR5 signaling *in vitro* is complicated by the absence of TLR5 on bone marrow-derived macrophages (Chandler et al., 1977; Hayashi et al., 2001; Means et al., 2003). *In vivo*, the absence of TLR5 does not alter the course of pulmonary infection (Archer et al., 2010). In fact, mice deficient for either TLR2, TLR4, TLR5, or TLR9 demonstrate no defect or only a slight defect in controlling pulmonary *L. pneumophila* infection (Archer et al., 2010; Hawn et al., 2007). Even mice triply deficient in TLR2, 4, and 9 show a minimal defect in control. This is in stark contrast to the dramatic phenotype observed in MyD88-deficient mice during infection, which are deficient in TLR signaling as well as IL-1 family signaling. No role has been determined for TLRs 3, 7, and 8 that recognize synthetic viral motifs. Likewise, no role has been investigated for TLRs 11, 12, or 13. In humans, certain mutations in TLR5 are associated with an increased prevalence of legionellosis (Hawn et al., 2003). This is in contrast to the phenotype of TLR5-deficient mice, indicating that the role of various immune pathways may differ between mouse models of *L. pneumophila* infection and clinical manifestations of disease (Hawn et al., 2007).

Various intracellular PRRs are activated during WT *L. pneumophila* infection, but not with bacteria lacking a T4SS (Berrington et al., 2010; Neild and Roy, 2004; Shin and Roy, 2008). This T4SS-dependent intracellular activation is thought to be important for the distinction between virulent and avirulent *L. pneumophila* infection (Shin et al., 2008). Nod1 and Nod2, which signal via Rip2, are activated in response to the cell well components diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively, in the cytosol of host cells (Girardin et al., 2003a; 2003b; Inohara et al., 2005). Deletion of Nod1 and Nod2 *in vivo* leads to different effects on pathogenesis; deletion of Nod1 enhances disease, while deletion of Nod2 limits infection (Berrington et al., 2010; Frutuoso et al., 2010). RIG-I and MDA5 are activated by WT *L. pneumophila* infection
(Monroe et al., 2009). Both RIG-I and MDA5 signal via MAVS to induce the production of IFNβ.

Additionally, intracellular pathways may sense the effects of the T4SS on host cell processes. Deletion of five of the effectors that inhibit protein translation alters the proinflammatory genes expressed in response to infection, notably the transcription of *Gmcsf* and *Il23* (Fontana et al., 2011). Perturbations in mTOR signaling may partially drive the response to protein translation inhibition (Ivanov and Roy, 2013). Interestingly, deletion and manipulation of the known intracellular signaling pathways does not fully explain the difference in immune responses between virulent *L. pneumophila* and bacteria lacking a functional T4SS (Shin et al., 2008). Whether the host directly senses other, specific components of the T4SS or if other effects on the host cell, such as manipulation of ER-Golgi trafficking, activate the immune response is not known.

**G. Inflammasome activation during Legionella pneumophila infection**

The inflammasome is a large, multi-protein complex that forms in the cytosol of cells upon induction by various infectious or stress-induced signals. Many different inflammasomes have been characterized, but all inflammasome activation leads to the same downstream consequences: release of IL-1 family members and an inflammatory form of cell-death known as pyroptosis (Lamkanfi and Dixit, 2009; Moltke et al., 2013). Activation of the inflammasome is a two-step process. First, NF-κB induction leads to the translation of many of the components of the inflammasome, including the upstream NLRs and downstream caspases and IL-1 family members (Rathinam et al., 2012). NLRs are thought to be sensors that either directly or indirectly recognize stress or infectious components. Some of these NLRs, such as NLRC4, can then directly oligomerize and recruit caspase-1 (Broz et al., 2010). Others, such as NLRP3, require
the adaptor protein ASC to bridge the interaction between NLR and caspase (Mariathasan et al., 2006; Martinon et al., 2002). Both caspase-1 and caspase-11 are involved in inflammasome activation during L. pneumophila infection (Akhter et al., 2012; Case et al., 2009; Casson et al., 2013; Ren et al., 2006; Zamboni et al., 2006). Various NLRs are activated by infection and, importantly, inflammasome activation is exquisitely dependent on the T4SS (Casson et al., 2013). L. pneumophila lacking a functional T4SS fail to activate the inflammasome and no release of IL-1 family members or cell death is observed. In vivo, mice lacking caspase-1 and caspase-11 have increased bacterial burdens compared to the WT counterparts, but IL-1 family members can still be detected during infection, suggesting that other enzymes or pathways can mimic inflammasome activation in vivo (Barry et al., 2013; Casson et al., 2013).

The earliest characterized inflammasome important for L. pneumophila infection is the Naip5/NLRC4 inflammasome (Derré and Isberg, 2004; Zamboni et al., 2006). Naip5, in conjunction with NLRC4, binds monomers of L. pneumophila flagellin (Kofoed and Vance, 2011). In C57BL/6 mice, this inflammasome is restrictive to growth and, thus, bacteria expressing flagellin are rapidly cleared in vivo and replicate poorly in macrophages derived from these mice in vitro (Molofsky et al., 2006; Ren et al., 2006). The discovery of Naip5 as an intracellular flagellin sensor occurred because although B6 mice are restrictive, A/J mice and their macrophages are permissive for replication of WT L. pneumophila. A/J mice have a polymorphism in the Naip5 gene, resulting in a hypomorphic allele (Beckers et al., 1995; Dietrich et al., 1995; Diez et al., 2003; Wright et al., 2003). Either deletion of Naip5 from mice or deletion of the L. pneumophila flagellin gene flaA, results in restored replication in mice and macrophages (Coers et al., 2007; Molofsky et al., 2006; Ren et al., 2006). As activation of this inflammasome
requires the T4SS, it is assumed the flagellin monomers are translocated into the cytosol of host cells, although this has never been fully demonstrated.

In the absence of the Naip5/NLRC4/flagellin axis, inflammasome activation still occurs in a less robust and less rapid fashion (Case et al., 2009; 2013; Casson et al., 2013). Part of this remaining response is controlled by NLRP3, an inflammasome that requires the adaptor ASC (Case et al., 2013; Casson et al., 2013). What exactly NLRP3 senses during infection has not been described, although a variety of other signals, such as intracellular reactive oxygen species, uric acid crystal, extracellular ATP, and other cellular stressors lead to NLRP3 activation (Leemans et al., 2011).

The interferon-inducible protein AIM2 recognizes cytosolic dsDNA, but is not normally activated by infection with *L. pneumophila* (Fernandes-Alnemri et al., 2009; Homung et al., 2009; Roberts et al., 2009). The AIM2 inflammasome is induced, however, during infection with the SdhA mutant of *L. pneumophila* (Aachoui et al., 2013; Creasey and Isberg, 2012). It is not known how AIM2 ligands enter the cytosol, but it is assumed that cytosolic degradation of bacteria leads to the release of genetic content.

More recently, caspase-11 has been demonstrated to be activated by *L. pneumophila* infection (Akhter et al., 2012; Barry et al., 2013; Case et al., 2013; Casson et al., 2013). The only known activator of caspase-11 is LPS as it can bind directly to caspase-11 in the absence of an NLR (Hagar et al., 2013; Kayagaki et al., 2013; Shi et al., 2014). Caspase-11 activation during *L. pneumophila* infection leads to the release of IL-1α and death of the host cell (Case et al., 2013; Casson et al., 2013). Interestingly, deletion of caspase-11 in murine macrophages ameliorates not only IL-1α release and cell death, but also diminishes IL-1β release, suggesting that caspase-11 activation feeds into
caspase-1-mediated responses (Case et al., 2013; Casson et al., 2013). It has not been directly shown that *L. pneumophila* LPS is the signal for caspase-11 activation.

H. The cytokine response to *Legionella pneumophila*

As a consequence of the various signaling cascades and PRRs activated by *L. pneumophila*, a variety of cytokines are produced during infection and many of these cytokines are critical for controlling and clearing infection. TNF, which binds the TNF receptor, is produced both *in vitro* and *in vivo* downstream of MyD88 and NF-κB signaling and is critical for limiting pulmonary infection (Brieland et al., 1998). *In vitro*, TNF restricts *L. pneumophila* growth in macrophages via an unknown mechanism (Coers et al., 2007). Importantly, human patients on TNF therapeutics that inhibit or limit TNF signaling are more susceptible to *L. pneumophila* infection (Aringer et al., 2009; Beigel et al., 2009; Jinno et al., 2009).

IL-12 is produced in response to *L. pneumophila* infection and, like TNF, is protective (Brieland et al., 1998; 2000). IL-12 leads to the production of IFNγ (Hsieh et al., 1993). Mice deficient for IFNγ contain high bacterial burdens, although whether IFNγ deficiency is fatal in mice has not been reported (Heath et al., 1996; Spörri et al., 2006). IL-18 can lead to the production of IFNγ from natural killer (NK) cells during *L. pneumophila* infection; however, blocking IL-18 has no impact on bacterial burdens (Archer et al., 2009; Brieland et al., 2000; Spörri et al., 2008). Blocking both IL-12 and IL-18 during infection leads to greater bacterial burdens than blocking either alone, suggesting synergy between these two cytokines (Brieland et al., 2000).

Type I interferon and IL-6 are produced in response to *L. pneumophila* infection. *In vitro*, type I interferon restricts bacterial replication in macrophages; however, the effect of
deleting the type I interferon receptor, IFNAR, has a mild to absent phenotype during *L. pneumophila* infection (Ang et al., 2010; Plumlee et al., 2009). Type I interferon signaling may be more important in the absence of IFNγ signaling (Lippmann et al., 2011). A role for IL-6 has not been demonstrated during *L. pneumophila* infection, but is protective in other models of bacterial pneumonia (Jones et al., 2006; Sutherland et al., 2008; van der Poll et al., 1997).

Beyond cytokines, chemokines are produced and play a role in controlling *L. pneumophila* infection. The chemokine KC is mildly protective during infection as it recruits neutrophils to the site of infection (Tateda et al., 2001b). Blockade of CXCR2, the receptor for KC, has a greater impact on infection than blocking KC alone (Tateda et al., 2001b). These data indicate a role for other chemokine ligands of CXCR2 during infection. Whether other chemokine receptors are critical for control of *L. pneumophila* infection, such as CCR2 or CXCR4 which recruit other immune cell types like monocytes, has not been investigated (Hasenberg et al., 2013).

Both IL-1α and IL-1β are produced during *L. pneumophila* infection *in vitro* and *in vivo*. IL-1 signaling *in vivo* rapidly recruits neutrophils to the airway space during infection (Barry et al., 2013; Casson et al., 2013; LeibundGut-Landmann et al., 2011). Deletion of the IL-1R or IL-1α or antibody-mediated neutralization of IL-1 is deleterious to the host as evidenced by increased bacterial burdens and delayed clearance (Barry et al., 2013; Casson et al., 2013). Of note, there is a case study of a patient taking Anakinra, an IL-1 antagonist, who became infected with *L. pneumophila* during therapy, suggesting a role for IL-1 in human as well as murine *L. pneumophila* infection (Scholtze et al., 2011).
I. The role of IL-1α and IL-1β during infection

IL-1α and β are part of a larger family of cytokines, known as the IL-1 family (Dinarello, 1996). IL-1β and IL-1α are the founding members of the IL-1 family and were first described in the 1980s (Auron et al., 1984; March et al., 1985). Unlike other conventional cytokines, IL-1 family members are translated by cytosolic ribosomes and do not traffic through the ER-Golgi network (Stevenson et al., 1992). The transcription and translation of IL-1α and β, like many cytokines, require the induction of NF-κB signaling. Once translated, these cytokines exist in a cytosolic, inactive pool in their pro-forms.

Translation of IL-1β and IL-1α alone does not lead to their activation. Instead, cleavage and activation of IL-1α and β require the activity of the inflammasome (Lamkanfi and Dixit, 2009). Cleavage is absolutely required for the release of IL-1β and its ability to bind the IL-1R (Black et al., 1988). IL-1α does not require cleavage for release and can bind and activate the IL-1R in either its full pro-form or mature form (Chen et al., 2007; Howard et al., 1991). The binding affinity to IL-1R is higher for pro- and mature IL-1α than for IL-1β (Symons et al., 1995).

The IL-1R is expressed on a variety of cell types, including epithelial cells, endothelial cells, astrocytes, and T cells (Dinarello, 2013). The IL-1R itself is comprised of two chains. IL-1R1 physically binds the ligands IL-1α and IL-1β, as well as a non-signaling competitive antagonist IL-1Ra (Greenfeder et al., 1995). The second chain in the receptor is the IL-1Racp - a common chain that, in part, also forms the receptors for IL-33 and IL-36. Signaling by the IL-1R requires MyD88, which is important for many of the TLRs (Weber et al., 2010). Induction of IL-1R signaling leads to the activation of NF-κB and MAPK pathways, ultimately leading to transcription of pro-inflammatory genes (Dinarello, 1996). IL-1R signaling on epithelial and endothelial cells leads to the production of IL-6 and various chemokines, including KC (Kirnbauer et al., 1989;
Nakamura et al., 1991; Schröder et al., 1990; Sironi et al., 1989). For immune cells, IL-1 signaling on DCs leads to CD86 and CCR7 expression and IL-1 on T cells leads to the activation of T cells as well as Th17 skewing (Lichtman et al., 1988; Pang et al., 2013; Veldhoen et al., 2006). In vivo, injection of IL-1 leads to the rapid recruitment of neutrophils to the site of injection as well as the production of IL-6 and certain chemokines (Dinarello, 1996). IL-6 production induced by IL-1 leads to the induction of fever in humans and other mammals. Beyond immune responses, injection of IL-1β can diminish sexual receptivity in female, but not in male rats, highlighting the broad and systemic responses that IL-1 imparts on host organisms (Yirmiya et al., 1995).

IL-1 and the IL-1R signaling have been shown to be protective in a variety of infectious models, including Salmonella enterica Typhimurium, Yersinia pseudotuberculosis, Staphylococcus aureus, and Mycobacterium tuberculosis, as well as others (Moltke et al., 2013). Interestingly, although IL-1α and IL-1β bind the same receptor, deletion of either IL-1α or IL-1β alone reduces resistance to M. tuberculosis in mice, suggesting a non-redundant role for the two cytokines (Mayer-Barber et al., 2011). In contrast, IL-1 has been shown to be deleterious to the host in a number of infectious models as well as models of autoinflammatory disease (Dinarello, 1996). For example, IL-1β increases bacterial burden during Burkholderia pseudomallei infection (Ceballos-Olvera et al., 2011). For autoinflammatory disorders, a role for IL-1 or inflammasome-associated genes has been demonstrated for cryopyrin-associated periodic syndrome, familial cold autoinflammatory syndrome, familial Mediterranean fever, rheumatoid arthritis, and others (de Jesus et al., 2015; Jesus and Goldbach-Mansky, 2014; Ting et al., 2006). Therapy for many of these syndromes and diseases relies on IL-1 antagonists, such as anakinra (Dinarello and van der Meer, 2013). Thus, IL-1 signaling is highly proinflammatory in a variety of settings. This inflammation can limit infection, but can
have pathological consequences Therefore, activation of the inflammasome and production of IL-1 family members is tightly regulated.

**J. Pulmonary immune responses**

The mammalian lung is a highly vascularized structure containing a variety of cell types. Alveolar macrophages are tissue resident macrophages of the lung. Many alveolar macrophages exist outside the body in the alveolar sacs (Hussell and Bell, 2014). During homeostasis they clear debris and surfactant from the airway space, facilitating respiration. Alveolar macrophages require GM-CSF for both their presence and function in the lung (Stanley et al., 1994). Their absence impairs lung function and in humans leads to a disease known as pulmonary alveolar proteinosis (Stanley et al., 1994).

Although thought to be free of association with other cells, a subset of alveolar macrophages were recently shown to have intimate contact with the airway epithelium and were termed sessile alveolar macrophages (Westphalen et al., 2014). These macrophages are defined by their localization and no markers currently distinguish between these two types of alveolar macrophages. Alveolar macrophages express TLRs and are known to make cytokines such as TNF (Hussell and Bell, 2014). During Legionnaire’s disease, alveolar macrophages harbor *L. pneumophila* as evidenced by detection of Gram-negative bacteria by electron microscopy in human patients as well as by detection of GFP+ *L. pneumophila* in a murine model of infection (Horwitz and Silverstein, 1981; LeibundGut-Landmann et al., 2011). Alveolar macrophages, then, are thought to be the primary reservoir of *L. pneumophila* during pulmonary infection. Whether or not other cell types harbor *L. pneumophila*, however, has not been fully investigated.
There are two main types of epithelial cells in the mammalian lung, termed airway epithelial cells (AEC) type I and type II. AECIs physically perform the act of respiration by governing gas exchange (Borok et al., 2002; Johnson et al., 2002). AECII produce surfactant to lower surface tension facilitating gas exchange (Fleming et al., 1994). AECI and AECII cells are often connected via gap junctions, and signaling cascades initiated in one cell can migrate via these junctions to adjacent cells (Guo et al., 1999). *In vitro*, epithelial cells can be infected with *L. pneumophila* and the bacteria can adhere to the outside of epithelial cells, but no evidence for epithelial cell infection has been demonstrated *in vivo* (Maruta et al., 1998; Mody et al., 1993; Prashar et al., 2012).

Epithelial cells produce chemokines, such as KC, during *L. pneumophila* infection, which are important to recruit immune cells to the site of infection (Tateda et al., 2001b).

Neutrophils are not generally present in the lung tissue itself, but due to the vascularized nature of the tissue these cells are found in vessels throughout the tissue. Neutrophils are considered highly inflammatory cells in most tissues, including the lung (Mizgerd, 2002). Neutrophils are often associated with tissue damage, as they are present at sites of damage to the epithelial barrier (Puljic et al., 2007). Neutrophils are recruited into the lung tissue and subsequently into the airway space itself by chemokines produced by epithelial cells during inflammation (Tateda et al., 2001a; 2001b). Neutrophils are highly bactericidal and *L. pneumophila* products can be detected in neutrophils during infection *in vivo* (LeibundGut-Landmann et al., 2011). Depletion of neutrophils with the antibody RB68C5 leads to a reduction in IL-18 and a subsequent decrease in IFNγ during *L. pneumophila* infection, ultimately leading to an increase in bacterial burdens (Spörri et al., 2008). Depletion with the RB68C5 clone, however, perturbs inflammatory monocytes, though the role of monocytes during infection has not been investigated on
its own (Daley et al., 2008). Thus, neutrophils are important for controlling infection, both for their ability to destroy live bacteria and their contribution to the cytokine milieu.

Inflammatory monocytes and natural killer (NK) cells are recruited to the lung tissue during infection (LeibundGut-Landmann et al., 2011; Spörri et al., 2008). NK cells respond to IL-12 and IL-18 produced during infection and rapidly produce IFNγ that can be detected as early as two days post infection in mice (Archer et al., 2010). This IFNγ production is critical for control of infection. The role of inflammatory monocytes during L. pneumophila infection has not been investigated; however, inflammatory monocytes are important in other models of bacterial infection (Serbina et al., 2008). Inflammatory monocytes are capable of producing a wide array of proinflammatory cytokines, including TNF, IL-6, and IL-12 (Serbina et al., 2008). These cells are capable of differentiating into other cells types, including dendritic cells (DC) (León et al., 2004; Yang et al., 2014). Inflammatory monocytes have recently been demonstrated to be bactericidal in certain models of infection (Narni-Mancinelli et al., 2011). The recruitment of inflammatory monocytes during other infections, and in other tissues, is often dependent on the expression of CCR2 on the monocytes and production of CCR2 ligands, such as CCL2, CCL7, and CCL12 in the infected site (Serbina et al., 2008). However, not all monocytes express CCR2; thus, certain subsets of monocytes are recruited independently of this pathway.

A variety of cells exist in the mammalian lung, but their roles in controlling L. pneumophila infection have not been investigated. Conventional DCs (cDC) exist in the lung during both homeostasis and infection (Ang et al., 2010). In vitro, cDCs rapidly undergo apoptosis when infected with virulent L. pneumophila in a manner that is independent of the inflammasome, suggesting that they may not be able to harbor L.
pneumophila in vivo (Nogueira et al., 2009). Eosinophils, although well characterized for their ability to exacerbate the symptoms of asthma in the lung, have no demonstrated role in either controlling or worsening L. pneumophila infection (Busse and Sedgwick, 1992; Stevens et al., 2007). B and T cells are recruited to the lung during infection and in other infectious models tertiary lymphoid structures (BALT) are formed weeks into pulmonary infection, which contain a large number of these cells (Sminia et al., 1989; Trunk and Oxenius, 2012). Antibody, and therefore B cell, responses are induced during L. pneumophila infection and passive transfer of immune sera protects mice from a low-dose challenge model of infection (Weber et al., 2012). T cells have been demonstrated to produce both IFNγ and IL-17 during L. pneumophila infection, but the contribution of either of these responses has not been fully investigated (Trunk and Oxenius, 2012).

K. Dissertation Aims

It is evident, then, that rapid and robust immune responses are mounted in response to L. pneumophila infection both in vitro and in vivo. In vitro, this response is diminished, but still present, in response to L. pneumophila lacking a functional T4SS. In vivo, the immune response to T4SS-deficient L. pneumophila is nearly undetectable and these bacteria are rapidly cleared from the host. This leads to two conclusions. First, the proinflammatory immune response to L. pneumophila both in vitro and in vivo is greatly enhanced by the presence of the T4SS. And second, although the protein translation inhibition mediated by effectors of the T4SS should limit the immune response against L. pneumophila, an immune response is still mounted and is vital for control and ultimate clearance of bacteria from the host. This paradoxical T4SS-dependent immune response to L. pneumophila leads to two questions. Which cells are targeted by the T4SS of L. pneumophila during infection? And how does the immune system overcome the T4SS-dependent block in protein translation to produce cytokines?
In chapter 3 of this dissertation, I investigate the cell types targeted by the T4SS of \textit{L. pneumophila} by using a fluorescence-based translocation assay. I find that alveolar macrophages and neutrophils are the primary reservoir for \textit{L. pneumophila} during pulmonary infection as they are both targeted by the T4SS and contain viable \textit{L. pneumophila}. Although the T4SS can actively inject dendritic cells \textit{in vitro}, I see no injection into dendritic cells \textit{in vivo}. Likewise, I see no injection into alveolar epithelial cells. Surprisingly, although only a small percentage of neutrophils harbor viable \textit{L. pneumophila}, neutrophils are the largest reservoir of viable bacteria \textit{in vivo}.

In chapter 4, I investigate the ability of injected cells and uninjected, bystander cells to produce proinflammatory cytokines in response to infection by combining our β-lactamase (BlaM)-based assay with intracellular cytokine staining. Injected cells are able to produce IL-1α and β, but are poor producers of the cytokines TNF, IL-6, IL-12p40, and do not increase expression of CD86. Instead, cells not targeted by the T4SS during infection, including non-targeted alveolar macrophages and neutrophils, produce TNF in response to \textit{L. pneumophila} infection \textit{in vivo} and increase expression of CD86. Importantly, this TNF production and CD86 expression is partially dependent on IL-1R signaling, suggesting that the IL-1 produced by alveolar macrophages and neutrophils during infection is critical for the response of uninfected, bystander cells. Together, these data suggest a coordinated effort between cells that harbor viable \textit{L. pneumophila} during infection and uninfected, bystander cells to produce a successful immune response in spite of pathogenic manipulation of host cell processes. Part of the communication between infected and uninfected cells depends on the production of IL-1α and β. This signaling pathway may be more broadly applicable to other infections, as IL-1 is produced in response to variety of bacterial pathogens and aids in bacterial clearance.
Figure 1-1. Bacterial pathogens inhibit host cell signaling and protein translation during infection. A number of bacterial pathogens encode virulence factors that inhibit host cell processes. NF-κB signaling is inhibited by virulence factors from Escherichia coli, Yersinia spp., and Shigella flexneri. MAPK signaling is also inhibited by proteins from Yersinia spp. and S. flexneri as well as Vibro parahaemolyticus and Bacillus anthracis. Downstream of receptor signaling Shigella dysenteriae, Pseudomonas aeruginosa, Corynebacterium diphtheria, and Legionella pneumophila encode virulence factors that inhibit host protein synthesis. Despite this, immune responses are still mounted against these pathogens as evidenced by proinflammatory cytokine production, indicating that hosts have mechanisms to bypass pathogenic manipulation.
Figure 1-2. Legionella pneumophila activates multiple immune pathways, but inhibits protein synthesis in infected cells. Infection with Legionella pneumophila activates multiple signaling pathways, leading to the induction of NF-κB and MAPK signaling. These pathways induce the transcription of proinflammatory genes. The T4SS effectors Lgt1, Lgt2, Lgt3, SidI, SidL, Pkn5, and Lpg1489 block the translation of these proteins; yet, TNF, IL-6, and IL-12 are produced during L. pneumophila infection. In tandem, bacterial products in the cytosol of infected cells activates the inflammasome, leading to the processing of mature IL-1 and death of the host cell. The coordination of all these events is poorly characterized.
Ethics statement
All experiments performed in this study were done so in accordance with the Animal Welfare Act (AWA), the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures (protocols #803465, #803459, and #804928).

Bacterial strains and plasmids
All experiments used Legionella pneumophila serogroup 1 strains. For in vitro studies, macrophages, dendritic cells, and epithelial cells were infected with Lp02 (rpsL, hsdR, thyA), a thymidine auxotroph derived from strain Lp01, or ΔdotA or ΔflaA isogenic mutant strains (Berger and Isberg, 1993). Certain experiments also used strains of L. pneumophila with deletions in Lgt1, Lgt2, Lgt3, SidI, and SidL on the Lp02 background (WTΔ5) or L. pneumophila with deletions in Lgt, Lgt2, Lgt3, SidI, SidL, Pkn5, and Lpg1489 on the ΔflaA background (Δ7ΔflaA) (Barry et al., 2013; Fontana et al., 2011). For in vivo studies, mice were infected with JR32-derived (rpsL, hsdR) ΔdotA or ΔflaA isogenic mutant strains (Marra and Shuman, 1989; Ren et al., 2006). For in vivo experiments requiring cell sorting, the aforementioned Lp02 strains were used. For in vitro and in vivo studies L. pneumophila was cultured on charcoal yeast extract agar containing 6.25µg/mL chloramphenicol for 48 hours at 37°C prior to infection (Feeley et al., 1979; Neild et al., 2005). For studies requiring motile L. pneumophila, 48 hour cultures grown on CYE agar were grown overnight in AYE broth containing chloramphenicol with shaking at 37°C until >50% of the bacteria were observed to be motile.
motile by light microscopy. Plasmids encoding M45 tagged β-lactamase-RalF fusion protein or M45-tagged β-lactamase were generated as follows. Briefly, the pJB1806 plasmid (RSF1010 ori, tdΔI, Amp’, Cm’) was first modified by cloning the icmR promoter and M45 epitope tag into the EcoRI and BamHI sites (Bardill et al., 2005). The mature TEM-1 β-lactamase gene (BlaM) was then amplified from a Y. pseudotuberculosis YopE-BlaM-encoding plasmid using primers that introduced a 5’ BglII site (5’-AATAAGATCTTGCACCCAGAAACGCTGGTG-3’) and 3’ BamHI site (5’-GCCTCACTGATTAAGGATCCATGCGGATCC-3’)(Brodsky and Medzhitov, 2008). The resulting PCR product was digested with BglII and BamHI and cloned into the BamHI site of the pJB1806 PicmR:M45 plasmid to create a plasmid encoding M45-tagged β-lactamase. To generate the plasmid encoding a translational fusion of M45-β-lactamase-RalF, RalF was amplified from Lp01 genomic DNA using primers that introduced BamHI sites at the 5’ and 3’ ends (5’-AATAGGATCCGGCATCCAGAAATTGAAAAAGCCC-3’) and (5’-GAAAAAGGTAGACAAATTAATTTTAAGGATCC-3’). The resulting PCR product was digested with BamHI and cloned into the BamHI site downstream of gene encoding M45-BlaM. The resulting plasmids were then electroporated into L. pneumophila and transformed colonies were selected for with chloramphenicol (Marra et al., 1992).

Mice

All mice were purchased from Jackson Laboratories. Mice were maintained in accordance with the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee. For infections, 8-12 week old male and female mice were anesthetized by intraperitoneal injection of a ketamine/xylazine/PBS solution at a dose of 100mg/kg ketamine and 10mg/kg xylazine. Mice were then infected intranasally with 40µl of a bacterial suspension containing 1-5x10^6 CFU L. pneumophila or PBS vehicle.
control. At the indicated time points after infection, mice were sacrificed. To isolate lung airway cells, bronchoalveolar lavage was performed 3-5 times with 1mL of cold PBS each time. Lungs were then excised and digested for 30 minutes at 37°C with occasional shaking in 5mL of PBS containing 5% FBS, 250U/mL of collagenase IV (Worthington Biochem), and 20U/mL DNAse I (Roche). Lungs were then mechanically homogenized and a single-cell suspension was obtained. To determine bacterial burden, lungs were mechanically homogenized in sterile, distilled H₂O and a portion of the lysate was spread onto CYE plates containing either chloramphenicol or streptomycin.

**Antibody-mediated neutralizations and depletions**

All antibodies were administered by intraperitoneal injection in 500µl PBS. For IL-1 antibody neutralizations 16 hours prior to infection, mice were given 200µg of either isotype antibody (hamster IgG), antibody against IL-1α (α-IL-1α) (ALF-161), IL-1β (α-IL-1β) (B122), or both (BioXcell).

**Cell culture**

For macrophages, C57BL/6 mouse bone marrow cells were differentiated in RPMI containing 30% L929 cell supernatant and 20% FBS at 37°C, 5% CO₂ in a humidified incubator. The macrophages were replated in RPMI containing 15% L929 cell supernatant and 10% FBS (Casson et al., 2013). For dendritic cells, bone marrow cells were differentiated in RPMI containing 10% FBS, 50µM β-mercaptoethanol, 2mM L-glutamine, and 20ng/mL GM-CSF (Peprotech) (Inaba et al., 1992). Semi-adherent dendritic cells were then isolated and replated in medium lacking GM-CSF. A549 cells and NIH/3T3 cells (ATCC) were cultured in DMEM containing 10% FBS (Wickremasinghe et al., 1999). For infections, cells were treated with 10-100ng/mL LPS.
from *E. coli* strain 055:B5 (Sigma), 0.4µg/mL PAM3CSK4, 10µl of bacterial suspension, or 10µL of PBS vehicle control.

**Isolation of inflammatory monocytes and alveolar macrophages**

Alveolar macrophages were harvested from the airways of mice by bronchoalveolar lavage in 5 x 1mL cold PBS. For intracellular cytokine staining, cells were centrifuged, counted, and resuspended at 10,000 cells per well in 150µl DMEM + 10% FBS.

Inflammatory monocytes were isolated from the bone marrow of mice using a magnetic negative selection kit (Miltenyi). Inflammatory monocytes were assayed for purity post selection and were consistently 90-95% CD45⁺, Ly6C<sup>hi</sup>, Ly6G⁻, CD11b<sup>int</sup> to CD11b<sup>hi</sup>. For intracellular cytokine staining, inflammatory monocytes were resuspended at 30,000 cells per well in 150µl DMEM with 10% FBS.

**Flow cytometry, fluorescence-based imaging flow cytometry, and cell sorting**

For *in vitro* experiments, infected cells were lifted and loaded with CCF4-AM (Invitrogen) per the manufacturer’s instructions. Cells were then washed and treated with Live/Dead Fixable Dead Cell Stain (Invitrogen). Bone marrow-derived dendritic cells were stained with antibodies specific for CD11c and MHCII (eBioscience). To stain for intracellular *L. pneumophila*, cells were fixed with BD Cytofix, permeabilized with BD Phosflow Perm Buffer III (BD Biosciences), and then stained with a rabbit polyclonal antibody against *L. pneumophila* followed by a rabbit-specific secondary antibody tagged to a fluorophore (Invitrogen). For *in vivo* studies, lung and airway cells were loaded with CCF4-AM and treated with the Live/Dead stain. Cells were then stained with antibodies specific for the cell surface antigens CD45, CD11c, Ly6G, Ly6C, NK1.1 (BioLegend), MHCII, CD19, CD3ε, CD31, CD326 (eBioscience), Siglec F, CD11b, and Ter119 (BD Biosciences). Data were collected on a LSR II flow cytometer (BD Biosciences) and post-collection
data were analyzed using FlowJo (Treestar). For fluorescent imaging experiments, data and images were collected on an Amnis ImageStream Mark II and data were analyzed using IDEAS software (EMD Millipore). Cells were gated on live singlets that had retained the CCF4-AM dye. Cell sorting experiments were performed on a FACS Aria II flow cytometer (BD Biosciences).

**Intracellular staining**

For *in vitro* experiments, infected cells were treated with 10µg/mL brefeldin A with or without 2µM monensin (Sigma) three hours prior to harvest. To stain for intracellular cytokines cells were fixed with BD Cytofix/Cytoperm (BD Biosciences), and then stained with antibodies specific for IL-6 (BioLegend), TNF, IL-1α and IL-1β (eBioscience). For *in vivo* studies, lung and airway cells were resuspended in RPMI or DMEM containing 10% FBS and 10µg/mL brefeldin A for 3 hours. Intracellular staining was performed as described above. Data were collected on a LSR II flow cytometer (BD Biosciences) and post-collection data were analyzed using FlowJo (Treestar). Cells were gated on live singlets that had retained the CCF4-AM dye. Cell sorting experiments were performed on a FACS Aria II flow cytometer (BD Biosciences).

**IL-1 Stimulation**

For treatment of cells with IL-1, mature recombinant IL-1α, IL-1β, or IL-1α and IL-1β (eBioscience) combined were added to cells at various concentrations. For intracellular staining of BMDCs, inflammatory monocytes, and alveolar macrophages, IL-1 was added at 100ng/mL for 6 hours with BFA added 3 hours prior to harvest. BMDCs were stained for CD11c, MHC II, and CD86. BMDCs were then fixed and stained for TNF. Inflammatory monocytes were stained for CD45, Ly6C, Ly6G, CD11b, Ter119, CD86.
and then fixed and stained for TNF. Alveolar macrophages were fixed and then stained for TNF.

**ELISA**

Harvested supernatants from cultured cells or BAL were assayed using capture and detection antibodies specific for IL-1α, IL-6, TNF, IL-12p70 (BioLegend), KC (R&D system), and IL-12p40 (BD Biosciences).

**RT-PCR**

Sorted bone marrow-derived macrophages were centrifuged and resuspended in RLT lysis buffer and RNA was isolated using an RNeasy kit as per kit’s protocol (Qiagen). RNA was then reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was then performed using the prepared cDNA with primers for *Il1b* (5’-GCA ACT GTT CCT GAA CTA AAC T-3’, 5’-ATC TTT TGG GGT CCG TCA ACT-3’), *Il1a* (5’-GCA CCT TAC ACC TAC CAG AGT AGT-3’, 5’-TGC AGG TCA TTT AAC CAA GTG G-3’), *Il6* (5’-GAC TTC CAT CCA GTT GCC TTC TTG G-3’, 5’-CCA GTT TGG TAG CAT CCA TCA TTT CT-3’), *Tnf* (5’-GAC GTG GAA GAG CAG GGA AGT-3’, 5’-TG TGT AGT GAG TAG TGT GAG-3’), or the housekeeping gene *Hprt* (5’-GT TGT GGA TAC AGG CCA GAC TTT GTT G-3’, 5’-GAG GTG AGT AGG CTG GCC TAT AGG CT-3’). Data were analyzed using by comparing the C₇ values obtained for each reaction and then comparing them with the appropriate samples’ housekeeping gene to determine relative expression.

**Immunoblot analysis**

*Legionella pneumophila* expressing the appropriate reporter plasmids were harvested from a two-day heavy patch and lysed. Lysates were then subjected to SDS-PAGE,
transferred to PVDF membrane, and probed with an anti-M45 epitope monoclonal antibody (Obert et al., 1994).

**Statistical analysis**

Plotting of data and statistical analysis were performed using Graphpad Prism software. Statistical significance was determined using the unpaired, two-tailed Student’s t test or one-way ANOVA with Tukey post-test. Differences were considered significant if the p value was <0.05.
CHAPTER 3

ALVEOLAR MACROPHAGES AND NEUTROPHILS ARE THE PRIMARY RESERVOIRS FOR LEGIONELLA PNEUMOPHILA AND MEDIATE CYTOSOLIC SURVEILLANCE OF TYPE IV SECRETION

This chapter appeared as a published peer-reviewed article titled “Alveolar Macrophages and Neutrophils Are the Primary Reservoirs for Legionella pneumophila and Mediate Cytosolic Surveillance of Type IV Secretion” by Alan M. Copenhaver, Cierra N. Casson, Hieu T. Nguyen, Thomas C Fung, Matthew M. Duda, Craig R. Roy, and Sunny Shin. Infection and Immunity, 2014.
Abstract

*Legionella pneumophila*, an intracellular pathogen responsible for the severe pneumonia Legionnaires’ disease, uses its *dot/icm*-encoded type IV secretion system (T4SS) to translocate effector proteins that promote its survival and replication into the host cell cytosol. However, by introducing bacterial products into the host cytosol, *L. pneumophila* also activates cytosolic immunosurveillance pathways, thereby triggering robust proinflammatory responses that mediate control of infection. Thus, the pulmonary cell types that *L. pneumophila* infects may act not only as an intracellular niche that facilitates its pathogenesis, but also contribute to the immune response against *L. pneumophila*. The identity of these host cells remains poorly understood. Here, we developed a strain of *L. pneumophila* producing a fusion protein consisting of β-lactamase fused to the T4SS-translocated effector RalF, which allowed us to track cells injected by the T4SS. Our data reveal that alveolar macrophages and neutrophils are both the primary recipients of T4SS-translocated effectors and harbor viable *L. pneumophila* during pulmonary infection of mice. Moreover, both alveolar macrophages and neutrophils from infected mice produced TNF and IL-1α in response to T4SS-sufficient, but not T4SS-deficient, *L. pneumophila*. Collectively, our data suggest that alveolar macrophages and neutrophils are both an intracellular reservoir for *L. pneumophila* and a source of proinflammatory cytokines that contribute to the host immune response against *L. pneumophila* during pulmonary infection.

Introduction

*Legionella pneumophila* is a Gram-negative bacterium found ubiquitously in fresh water environments, where it is often found in association with its natural host, protozoan amoebae (Fields, 1996). *L. pneumophila* has recently become a human pathogen due to modern technologies, such as cooling towers and air conditioners, which can aerosolize
fresh water contaminated with *L. pneumophila* (Fraser et al., 1977; McDade et al., 1977; Phin et al., 2014). Humans can then inhale these contaminated droplets, thus allowing *L. pneumophila* to gain access to the pulmonary airway. *L. pneumophila* infection can lead to a severe bacterial pneumonia known as Legionnaires’ disease (Fraser et al., 1977), with mortality rates approaching 30% (Domínguez et al., 2009).

Once in the lung, *L. pneumophila* encounters a specialized subset of pulmonary phagocytes called alveolar macrophages (Chandler et al., 1977). Following phagocytosis, the *Legionella*-containing phagosome avoids endocytic maturation and bacterial degradation and is converted into an ER-derived vacuole that supports bacterial replication (Shin and Roy, 2008). To establish infection, *L. pneumophila* utilizes its type IV secretion system (T4SS), encoded by the *dot/icm* genes, to translocate approximately 300 effector proteins into the host cell cytosol (Berger and Isberg, 1993; Ensminger and Isberg, 2009; Hubber and Roy, 2010; Marra et al., 1992; Nagai, 2002; Roy et al., 1998; Segal et al., 1998; Vogel et al., 1998). Many of these effector proteins are thought to be involved in recruiting ER-derived vacuoles to the *Legionella*-containing vacuole or preventing endocytic maturation (Hubber and Roy, 2010). Other effector proteins modulate host cell processes such as autophagy or host protein synthesis (Barry et al., 2013; Belyi et al., 2006; 2008; Choy et al., 2012; Shen et al., 2009). These virulence activities ultimately prevent destruction of *L. pneumophila* and allow for its replication within host cells. The T4SS is essential for the ability of *L. pneumophila* to survive and replicate within host cells, as *L. pneumophila* mutants lacking a functional T4SS do not replicate and reside in phagosomes that mature along a canonical endocytic pathway (Berger and Isberg, 1993; Roy et al., 1998).
While the Dot/Icm T4SS is essential for *L. pneumophila* to survive intracellularly and to cause disease, cytosolic immune surveillance systems activate host defense responses to T4SS activity that are critical for the control of *L. pneumophila* infection (Shin, 2012). For example, the NAIP5/NLRC4 inflammasome detects T4SS-dependent delivery of flagellin, leading to the caspase-1-dependent secretion of IL-1 family cytokines and pyroptotic cell death (Lightfield et al., 2008; Molofsky et al., 2006; Ren et al., 2006). Cytosolic detection of T4SS activity is also required for the robust secretion of inflammasome-independent cytokines, such as TNF (Fontana et al., 2011; Shin et al., 2008; Spörri et al., 2006). The IL-1 of family cytokines and TNF are critical for host defense against *L. pneumophila* (Barry et al., 2013; Brieland et al., 1995; Casson et al., 2013; LeibundGut-Landmann et al., 2011). Thus, the cells that interact with *L. pneumophila* in the lung and receive T4SS-translocated effectors may have a dual role during *in vivo* infection, in that they can enable intracellular survival of the pathogen and also contribute directly to the immune response by detecting T4SS-translocated products. However, the identities of the pulmonary cell types that interact with *L. pneumophila* and receive T4SS-translocated effectors are poorly understood.

Alveolar macrophages are thought to be the primary cell type infected by *L. pneumophila* and to support bacterial replication *in vivo* (Nash et al., 1984). However, it is unknown whether other immune phagocytes in the lung, such as neutrophils, inflammatory monocytes, or dendritic cells, may also receive T4SS-translocated effectors and contribute to the immune response or support *L. pneumophila* survival. Previous studies have demonstrated that in addition to alveolar macrophages, *L. pneumophila* can be detected in neutrophils during pulmonary infection (LeibundGut-Landmann et al., 2011). Neutrophils are thought to be highly bactericidal, and their presence in the lung and airway space during pulmonary *L. pneumophila* infection correlates with lower bacterial
burden (Barry et al., 2013; Casson et al., 2013; Nauseef, 2007; Tateda et al., 2001a; 2001b). Whether *L. pneumophila* can survive within neutrophils and translocate T4SS effectors into these cells during pulmonary infection is unknown. *L. pneumophila* can be taken up by a wide variety of cell types *in vitro*, such as neutrophils, bone marrow-derived dendritic cells, type I and type II alveolar epithelial cells, endothelial cells, and plasmacytoid dendritic cells (Ang et al., 2010; Horwitz and Silverstein, 1981; Maruta et al., 1998; Mody et al., 1993; Neild and Roy, 2003). However, the efficiency of *L. pneumophila* replication within these cell types varies greatly, and whether these cell types are injected by the T4SS or productively infected *in vivo* is unknown. We thus decided to investigate which cell types receive T4SS-translocated effectors and therefore may support *L. pneumophila* survival and contribute to cytosolic immunosurveillance during pulmonary infection.

Using a FRET-based reporter of T4SS translocation, we were able to detect effector translocation into macrophages, dendritic cells, and airway epithelial cells *in vitro*. We also demonstrate that only T4SS-injected cells contain viable *L. pneumophila*, whereas infected cells that have not received T4SS effectors do not contain viable bacteria. *In vivo*, alveolar macrophages and neutrophils in the airway space and lung tissue were the primary recipients of T4SS-translocated effectors and harbored viable bacteria. Consistent with the critical role of immune sensing of T4SS activity in triggering host cytokine production, alveolar macrophages and neutrophils from mice infected with T4SS-competent *L. pneumophila*, but not T4SS-deficient bacteria, secreted the cytokines TNF and IL-1α, which are known to be important for immune-mediated clearance of infection (Brieland et al., 1995; Casson et al., 2013; LeibundGut-Landmann et al., 2011). We did not observe T4SS-mediated injection into other lung cell populations, including airway epithelial cells and dendritic cells, suggesting that these
cells are neither a primary intracellular niche for *L. pneumophila*, nor do they directly participate in cytosolic immunosurveillance of T4SS activity during lung infection. Collectively, our data indicate that alveolar macrophages and neutrophils play a dual role as both an intracellular niche and immune mediator during pulmonary *L. pneumophila* infection.

**Results**

*A reporter system tracks translocation of type IV secretion system effectors by Legionella pneumophila into mammalian cells*

*L. pneumophila* uses its type IV secretion system (T4SS) to translocate effector proteins into the cytosol of host cells. To track this translocation, we constructed a plasmid in which the well-characterized *L. pneumophila icmR* promoter drives transcription of a gene encoding a translational fusion of the mature TEM-1 BlaM and the well-characterized T4SS effector protein RalF and introduced this plasmid into *L. pneumophila* (Figure 3-1A & B) (Gal-Mor et al., 2002; Knapp et al., 2003; Zlokarnik et al., 1998). We chose RalF because it is translocated into the cytosol of infected cells immediately following intimate interaction of *L. pneumophila* with host cells (Nagai et al., 2005; Ninio and Roy, 2007). Following infection of host cells by bacterial strains expressing the BlaM-RalF fusion protein, the enzymatic activity of translocated BlaM-RalF was detected in host cells by means of the membrane-permeable BlaM substrate CCF4-AM (Zlokarnik et al., 1998). CCF4-AM consists of coumarin joined to fluorescein by a β-lactam ring. When excited at 409nm, fluorescence resonance energy transfer (FRET) between coumarin and fluorescein results in green fluorescence emission at 518nm. T4SS-injected BlaM will cleave the CCF4-AM substrate in the host cytosol and eliminate FRET, thus resulting in blue fluorescence emission at 447nm.
We generated *L. pneumophila* strains expressing either BlaM or BlaM-RalF and infected C57BL/6 bone marrow-derived macrophages (BMDM) with these strains for 8 hours. Following infection, the cells were loaded with CCF4-AM and analyzed by flow cytometry to determine whether blue fluorescence emitted by cleaved CCF4-AM was detected (Figure 3-1C). Approximately 20-25% of macrophages infected with BlaM-RalF-expressing WT *L. pneumophila* and *L. pneumophila* lacking flagellin (ΔflaA), which evade NAIP5 inflammasome responses, were positive for blue fluorescence resulting from cleaved CCF4-AM, but not following infection with strains lacking a functional T4SS (ΔdotA). This indicates that CCF4-AM is efficiently cleaved only by BlaM-RalF translocated by T4SS-sufficient bacteria and that BlaM-RalF remaining within bacteria does not generate a detectable signal in this assay. The frequencies of injected cells in WT and ΔflaA *L. pneumophila* infections were comparable, although the frequency of injection was consistently lower in WT infections (Figures 3-1C, 3-2A, & 3-5A). The robust detection of injection by WT *L. pneumophila* is surprising considering that a higher percentage of vacuoles containing WT *L. pneumophila* fail to avoid rapid endocytic maturation and that flagellin induces NAIP5-dependent cell death in C57BL/6 macrophages (Amer and Swanson, 2005; Amer et al., 2006; Byrne et al., 2013; Molofsky et al., 2006; Ren et al., 2006). Following infection with *L. pneumophila* strains expressing BlaM alone, we found that a much lower percentage of macrophages became positive for blue CCF4-AM fluorescence compared to macrophages infected with *L. pneumophila* expressing BlaM-RalF (Figure 3-1C). Importantly, this small percentage of CCF4-AM positive cells was still dependent on infection with T4SS-sufficient bacteria, suggesting that BlaM lacking a canonical T4SS signal sequence may be inefficiently delivered into the host cytosol by the T4SS.
**T4SS-injected host cells contain viable Legionella pneumophila.**

The T4SS is essential for the survival of *L. pneumophila* within host cells. To determine whether cells injected with BlaM-RalF contain *L. pneumophila*, we infected BMDMs with these reporter strains and loaded the cells with CCF4-AM. After loading, the macrophages were fixed, permeabilized, and stained with an antibody specific for *L. pneumophila* (Figure 3-2A). Infection with all three strains (WT, ΔdotA, ΔflaA) of *L. pneumophila* resulted in macrophages staining positive for the presence of bacteria. 90-100% of cells that were positive for BlaM-RalF injection were also positive for *L. pneumophila* staining in both the WT and ΔflaA strains. With both strains, we detected a subset of cells that was positive for *L. pneumophila* but translocation of BlaM-RalF was not within a detectible range, revealing heterogeneity in BlaM-RalF translocation at the single cell level. The percentage of cells positive for *L. pneumophila* but negative for BlaM-RalF translocation could result from bacteria that failed to successfully translocate T4SS effectors into the host cell, either because they were non-viable, were not in the transmissive phase, or failed to efficiently evade rapid phagosomal maturation.

To determine whether the *L. pneumophila* associated with injected or uninjected macrophages were intact or degraded, we infected macrophages with our reporter strains of *L. pneumophila* and analyzed these macrophages with fluorescence-based imaging flow cytometry (Figure 3-2B and Figures 3-3A). The majority of macrophages infected with the ΔdotA strain showed dim *L. pneumophila* staining, with multiple small puncta present per cell (Figure 3-2C). Because the ΔdotA mutants are unable to evade endocytic maturation due to their lack of a functional T4SS, punctate staining could result from bacteria that were degraded. Alternatively, punctate staining could represent uninfected cells that had phagocytosed bacterial debris. When we infected macrophages with ΔflaA *L. pneumophila* encoding a functional T4SS, we again could identify T4SS-
injected and uninjected cells. Many of the uninjected cells stained positive for intracellular *L. pneumophila* (Figure 3-3B) as determined by analysis of the images obtained of infected cells using fluorescent-based imaging flow cytometry, but the majority of these cells exhibited dim, punctate staining similar to the staining seen for Δ*dotA*-infected macrophages (Figure 3-2B & C). This may represent cells containing bacteria that had not successfully evaded endocytic maturation or uninjected cells that had phagocytosed bacterial debris. In contrast, the majority of injected cells showed a single bright punctum of *L. pneumophila* staining, indicating the presence of an intact bacterium that had not been transported to a hydrolytic compartment.

To test whether injected macrophages contain viable *L. pneumophila*, we sorted infected macrophages that were either positive or negative for the cleaved CCF4-AM signal, lysed the macrophages, and enumerated bacterial CFUs in these distinct cell populations (Figure 3-2D). T4SS-injected cells recovered from a Δ*flaA* *L. pneumophila* infection contained the vast majority (nearly 6 bacteria for every injected BMDM) of viable *L. pneumophila* as determined by CFU count. Uninjected cells from the same infection contained a minimal number of viable *L. pneumophila* (less than 1 bacterium for each uninjected BMDM), comparable to the number of viable bacteria recovered from a Δ*dotA* infection. To exclude the possibility that the uninjected cells contained viable *L. pneumophila* that lost the BlaM-RalF reporter plasmid encoding chloramphenicol resistance, we also plated cell lysates in the presence or absence of chloramphenicol (Figure 3-4A). The CFUs obtained on plates with and without chloramphenicol were indistinguishable, suggesting that the plasmid is stably maintained during *in vitro* infection in the absence of antibiotics. Collectively, our data indicate that viable bacteria are associated primarily with cells that have received translocated BlaM-RalF, whereas uninjected cells are either not infected or contain non-viable bacteria.
Translocation by the type IV secretion system can be detected in dendritic cells and alveolar epithelial cells in vitro

*L. pneumophila* can infect a variety of cell types *in vitro*, including dendritic cells and airway epithelial cells (Maruta et al., 1998; Mody et al., 1993; Neild and Roy, 2003). We thus examined whether T4SS-mediated translocation into these cell types could be detected using the β-lactamase reporter system. At a given MOI, as compared to infected BMDMs, we detected a much lower frequency of T4SS-mediated injection into bone marrow-derived dendritic cells (BMDCs) infected with WT or ΔflaA *L. pneumophila* (Figure 3-5A & B). We also infected A549 cells, an alveolar epithelial cell line, and detected a low frequency of injection into these cells (Figure 3-5C).

Other researchers have noted an increase in bacterial uptake by host cells when *L. pneumophila* is grown under conditions that promote bacterial motility (Ren et al., 2006). Indeed, infection of macrophages with motile *L. pneumophila* resulted in a large increase in the frequency of injected macrophages, as the percentage of injected cells increased from 10% to more than 80% (Figure 3-6B). In contrast, in A549 cells, we did not observe an increase in injection regardless of bacterial motility in that 1.3% of cells infected with non-motile or motile *L. pneumophila* were injected (Figure 3-6A). For all cell types, the percentage of cells injected by the T4SS of *L. pneumophila* increased over time (Figure 3-5). In all instances, cleaved CCF4-AM signal required expression of a functional T4SS, suggesting that the β-lactamase reporter operates in a T4SS-dependent manner in a variety of cell types. As we observed more robust injection into macrophages and dendritic cells than into non-phagocytic alveolar epithelial cells, these data suggest that both increased cell contact and efficient uptake by professional phagocytes contribute to the ability of *L. pneumophila* to efficiently translocate effector proteins.
Legionella pneumophila translocates bacterial effectors into alveolar macrophages and neutrophils during pulmonary infection

Our data suggest that the L. pneumophila T4SS can translocate effectors into alveolar epithelial cells, dendritic cells, and macrophages during in vitro infection. During pulmonary infection, replicating L. pneumophila can be detected in alveolar macrophages (Nash et al., 1984), indicating that alveolar macrophages receive T4SS-translocated effectors. However, whether alveolar epithelial cells, dendritic cells, and other cell types receive T4SS-translocated effectors in vivo has not been investigated. To identify the cells that receive translocated effectors during a permissive model of in vivo infection, we intranasally infected C57BL/6 mice with ΔflaA L. pneumophila expressing BlaM-RalF, as WT L. pneumophila do not establish a productive infection in mice that encode a functional NAIP5 allele as CFUs do not increase and are cleared from C567BL/6 mice (Figure 3-4D) (Molofsky et al., 2006; Ren et al., 2006). In this model, similar to WT L. pneumophila infection of A/J mice expressing a hypomorphic NAIP5 allele, the lungs of C57BL/6 mice exhibit approximately a one-log increase in ΔflaA L. pneumophila CFUs by 24-48 hours post-infection (Figure 3-4C & D) (Case et al., 2009). The mice are subsequently able to control infection, with minimal bacterial CFUs detected in the lungs by 5 days post-infection (Figure 3-4C & D) (Molofsky et al., 2006). Expression of the plasmid containing BlaM-RalF did not affect the replication of ΔflaA L. pneumophila in vivo (Figure 3-4C). After intranasal inoculation with L. pneumophila, we performed bronchoalveolar lavage to isolate cells from the airway space at various time points and loaded them with CCF4-AM to detect T4SS-mediated injection of BlaM-RalF. At four hours post-inoculation, we detected T4SS-mediated translocation of β-lactamase activity in nearly 50% of cells recovered from the airway of mice infected with ΔflaA L. pneumophila (Figure 3-7A). Greater than 95% of the T4SS-
injected cells were alveolar macrophages, as indicated by their expression of CD11c and Siglec F (Stevens et al., 2007; Sung et al., 2006). Similar results were obtained with WT L. pneumophila at this time point (data not shown). Consistent with our in vitro data, we did not observe injection of BlaM-RalF in mice infected with the ΔdotA strain, which is unable to translocate effectors into host cells and cannot establish a productive infection in vivo.

At later times post-infection, we detected recruitment of a large population of neutrophils to the airway space of ΔflaA L. pneumophila-infected mice that did not occur in mice infected with ΔdotA L. pneumophila (Figures 3-8A & B), consistent with previous studies indicating that neutrophil recruitment is T4SS-dependent (Barry et al., 2013; Casson et al., 2013; Frutuoso et al., 2010; LeibundGut-Landmann et al., 2011). When we identified cells injected by L. pneumophila in the airway space at 24 hours post-infection, we again identified alveolar macrophages as being positive for T4SS-mediated injection, but we could also identify injected cells that expressed high levels of Ly6G and were negative for MHC class II (Figure 3-7B). We determined these injected Ly6G⁺ cells to be neutrophils, as they expressed low levels of Ly6C, a cell surface marker highly expressed on inflammatory monocytes (Figure 3-3C) (Fleming et al., 1993; Sunderkötter et al., 2004). The frequency of injected neutrophils was much lower than that of injected alveolar macrophages (Figure 3-7C). However, due to the large influx of neutrophils, the total number of injected neutrophils was comparable to or greater than the total number of injected alveolar macrophages at 24, 48, and 72 hours post-infection (Figure 3-7D).

As we could detect robust T4SS-mediated injection of BlaM-RalF into cells of the airway space, we wanted to determine whether cells within the lung interstitium were injected by L. pneumophila as well. Notably, we again observed T4SS-mediated injection into
alveolar macrophages and neutrophils within lung homogenates (Figure 3-9A). As in the
airway space, we detected a large influx of neutrophils into the lung tissue of ΔflA-
infected mice, but not in mice infected with the ΔdotA strain of *L. pneumophila* (Figures
3-8C & D). Though *in vitro* we observed T4SS-mediated injection into bone marrow-
derived dendritic cells as well as A549 alveolar epithelial cells (Figure 3-5B & C), we did
not detect injection into lung dendritic cells or CD326⁺ airway epithelial cells, suggesting
that *L. pneumophila* does not efficiently infect or translocate effectors into these cell
types during a permissive mouse model of infection (Figure 3-9A). We also did not
observe injection into inflammatory monocytes, plasmacytoid dendritic cells, eosinophils,
B cells, T cells, NK cells, or endothelial cells within the lung tissue at any time assayed
post infection (Figure 3-3C & D). The frequency of T4SS-injected neutrophils in the lung
tissue was much lower than that seen in alveolar macrophages, similar to what we
observed in the airway space (Figure 3-9B). At four hours post-infection, the majority of
cells receiving T4SS-translocated effectors in the lung tissue were alveolar
macrophages, but at later times, many of the T4SS-injected cells were neutrophils
(Figure 3-9C). Importantly, *L. pneumophila* recovered at 48 and 72 hours post-infection
retained the reporter plasmid, indicating that the plasmid is stably maintained during *in
vivo* infection even in the absence of antibiotic selection (Figure 3-4B). A previous study
examining a non-permissive model of C57BL/6 mice infected with WT *L. pneumophila*
also found that CD45-negative cells or lung epithelial cells did not appear to have taken
up *L. pneumophila*, with alveolar macrophages appearing to be the primary cells infected
at early time points post-infection, followed by infection of recruited neutrophils at one
day post-infection (LeibundGut-Landmann et al., 2011), suggesting that in both
permissive and non-permissive mouse models, similar lung cell types are infected.
Neutrophils and alveolar macrophages in the lungs of infected mice harbor viable *Legionella pneumophila* bacteria and produce cytokines

Alveolar macrophages are thought to be the primary cell type that is infected by *L. pneumophila* and supports bacterial replication (Nash et al., 1984). A previous study using a non-permissive model of C57BL/6 mice infected with WT *L. pneumophila* also found that recruited neutrophils take up *L. pneumophila* in the lung, but whether *L. pneumophila* could translocate effectors into neutrophils or survive within these cells was not examined (LeibundGut-Landmann et al., 2011). As we observed that both alveolar macrophages and neutrophils in *L. pneumophila*-infected lungs were injected by the T4SS, we sought to determine whether in addition to alveolar macrophages, neutrophils also contained viable bacteria. We therefore sorted total alveolar macrophages and neutrophils from the lungs of mice infected intranasally with *L. pneumophila* and enumerated bacteria from lysed cells. As a comparison, we also sorted total inflammatory monocytes, a population of cells negative for T4SS injection, from these infected mice as well. As expected, alveolar macrophages isolated twenty-four hours after infection contained viable bacteria (Figure 3-10A). In contrast, inflammatory monocytes contained very few viable *L. pneumophila*, consistent with the lack of observed T4SS-dependent translocation into these cells (Figure 3-10A). Interestingly, neutrophils from Δ*flaA*-infected mice contained viable bacteria at a frequency consistent with the extent of injection, suggesting that injected neutrophils harbor viable bacteria in the airway and lung tissue. Although the absolute frequency of viable *L. pneumophila* in alveolar macrophages was greater than within neutrophils (nearly 10 bacteria per 100 alveolar macrophages vs. 1 bacterium per 100 neutrophils), the higher absolute numbers of neutrophils present during infection results in the unexpected finding that neutrophils actually contain nearly twice as many viable bacteria as alveolar macrophages (Figure 3-10B). To examine whether this was also the case at the peak of
pulmonary bacterial load, we sorted alveolar macrophages and neutrophils from mice 48 hours PI (Figure 5C &D). As with 24 hours PI, although a higher frequency of alveolar macrophages contained viable *L. pneumophila*, in total there were more *L. pneumophila* in neutrophils than alveolar macrophages.

The presence of neutrophils in the airway space during infection correlates with lower bacterial burden, thought to be in part due to their potent bactericidal activity (Barry et al., 2013; Casson et al., 2013; LeibundGut-Landmann et al., 2011). However, as our data suggest that *L. pneumophila* inject and survive within neutrophils, thus potentially activating cytosolic immunosurveillance pathways within these cells, we next examined whether or not infected neutrophils may also contribute to the T4SS-dependent production of pro-inflammatory cytokines important for bacterial clearance. To test this, following intranasal infection with either ΔdotA or ΔflaA *L. pneumophila*, we measured cytokines secreted by alveolar macrophages isolated at four hours post-infection or neutrophils isolated at twenty-four hours post-infection. Alveolar macrophages from ΔflaA-infected mice secreted TNF and IL-1α, whereas macrophages from ΔdotA-infected mice did not. Interestingly, neutrophils from mice infected with ΔflaA *L. pneumophila* also secreted substantial amounts of TNF and IL-1α, whereas neutrophils from mice infected with ΔdotA *L. pneumophila* did not secrete detectable levels of IL-1α and secreted significantly less TNF, which correlates with the lack of detectable cytokine production observed during pulmonary infection with ΔdotA *L. pneumophila* (Fig 3-10E & F). Intriguingly, these data demonstrate that in addition to alveolar macrophages, neutrophils also produce proinflammatory cytokines in the context of T4SS-competent *L. pneumophila* infection. This indicates that in addition to their potent bactericidal activity, neutrophils may contribute to the control of infection by other immune effector mechanisms such as cytokine production.
Conclusion

Here, I describe the creation of a sensitive and faithful fluorescent reporter to track the activity of the T4SS of L. pneumophila within in host cells. Using this system, I am able to detect injection by the T4SS into cells both in vitro and in vivo. In vitro, injected harbor viable L. pneumophila, demonstrating that this fluorescent detection system correlates with active infection in host cells. Cells not injected by the T4SS do not contain viable L. pneumophila. Injection is detectable in macrophages, dendritic cells, and alveolar epithelial cells in vitro. These data indicate that injection can occur into a variety of cell types. In a pulmonary model of L. pneumophila infection in vivo, however, injection is only detected in alveolar macrophages and neutrophils at multiple times PI. Importantly, viable CFUs are obtained from these cell types, indicating that L. pneumophila are able to survive and potentially replicate within these cells. Beyond alveolar macrophages and neutrophils being a niche for L. pneumophila in vivo, the cells also secrete IL-1α and TNF in response to infection with virulent, but not avirulent infection. These data indicate a dual role for phagocytic cells during pulmonary L. pneumophila infection as both intracellular niche and important activators of subsequent immune responses.

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Figure 3-1. β-lactamase translocation into cells requires fusion to RalF and a functional type IV secretion system. (A) Schematic of the BlaM-RalF expression plasmid. β-lactamase (BlaM) is tagged with the M45 peptide and fused to the N-terminus of the effector protein RalF. The icmR promoter drives the expression of this fusion protein. (B) Lysates from WT, ΔdotA, and ΔflaA L. pneumophila expressing either β-lactamase (BlaM) alone or Blam-RalF fusion reporter plasmids were blotted with an α-M45 antibody to detect reporter protein expression. (C) Bone marrow-derived macrophages (BMDMs) were treated with PBS (Unfx) or infected with WT, ΔdotA, and ΔflaA strains of Legionella pneumophila (Lp) expressing BlaM or BlaM-RalF for 8 hours with an MOI=5. Cells were then loaded with CCF4-AM and analyzed by flow cytometry. Cells positive for cleaved CCF4-AM (blue) fluorescence are gated and the frequency of injected cells for each condition is denoted within the gate. Bar graph shows mean ± standard error of the mean (SEM) of triplicate wells. Representative of three independent experiments.
Figure 3-2. Viable Legionella pneumophila are predominantly associated with macrophages positive for T4SS-dependent translocation. (A) BMDMs were left untreated (Unfx) or infected with WT, ΔdotA, or ΔflaA Lp for 8 hours with an MOI=5. Cells were then loaded with CCF4-AM, fixed, permeabilized, and stained with a polyclonal antibody against Lp. Cells were analyzed by flow cytometry. The percentages of cells positive for Lp as determined by staining and cells positive for T4SS injection are denoted within the gates. (B & C) Cells were treated similarly to (A), but infected with an MOI=10 for 4 hours and analyzed using an Amnis ImageStream imaging flow cytometer. (D) BMDMs were left untreated, treated with LPS, or infected with ΔdotA or ΔflaA Lp for 8 hours with an MOI=5 in the presence of exogenous thymidine (100µg/mL) and loaded with CCF4-AM. Samples infected with ΔdotA were sorted in bulk for loaded cells. Cells infected with ΔflaA were then sorted based on uncleaved or cleaved CCF4-AM signal, lysed, and plated on CYE plates. CFUs were then enumerated. Bar graphs show mean only or mean ± SEM of triplicate samples. Representative of 2 independent experiments. ns=not significant.
Figure 3-3. Gating strategies for fluorescence-based imaging flow cytometry and conventional flow cytometry. (A & B) BMDMs were left untreated or infected with ΔdotA or ΔflaA Lp for 8 hours with an MOI=10. Cells were then loaded with CCF4-AM, fixed, permeabilized, and stained with an antibody against Lp. Cells were then analyzed using the Amnis ImageStream imaging flow cytometer. Focused singlets that took up CCF4-AM were selected as shown in (A) and the frequency of injected (cleaved) cells was determined by gating as well as the amount of staining for Lp in either uninjected (uncleaved) or injected cells. The frequency of injection and Lp+ cells was then determined as shown in (B). (C & D) Murine lung was homogenized and single cell suspensions were stained with extracellular markers to identify various populations of immune cells including alveolar macrophages (AMΦ) (CD45+, Ly6G−, Ly6C−, CD11c+, Siglec F−), neutrophils (NΦ) (CD45+, Ly6G+, Ly6Clow), inflammatory monocytes (iMC) (CD45+, Ly6G−, Ly6C+), conventional DCs (cDC) (CD45+, Ly6G−, Ly6C−, CD11c+, Siglec F−, MHCII+) T cells (CD45+, CD3ε+, CD19−), B cells (CD45+, CD3ε−, CD19+) and NK cells (CD45−, CD3ε−, CD19−, NK1.1+). Airway epithelial cells (AEC) (CD45−, CD31+, CD326+) and lung endothelial cells (LEC) (CD45+, CD31+, CD326−) were also identified. Gates shown are representative of the gates used throughout this study.
Figure 3-4. The BlaM-RalF expression plasmid is stably maintained during both in vitro and in vivo infection. (A) BMDMs were infected with ΔdotA or ΔflaA Lp for 8 hours with an MOI=5. Cells were then sorted based on cleaved CCF4-AM signal, lysed, and plated on CYE agar plates containing either streptomycin or chloramphenicol. CFUs were then enumerated. Bar graph shows mean ± SEM. N=3. (B) Mice were infected intranasally with ΔdotA or ΔflaA Lp for 48 or 72 hours. The lungs of the infected mice were then homogenized and the cells were lysed and plated on CYE plates as in (A). Points represent individual mice with the mean denoted as a line. N=3-4 mice per group. (C) Mice were infected with infected intranasally with 1x10^6 JR32 ΔflaA, 5x10^6 JR32 ΔflaA, or 5x10^6 JR32 ΔflaA expressing the BlaM-RalF plasmid. CFUs were enumerated on CYE plates containing streptomycin. N=3 mice per group. (D) Mice were infected with 5x10^6 CFUs of BlaM-RalF-expressing strains of JR32 WT, ΔdotA, or ΔflaA L. pneumophila and bacterial burdens were enumerated. N= 2-4 mice per group.
Figure 3-5. Legionella pneumophila T4SS-dependent translocation is detected in dendritic cells and alveolar epithelial cells during in vitro infection. BMDMs (A), bone marrow-derived dendritic cells (BMDCs) (B), and A549 cells (C) were left untreated or infected with WT, ΔdotA, or ΔflaA Lp for 4, 8, or 12 hours with an MOI=5. Cells were then loaded with CCF4-AM and analyzed for injection by flow cytometry. Representative plots show injection 8 hours post infection. Graphs show mean ± SEM of triplicate wells. Representative of 2 independent experiments, N=3.
Figure 3-6. **Bacterial motility increases the frequency of T4SS-injected macrophages, but not T4SS-injected alveolar epithelial cells.** A549 cells (A) and BMDMs (B) were left untreated, treated with LPS, or infected with WT, ΔdotA, or ΔflaA Lp at MOI=5 for 4 hours that were either non-motile or grown under conditions that induce motility. Cells were then loaded with CCF4-AM and analyzed for injection by flow cytometry. Bar graphs are mean ± SEM. Representative of 2 independent experiments, N=3.
Figure 3-7. Alveolar macrophages and neutrophils in the airway space are injected by the *Legionella pneumophila* T4SS. C57BL/6J mice were infected intranasally with PBS vehicle control, Δ*dotA Lp*, or Δ*flaA Lp*. At 4 (A) or 24 (B) hours post infection, cells in the airway space were isolated, enumerated, loaded with CCF4-AM, and stained for cell surface markers. Cells were then analyzed for injection by flow cytometry and cell surface marker expression. Representative of 2 independent experiments, N=4 mice per group. (C & D) Alveolar macrophages (MΦ) and neutrophils from the airway space of mice infected with Δ*flaA Lp* were isolated at 4, 24, 48, or 72 hours post infection and loaded with CCF4-AM. The percentage (C) and total number (D) of cells injected by *Lp* in each population was quantified. Graphs show mean ± SEM. N=3-4 mice per group.
Figure 3-8. Infection with ΔflaA Legionella pneumophila recruits immune cells to the lungs of infected mice. Mice were infected intranasally with ΔflaA (A & C) or ΔdotA (B & D) Lp. At various times post infection, airway space cells (A & B) and lung cells (C & D) were separately isolated, enumerated, and stained for extracellular markers. The frequency and number of alveolar macrophages, neutrophils, monocytes, conventional DCs, T cells, B cells, and NK cells was calculated. Graphs show mean ± SEM. N=3-4 mice per group.
Figure 3-9. The Legionella pneumophila T4SS injects alveolar macrophages and neutrophils in the lung. (A) Mice were infected intranasally with ΔdotA or ΔflaA L. pneumophila for 24 hours. Lung cells were then isolated, loaded with CCF4-AM, and stained. Flow plots are pre-gated on the denoted cell populations. Representative of 3 independent experiments, N=4 mice per group. (B & C) Mice were infected with ΔflaA Lp and lung cells were isolated at various times post infection. The percentage (B) and total number (C) of cells injected by Lp in each population was quantified. Graphs show mean ± SEM. N=3-4 mice per group. cDC=conventional dendritic cell.
Figure 3-10. Alveolar macrophages and neutrophils from infected mice contain viable Legionella pneumophila and secrete cytokines. (A+C) Mice were intranasally infected with ΔdotA or ΔflaA Lp. At 24 (A) or 48 (B) hours post-infection, the lungs and airway space were homogenized and single cell suspensions were stained for cell surface markers to identify alveolar macrophages, neutrophils and inflammatory monocytes. These cell populations were sorted using a flow cytometer, lysed, and plated on CYE agar to enumerate L. pneumophila CFUs. (B) The frequency of viable bacteria per cell type in (A) was then multiplied by the frequency of the appropriate cell type found in a total of 10^6 lung cells. (D) Similarly, the frequency of viable bacteria per cell type in (C) was multiplied by the frequency of the appropriate cell type found in a total of 10^6 lung cells. (E) Alveolar macrophages were isolated by bronchoalveolar lavage 4 hours after intranasal infection with ΔdotA or ΔflaA Lp and cultured overnight. Supernatants were collected and TNF and IL-1α concentrations were determined by ELISA. (F) Neutrophils were isolated and sorted at 24 hours PI from mice treated as in (A) and then cultured overnight as in (C). Graphs show mean ± SEM. *p<0.05, ***p<0.0005
CHAPTER 4

IL-1 INDUCES INNATE BYSTANDER CYTOKINE PRODUCTION TO BYPASS

BACTERIAL BLOCKADE OF HOST PROTEIN SYNTHESIS

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Abstract
The innate immune system is critical for host defense against microbial pathogens, yet many pathogens utilize virulence factors to impair immune cell function. Here, we employed the intracellular bacterium *Legionella pneumophila* to understand how the immune system overcomes pathogenic activities. *L. pneumophila* utilizes a type IV secretion system (T4SS) to translocate bacterial effectors that potently block host translation in infected host cells. Despite this translational block, virulent *L. pneumophila* elicits a robust inflammatory response, but the basis for this is poorly understood. Single cell analysis of cytokine production by infected and uninfected cells revealed that infected macrophages produce IL-1α and IL-1β but poorly synthesize the cytokines IL-6, TNF, and IL-12, which are critical for controlling *L. pneumophila* infection. Intriguingly, uninfected bystander immune cells produced IL-6, TNF, and IL-12, and IL-1 receptor (IL-1R) signaling was required for this cytokine production. Thus, our data demonstrate functional heterogeneity in production of critical protective cytokines in the response to bacterial infection. These data suggest collaboration between infected and uninfected cells that enables the immune system to bypass protein synthesis inhibition and generate a robust immune response against *L. pneumophila*.

Significance Statement
Pathogens use virulence factors to inhibit key immune cell functions and would be expected to impair immune responses to infection. However, immune responses are still activated during infection, suggesting that the immune system has evolved mechanisms for overcoming pathogenic activity. Here, we demonstrate that cells infected with *L. pneumophila* produce IL-1 despite a pathogen-imposed host translational block, but are unable to robustly produce other critical cytokines. IL-1R signaling allowed uninfected bystander cells to produce protective cytokines. Our data thus demonstrate a key role for
communication between infected and uninfected bystander cells in overcoming pathogenic activities. This mechanism of immune activation has broad significance for our understanding of how successful immune responses are generated against pathogens.

**Introduction**

Initiation of innate immune responses to microbial pathogens is thought to involve the direct recognition of pathogen-associated molecular patterns (PAMPs) by membrane-bound and cytosolic pattern recognition receptors (PRRs) on infected cells (Janeway and Medzhitov, 2002; Vance et al., 2009). However, many pathogens utilize virulence factors that interfere with essential immune signaling processes, including NF-κB and MAPK signaling and host protein synthesis (Krachler et al., 2011; Roy and Mocarski, 2007; Viboud and Bliska, 2005). Such virulence factors limit cell-intrinsic immune activation of infected cells. The mechanisms that enable the host to circumvent pathogen manipulation of host cell signaling processes remain poorly understood.

The Gram-negative bacterium *Legionella pneumophila* encodes a specialized Dot/Icm type IV secretion system (T4SS) that injects bacterial effector proteins into host cells to facilitate bacterial intracellular survival and replication (Berger and Isberg, 1993; Hubber and Roy, 2010; Marra et al., 1992). A subset of effector proteins, Lgt1, Lgt2, Lgt3, SidI, SidL, Pkn5, and Lpg1489, block host protein synthesis, in part by targeting translational elongation factors (Barry et al., 2013; Belyi et al., 2006; 2008; Shen et al., 2009). Furthermore, translational initiation is inhibited during infection due to diminished mTOR signaling (Ivanov and Roy, 2013). These activities result in a greater than 90% block in host translation in infected host cells (Fontana et al., 2011; McCusker et al., 1991). Nevertheless, infection by *L. pneumophila* leads to the robust production of pro-
inflammatory cytokines, such as TNF, IL-6, IL-1α, IL-1β, and IL-12 (Barry et al., 2013; Casson et al., 2013; LeibundGut-Landmann et al., 2011; Shin et al., 2008; Spörri et al., 2006). Moreover, the presence of the T4SS paradoxically enhances levels of cytokine production in response to *L. pneumophila*, suggesting that much of the cytokine response against *L. pneumophila* is mediated by cytosolic immune sensing of bacterial ligands and virulence activities (Fontana et al., 2011; 2012; Krachler et al., 2011; Roy et al., 1998; Shin et al., 2008; Spörri et al., 2008).

It is unclear, then, how the host is able to mount a pro-inflammatory cytokine response when *L. pneumophila* infection potently blocks host protein synthesis. At the population level, decreased host protein synthesis leads to preferential translation of the most abundant cytokine transcripts (Ivanov and Roy, 2013). At the single cell level, directly infected cells overcome the translational block and selectively synthesize IL-1α and IL-1β through a mechanism involving MyD88-dependent transcriptional expression of IL-1α and IL-1β (Asrat et al., 2014). However, whether mechanisms that enable selective translation of IL-1 also apply more broadly to other key cytokines and immune effector proteins is unclear. Alternatively, as uninfected bystander cells are present both *in vitro* and *in vivo* during infection, we considered the possibility that these bystander cells might somehow respond to infection and produce cytokines instead (Copenhaver et al., 2014). Here, by tracking immune responses in *L. pneumophila*-infected cells at the single cell level, we demonstrate that although infected cells that have received T4SS effectors are able to synthesize IL-1α and IL-1β, they are poor producers of other key cytokines. Instead, bystander cells that have not received T4SS effectors are the primary producers of TNF, IL-6, IL-12, and the costimulatory molecule CD86 during both *in vitro* and *in vivo* infection. Importantly, loss of IL-1R signaling leads to reduced bystander cytokine production and increased bacterial burden *in vivo*, suggesting that the IL-1
released from infected cells mediates production of pro-inflammatory cytokines by bystander cells. Overall, our data suggest that release of IL-1 by infected cells signals the presence of virulent infection, enabling the host to generate a robust innate immune response despite a pathogen-imposed translational block.

Results

**Cells that have not received bacterial effectors are the primary producers of pro-inflammatory cytokines during *in vitro* infection**

Infection with virulent *L. pneumophila* expressing a type IV secretion system (T4SS) leads to an enhanced cytokine response despite bacterial inhibition of host translation. How this enhanced T4SS-dependent cytokine response is generated remains unclear. We considered the possibility that directly infected macrophages possess cell-intrinsic mechanisms for overcoming the translational block to selectively synthesize cytokines. Alternatively, cytokines may be produced by bystander cells that are uninfected or have taken up bacteria that failed to translocate effector proteins (Copenhaver et al., 2014). To determine whether T4SS-injected cells or uninfected bystander cells produce cytokines during infection, we utilized a fluorescence-based system that detects a translocated *L. pneumophila* effector protein (RalF) fused to β-lactamase (BlaM) (Copenhaver et al., 2014; Knapp et al., 2003). In the absence of BlaM activity, 409nm excitation of the cell-permeable BlaM fluorescent substrate CCF4-AM results emission of green fluorescence at 518 nm. However, T4SS-translocated BlaM-RalF results in cleavage of CCF4-AM and a shift in emission to blue fluorescence at 447 nm. This system enables robust discrimination of productively infected and uninfected cells within tissues *in vivo* or in cultured cells *in vitro* (Copenhaver et al., 2014).
We infected bone marrow-derived macrophages with *L. pneumophila* encoding the BlaM-RalF reporter. As flagellin delivered by the T4SS into the host cell cytosol induces rapid cell death via NAIP5 detection and inflammasome activation, we infected macrophages with the virulent ΔflaA strain lacking flagellin (Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). Following infection, macrophages were loaded with CCF4-AM and intracellularly stained for various cytokines at multiple time points post-infection. (Figure 4-1 & 4-2). Macrophages infected with avirulent *L. pneumophila* lacking the Dot/Icm T4SS (ΔdotA) demonstrated no BlaM-RalF activity as expected. In the virulent ΔflaA infection, we found that TNF is rapidly produced in response to infection, but that T4SS-injected cells were poor producers of TNF at all time points assayed (Figure 4-1A). Instead, the majority of cells robustly producing TNF were cells that had not received T4SS effectors. Likewise, T4SS-injected cells poorly produced IL-6. Instead, IL-6 was primarily produced by uninjected cells (Figure 4-1B). Similar findings were obtained for IL-12 and CD86 (Figure 4-2). In contrast, IL-1α and IL-1β were robustly produced by both T4SS-injected cells and uninjected cells (Figure 4-1C &D), indicating that T4SS-injected cells produce IL-1α and IL-1β despite the translational block imposed by *L. pneumophila* effector proteins, consistent with recent findings (Asrat et al., 2014).

To determine whether the decreased cytokine production in T4SS-injected cells is due to decreased translation or transcription, we performed qRT-PCR on sorted T4SS-injected and uninjected cells (Figure 4-1E). Both injected and uninjected cells displayed marked increases in transcript levels for the cytokines IL-1α, IL-1β, TNF, and IL-6 relative to uninfected cells, with injected cells consistently exhibiting a greater increase in cytokine transcript levels, consistent with recent findings (Asrat et al., 2014). Thus, the decreased production of immune proteins by injected cells is not due to a lack of transcriptional
activation and most likely is a direct consequence of the translational block. Taken together, these data suggest that although productively infected cells can bypass the translational block to produce IL-1α and IL-1β, they poorly translate TNF, IL-6, IL-12, and CD86. Instead, bystander cells preferentially produce these proteins.

There are currently seven identified effectors in virulent L. pneumophila that inhibit protein translation in host cells (Barry et al., 2013; Belyi et al., 2013). To determine if these effectors prevent cytokine production in injected cells, we infected BMDMs with strains of L. pneumophila that lack either five of the known effectors (Lgt1, Lgt2, Lgt3, SidI, SidL) (Δ5) or all seven (Δ5 and Pkn5 and Lpg1489) (Δ7ΔflaA) of the known effectors. At four hours PI, cells injected by the Δ5 or Δ7ΔflaA strains produced more TNF than cells injected by their parental WT counterparts (Figure 4-3A &D). This increase occurred both in the frequency of TNF-producing injected cells as well as the gMFI of injected cells producing TNF. At 16 hours PI, however, there was no difference in the frequency of TNF-producing injected cells in either the Δ5 or Δ7ΔflaA strains and gMFI was only different in the Δ7ΔflaA strain compared to the parent strain (Figure 4-3B &E). IL-6 production by injected cells was also unaffected by the presence or absence of these effectors (Figure 4-3C &F). These data indicate that T4SS effectors that block protein translation effect early, but not later cytokine production in infected cells.

**Bystander alveolar macrophages and neutrophils are predominant producers of TNF during L. pneumophila infection**

Our *in vitro* findings indicate that many key protective cytokines are primarily generated by bystander macrophages during infection by pathogens that interfere with cell intrinsic immune defense. *In vivo*, multiple cell populations exist, and distinct immune populations may possess alternative mechanisms for overcoming pathogen-induced translational
blockade. We therefore intranasally infected WT mice with \textit{L. pneumophila} strains expressing BlaM-RalF and examined TNF production in airway-resident alveolar macrophages and recruited neutrophils, as they are the primary cell types that receive \textit{L. pneumophila} T4SS effectors (Copenhaver et al., 2014). Following infection with \textit{ΔflaA} \textit{L. pneumophila}, we observed a significant increase in both the percentage and total numbers of TNF-producing airway alveolar macrophages and neutrophils (Figure 4-4), consistent with previous findings showing that cytosolic immune sensing of T4SS activity is required for maximal TNF production (Shin et al., 2008; Spörri et al., 2006). We observed that T4SS-injected alveolar macrophages and neutrophils did not produce TNF. Instead, TNF was produced almost exclusively by uninjected alveolar macrophages and neutrophils (Figure 4-4). These data, along with our data in macrophages \textit{in vitro}, indicate that infected cells are poor producers of proinflammatory cytokines and that uninfected cells are the major TNF-producers.

In contrast, both injected and uninjected alveolar macrophages produced IL-1\(\alpha\) and IL-1\(\beta\), in agreement with our \textit{in vitro} findings (Figure 4-5). PBS vehicle control and avirulent \textit{ΔdotA} infection yielded no significant increase in TNF-positive alveolar macrophages or neutrophils (Figure 4-4), corroborating with previous findings that TNF is undetectable in the bronchoalveolar lavage fluid or serum isolated from similarly treated mice (Shin et al., 2008; Spörri et al., 2006). Consistent results were also obtained from alveolar macrophages and neutrophils isolated from lung tissue (Figure 4-6). These data indicate that although cytosolic immune sensing of T4SS-translocated bacterial products is critical to elicit TNF production, TNF is produced by uninjected bystander alveolar macrophages and neutrophils that have not received T4SS effectors.
Bystander inflammatory monocytes and dendritic cells produce TNF and CD86 during *L. pneumophila* infection

Inflammatory monocytes and conventional dendritic cells are also recruited to the lung during *L. pneumophila* infection. In contrast to alveolar macrophages and neutrophils, these cell populations are not productively infected by *L. pneumophila* and do not receive T4SS effectors (Copenhaver et al., 2014; LeibundGut-Landmann et al., 2011). Even so, inflammatory monocytes and dendritic cells produced significant amounts of TNF and were the primary cell types in the lung responsible for producing TNF during \(\Delta\)flaA *L. pneumophila* infection (Figures 4-7A & B). In contrast, inflammatory monocytes and dendritic cells from \(\Delta\)dotA-infected mice did not exhibit an increase in TNF production compared to cells from PBS-infected mice. Inflammatory monocytes and dendritic cells also substantially increased expression of CD86 during infection with virulent \(\Delta\)flaA *L. pneumophila* compared to PBS or \(\Delta\)dotA infection, and were the primary cell types expressing CD86 (Figure 4-7C & D). Alveolar macrophages and neutrophils did not increase expression of CD86 during \(\Delta\)flaA infection (Figure 4-8). These data indicate that although inflammatory monocytes and dendritic cells are not productively infected and do not receive T4SS-translocated products, they are the primary cell types that express TNF and CD86 during virulent *L. pneumophila* infection as measure by intracellular cytokine staining.

**IL-1 signaling is critical for activating bystander immune cells to produce cytokines and express CD86**

Maximal immune responses to *L. pneumophila* require cytosolic sensing of T4SS activity (Shin et al., 2008), yet our data indicate that the majority of cytokine- and CD86-producing cells are bystander innate immune cells that have not received T4SS effectors. Given that T4SS-injected cells are capable of producing IL-1\(\alpha\) and IL-1\(\beta\), we
considered the possibility that IL-1α and IL-1β released by infected cells might play a role in instructing bystander cells to produce other key inflammatory cytokines. Notably, IL-1α and IL-1β can elicit expression of other cytokines (Dinarello, 1996; Kohase et al., 1987), and IL-1R signaling is critical for innate immune control of L. pneumophila infection (Barry et al., 2013; Casson et al., 2013; LeibundGut-Landmann et al., 2011; Mascarenhas et al., 2015). The crucial role of IL-1 in early host defense has been primarily attributed to IL-1-dependent expression of neutrophil-attracting chemokines by epithelial cells and subsequent neutrophil recruitment to the site of infection (Barry et al., 2013; Casson et al., 2013; LeibundGut-Landmann et al., 2011; Mascarenhas et al., 2015). We hypothesized that IL-1R signaling would also be critical for instructing bystander innate immune cells to produce cytokines. We therefore intranasally infected WT or Il1r1−/− (IL-1R−/−) mice with ΔflaA L. pneumophila and assayed TNF production 24 hours later. Critically, IL-1R−/− mice exhibited significant reductions in both the percentages and total numbers of TNF-producing alveolar macrophages, neutrophils, dendritic cells and inflammatory monocytes compared to WT mice, in which large numbers of the aforementioned cells produced TNF (Figure 4-9 & 4-10). Although a percentage of IL-1R−/− dendritic cells and inflammatory monocytes remained positive for TNF, it was only minimally higher than the basal percentage of TNF-positive cells observed in WT or IL-1R−/− mice infected with avirulent ΔdotA L. pneumophila (Figure 4-11). Consistent with the decreased numbers of TNF-producing cells in IL-1R−/− mice, TNF levels in the bronchoalveolar lavage of IL-1R−/− mice were significantly reduced compared to WT mice (Figure 4-11E). IL-12p40 levels in IL-1R−/− mice were also significantly reduced (Figure 4-9E). Furthermore, IL-1R−/− mice also exhibited a significant decrease in the percentages and total numbers of CD86-expressing inflammatory monocytes and dendritic cells compared to WT controls (Figure 4-10). In contrast, IL-6 levels were unaffected, indicating that IL-1 signaling is not required for production of all responses by
bystander cells during *L. pneumophila* infection (Figure 4-9E). Finally, injection of neutralizing antibodies against IL-1α and IL-1β resulted in a significant decrease in the levels of TNF and IL-12p40, but not IL-6, with neutralization of IL-1α and IL-1β together having the greatest effect (Figure 4-12). Overall, these data indicate a crucial role for IL-1 signaling in directing the optimal production of the pro-inflammatory cytokines TNF and IL-12p40 as well as the costimulatory molecule CD86 by bystander innate immune cells during pulmonary *L. pneumophila* infection.

BMDMs are reported to not express the IL-1R and should therefore not respond to IL-1 stimulation *in vitro* (Demuth et al., 1996). To see, however, whether IL-1R signaling occurs during *L. pneumophila* infection *in vitro*, we infected WT or IL-1R<sup>−/−</sup> BMDMs with *L. pneumophila* and assayed for cytokine production by flow as well as be ELISA (Figure 4-13A &B). An equal frequency of IL-1R<sup>−/−</sup> bystander BMDMs produced TNF in response to *L. pneumophila* as compared to WT cells (Figure 4-13A) and IL-6 production in response to infection was also equivalent between the two genotypes of BMDMs (Figure 4-13B). Indeed, treating BMDMs with varying concentrations of recombinant IL-1α, IL-1β, or both combined did not elicit TNF or IL-6 production from these cells (Figure 4-13C). Treatment of NIH/3T3 cells, an epithelial cell line known to express the IL-1R, elicited IL-6 production, demonstrating that the recombinant IL-1 is competent to induce signaling (Figure 4-13D). These data, along with the decreased, but present cytokine response to *L. pneumophila* infection from IL-1R<sup>−/−</sup> mice *in vivo*, suggest that additional signals may drive bystander cytokine responses during infection.

To determine if IL-1 signaling is sufficient to drive TNF production on other innate cell types, we treated alveolar macrophages, bone marrow-derived dendritic cells (BMDCs) or inflammatory monocytes isolated from mouse bone marrow and treated with IL-1α, IL-
1β, or both in combination. A small, but consistent frequency of BMDCs stained for TNF production in response to IL-1 treatment (Figure 4-14A). These BMDCs also increased expression of CD86 in response to IL-1 treatment (Figures 4-14B). BMDCs also produced a low level of TNF and IL-6 in response to IL-1 treatment as measured by ELISA (Figure 4-14D). Alveolar macrophages also produced TNF in response to IL-1 treatment (Figure 4-15A). Importantly, this response was dependent on expression of the IL-1R, as alveolar macrophages from IL-1R−/− mice did not produce TNF when treated with IL-1 (Figure 4-15B). Inflammatory monocytes were unresponsive to IL-1 treatment as measured by TNF production and CD86 expression (Figure 4-16). We were also unable to see increased CD86 expression in response to LPS on inflammatory monocytes, suggesting that inflammatory monocytes may not express CD86 ex vivo (Figure 4-16A). Together, these data indicate that some innate cell populations, such as dendritic cells and alveolar macrophages, may directly respond to IL-1 signaling during *L. pneumophila* infection in a cell-intrinsic manner. Other cells types, however, do not respond to IL-1 and may require either additional signals to produce cytokines or require an intermediate cell type to respond to IL-1.

**Conclusion**

Cells targeted by the T4SS of *L. pneumophila* were poor producers of the cytokines TNF, IL-6, IL-12p40, and fail to increase expression of CD86 upon infection. Instead, uninjected, bystander cells produced the majority of these cytokines *in vitro and in vivo*. Injected cells were still able to produce IL-1α and IL-1β during infection and IL-1 signaling was important *in vivo* for the production of cytokines by bystander cells including uninjected alveolar macrophages, neutrophils, inflammatory monocytes, and dendritic cells. The 7 currently known T4SS effectors that block protein translation were important for preventing TNF production in injected cells early, but not later during
infection in vitro. Deleting these effectors did not affect the production of IL-6 by injected cells, suggesting that other effectors or a host-driven response prevent IL-6 and late TNF production by injected cells. BMDMs did not respond to IL-1 stimulation in vitro and do not require IL-1R signaling to produce bystander cytokines. IL-1R−/− mice in vivo produced less TNF and IL-12 during L. pneumophila infection, but still produced a small amount of cytokine. Together, these data suggest that other mechanisms besides IL-1 signaling drive bystander cytokine production. Both alveolar macrophages and BMDCs were able to produce TNF in response to IL-1 stimulation and BMDCs also increased expression of CD86. Inflammatory monocytes, however, did not respond to IL-1. Thus, IL-1 signaling is sufficient in some, but not all cell types shown to produce bystander cytokines in vivo. Together, these data detail a mechanism by which cells infected with a pathogen that blocks host protein synthesis are able to communicate to uninfected cells to initiate a protective immune response.

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Figure 4-1. *T4SS-injected and uninjected macrophages produce different cytokines* in vitro. (A-D) Bone marrow-derived macrophages (BMDMs) were infected with Lp02 strains of *L. pneumophila* for 4, 8, 12, or 16 hours. BMDMs were treated with brefeldin A and monensin starting at 3 hours prior to harvest. Cells were then loaded with CCF4-AM, fixed, and then stained with antibodies against TNF (A), IL-6 (B), IL-1α (C), and IL-1β (D). Plots show cytokine production at 16 hours post infection (PI). Line graphs show ΔflaA-infected macrophages where total frequency of cytokine producing cells as well as the contribution of injected and uninjected cells to cytokines are shown. N=3 wells per condition per timepoint. Graphs show mean ± SEM. Representative of 3 independent experiments. (E) BMDMs were infected with Lp02 ΔflaA *L. pneumophila* for 8 hours. Cells were then loaded with CCF4-AM and sorted based upon cleavage of the dye, or were collected after going through the sorter without separating the cells based on dye cleavage (bulk). Sorted cells were then lysed and relative abundance of transcripts for proinflammatory cytokines were assayed using RT-PCR. Bar graphs show mean relative abundance of 2 separate wells. Statistics represent the results of Tukey’s post test between ΔflaA uninjected and ΔflaA injected columns. Representative of 3 independent experiments. ***p<0.0005
Figure 4-2. *IL-12p40* and *CD86* are expressed by bystander cells during infection. (A) Bone marrow-derived macrophages (BMDMs) were infected with Lp02 strains of *L. pneumophila* at an MOI = 5 for 4, 8, 12, or 16 hours. BMDMs were treated with brefeldin A and monensin starting at 3 hours prior to harvest. Cells were then loaded with CCF4-AM, fixed, and then stained with antibodies against *IL-12p40*. Plots show cytokine production at 16 hours post infection (PI). Line graph shows Δ*flaA*-infected macrophages where total = the total frequency of cells positive for the given cytokine. Injected and uninjected = the relative contribution of each subset of cells to the total frequency of cytokine positive cells. N=3 wells per condition per timepoint. Line graphs show mean ± SEM. (B) Cells infected as in (A) were harvested 8 hours PI, loaded with CCF4-AM and stained for *CD86*. Bar graph shows mean ± SEM.
Figure 4-3. Type IV secretion system effectors that block protein translation inhibit early TNF production in injected cells. BMDMs were treated with LPS or infected with Lp02 WT, ΔdotA, ΔflaA, Δ5, or Δ7ΔflaA strains of L. pneumophila at an MOI = 5, infected for the indicated amount of time, loaded with CCF4-AM, and stained for ICS. (A & B) Representative flow plots showing TNF production 4 (A) or 16 (B) hours PI. The frequency of TNF-producing cells and the geometric MFI (gMFI) of TNF-producing injected cells are quantified in (D) for 4 hours and (E) for 16 hours. (C) Plots showing IL-6 production at 16 hours PI. The frequency of IL-6-producing injected cells is quantified in (F). Representative of 2 independent experiments. Graphs show individual wells and mean. ANOVA was performed and statistics show significance between Δ5 and WT or Δ7ΔflaA and ΔflaA as determined by Tukey post test. *p<0.05, **p<0.005, ***p<0.0005, ns=not significant.
Figure 4-4. **Bystander alveolar macrophages and neutrophils produce TNF during L. pneumophila infection.** Mice were infected and cells were harvested from the bronchoalveolar lavage and lung tissue, treated with BFA, loaded with CCF4-AM, stained, fixed and stained with an antibody against TNF. (A-D) Representative plots from alveolar macrophages (A) and neutrophils (C) collected from the BAL 24 hours PI and quantified in (B & D). Graphs show individual mice and mean from 3 pooled independent experiments. Graphs on far right show the relative contribution of uninjected and injected cells to TNF production from ΔflaA-infected mice. N=2-3 mice per group per experiment. Student’s T-tests were performed for total TNF producing cells between ΔdotA and ΔflaA infected mice. **p<0.005, ***p<0.0005.
Figure 4-5. **T4SS-injected alveolar macrophages produce IL-1 during pulmonary infection.** Mice were infected with $5 \times 10^6$ CFU of JR32 $\Delta$dotA or $\Delta$flaA L. pneumophila intranasally or given PBS. 24 hours PI, cells were harvested from the lung tissue, treated with BFA, loaded with CCF4-AM, stained, fixed and stained with an antibody against IL-1α (**A**) and IL-1β (**B**). Representative plots are shown. N=2-3 mice per group. Representative of 2 independent experiments.
Figure 4-6. Bystander alveolar macrophages and neutrophils from the lung produce TNF during infection. Alveolar macrophages (A) and neutrophils (C) were harvested from the lung tissue of infected mice and stained for TNF production. Representative flow plots are shown. Graphs show individual mice and mean from 3 pooled independent experiments. (B & D) Graphs on far right show the relative contribution of uninjected and injected cells to TNF production from ΔflaA-infected mice. N=2-3 mice per group per experiment. Student’s T-tests were performed for total TNF producing cells between ΔdotA and ΔflaA infected mice. ns=not significant, **p<0.005, ***p<0.0005.
Figure 4-7. Bystander inflammatory monocytes and conventional dendritic cells produce TNF and express CD86 during infection. Cells were harvested from the lung tissue of mice, treated with BFA, loaded with CCF4-AM, stained, fixed and stained with an antibody against TNF (A & B) or CD86 (C & D). Representative plots are shown. Graphs show individual mice and mean from 3 pooled independent experiments. Student’s T-test were performed between ΔdotA and ΔflaA infected mice. **p<0.005, ***p<0.0005.
Figure 4-8. *Alveolar macrophages and neutrophils do not express CD86 during infection.* 24 hours PI, cells were harvested from the lung tissue, treated with BFA, loaded with CCF4-AM, stained with an antibody against CD86, and fixed. Representative plots for alveolar macrophages (A) and neutrophils (B) are shown. N=2-3 mice per group. Representative of 3 independent experiments.
Figure 4-9. **IL-1 signaling induces cytokine production by bystander cells.** Cells were harvested from the lungs of infected WT or IL-1R-/- (IL-1R^-/-) mice, treated with BFA, loaded with CCF4-AM, stained, fixed and then stained with an antibody against TNF. Representative plots from alveolar macrophages (A & B) and conventional dendritic cells (C & D) are shown. Graphs show individual mice and mean pooled from 4 independent experiments. (E) BAL fluid was collected from mice and cytokine levels were measured by ELISA. Graphs show individual mice and mean pooled from 3 independent experiments. N=2-4 mice per group. Student’s T-test were performed between pooled groups. ns=not significant, *p<0.05, **p<0.005, ***p<0.0005.
Figure 4-10. *IL-1 signaling leads to the expression of CD86 and TNF by bystander cells.* Cells were harvested from the lungs of infected WT or *Il1r1* -/- (IL-1R-/-) mice, treated with BFA, loaded with CCF4-AM, stained with an antibody against TNF (A & B) or CD86 (C & D). Representative flow plots from various cell populations are shown. Graphs show individual mice and mean pooled from 4 independent experiments. N=2-4 mice per group. Student’s T-test were performed between pooled groups. *p<0.05, **p<0.005, ***p<0.0005.
Figure 4-11. Lack of IL-1 signaling does not alter the production of TNF by avirulent \( L. \) pneumophila infection. Cells were harvested from the lungs of infected WT or \( Il1r1^{--} \) (IL-1R) mice, treated with BFA, loaded with CCF4-AM, stained, fixed and stained with an antibody against TNF. Representative flow plots showing TNF production from inflammatory monocytes (A) or conventional dendritic cells (B) from the lung. Bar graphs on right show individual mice and pooled mean from 3 independent experiments.
Figure 4-12. Both IL-1α and IL-1β contribute to cytokine production during L. pneumophila infection. Mice were then infected with JR32 ΔflaA L. pneumophila intranasally. 24 hours PI, BAL and lungs were harvested and CFUs were plated for viable L. pneumophila from lung homogenates. Graphs show individual mice and mean. Statistics are one-way ANOVA with the stats representing the significance of the Tukey post-test between isotype mice and the groups of interest. ns=not significant, **p<0.05, ***p<0.005, ****p<0.0005.
Bone marrow-derived macrophages are unresponsive to IL-1 treatment. (A) BMDMs from WT or IL-1R−/− mice were left untreated or infected with the Lp02 ΔflaA strain of L. pneumophila at an MOI = 5, infected for 4 hours, loaded with CCF4-AM, and stained for TNF. (B) BMDMs from WT or IL-1R−/− mice were infected with the Lp02 ΔdotA or ΔflaA strain of L. pneumophila at an MOI = 5 and IL-6 production was measured by ELISA 24 hours PI. (C) BMDMs were treated with 100-100,000 pg/mL of recombinant IL-1α, IL-1β, or IL-1α and IL-1β combined, or 100ng/mL LPS for 24 hours. TNF and IL-6 were then measured by ELISA. (D) NIH/3T3 cells were treated with 10-100ng/mL of recombinant IL-1α, IL-1β, or IL-1α and IL-1β combined, or 100ng/mL LPS for 24 hours and IL-6 was measured by ELISA. Graphs show mean ± S.E.M. Representative of two independent experiments.
Figure 4-14. Bone marrow-derived dendritic cells produce cytokines and express CD86 in response to IL-1 treatment. (A & B) BMDCs were treated with LPS, PAM3CSK4, or 100ng/mL IL-1α, IL-1β, or both for 6 hours. 3 hours prior to harvest, cells were treated with BFA. Cells were then fixed and stained for CD86 (A) or TNF (B). Representative flow plots are shown. (C) Quantification of plots shown in (A & B). (D) BMDCs were treated as in (A & B) for 24 hours. Supernatants were then harvested and TNF and IL-6 production were measured by ELISA. Graphs show individual wells and mean. ANOVA was performed and statistics show significance between untreated cells and the appropriate condition as determined by Tukey post test. *p<0.05, **p<0.005, ***p<0.0005, ns=not significant.
Figure 4-15. **Alveolar macrophages produce TNF in response to IL-1 treatment.** (A & B) Alveolar macrophages from WT (A) or IL-1R\(^{-/-}\) (B) mice were isolated and seeded in culture at 10,000 cells per well in 150µl DMEM + 10% FBS. Cells were then treated with 100ng/mL LPS, IL-1\(\alpha\), IL-1\(\beta\), or both for 6 hours. 3 hours prior to harvest, BFA was added to the wells. Cells were then fixed and then stained for TNF. (C) Quantification of (A & B). Graphs show mean of two independent mice.
Inflammatory monocytes do not respond to IL-1 treatment. (A & B) Inflammatory monocytes were isolated from the bone marrow of WT (A) or IL-1R⁻/⁻ (B) mice. Cells were then treated with 100ng/mL LPS, IL-1α, IL-1β, or both for 6 hours. 3 hours prior to harvest, BFA was added to the wells. Cells were then fixed and then stained for TNF and CD86. (C) Quantification of (A & B). (D) Gating strategy for isolated inflammatory monocytes. Monocytes are Ly6C<hi>, CD11b<int> to CD11b<hi> and do not express Ly6G. Monocytes were 90-95% pure. Graphs show mean of two independent mice. Representative of two independent experiments.
A. Alveolar macrophages and neutrophils are targeted by the type IV secretion system of \textit{L. pneumophila}

\textit{Legionella pneumophila} uses its T4SS to inject a large number of effector proteins into the cytosol of host phagocytes (Chen et al., 2010). The T4SS is necessary for intracellular replication and pathogenesis as \textit{L. pneumophila} mutants lacking a functional T4SS fail to establish a replicative niche and do not cause pathology in mice (Barry et al., 2013; LeibundGut-Landmann et al., 2011; Marra et al., 1992; Roy et al., 1998). In addition to being required for \textit{L. pneumophila} pathogenesis, T4SS activity potently activates multiple cytosolic immunosurveillance pathways (Casson and Shin, 2013; Losick and Isberg, 2006; Moltke et al., 2013; Shin et al., 2008). Thus, cells that interact with \textit{L. pneumophila} and receive T4SS-translocated effectors serve as a potential replicative niche, but may also contribute to the immune response against \textit{L. pneumophila}. However, the precise identity of such cells is unknown. I, therefore, set out to identify host cells that receive T4SS-translocated effectors during infection with \textit{L. pneumophila}. BlaM reporter systems have been used during \textit{in vivo} infection with \textit{Yersinia pseudotuberculosis} (Harmon et al., 2010; Maldonado-Arocho et al., 2013), \textit{Yersinia pestis} (Pan et al., 2009; Pechous et al., 2013), \textit{Yersinia enterocolitica} (Köberle et al., 2009), \textit{Salmonella typhimurium} (Geddes et al., 2007; Yoon et al., 2011), and \textit{Pseudomonas aeruginosa} (Diaz and Hauser, 2010; Kung et al., 2012) to detect the translocation of effectors into host cells by the type III and type IV secretion systems. In chapter 3, I demonstrate that by using β-lactamase (BlaM) translationally fused to the T4SS-translocated effector protein RalF, I can successfully track injection by the T4SS
into host cells during both \textit{in vitro} and \textit{in vivo} infection, and I describe the first use of this BlaM reporter during \textit{in vivo} pulmonary infection with \textit{L. pneumophila}.

I observed robust T4SS-mediated injection into alveolar macrophages at four hours post infection, consistent with previous observations that these cells are the primary cell type infected by \textit{L. pneumophila} during pulmonary infection in human patients (Nash et al., 1984). At later time points post infection, I find that in addition to alveolar macrophages, a large number of the cells injected by \textit{L. pneumophila} \textit{in vivo} are neutrophils (Figure 5-1). This is likely due to the influx of neutrophils into the lungs and airway space during infection (Ang et al., 2012; Berrington et al., 2010; Brieland et al., 1994; Frutuoso et al., 2010; Winn and Myerowitz, 1981; Winn et al., 1978). Other researchers have shown that neutrophils contain intracellular \textit{L. pneumophila} in a non-permissive mouse model of pulmonary infection, but it was not examined whether \textit{L. pneumophila} could survive within neutrophils or whether neutrophils are capable of receiving T4SS-translocated effectors (LeibundGut-Landmann et al., 2011). \textit{In vitro} studies have suggested that \textit{Legionella} species are resistant to the highly bactericidal activity of neutrophils but cannot replicate within these cells (Horwitz and Silverstein, 1981; Weinbaum et al., 1983). Thus, I initially presumed that although neutrophils might be injected during \textit{in vivo} infection, the majority of bacteria would eventually be cleared due to a failure to replicate in these cells, and I would not be able to detect any appreciable number of viable bacteria within these cells. I obtained viable \textit{L. pneumophila} in numbers that roughly corresponded with the frequency of injection seen with our reporter system, suggesting that \textit{L. pneumophila} can survive within neutrophils during \textit{in vivo} infection. Unexpectedly, given the large number of neutrophils that enter the lung, the total number of \textit{L. pneumophila} CFUs harbored by neutrophils is greater than the total number of \textit{L. pneumophila} CFUs found within the alveolar macrophage population 24 and 48 hours
Given the large numbers of infected neutrophils that I observed, it would be of interest to determine whether \textit{L. pneumophila} could establish an ER-derived vacuole and successfully replicate within neutrophils, as this could represent another intracellular niche for \textit{L. pneumophila}. Most bacteria are not thought to survive or replicate within neutrophils, but there are a few exceptions, including \textit{Neisseria gonorrhoeae} (Simons et al., 2005), \textit{Anaplasma phagocytophilum} (Chen et al., 1994; Ohashi et al., 2002), and pathogenic \textit{Escherichia coli} (Nazareth et al., 2007).

\textbf{B. Non-phagocytic cells are not targeted by \textit{L. pneumophila} for injection}

As I was only able to detect robust T4SS-dependent injection into alveolar macrophages and neutrophils, I conclude that phagocytic cells in the airway space are the primary recipients of T4SS-translocated effectors during pulmonary \textit{L. pneumophila} infection. Whether or not T4SS-injected cells survive infection and traffic to other organs including lymph nodes is unknown. However, previous studies have reported that alveolar macrophages do traffic to lymph nodes when given allergic stimuli (Kirby et al., 2009). Thus, it would be of interest to investigate whether T4SS-injected alveolar macrophages either induce adaptive immunity or participate in the dissemination of infection to other organs (Kirby et al., 2009). Surprisingly, I was unable to detect T4SS-injected conventional dendritic cells during \textit{in vivo} infection. Dendritic cells undergo rapid apoptosis in response to \textit{L. pneumophila} T4SS activity (Nogueira et al., 2009), which could account for why I do not detect as robust injection in dendritic cells as compared to macrophages \textit{in vitro} and \textit{in vivo}, although the number of DCs increased during infection. Further experiments to determine whether injection could be detected in DCs lacking apoptotic regulators such as BAX and BAK, thus, resistant to \textit{L. pneumophila}-induced apoptosis, could be fruitful. However, C57BL/6-derived macrophages undergo rapid pyroptosis in response to WT \textit{L. pneumophila} infection, yet I still detect injection in
this cell type, suggesting that *L. pneumophila*-induced cell death is an insufficient explanation to account for the lack of detectable injection in DCs (Case et al., 2009). Given that phagocytosis is required for T4SS-mediated translocation, another possibility is that dendritic cells in the lung may not efficiently phagocytose *L. pneumophila* (Charpentier et al., 2009). Alternatively, pulmonary dendritic cells and *L. pneumophila* may be spatially separated during *in vivo* infection. Infection may occur primarily in the airway space itself, and thus only neutrophils and alveolar macrophages are available targets for translocation. To what extent infection occurs in the lung tissue itself has not been fully investigated. Histological studies would help elucidate the temporal and spatial constraints present during *L. pneumophila* infection.

Although I could only detect T4SS-mediated translocation in pulmonary phagocytic cells, other infectious models using different pathogens have observed translocation into a larger variety of cells. For instance, studies using an intraperitoneal model of *Yersinia pseudotuberculosis* demonstrate translocation mediated by the type three secretion system into splenic phagocytes as well as splenic B and T cells (Köberle et al., 2009). In an infectious model using the parasite *Toxoplasma gondii* that does not encode a secretion system but does inject proteins into infected cells, injects phagocytic cells, non-immune cells, and even injects neurons (Koshy et al., 2012). Interestingly, in the *T. gondii* model, injection is detected in cells that do not harbor intracellular parasites. I did not assay whether *L. pneumophila* also injects cells it does not invade, but the data obtained via imaging flow cytometry suggest that nearly all injected cells contain *L. pneumophila* and it has not been demonstrated that *L. pneumophila* can egress from an infected host cells without subsequent death of the cell.
The experiments performed in this study looked at cells targeted by the T4SS of *L. pneumophila* in immunocompetent hosts. Infections of immunocompromised mice, such as mice lacking IFNγ and MyD88, result in higher bacterial burdens, bacterial dissemination to peripheral organs, and sometimes death of the host organism (Archer et al., 2009; Spörri et al., 2006). Higher bacterial burdens would require increased bacterial burdens in alveolar macrophages and neutrophils either in the frequency of infected cells, or an increase in the number of viable bacteria in a single cell. However, MyD88-deficient mice fail to mount an appropriate immune response and have delayed and reduced recruitment of cells such as neutrophils to the site of infection (Archer and Roy, 2006). Whether other cell types, such as epithelial or dendritic cells, harbor viable *L. pneumophila* in immunocompromised hosts remains to be seen. For dissemination, other cell types, such as tissue resident macrophages in the spleen and liver, may harbor viable *L. pneumophila*; however, I did not look at a model of dissemination. It will be important to investigate targeting of the T4SS in these models as they mimic the increased bacterial burdens and dissemination seen in immunocompromised human patients, which would lead to a better understanding of clinical disease (Lowry and Tompkins, 1993).

Using the A549 alveolar epithelial cell line during *in vitro* infection, I detected a low percentage of T4SS-injected cells under conditions using both nonmotile and motile bacteria. Although the *L. pneumophila* were centrifuged onto A549 cells *in vitro*, motility may alter the contact between host cells and *L. pneumophila* in a way that facilitates bacterial entry. Many researchers utilize A549 cells as a model for *L. pneumophila* infection and can detect productive bacterial replication within these cells (Maruta et al., 1998). However, these studies either use higher MOIs than those used in this study or opsonize the bacteria prior to infection. These discrepancies in technique may explain
why I am unable to detect higher percentages of injected alveolar epithelial cells during \textit{in vitro} infection. I was also unable to detect robust T4SS-dependent translocation into airway epithelial cells or other non-phagocytic cells during \textit{in vivo} infection. Utilizing a non-permissive model of C57BL/6 mice infected with WT \textit{L. pneumophila}, other researchers found that lung epithelial cells did not appear to contain \textit{L. pneumophila} (LeibundGut-Landmann et al., 2011). Our data argue against a direct role for airway epithelial cells in cytosolic sensing of \textit{L. pneumophila} T4SS activity during pulmonary infection. Airway epithelial cells have been shown to indirectly respond to \textit{L. pneumophila} infection by producing the chemokine CXCL1 in response to IL-1 produced by macrophages (LeibundGut-Landmann et al., 2011).

\textbf{C. Alveolar macrophages and neutrophils produce cytokines in response to virulent \textit{L. pneumophila} infection}

I found that alveolar macrophages secreted TNF and IL-1α four hours PI \textit{in vivo}. Both TNF and IL-1α are important for controlling \textit{L. pneumophila} infection. The inflammasome-regulated cytokines IL-1α and IL-1β are critical for neutrophil recruitment to the lung airway during \textit{L. pneumophila} infection through a mechanism involving the IL-1R-dependent induction of CXCL1 from alveolar epithelial cells (Barry et al., 2013; Casson et al., 2013; LeibundGut-Landmann et al., 2011). It is unclear whether other cells in the lung produce cytokines so early during infection. However, as IL-1α production \textit{in vivo} is T4SS-dependent and I could only detect T4SS injection into alveolar macrophages at 4 hours post-infection, our data would suggest that during the first few hours of infection, alveolar macrophages are the primary source of IL-1α, consistent with another study indicating that hematopoietic cells are an early source of IL-1α (Barry et al., 2013).
At 24 hours post-infection, I found that neutrophils recruited to the lungs of mice infected with \(\Delta flaA\) *L. pneumophila* secrete the proinflammatory cytokines TNF and IL-1\(\alpha\), but not mice infected with \(\Delta dotA\) *L. pneumophila*. These data indicate that, like alveolar macrophages, neutrophils secrete cytokines in response to cytosolic sensing of T4SS-translocated bacterial products. It has previously been reported that neutrophils can secrete cytokines, but the signaling pathways that control cytokine production and secretion in neutrophils are poorly understood. Neutrophils are known to release TNF-containing granules in response to a variety of stimuli, including various bacterial infections (Bennouna et al., 2003; Tsuda et al., 2004). Previous research has demonstrated that neutrophils can release IL-1\(\alpha\) in a model of sterile inflammation or IL-1\(\beta\) independently of caspase-1 and caspase-11 in a mouse model of arthritis and during bacterial infection (Guma et al., 2009; Karmakar et al., 2012; Rider et al., 2011). In an intravenous infection model of *L. pneumophila* infection, splenic neutrophils were shown to produce IL-18, an IL-1 family cytokine, which induces IFN-\(\gamma\) production from NK cells (Spörri et al., 2008). Previous studies demonstrated that IL-1\(\alpha\) secretion is regulated by both inflammasome-dependent and –independent pathways during *in vivo* WT *L. pneumophila* infection (Barry et al., 2013; Casson et al., 2013), but it is unknown which of these pathways are used by macrophages and neutrophils to secrete IL-1\(\alpha\) *in vivo*. It would be of interest to determine the host and bacterial components required for release of IL-1 and other cytokines from macrophages and neutrophils in response to *in vivo* infection with *L. pneumophila*.

Overall, our study is the first to define the cell types that receive T4SS-translocated effectors during pulmonary *L. pneumophila* infection. I reveal that both alveolar macrophages and neutrophils receive translocated effector proteins, harbor viable bacteria, and respond to infection by producing inflammatory cytokines. Collectively, our
data indicate that alveolar macrophages and neutrophils not only provide an intracellular reservoir for *L. pneumophila*, but also provide an important source of proinflammatory cytokines that contribute to a successful host immune response during pulmonary *L. pneumophila* infection.

**D. Bystander cytokine production by uninjected cells**

Infection with *L. pneumophila* leads to robust pro-inflammatory cytokine production. Much of this cytokine response is dependent on cytosolic sensing of T4SS activity (Fontana et al., 2011; Shin et al., 2008), although paradoxically, several T4SS effector proteins introduced into infected cells potently block host protein synthesis (Barry et al., 2013; Belyi et al., 2006; 2008; Fontana et al., 2012; Ivanov and Roy, 2013; McCusker et al., 1991; Shen et al., 2009). In chapter 4, I set out to examine how a robust cytokine response is generated despite this translational block. Our data indicate that cells receiving T4SS effectors can synthesize IL-1α and IL-1β, but are poor producers of the key immune proteins TNF, IL-6, IL-12, and CD86. Instead, bystander immune cells that have not received T4SS effectors produce the majority of these immune signals *in vitro* and *in vivo*. Furthermore, IL-1 signaling is required for robust production of TNF and other immune proteins by bystander cells. Thus, our data suggest that detection of PAMPs is inadequate to trigger a robust immune response in bystander cells. Instead, a cytokine released by infected cells signals to uninfected bystander cells to bypass the pathogen-imposed translational block and enable production of a robust inflammatory cytokine response.

Why TLR signaling alone is insufficient to drive cytokine responses *in vivo* is a conundrum. *In vitro* and *in vivo*, the use of purified TLR ligands can induce robust cytokine production and cellular recruitment to the site of delivery. It must be noted,
however, that many LPS instillation protocols use an amount of LPS that can equate to a number of bacteria on the order of $10^{10}$ CFUs (if 0.1 ng of LPS = $10^5$ CFU of *E. coli*, then 10-100 µg of LPS would be equivalent to nearly one million times more bacteria) (Arndt et al., 2005; Raetz, 1986; Szarka et al., 1997). Assuming equivalent calculations for *L. pneumophila*, this dose is equal to a 10,000-fold increase in the number of *L. pneumophila* we detect even during the peak of virulent pulmonary infection. Thus, TLR signaling can induce cytokine production alone, but perhaps only in response to large amounts of TLR ligands. *L. pneumophila* LPS is a poor stimulator of TLR4 (Lettinga et al., 2002) and infection itself does not appear to induce the required amount of TLR stimulation to drive cytokine responses *in vivo*.

Beyond the total amount of TLR ligands present, tolerance to TLR-mediated signaling, specifically tolerance to LPS or endotoxin is a well-described, multi-faceted phenomenon (Biswa and Lopez-Collazo, 2009). Upon repeated, low exposure to LPS, cells exhibit milder and milder cytokine and recruitment responses. This tolerance is mediated by a variety of mechanisms, including increased expression of negative regulators of TLR signaling (Liew et al., 2005; Nimah et al., 2005), decreased expression of TLRs and downstream signaling pathways (Biswa and Tergaonkar, 2007; Medvedev et al., 2000), and changes in the chromatin containing proinflammatory genes (Chan et al., 2005; Foster et al., 2007). Cells of the innate immune system patrol barrier sites and cells such as alveolar macrophages are exposed to the outside of the body, these cells may be in contact with a low, constant level of TLR ligands that may induce and maintain a state of tolerance in these cells. Indeed, a role for TLR tolerance in maintaining intestinal homeostasis has been demonstrated (Abreu et al., 2002; Singh et al., 2005). Likewise, research has demonstrated that innate immune cells of the lamina propria do not produce TNF or IL-6 in response to TLR stimulation, suggesting that cells at other barrier
sites may behave in a similar fashion (Franchi et al., 2012). As these cells may be
tolerized, other signals such as IL-1 may be required either to act synergistically with
TLR and other PRR signaling to overcome this tolerance or may sensitize innate
immune cells to the presence of bacterial PAMPs. It would be of interest to investigate
whether TLR signaling is enhanced by the presence of IL-1. Treating cells with purified
TLR ligands or avirulent T4SS-deficient L. pneumophila in the presence or absence of
IL-1 will help determine whether synergistic effects occur with this combination of stimuli.

E. Injected cells produce IL-1α and IL-1β during protein translations inhibition

Cells targeted by the T4SS of L. pneumophila still transcribe many proinflammatory
genres, such as Il6, Tnf, Il1a, and Il1b. These cells, however, are unable to translate IL-6
and TNF during virulent infection, but are able to translate IL-1α and IL-1β (Figure 5-2).
The inability of injected cells to produce TNF is partly due to effector-mediated protein
translation inhibition, as cells injected by mutants of L. pneumophila lacking either 5 or 7
of the protein translation inhibiting effectors were able to produce TNF during infection.
At later times, however, injected cells infected with these mutants produced just as little
TNF and IL-6 as their WT infected counterparts. These data indicate that part of the
inability of injected cells to produce certain proinflammatory cytokines is effector driven.
Why injected cells are unable to produce TNF and IL-6 later in infection remains to be
elucidated. Given the highly redundant nature of the T4SS effectors, other effectors that
inhibit host protein translation may exist. Alternatively, later protein translation may be a
host-driven response depending on mTOR or some other stress-induced pathway.
Future experiments will focus on altering mTOR signaling during L. pneumophila
infection and assaying for cytokine production from infected cells to determine whether a
mTOR-mediated host response is responsible for the block in protein translation.
Alternatively, infections using T4SS-deficient L. pneumophila in combination with mTOR
inhibitors such as rapamycin may elucidate whether translation inhibition during \( L. \) pneumophila infection is host cell-driven.

It is intriguing that infected cells are able to effectively translate IL-1 but not other cytokines that I assayed. Unlike conventional cytokines, IL-1\( \alpha \) and IL-1\( \beta \) are translated by cytosolic ribosomes in a pro-form and are secreted via an unknown mechanism requiring the activation of the inflammasome (Rathinam et al., 2012; Stevenson et al., 1992). MyD88 and altered mTOR signaling participate in the selective translation of proinflammatory cytokines during \( L. \) pneumophila infection via unknown or poorly understood mechanisms (Asrat et al., 2014; Brieland et al., 1995; 1998; Byrd and Horwitz, 1989; Casson et al., 2013; Copenhaver et al., 2014; Ivanov and Roy, 2013; LeibundGut-Landmann et al., 2011; Spörri et al., 2006). Whether MyD88 and altered mTOR signaling occurs directly in infected cells or influences cytokine production in bystander cells remains to be determined. Previous research has suggested that mRNA abundance may determine which transcripts are selected for translation when translation is inhibited (Asrat et al., 2014; Ivanov and Roy, 2013). My data, however, do not demonstrate a large difference in the total abundance between transcripts that are translated (\( \text{Il1a, Il1b} \)) and transcripts that are not (\( \text{Il6, Tnf} \)). Thus, it is unlikely that mRNA fully explains the mechanism of selective translation.

During periods of protein synthesis blockade, not all proteins remain untranslated. Host cells target key mRNAs to ribosomes during protein synthesis inhibition induced during the unfolded protein response, which allows for at least minimal translation of key proteins. Targeting of key mRNAs to the ribosome during protein synthesis inhibition requires the presence of unique 5’ and 3’ untranslated regions (UTR) on the mRNA of interest (Vivinus et al., 2001). Host cells also segregate cytosolic non-essential mRNAs.
into structures called stress granules (Thomas et al., 2011). These granules associate mRNAs with the mRNA degradation machinery, depleting the cell of non-essential mRNAs to translate. Sequestration and clearance of non-key mRNAs prevents their translation and decreases the competition key mRNAs have to overcome to be translated. Thus, even when global protein production is decreased, cells can preferentially target certain mRNAs for translation. It is known that the mRNAs encoding the proinflammatory cytokines IL-6 and TNF have AU-rich UTRs, but these UTRs have been implicated in the instability of these mRNAs (Villarino et al., 2011). In contrast, the mRNA encoding IL-1β has been previously shown to be more stable than the mRNA for TNF (Chen et al., 2006). This raises the possibility that inflammasome-related mRNAs, due to their unique UTR sequences, are preferentially translated during L. pneumophila infection. Future work will investigate whether certain cytokine mRNAs, such as IL-1α and IL-1β contain regulatory elements in their UTRs that spare them from the effects of global suppression of protein synthesis and if mRNA regulation of IL-1 is important for its production during infection with L. pneumophila.

F. IL-1 induces TNF production and CD86 expression in dendritic cells and alveolar macrophages

My data indicate that uninfected bystander cells play a critical role in host defense during L. pneumophila infection. These cells both directly or indirectly sense the IL-1 released by infected cells and respond by producing TNF and other immune proteins. IL-1 signaling utilizes MyD88 and leads to the activation of NF-κB as well as MAPK. In epithelial cells, IL-1 induces IL-6 production as well as chemokine production. TNF is not induced in response to IL-1 by epithelial cells; however. These data indicate that epithelial cells are incapable of producing TNF to a variety of stimuli that induce the production of other cytokines. IL-1-induced TNF production is not a well-characterized
phenomenon, but human PBMCs have been shown to produce TNF in response to treatment with IL-1α as well as IL-1β (Kim et al., 2013). As these experiments were performed with whole PBMCs, it is unclear which cell types specifically respond to IL-1, but it implies at least one cell type expresses the IL-1R and can produce TNF in response to IL-1 signaling.

Expression of the IL-1R in vivo is poorly characterized. Epithelial cells and T cells are known to express the IL-1R and the response to IL-1 stimulation is best characterized in these cell types (Dinarello, 1996). Bone marrow-derived macrophages fail to respond to IL-1 either alone or in combination with other signals. Bone marrow-derived macrophages also do not express TLR5 and do not respond to extracellular flagellin, even though various subsets of macrophages in vivo do (Means et al., 2003). I detected a low, but evident production of TNF by alveolar macrophages in response to IL-1 stimulation ex vivo. It is possible that ex vivo culture diminishes the responsiveness of cells to IL-1 stimulation, although cells were able to produce robust amounts of TNF in response to LPS, ruling out broad inability of the cells to function. These data indicate that alveolar macrophages express the IL-1R. IL-1 stimulation is known to activate alveolar macrophages in vivo, but TNF production in response to IL-1 has never been assayed until this study (Hussell and Bell, 2014). Only a small frequency of alveolar macrophages produced TNF in response to IL-1 stimulation ex vivo. It is unclear whether all alveolar macrophages express the IL-1R ubiquitously or if only a subset of cells express the receptor. Although an antibody exists to stain for the IL-1R1 (CD121a) chain of the IL-1R, staining for this receptor in vivo has proved difficult and not definitive in mice (data not shown), but staining of human T cells demonstrates heterogeneous IL-1R expression on subsets of activated T cells (Dower et al., 1986; Lee et al., 2010). Likewise, it is unclear whether TLR signaling or some other infectious cue can induce IL-
1R expression on alveolar macrophages. Activation of T cells can increase expression of the IL-1R on the surface, suggesting that similar activation of other cell types might also result in increased receptor expression and responsiveness to IL-1 (Lee et al., 2010). Future work will focus on assaying IL-1R expression on various innate immune cell types during *L. pneumophila* infection to address whether receptor expression is altered during infection.

Bone marrow-derived dendritic cells, like alveolar macrophages, also responded to IL-1 stimulation. These cells produced TNF and increased surface expression of CD86. Again, only a small portion of dendritic cells produced TNF *in vitro*. These data suggest that bone marrow-derived dendritic cells express the IL-1R at a resting state. It would be of interest to determine whether dendritic cells from the lung or other organs also express the IL-1R and respond in a similar fashion. Of note, dendritic cells transferred into the lungs of mice increase expression of CD86 upon repeated intranasal treatment with IL-1 which indicates that dendritic cells may express the IL-1R and are activated upon IL-1 stimulation (Pang et al., 2013).

Inflammatory monocytes isolated from the resting bone marrow pool did not respond to IL-1 stimulation *ex vivo*. This is in stark contrast to the massive reduction in TNF-producing monocytes in IL-1R−/− mice. It is still unclear whether inflammatory monocytes express the IL-1R at a basal state or express it during infection or tissue egress. Experiments in this study use bone marrow-derived monocytes, which are considered a resting pool of monocytes. Many recent advances in the description and analysis of inflammatory monocytes have described several subsets of monocytes that express different chemokine receptors, including CCR2 and CX3CR1 and vary in their functional capacities (Hohl et al., 2009; Narni-Mancinelli et al., 2011; Yang et al., 2014). Thus,
future work should determine the phenotype of the inflammatory monocytes recruited to the lung during *L. pneumophila* infection as well as their expression of various receptors, including IL-1R. If inflammatory monocytes do express the IL-1R, then their lack of a response to IL-1 stimulation alone could indicate that they require additional signals, such as TLR signaling or chemokines, to produce cytokines. It is also unclear whether inflammatory monocytes are responding directly to IL-1R signaling in a cell-intrinsic manner, or are responding to a signal produced in response to IL-1 made by another cell type. Chimeric mice, in which a mix of WT and IL-1R-deficient innate immune cells exist, will be useful to determine the direct and indirect role of IL-1 signaling on inflammatory monocytes as well as other innate immune cell types. There is some evidence for this cell-extrinsic response to IL-1 as neutrophils are recruited to sites of inflammation by chemokine production from epithelial cells induced by IL-1 signaling. Thus, we find that IL-1R signaling is necessary, but not sufficient, to drive optimal cytokine production from monocytes during *L. pneumophila* infection.

I was unable to determine a role for IL-1 signaling directly on neutrophils during infection. Neutrophils were originally thought to migrate towards IL-1 and do express some inducible level of IL-1R (Fasano et al., 1991). However, instead of migrating directly in response to IL-1, neutrophils respond to the aforementioned chemokine products of IL-1 signaling on epithelial cells (Tateda et al., 2001b). Neutrophils are a source of IL-1α during *L. pneumophila* infection, but unfortunately, we were unable to induce cytokine production from neutrophils isolated from the resting bone marrow pool ex vivo in response to any stimuli, including LPS (data not shown). It is possible that neutrophils do respond to IL-1, but as my culturing systems are unable to demonstrate neutrophil activation in response to any stimuli, I am currently unable to determine a direct role IL-1 signaling on neutrophils during infection.
Together, these data indicate that in certain cell types, IL-1 signaling is sufficient to drive a low-level cytokine response. However, not all cells are activated directly by IL-1 and IL-1 does not induce robust cytokine production. Yet, a lack of IL-1R affects cytokine production from neutrophils, alveolar macrophages, dendritic cells, and inflammatory monocytes. These data argue for a complex model of IL-1 signaling in vivo during L. pneumophila infection (Figure 5-3). It is unclear what other signaling pathways might be important for bystander cytokine production in vivo.

Unlike other models, my data indicate IL-1 signaling is partially required for the bystander cytokine response to L. pneumophila in vivo. There are multiple possible benefits to regulating cytokine production in this way. First, it ensures the production of pro-inflammatory cytokines during infection with a pathogen that limits host protein translation. Second, it may be a strategy that the immune system uses to avoid inappropriate responses to avirulent bacteria and instead respond only to virulent pathogens when it encounters a second signal, such as IL-1, that is specifically released in response to virulent pathogens (Fontana and Vance, 2011; Franchi et al., 2012). Third, cytokine production by bystander cells may provide a means of amplifying early immune signals, allowing for a more rapid and robust response. Bystander activation is likely to be a common strategy employed by the immune system for overcoming pathogen virulence mechanisms, and multiple mechanisms may exist for activating bystander cells by different cell types (Ablasser et al., 2013; Dolowschiak et al., 2010; Dreux et al., 2012; Kasper et al., 2010; Patel et al., 2009). Notably, during influenza A virus infection, IL-1 signaling activates bystander dendritic cells and enables subsequent priming of naïve CD8\(^+\) T cells (Pang et al., 2013). This study, along with our finding that uninfected bystander cells upregulate the costimulatory molecule CD86 (Figures 3 &
S5), has implications for understanding how a T cell response is generated during \textit{L. pneumophila} infection. This implies that bystander cells may be key antigen presenting cells, perhaps following uptake of dead or dying infected cells (Trunk and Oxenius, 2012; Yrlid and Wick, 2000).

Our findings also elucidate another critical function for IL-1 in early innate immune defense. In addition to the well-established role of IL-1 in eliciting production of neutrophil-attracting chemokines and the subsequent recruitment of neutrophils to the site of infection (Barry et al., 2013; Casson et al., 2013; LeibundGut-Landmann et al., 2011; Mascarenhas et al., 2015), my data indicate that IL-1 mediates production of cytokines by bystander innate immune cells (Figure 4-9 & 4-10). Immune signals other than IL-1 also likely contribute to T4SS-dependent proinflammatory cytokine production \textit{in vivo}, as IL-1 signaling is required for the maximal production of TNF and IL-12, but is dispensable for IL-6 production during \textit{L. pneumophila} infection (Figure 4). The nature of these other immune signals and whether they can compensate for the absence of IL-1 in certain infection settings remains to be determined. Future work will focus whether other IL-1 family members, including IL-18 and IL-36 also contribute to bystander responses to \textit{L. pneumophila} infection (Spörri et al., 2008; Towne et al., 2011; Vigne et al., 2011). Both IL-18 and IL-36 signal via MyD88 downstream of their receptors (Dinarello, 2013). It is of interest to determine whether these other MyD88-dependent signals contribute to the immune response against \textit{L. pneumophila} infection as it is currently unclear which signal or signals contribute to the defect in MyD88-deficient mice.

\textbf{G. Bystander signaling occurs in other models of infection}

Other pathways to activate immune responses have been described for other pathogens. The proinflammatory form of cell death known as necroptosis may activate
immune responses to *Y. pseudotuberculosis* in vivo from uninfected bystander cells (Philip et al., 2014). Necroptosis has not been demonstrated during *L. pneumophila* infection as inhibiting pyroptosis prevents cell death (Casson et al., 2013). Inhibiting the production of reactive oxygen species (ROS) in intestinal epithelial cells infected with *Listeria monocytogenes* prevents CXCL2 production by neighboring uninfected cells (Dolowschiak et al., 2010). These data indicate that bystander epithelial cells are important for immune responses to *L. monocytogenes* and that ROS activates uninfected bystander cells. Similar experiments performed in macrophages infected with *L. pneumophila*, however, did not produce similar results (data not shown). These data suggest that ROS production does not play a role in bystander activation during *L. pneumophila infection*. Gap junction signaling is important for the propagation of bystander responses to *S. flexneri* and vaccinia virus infection in epithelial cells (Ablasser et al., 2013; Kasper et al., 2010). Inhibition of gap junction signaling during *L. pneumophila* infection in vitro, however, did not alter bystander cytokine production in macrophages (data not shown). Thus, a variety of signaling pathways initiated by infected cells are able to activate uninfected bystander cells to produce cytokines or chemokines important for controlling infection. These pathways are not important for all infections and others pathways may exist to induce bystander activation (Dolowschiak et al., 2010).

*In vitro*, TNF and IL-6 are still produced by bystander bone marrow-derived macrophages in the absence of IL-1 signaling, indicating that other signals are released by injected cells to activate bystanders. Since injected cells, in spite of translational inhibition, synthesize IL-1α and IL-1β, other cytokines may be translated in injected cells. It remains to be seen whether IL-1α and IL-1β are the sole proteins produced robustly by injected cells. IL-18 is also released by the inflammasome, but the reagents for detecting
IL-18 are poor and often do not recognize all forms of IL-18 released from cells (Shida et al., 2001). Importantly, many inflammasome components are poorly expressed in resting cells and require NF-κB signaling for their expression (Lamkanfi and Dixit, 2009). As IL-1α and IL-1β require the inflammasome for their release, these inflammasome components must be present in injected cells, indicating that these proteins may be translated in injected cells. Experiments to determine which proteins injected cells translate will be performed, including experiments to isolate polysomes to sequence which transcripts are associated with active ribosomes for translation.

H. Final Conclusions

I have identified a system used by the innate immune system to ensure the generation of proinflammatory cytokines during infection with an intracellular bacterial pathogen. My findings demonstrate the existence of heterogeneity in the production of critical protective cytokines by innate immune cells during the early response to bacterial infection and define a collaboration between infected and uninfected cells that enables the immune system to bypass protein synthesis inhibition and generate a robust immune response against *L. pneumophila*. As a variety of pathogens, including *Yersinia spp.*, *V. parahaemolyticus*, *S. flexneri*, and others limit immune responses by inhibiting host cells signaling or protein translation, uninflated bystander cells may be required to carry out and amplify the early innate signals that eventually confer protective immunity (Fontana and Vance, 2011; Krachler et al., 2011). These bystander responses can be mediated by a number of pathways, including gap junctions, ROS production, cell death, and IL-1 (Dolowschiak et al., 2010; Kasper et al., 2010; Philip et al., 2014). IL-1 is produced in response to a variety of infectious pathogens, and beyond its known role in recruiting inflammatory cells to sites of infection, may orchestrate the production of bystander cytokines in response to these pathogens (Dinarello, 1996). Therefore, the ability of IL-1
to induce bystander cytokine production and cell activation may be a key pathway to bypass pathogen-induced manipulation of host cell processes during immune responses, ensuring the production of a successful immune response and eventual control of infection.
Figure 5-1. Alveolar macrophages and neutrophils are targeted by the T4SS of L. pneumophila and contain viable bacteria during pulmonary infection. A variety of cell types exist within the mammalian lung including alveolar macrophages (AMΦ), dendritic cells (DC), natural killer (NK) cells, B cells, T cells, eosinophils (EΦ), and inflammatory monocytes (iMC) and neutrophils (NΦ). At 4 hours PI, alveolar macrophages in the airway space contain viable L. pneumophila and are targeted by the T4SS for injection. Activation of the immune response to virulent infection recruits inflammatory monocytes to the lung tissue and neutrophils to the tissue and airway space. Neutrophils, like alveolar macrophages, also contain viable bacteria at later times PI and are injected by the T4SS; whereas, inflammatory monocytes and DCs are not.
Figure 5-2. T4SS effectors block the production of cytokines in infected cells, yet IL-1α and IL-1β are still translated during infection. Cells infected with *L. pneumophila* activate several signaling pathways that induce the activation of NF-κB and MAPK. These pathways lead to the transcription of proinflammatory genes such as *Il6, Tnf, Il1a,* and *Il1b*. The T4SS effectors Lgt1, Lgt2, Lgt3, SidI, SidL, Pkn5, and Lpg1489 block early translation of TNF, but do not inhibit synthesis of TNF and IL-6 later post infection. Instead, TNF and IL-6 are inhibited by an unknown mechanism, either another bacterial effector or a host-driven response. IL-1α and IL-1β, however, are translated and activation of caspase-1 as well as capase-11 leads to the release of active IL-1 from infected cells. Whether infected cells release other inflammatory cues is unknown.
Figure 5-3. Direct and indirect IL-1 signaling induces bystander cytokine production during *L. pneumophila* infection from uninfected innate host cells. Alveolar macrophages (AMΦ) and neutrophils (NΦ) infected with virulent *L. pneumophila* produce IL-1α and IL-1β, but do not produce TNF, IL-6, IL-12, or express CD86. Certain bystander cell types, such as uninfected alveolar macrophages and dendritic cells, can directly respond to IL-1 signaling in a cell intrinsic manner and produce TNF and potentially IL-12 as well as express CD86. Other cell types, such as uninfected neutrophils and inflammatory monocytes (iMC), do not demonstrably respond directly to IL-1, yet the absence of IL-1R signaling during infection impairs their ability to produce cytokines. Other cells, such as airway epithelia cells (AEC) produce chemokines like KC in response to IL-1 signaling and may produce other signals that act to stimulate cytokine production from inflammatory monocytes and neutrophils. This indirect response to IL-1 may explain the requirement for IL-1R signaling from these cell types. Whether other signals are involved in the activation of bystander cells during *L. pneumophila* infection has not been determined.


