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By

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By Kevin Christopher Barry

# Abstract

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The innate immune system is the first line of defense against pathogens. Innate immune receptors, termed pattern recognition receptors, are germline-encoded receptors that recognize conserved microbial products and activate an immune response. Examples of these microbial products, termed pathogen-associated molecular patterns, are components of the bacterial outer membrane, such as lipopolysaccharide or bacterial lipoproteins, and microbe-derived nucleic acids. Importantly these molecular patterns are not just found on pathogens, but are also encoded by harmless commensal microbes as well. It has become clear in recent years that the innate immune system distinguishes pathogens from harmless commensals and preferentially responds to pathogens. It has been established that one mechanism by which the innate immune system makes this distinction is through the recognition of activities that are associated with the pathogenic lifestyle, termed patterns of pathogenesis, such as access to the host cytosol and microbial growth. Recently, translation inhibition induced by pathogenic microbes has been shown to be important for the induction of immune responses, and thus has been termed a novel pathogen-associated activity.

*Legionella pneumophila* is a gram-negative intracellular bacterial pathogen that is the causative agent of a severe pneumonia called Legionnaires' Disease. After inhalation of aerosolized bacteria, *L. pneumophila* can infect and replicate within lung alveolar macrophages. Intracellular replication of *L. pneumophila* in macrophages *in vitro,* and virulence of *L. pneumophila* in animal models, requires a Type IV secretion system (T4SS) called the Dot/Icm system, which secretes bacterial effector proteins into the host cytosol. These effectors, greater than 270 of which have been identified, are believed to be critical for establishment of the *Legionella*-containing vacuole, the specialized membrane-bound intracellular compartment in which *L. pneumophila* replicates. In addition to its essential role in facilitating intracellular bacterial replication, the *L. pneumophila* T4SS is also associated with a strong block in host protein synthesis and the induction of several potent innate immune responses.

The over-arching goal of this thesis is to expand our knowledge of the mechanisms by which the innate immune system distinguishes pathogenic microbes from non-pathogenic microbes. In the first chapter of this thesis I will review the current state of the field. In the second chapter of this thesis I will describe studies using *L. pneumophila* infection *in vivo* where I found an important role for the often-overlooked cytokine, interleukin-1α (IL-1α), in initiating the immune response to virulent *L. pneumophila*. I was able to demonstrate, consistent with previous studies, that signaling through the interleukin-1 receptor (IL-1R) is important for the

recruitment of protective neutrophils to the lungs of mice, but unlike previous studies, we could show that the early recruitment of these cells required IL-1α. I was further able to characterize the molecular mechanism by which the innate immune system is able to produce IL-1α specifically in response to virulent infection. I found that host protein synthesis is inhibited by T4SS$^{+}$, but not T4SS$^{-}$, *L. pneumophila*. I was further able to show that translation inhibition in concert with signaling via the innate immune receptors the toll-like receptors (TLRs) induced sustained and massive induction of *Il1a* transcript. I proposed that this massive induction of *Il1a* transcript overcame the *L. pneumophila* induced block in host protein synthesis and permitted the enhanced production and release of IL-1α. Thus, these studies demonstrated that IL-1α, a cytokine I showed to be important for protecting the host from *L pneumophila* infection *in vivo*, was preferentially made in response to T4SS$^{+}$ *L. pneumophila*. Moreover, I linked the production of IL-1α to the sensing of the pathogen-induced block in host protein synthesis. These studies also identified five known and two novel bacterial effectors that block host protein synthesis, but deletion of all seven of these effectors did not affect the *L. pneumophila* induced block in host protein synthesis. I hypothesized that other mechanisms, possibly host stress induced by intracellular bacterial infection, could induce this block in translation. Thus, taken together, the experiments described in the second chapter of this thesis identify a novel inflammatory response to *L. pneumophila in vivo* and further support a model in which pathogen-induced translation inhibition can allow the immune system to detect a pathogen and respond appropriately.

In the third chapter of this thesis I set out to further characterize the molecular mechanism of IL-1α production and translation inhibition induced by T4SS$^{+}$ *L. pneumophila*. As deletion of the seven *L. pneumophila* effectors that block host protein synthesis did not relieve the block in host protein synthesis induced by *L. pneumophila,* I set out to determine if the residual block in host protein synthesis by the Δ*7 L. pneumophila* mutant was at the level of translation initiation or elongation. Using a deep sequencing technique called ribosome profiling in concert with RNAseq of total mRNA, I was able to look at translation in *L. pneumophila* infected macrophages globally and with nucleotide resolution. I found through these analyses that T4SS$^{+}$ *L. pneumophila* blocks translation elongation, but the residual translation inhibition induced by Δ*7 L. pneumophila* was at the level of translation initiation. The vast majority of translational control by the host is at the level of translation initiation. Thus, the Δ*7 L. pneumophila* induced block in translation initiation suggests that a host stress response could be blocking translation in response to the stresses of being infected by an intracellular pathogen. In the third chapter of this thesis I assay a number of host stress response pathways after *L. pneumophila* infection and see no role for these pathways in Δ*7 L. pneumophila* induced translation inhibition. I proposed that these data suggest that an unknown stress response pathway may be activated or, alternatively, a novel bacterial effector could be blocking translation initiation.

The studies described in the third chapter of this thesis also undertook analyses of ribosome profiling and RNAseq data to further test the model that inflammatory cytokines are made in response to pathogens by the massive induction of transcripts in response to the pathogen-associated activity of blocking host protein synthesis along with TLR signaling. The data presented in the third chapter support a model that the induction of cytokine transcripts via sensing of the pathogen-associated activity of translation inhibition and TLR activation overcomes the block in host protein synthesis and allows the infected cell to preferentially respond to pathogens with the production of inflammatory cytokines. I further describe

experiments that suggest diverse intracellular bacterial pathogens such as *Listeria monocytogenes* also induce a block in host protein synthesis and that this activity may be a broadly applicable pathogen-associated activity. Lastly, the studies presented in the third chapter of this thesis provide evidence that, at least in response to virulent *L. pneumophila*, the majority of control of gene expression in response to pathogenic infection is controlled at the level of mRNA induction.

The studies presented in this thesis lend credence to the proposal that translation inhibition is a pathogen-associated activity encoded by diverse intracellular bacterial pathogens. They also support a model by which translation inhibition is sensed by host innate immune cells to induce massive mRNA induction of inflammatory cytokines allowing for a specific inflammatory response to pathogens. Lastly, these studies link translation inhibition to an important role in protecting the host from pathogenic infection *in vivo.*

For
Caitlin DeJong, my greatest discovery in graduate school

and for
Ronald and Nancy Barry, who taught me that anything is possible

and for
Kimberly and Justin Neff, my partners in crime and role models.

In memory of
Jesse Franklin Martin, Ruth Wadine Martin
and
Mary Jane Barry

# Table of Contents

# List of Figures

# List of Tables

## List of Abbreviations

PAMPs: Pathogen-associated molecular patterns
PRRs: Pattern recognition receptors
TLRs: Toll-like receptors
CLRs: C-type lectin receptors
LRRs: Leucine-rich repeats
NLRs: Nucleotide-binding domain, leucine-rich repeat (LRR)- containing receptors
RLRs: Retinoic acid-inducible gene 1(RIG-I)-like receptors
AIM-2: Absent in melanoma 2
ALRs: AIM-2-like receptors
LPS: Lipopolysaccharide
MAMPs: Microbe-associated molecular patterns
DAMPs: Damage-associated molecular patterns
ETI: Effector triggered immunity
Avr: Avirulence
R: Resistance
BMDCs: Bone marrow derived dendritic cells
Met-tRNAi: initiator tRNA
eIF2-Met-tRNAi: ternary complex
GAP: GTPase activating protein
GEF: Guanine nucleotide exchange factor
dsRNA: double-stranded RNA
ER: endoplasmic reticulum
DT: diphtheria toxin
ExoA: ExotoxinA
ChxA: Cholix toxin
SLO: streptolysin O
SLS: streptolysin S
T4SS: Type IV secretion system
DOT/ICM: defect in organelle trafficking/intracellular multiplication
T3SS: Type III secretion system
SPI-1: *Salmonella* pathogenicity island 1
SCV: *Salmonella* containing vacuole
SPI-2: *Salmonella* pathogenicity island 2
LLO: listeriolysin O
PLC: phospholipase C
BAL: Bronchoalveolar lavage
CFU: Colony forming unit
ELISA: Enzyme linked immunosorbent assay
BALF: Bronchoalveolar lavage fluid
BMDM: Bone marrow derived macrophage
Pam3: Pam3CSK4
AECs: Airway epithelial cells
CrPV: Cricket paralysis virus
IRES: Internal ribosome entry site

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Dan Portnoy has also been an incredibly helpful mentor and collaborator. Through the Barton/Portnoy/Vance tri-lab my research and scientific development has benefited from Dan's comments and discussions. I am very grateful for the collaborations we have undertaken and cannot wait to continue these studies. I thank Dan for the meetings, discussions, and wonderful collaborations.

Another incredibly important scientific collaborator has been Nick Ingolia. Much of the work described in the third chapter of my thesis would not have been possible without the guidance and expertise offered by Nick. As I was trying to understand a brand new field, Nick made me feel like no question was too insignificant. I am incredibly grateful for Nick's patience and helpfulness as I attempted to learn the basics of bioinformatics, deep sequencing, and ribosome profiling.

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Ayres and Jordan Price. Janelle pushed me to be my best in the beginning of my career in the Vance Lab. Janelle always pushed me to the edge of my knowledge and she was also quite good at grilling me about my project before my qualifying exam. Being around Janelle's scientific vision and drive made me a better scientist. When Janelle moved on to a faculty position Jordan Price became my baymate. It has been a pleasure to be Jordan's baymate. We have had many great discussions and things are never dull (Bob'ra!). Jordan has an amazing way of thinking about complex interactions between microbes and the host and it has been such a pleasure to discuss science with him. I thank Jordan for all of the discussions and the fun!

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Growing up I remember being so in awe of my sister, Kimberly (Barry) Neff. I am still in awe of my sister as everything she does is done with such poise, passion and excellence. Looking up to my sister all of these years has been a huge motivator to better myself as well. Kimberly has been the best role model I could ever ask for. My brother (in-law) Justin has also been an incredible role model. Justin's successes in music and business are numerous. These two have really given me something to strive to be like and I thank them for this high bar along with a lot of love and support.

My parents, Ron and Nancy Barry taught me that I can be anything I want as long as I put my mind to it. My parents taught me that believing in yourself and hard work will make you succeed. Their lessons are what have helped me reach my goals of earning a PhD. They have supported me through this journey in every possible way. I cannot thank them enough for their love, support, and advice throughout my graduate work. My parents seeded my interest in science and discovery and are a huge reason why I was able to achieve this milestone in my life.

I had many amazing experiences in graduate school, but one of the best things to happen to me at Cal was meeting my wife, Caitlin DeJong. Caitlin is my classmate, colleague and best friend. I could not have imagined making it through graduate school without her love and support. Graduate school was truly an amazing journey, mostly because I got to walk the journey with her.

# Chapter 1: Introduction

## Section 1.1: Innate immune recognition of pathogens

### Section 1.1.1: Origins of the innate immune system

In 1989 Charles A. Janeway published his introduction to the 1989 Cold Spring Harbor Symposium in which he described the immunologist's "dirty little secret", the fact that researchers had to mix antigen with adjuvant, a concoction of oil and heat killed *Mycobacterium tuberculosis*, in order to induce detectable responses from lymphocytes (Janeway, 1989). Janeway noted that while lymphocytes were thought to be the key cells discerning self from non-self, B and T cells responded poorly to non-self antigen in the absence of adjuvant. This led Janeway to propose that "the immune system has evolved specifically to recognize and respond to infectious microorganisms and that this involves recognition not only of specific antigenic determinants, but also of certain characteristics or patterns common on infectious agents but absent from host" (Janeway, 1989). Janeway continued to propose that pathogens encode conserved molecular patterns, which he termed pathogen associated molecular patterns or PAMPs, that non-clonal, germline encoded immune receptors could recognize, receptors he referred to as pattern recognition receptors (PRRs) (Janeway, 1989). Amazingly, Janeway even proposed that the immune system did not simply evolve to distinguish self from non-self, as was the belief at the time, but that it evolved to discriminate "noninfectious self from infectious non-self" (Janeway, 1989). In essence, Janeway proposed, without much, if any experimental evidence, that recognition of PAMPs by germline encoded PRRs would lead to the activation of B and T cells allowing the immune system to distinguish noninfectious self antigens from infectious non-self antigens. Eight years after hypothesizing the existence of PRRs, a paper was published that showed that loss-of-function mutations in the *Drosophila* gene encoding the protein Toll left adult flies highly susceptible to fungal infection, and that Toll signaling induced production of antimicrobial genes (Lemaitre et al., 1996). The following year a postdoctoral fellow in Janeway's laboratory, Ruslan Medzhitov, identified a human homologue of *Drosophila* Toll and was able to demonstrate that human Toll could activate the transcription factor NF-κB and induce expression of cytokines and co-stimulatory molecules known to activate lymphocytes, confirming one of Janeway's main hypotheses, that PRRs would lead to activation of lymphocytes (Medzhitov et al., 1997). Bruce Beutler and colleagues later showed that the gene *Tlr4* detected bacterial lipopolysaccharide demonstrating, for the first time, that mammalian Toll-like receptors recognized PAMPs and were truly PRRs (Poltorak et al., 1998). Now, 26 years after Janeway predicted the existence of the innate immune system, five families of PRRs have been identified including the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), nucleotide-binding domain, leucine-rich repeat (LRR)- containing receptors (NLRs), RIG-I-like receptors (RLRs), and the AIM2-like receptors (ALRs) (Brubaker et al., 2015). The human TLR family of PRRs consists of 10 proteins, TLR1-10 and the mouse family of TLRs has 12 proteins, TLR1-9 and 11-13 (Brubaker et al., 2015). Considerable progress has been made in identifying the molecular components involved in pattern recognition as can be seen from the incredible diversity of receptors described. Not only has the field identified vast numbers of novel PRRs but more recent studies have begun to demonstrate that while Janeway's vision gave birth to an entire field of research, the PRR system cannot explain all of the responses and distinctions that the immune system can detect. As will be described in more detail below, one

major limitation of the PRR system is the inability of this model to explain the mechanism by which the innate immune system distinguishes not just non-infectious self from infectious non-self, but noninfectious from infectious microbes.

**Section 1.1.2: Brief overview of the PRRs, focusing on TLRs and NLRs**

The five families PRRs described above can be separated into two major classes: the membrane-bound receptors, comprised of the TLRs and CLRs; and the cytosolic receptors, the NLRs, RLRs, and ALRs. As Janeway predicted, PRRs recognize conserved molecular patterns of microbes or PAMPs. PAMPs are incredibly diverse and can range from components of the microbe outer membrane or cell wall, such as lipopolysaccharide (LPS), bacterial lipoproteins, and zymosan, to microbial nucleic acids such as double-stranded RNA and DNA (Brubaker et al., 2015). While PAMPS are diverse, they do share a number of common features: they are essential for microbe survival, conserved among many types of microbes, abundant, and can be difficult for the microbe to modify. However, as will be discussed below, there are some widely recognized issues with the PAMPs nomenclature and specific examples of pathogens modifying PAMPs to avoid activation of host PRRs exist (Akerley et al., 1995; Jones et al., 2012; Montminy et al., 2006; Shen and Higgins, 2006; Wolfgang et al., 2004). More details of the PRRs and their cognate ligands have been discussed in detail elsewhere (Barbalat et al., 2011; Brubaker et al., 2015; Kawasaki and Kawai, 2014; Martinon et al., 2002; Takeda et al., 2003; von Moltke et al., 2012a) and thus I will only briefly discuss two classes of PRRs, the TLRs and the NLRs.

The TLRs are a broad class of transmembrane receptors that recognize microbial products ranging from nucleic acids to components of microbial cell walls (Barbalat et al., 2011; Brubaker et al., 2015; Kawasaki and Kawai, 2014). A subset of TLRs are capable of signaling from the cells surface, i.e., TLR1-2, TLR4-6, and TLR10, while TLR3, TLR7-9, and TLR11-12 signal from within endosomal compartments (Barbalat et al., 2011; Brubaker et al., 2015; Kawasaki and Kawai, 2014). Lipopolysaccharide (LPS), a component of the Gram-negative bacterial outer membrane, is recognized by TLR4, while bacterial lipoproteins, produced by both Gram-negative and Gram-positive bacteria, and zymosan, a component of the fungal cell wall, are recognized by TLR2 along with TLR1 or TLR6 (Brubaker et al., 2015; Kawasaki and Kawai, 2014). Bacterial flagellin is recognized by TLR5, whereas TLR3, TLR7 and TLR9 recognize microbial nucleic acids (Barbalat et al., 2011; Brubaker et al., 2015). Sensing of nucleic acids by TLRs occurs in endosomal compartments and this localization has been shown to be an important mechanism to limit the response of TLRs to host-derived nucleic acids (Barbalat et al., 2011; Ewald et al., 2008; Mouchess et al., 2011). Activation of PRRs through recognition of their cognate ligands leads to downstream signaling and the activation of a number of transcription factors, including NF-κB, IRF3 and 7, and the mitogen-activated kinase (MAPK) family members (Barbalat et al., 2011; Brubaker et al., 2015; Kawasaki and Kawai, 2014). Transcriptional responses initiated by TLRs can lead to the production of Type I IFNs, inflammatory cytokines, and other important innate immune molecules (Barbalat et al., 2011; Brubaker et al., 2015; Kawasaki and Kawai, 2014).

The NLR family of PRRs is a well-studied class of receptors localized to the cytosol (Martinon et al., 2009; von Moltke et al., 2012a). Upon activation, a subset of the NLRs form a cytosolic multiprotein complex called the inflammasome (Martinon et al., 2009; von Moltke et al., 2012a). The inflammasome is a high-molecular-weight protein complex that forms in the

cytosol and serves as a platform for the recruitment and autoproteolytic activation of certain caspases, the best characterized being CASP1 (Martinon et al., 2009; von Moltke et al., 2012a). There are multiple distinct inflammasomes that can be formed in response to a variety of infectious agents, ranging from the recognition of bacterial flagellin or components of the bacterial type III secretion system (Kofoed and Vance, 2011; Tenthorey et al., 2014) to proteolytic cleavage of the NLR by a pathogen encoded protease (Chavarria-Smith and Vance, 2013; Ewald et al., 2014). The activation of CASP1 by various inflammasomes leads to at least three known effects: the cleavage of the inflammatory cytokines IL-1β and IL-18 into their mature signaling competent forms, a lytic form of cell death known as pyroptosis, and the production of inflammatory signaling lipids (eicosanoids) (Martinon et al., 2009; von Moltke et al., 2012a; von Moltke et al., 2012b).

**Section 1.1.3: Complexities of the PRR system**

The PRRs of the innate immune system have been shown to recognize and respond to pathogens and induce immune responses. However, it is has become clear that PRRs alone cannot explain specific innate immune responses to pathogens. One major issue with the PRR system is the discovery of pathogens that have evolved mechanisms to modify PAMPs to make them less stimulatory or even invisible to PRRs. *Yersinia pestis* has been shown to modify its LPS to be less stimulatory to TLR4 (Montminy et al., 2006) and several bacterial pathogens can downregulate flagellin expression within hosts to limit activation of PRRs (Akerley et al., 1995; Jones et al., 2012; Shen and Higgins, 2006; Wolfgang et al., 2004). Additionally, a diverse population of commensal microbes colonizes humans and other animals (Guarner, 2014; Yoon et al., 2015). These commensal microbes are generally non-pathogenic and beneficial to the host (Guarner, 2014; Yoon et al., 2015). Beneficial microbes also possess the same conserved molecular patterns that pathogens encode leading some to propose that these patterns should instead be referred to as microbe associated molecular patterns, or MAMPs (Benko et al., 2008; He et al., 2007; Mackey and McFall, 2006). Thus, PRRs alone are not sufficient to explain how the immune system distinguishes pathogen from non-pathogen. Importantly, altered recognition of the intestinal microbiota has been linked to human diseases such as inflammatory bowel disease (Kostic et al., 2014), suggesting that it is vitally important to the host that the innate immune system distinguish pathogenic microbes from non-pathogenic microbes and only mount immune responses against pathogens. While PRRs are incredibly important for protecting the host from infection, these receptors alone cannot explain how the innate immune system can distinguish pathogenic and nonpathogenic microbes.

**Section 1.1.4: Complementary models to the PRR system**

The 'danger' or damage model has been proposed to explain how the innate immune system distinguishes pathogen from non-pathogen. The damage model suggests that pathogenic infection can lead to cellular damage that induces lytic forms of cell death like necrosis or pyroptosis; this lytic cell death leads to the release of intracellular contents, including DNA, ATP, uric acid, and DNA binding proteins, such as HMGB1, into the extracellular space where they can be recognized by PRRs and induce inflammation (Kono and Rock, 2008; Matzinger, 1994; Scaffidi et al., 2002; Shi et al., 2003). The molecules released from lytic cells have been named damage-associated molecular patterns (DAMPs) (Kono and Rock, 2008; Matzinger,

1994, 1998, 2002). In the damage model, cellular damage should only be induced by pathogens, as production of DAMPs is the signal to the host that a pathogen is present. However, DAMPs play an important role in in inflammation induced by physical or chemical stress, in the absence of infection, known as sterile inflammation. Further, immune responses themselves can lead to cellular damage and lytic cell death so it is unclear how DAMP-triggered immune responses would allow for specific detection of pathogens. It is clear that DAMPs can play a role in inducing sterile inflammation but it appears unlikely that DAMP-triggered immune responses can provide specific signals that allow the immune system to distinguish pathogenic infection. It remains to be seen if DAMP signaling can play a protective role during infection or it may just be involved in inducing immune pathology.

Two other models have been proposed for how the innate immune system distinguishes pathogen from non-pathogen; effector triggered immunity (reviewed in (Rajamuthiah and Mylonakis, 2014; Stuart et al., 2013) and patterns of pathogenesis (reviewed in (Vance et al., 2009). These two models are not mutually exclusive and in fact share many of the same characteristics. Effector triggered immunity (ETI) was first described in plants. In plants, ETI originated as the gene-for-gene theory in which pathogens encoded avirulence (Avr) genes and plants encoded resistance (R) genes (Muthamilarasan and Prasad, 2013). Avr genes, also known as bacterial effector genes, are proteins that are injected into the host cell through a variety of different bacterial secretion systems (Chang et al., 2014; Chisholm et al., 2006; Desvaux et al., 2009; Tseng et al., 2009). Effector proteins are injected across the host cell wall and encode diverse enzymatic functions including transcriptional regulation, phosphorylation, ubiquitylation, AMPylation, ADP-ribosylation, and proteolysis (Deslandes and Rivas, 2012; Salomon and Orth, 2013). The plant R proteins can directly interact with effector proteins or recognize effector-induced modifications on accessory-proteins to directly modulate transcriptional responses (Muthamilarasan and Prasad, 2013). ETI in plants induces the MAPK cascade, as well as other transcriptional responses, leading to the induction of a number antimicrobial genes as well as host repair genes (Muthamilarasan and Prasad, 2013). In metazoans, effector triggered immunity is broadly defined as a response to pathogen encoded virulence factors, effectors, and/or cellular damage or modification of the host cell (Rajamuthiah and Mylonakis, 2014; Stuart et al., 2013). Unlike plants, all known examples of ETI in animals have demonstrated that the activity of bacterial effectors are sensed and not the effectors themselves. Examples of effector-triggered immunity in animals are modification of host signaling molecules, inhibition of host translation, pore-forming toxins, and reorganization of the host cytoskeleton (Reviewed in(Rajamuthiah and Mylonakis, 2014; Stuart et al., 2013). In general these effector activities were shown to induce or increase transcriptional responses to pathogens, leading to increased production of inflammatory cytokines and antimicrobial products, much like ETI in plants (Reviewed in (Rajamuthiah and Mylonakis, 2014; Stuart et al., 2013). It has also been suggested that ETI induces immune responses to pathogens independently of the PRR system. As will be discussed in more detail below, while ETI alone can induce transcriptional responses it is intriguing to hypothesize that signals from ETI and PRRs can work synergistically. In fact it has been proposed that the two signals from ETI and PRRs allow the innate immune system to distinguish pathogens and nonpathogens and respond appropriately (reviewed in (Fontana and Vance, 2011).

Patterns of pathogenesis are a collection of strategies that pathogens use in order to replicate within and be transmitted among their hosts (Vance et al., 2009). These strategies can result in disease symptoms in the host. This model suggests that there are certain activities and strategies that pathogens use for survival and that the immune system is capable of recognizing

these activities. It should be noted that some patterns of pathogenesis could also be classified as ETI, although many pathogenic activities can be induced independent of classically defined bacterial effectors. Thus, the patterns of pathogenesis model encompasses a broad range of activities associated with the pathogenic lifestyle, not just those activities of classically defined effectors or toxins. Examples of patterns of pathogenesis are growth within the host, access to the host cytosol (either directly or via a bacterial secretion system), and disruption of the actin cytoskeleton. Disruption of the host actin cytoskeleton can be achieved by different mechanisms depending on the pathogen inducing this activity. For example many species of bacteria, including *Listeria*, *Shigella*, *Mycobacterium marinum,* and Rickettsial species directly accesses the host cytosol where they grow and use the host cytoskeleton for actin-based motility (Gouin et al., 2005; Welch and Way, 2013). In these cases, proteins associated with the surface of the pathogen recruit key regulators of the actin cytoskeleton to initiate rounds of actin polymerization within the host cell (Gouin et al., 2005; Welch and Way, 2013). Other pathogens, such as *Yersinia* also disrupt the actin cytoskeleton but do so by translocating a bacterial effector, YopE, into the host cytosol to disrupt macrophage phagocytosis (Black and Bliska, 2000) or *Salmonella*, which utilizes effector proteins to remodel the actin cytoskeleton and facilitate bacterial entry (Patel and Galan, 2005). Thus, this example of a pattern of pathogenesis encompasses effector triggered immune responses as well as other activities that are not technically classified as ETI.

Effector triggered immunity and patterns of pathogenesis both have a common hypothesis: pathogens encode activities, whether it is through effector proteins or some other strategy, that allows the pathogen to infect, replicate and spread within their hosts. Importantly, both models also agree that these activities are not present in harmless, non-pathogenic microbes, and that the immune system detects these pathogenic activities, directly or indirectly, to induce an appropriate immune response only to pathogens. While the PRR system alone does not explain how pathogens can be distinguished from non-pathogenic microbes, it has recently been suggested that PRRs in concert with detection of pathogenic activities, a two signal model, can provide the immune system with the information to make this important distinction (Reviewed in (Fontana and Vance, 2011). While there are clearly examples of innate immune responses to only one signal, it is intriguing to hypothesize that PRR signaling along with ETI or a pattern of pathogenesis signal could provide the contextual cue to the cell that it is infected with a pathogen and should respond with an appropriate inflammatory response.

Translation inhibition has recently been shown to be a novel pattern of pathogenesis that has been suggested, along with PRR stimulation, to induce a preferential inflammatory response to pathogens (Barry et al., 2013; Fontana et al., 2011; Fontana et al., 2012). Translation inhibition by microbial pathogens will be discussed in much more detail below. However, it is interesting to note that translation inhibition can be directly blocked by a pathogen, as is the case of *L. pneumophila* effectors that are secreted into the cell and block host protein synthesis (Fontana et al., 2011), or it can be induced by host stress responses induced by pathogens such as has been demonstrated by the pore-forming toxins that lead to cellular stress responses and blocks in host protein synthesis (Baruch et al., 2014a; Baruch et al., 2014b; Baruch et al., 2014c; Kloft et al., 2010; Pillich et al., 2012; Shrestha et al., 2012; Tattoli et al., 2012a; Tattoli et al., 2012b; Tattoli et al., 2013). Translation inhibition and cellular stress responses induced by pathogens are now being recognized as important pathogen-associated activities that are sensed by the immune system and in a two signal model with PRR activation provides the contextual cues that tell the cell that it is infected with a pathogen. Below I will discuss in detail common

host derived pathways that modulate translation, as well as what is currently known about bacterial induction of translation inhibition and how this activity can induce immune responses specifically to pathogens.

## Section 1.2: Global regulation of gene expression

Gene expression is a multistep process that controls the levels of proteins found in a cell. Gene expression is the net effect of four processes: the rate of gene transcription, mRNA degradation, translation of protein, and protein degradation. Each part of gene expression is regulated to provide the proper levels of gene products in a cell. The balance between transcription and mRNA degradation as well as translation and protein degradation controls the levels of mRNA and protein in a cell, respectively. Many studies have looked at the regulation of gene expression at the individual gene level. However, it has not been until recently that studies have begun to try to measure the regulation of gene expression at the global scale (Jovanovic et al., 2015; Li et al., 2014; Schwanhausser et al., 2011). Much of this has been due to advances in next generation sequencing (reviewed in (Buermans and den Dunnen, 2014; van Dijk et al., 2014) and protein identification technologies (reviewed in (Breker and Schuldiner, 2014) that allow researchers to track the many different components of gene expression globally. One intriguing question, especially in the field of immunology where we tend to use transcriptional induction as a readout of protein expression, is: what is the relative contribution of transcription and translation to global protein levels in a cell? This question can be addressed in steady state or in response to cellular perturbations, e.g. pathogenic infection or innate immune system activation. A number of studies have attempted to address the role of transcription in controlling protein levels at steady state; however, the contribution of transcription remains debated (Breker and Schuldiner, 2014; de Sousa Abreu et al., 2009; Li et al., 2014; Maier et al., 2009; Schwanhausser et al., 2011; Vogel and Marcotte, 2012). One study proposed that at steady state protein synthesis rates contributed most to final protein levels in a cell (Schwanhausser et al., 2011) while a more recent study re-analyzed these data and concluded that mRNA levels may control as high as 84% of the variation in protein levels at steady state (Li et al., 2014). Recently, a study was undertaken to clear up the debate over the role of mRNA abundance in protein levels at steady state (Jovanovic et al., 2015). In this study the authors tracked mRNA abundance and degradation as well as protein synthesis and degradation and generated an integrated experimental and computational strategy to quantitatively assess how protein levels are maintained in a cell (Jovanovic et al., 2015). Using experimental datasets from murine bone marrow derived dendritic cells (BMDCs) at steady state the authors were able to explain ~79% of the variance of protein levels (Jovanovic et al., 2015). This study concluded that mRNA levels explained 59-68% of the protein level variance while translation rates (18-26%) and protein degradation (8-22%) combined explained less variation than mRNA levels (Jovanovic et al., 2015). Interestingly, this study also looked at the variation in protein levels explained by mRNA levels after activation of BMDCs with LPS and found, even more strikingly than in steady state, that mRNA levels explain ~87-92% of protein fold changes in BMDCs after LPS treatment for 12hrs (Jovanovic et al., 2015). Taken together, these data suggest that while mRNA levels may not be the only method of controlling protein levels at steady state, in response to innate immune stimulation BMDCs almost entirely control the induction of genes at the level of transcriptional induction. Importantly, no study has measured the role of mRNA levels in controlling protein induction in response to a pathogenic infection.

## Section 1.3: Translation and translational control in eukaryotic cells

### Section 1.3.1: The molecular mechanism of eukaryotic translation

As described previously, protein levels in a cell can be controlled at multiple steps. Transcription will produce mRNAs and regulating translation of these mRNAs can be a powerful post-transcriptional tool a cell can use to control gene expression. Translation can be broken into four phases: initiation, elongation, termination and recycling (Hershey et al., 2012). Each of these four phases requires a specific subset of host proteins that have a multitude of regulatory networks controlling their activity (Hershey et al., 2012). Within eukaryotic cells, the vast majority of translation control occurs at the level of initiation (Hershey et al., 2012), although one notable exception is the control of translation elongation by the energy sensor AMP-activated protein kinase (AMPK) (Leprivier et al., 2013). Translation initiation begins with the formation of the ternary complex which consists of the heterotrimeric guanine nucleotide binding protein eIF2 (eIF2α, β and γ) protein bound to GTP and the initiator methionine tRNA (Met-tRNAi; (Hinnebusch and Lorsch, 2012; Jackson et al., 2010). The ternary complex (eIF2-Met-tRNAi) is then recruited to the 40S ribosomal subunit through interactions with the initiation factors eIF1, eIF1A, and eIF3 to generate the 43S preinitiation complex (Hinnebusch and Lorsch, 2012; Jackson et al., 2010). Facilitated by the cooperative actions of eIF4B, and the eIF4F complex, which consists of the 7-methylguanosine ($m^7G$) cap-binding protein eIF4E, the RNA-helicase eIF4A and the large molecular scaffold eIF4G, to open any secondary structure on the 5' end of transcripts, the 43S preinitiation complex is loaded onto mRNA (Hinnebusch and Lorsch, 2012; Jackson et al., 2010). The 43S complex then scans the mRNA downstream of the cap until it finds the start codon. Recognition of the start codon by the Met-tRNAi induces a conformational change in the 43S complex that locks it onto the start site and causes eIF1 to be displaced (Hinnebusch and Lorsch, 2012; Jackson et al., 2010). eIF1 displacement allows eIF5, an eIF2 GTPase-activating protein (GAP), to bind to the eIF2β subunit of eIF2 and induce the GTPase activity of eIF2γ to hydrolyze GTP and release inorganic phosphate ($P_i$) (Hinnebusch and Lorsch, 2012; Jackson et al., 2010). GDP bound eIF2 has a reduced affinity for the Met-tRNAi and is then partially lost from the complex. Following start site recognition, the 60S ribosomal subunit joins the 48S initiation complex in a mechanism mediated by eIF5B (Hinnebusch and Lorsch, 2012; Jackson et al., 2010). The complete 80S ribosome then commences polypeptide chain elongation (Dever and Green, 2012; Hinnebusch and Lorsch, 2012; Jackson et al., 2010). Translation elongation is controlled by a number of eukaryotic elongation factors. eEF1A, a guanine nucleotide binding protein, delivers aminoacylated tRNAs to the ribosome (Dever and Green, 2012). Codon recognition by the tRNA induces eEF1A hydrolysis of GTP, release of eEF1A from the ribosome, and allows the aminoacyl-tRNA to fit in the A-site of the ribosome (Dever and Green, 2012). eEF1A activity is regulated by eEF1B, a guanine nucleotide exchange factor (GEF) that induces the recycling of eEF1A into the active GTP bound state (Dever and Green, 2012). Another elongation factor, eEF2, is also a guanine nucleotide binding protein and is required to promote ribosome translocation following peptide bond formation (Dever and Green, 2012; Leprivier et al., 2013). Following translation elongation the ribosome must terminate translation and then recycle factors to translate other messages. As most regulation of translation occurs at the level of translation initiation, and to a lesser degree elongation (Hershey et al., 2012), and other groups have thoroughly reviewed the

final two steps of translation (Dever and Green, 2012), there will be no discussion of the final two steps of translation here.

**Section 1.3.2: Translational control by the eIF2α kinases**

As already described, eIF2 is an important initiation factor that controls the recruitment of the initiator tRNA into the ternary complex and subsequently controls the formation of the preinitiation complex as well as the 48S complex. eIF2 activity is regulated by the GEF, eIF2B which allows inactive eIF2-GDP to be recycled into the active GTP bound form (reviewed in (Hinnebusch and Lorsch, 2012; Jackson et al., 2010; Sonenberg and Hinnebusch, 2009). eIF2 is a heterotrimeric protein consisting of eIF2α, eIF2β and eIF2γ. The eIF2β subunit has been shown to be important for directly binding to other initiation factors, the eIF2γ subunit contains the GTPase activity of eIF2 while the eIF2α subunit is thought to be the regulatory subunit of this heterotrimeric protein (reviewed in (Hinnebusch and Lorsch, 2012; Jackson et al., 2010; Sonenberg and Hinnebusch, 2009). eIF2α can be inactivated by phosphorylation by the eIF2α kinases (Donnelly et al., 2013; Wek et al., 2006). Phosphorylated eIF2α (eIF2α-S51p) acts a competitive inhibitor of eIF2B, as eIF2α-S51p has a higher binding affinity for eIF2B, but eIF2B is unable to induce eIF2α-S51p to exchange GDP for GTP (Hinnebusch and Lorsch, 2012; Krishnamoorthy et al., 2001; Pavitt et al., 1998; Sonenberg and Hinnebusch, 2009; Sudhakar et al., 2000). One implication of eIF2α-S51p inhibition of eIF2B activity is that the amount of translation inhibition is directly related to the ratio of phospho-eIF2α:eIF2B and since it is thought that eIF2B is limiting in cells, low levels of eIF2α phosphorylation can have drastic inhibitory effects on translation rates (Kaufman, 1999).

Protein kinase double-stranded RNA-dependent (PKR), general control non-derepressible-2 (GCN2), PKR-like ER kinase (PERK), and heme-regulated inhibitor (HRI) are the four serine/threonine kinases that make up the eIF2α kinase family (reviewed in (Baird and Wek, 2012; Donnelly et al., 2013; Wek et al., 2006). PKR, PERK, HRI and GCN2 are activated by diverse stimuli and upon activation directly phosphorylate serine 51 of eIF2α resulting in global blockade of host protein synthesis. It is important to note that targets besides eIF2α have been identified for some of these kinases and thus there may be roles outside of translation regulation for these proteins (Donnelly et al., 2013). PKR was originally discovered as the kinase that phosphorylates eIF2α in response to viral infection, leading to a block in global translation and inhibition of viral growth (Donnelly et al., 2013). However, PKR has later been shown to respond to a number of other stimuli including oxidative stress, ER stress, as well as cytokine and growth factor signaling (Donnelly et al., 2013). Appropriately named, PKR is known to bind dsRNA and it has been proposed that the binding of dsRNA causes PKR to dimerize leading to autophosphorylation, stabilization of the dimer, and activation of the PKR dimer to phosphorylate eIF2α (Donnelly et al., 2013). Importantly, PKR is not constitutively expressed and *Pkr* transcription is induced upon signaling by the antiviral type-I interferon (IFN) cytokines (Donnelly et al., 2013). Similar to PKR, the kinase GCN2 has been suggested to respond to viral infection through binding to viral RNA (Donnelly et al., 2013). However, the primary stimulus for GCN2 is uncharged tRNAs that are induced in response to amino acid deprivation (Baird and Wek, 2012; Donnelly et al., 2013). Uncharged tRNAs are bound by GCN2 with a higher affinity than charged tRNA and the binding of these uncharged tRNAs leads to activation of GNC2 kinase activity and phosphorylation of its only characterized target, eIF2α (Baird and Wek, 2012; Donnelly et al., 2013). Similar to GCN2, PERK is primarily activated by

a cellular stress, the accumulation of unfolded proteins in the endoplasmic reticulum (ER), a phenomenon termed ER stress (Baird and Wek, 2012; Donnelly et al., 2013). Through PERK-dependent phosphorylation of eIF2α global translation rates are reduced, reducing the entry of nascent polypeptides into the ER lumen and allowing time for misfolded proteins to be re-folded in the ER (Baird and Wek, 2012; Donnelly et al., 2013). Finally, HRI has been shown to respond to heme deprivation as well as oxidative heat stress in erythroid tissues (Donnelly et al., 2013; Wek et al., 2006). Clearly, the eIF2α family of kinases are able to respond to a variety of inputs and induce translation blockade through targeting eIF2α.

**Section 1.3.3: Translational control by mechanistic/mammalian target of rapamycin**

Mechanistic target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that is an integral regulator of cellular metabolism and growth (reviewed in(Showkat et al., 2014). mTOR responds to a variety of inputs including mitogens, nutrients, energy status, and stress signals (Reviewed in (Efeyan et al., 2012; Kim et al., 2013; Showkat et al., 2014; Thoreen, 2013; Zoncu et al., 2011b). mTOR is the catalytic subunit of two complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 is defined by interaction with the accessory protein regulatory-associated protein of mTOR (RAPTOR) and has important roles in sensing growth factors and nutrient abundance, and regulating cell growth and metabolism (Showkat et al., 2014). mTORC2 interacts with the accessory protein rapamycin-insensitive companion of mTOR (RICTOR) and is thought to only respond to growth factor signals leading to regulation of cytoskeletal organization and cell survival (Showkat et al., 2014). mTORC1 has also been well characterized to regulate translation by targeting eIF4E-binding protein 1 and 2 (4E-BP1 and 2) and ribosomal protein S6 kinase (S6K1) (Showkat et al., 2014). Amino acids have long been known to activate mTOR and recent work has shown a role for amino acid sensing in the lysosome in controlling mTOR activation (Zoncu et al., 2011a; Zoncu et al., 2011b). mTOR has been shown to require a small group of GTPases, called the Rag proteins, to respond to amino acids (Sancak et al., 2008). The Rag GTPases are active in a GTP-bound stated and inactive in a GDP-bound state. Importantly, amino acid levels in the cell control the state of the nucleotide loading of the Rag GTPases and only in the presence of amino acids do Rag GTPases adopt an active confirmation where they directly bind and activate mTORC1 (Kim et al., 2013; Zoncu et al., 2011b). The Rag proteins localize to the lysosomal surface and require a complex of proteins termed the Ragulator for this localization (Bar-Peled et al., 2012). In amino acid replete conditions the Rag GTPases are active, recruit mTOR to the lysosome, and activate mTOR. Interestingly, the concentration of amino acids within the lysosome, not in the cytosol, leads to a currently unknown activating signal that is transmitted to the Rag GTPases via an interaction between the vacuolar ATPase and the Ragulator; this interaction in turn recruits mTOR to the lysosome surface (Zoncu et al., 2011a). The details of lysosomal sensing of amino acids in controlling mTOR activity have been reviewed elsewhere (Kim et al., 2013; Zoncu et al., 2011b).

mTOR activation controls translation at both initiation and elongation through many downstream targets. In amino acid replete conditions mTOR is active and phosphorylates two downstream targets inactivating 4E-BP1 and activating S6K1. Active mTOR (nutrient replete state) phosphorylates 4E-BP1 in many sites; the best-characterized phosphorylation sites on 4E-BP1 are T37, T46, S65, T70, S83, S101 and S112 (Showkat et al., 2014). There has been a debate over which phosphorylation events require mTOR but phosphorylation of S65 and T70

has been shown to be serum dependent and inhibited by the mTOR inhibitors rapamycin and Torin1, consistent with an important role for mTOR in regulating these phosphorylation events (Showkat et al., 2014). 4E-BP1 interactions with eIF4E have been shown to be inhibited by phosphorylation at residues T37, T46, S65 and T70 (Showkat et al., 2014). In a hypo-phosphorylated state, induced via depletion of amino acid and loss of mTOR activity, 4E-BP1 is capable of binding to eukaryotic initiation factor E (eIF4E), the limiting factor of the eukaryotic initiation factor 4F initiation complex (eIF4F), thus blocking cap-dependent translation initiation (Ma and Blenis, 2009; Showkat et al., 2014; Thoreen, 2013). S6K1 can also play an important role in regulating cap-dependent translation initiation. S6K1 is phosphorylated at T389 by mTOR allowing it to be fully activated through subsequent phosphorylation events catalyzed by other kinases (Showkat et al., 2014). Activated S6K1 has a number of downstream targets that are important for controlling translation in the cell including ribosomal protein S6 (rpS6), eIF4B, PDCD4, and eukaryotic elongation factor 2 kinase (eEF2K). The first identified substrate of S6K1, rpS6, is phosphorylated at five sites in the C-terminal region of the protein (Ferrari et al., 1991). Knock-in mice replacing these five phosphorylation sites with alanines showed defects in cell growth consistent with these five phosphorylation sites being important for activating rpS6 (Ruvinsky et al., 2005). However, Ruvinsky *et al.* also demonstrate that the lack of phosphorylation of rpS6 has no effect on global translation rates or the translation of a specific subset of mRNAs as previously described (Ruvinsky et al., 2005). Thus, phosphorylation of rpS6 does not seem to play a role in regulating global translation in response to mTOR-dependent activation of S6K1. Active S6K1 can also phosphorylate eIF4B, a member of the eIF4F cap binding complex, at S422, increasing the affinity of eIF4B for eIF4A at the translation initiation complex where it functions as a cofactor for eIF4A and increases its processivity (Holz et al., 2005). Active S6K1 phosphorylates PDCD4, a tumor suppressor that is a negative regulator of eIF4A (Yang et al., 2003), and targets the PDCD4 for degradation (Dorrello et al., 2006). Thus, in the presence of amino acids, or other mitogen signals that activate mTOR, mTOR activates S6K1 leading to increased translation initiation through two independent mechanisms that converge on controlling the formation of an active eIF4F complex. Furthermore, mTOR activation of S6K1 has been shown to promote translation elongation as well, as S6K1 directly phosphorylates eukaryotic elongation factor 2 kinase (eEF2K), inactivating the kinase which when active inhibits translation elongation by inactivating eukaryotic elongation factor 2 (eEF2) (Wang et al., 2001).

**Section 1.3.4: Translational control by the AMP-activated kinase**

The vast majority of translation regulation in eukaryotic cells occurs at the level of initiation (Hershey et al., 2012). One important exception is the control of translation elongation through regulation of eukaryotic elongation factor 2 (eEF2) (Leprivier et al., 2013). In response to nutrient deprivation the ratio of AMP:ATP or ADP:ATP increases and activates the energy sensor AMP-activated protein kinase (AMPK; (Hardie, 2011). Activated AMPK then phosphorylates and activates the eukaryotic elongation factor 2 kinase (eEF2K) leading to the phosphorylation and inactivation of elongation factor 2 (eEF2; (Leprivier et al., 2013) thus inducing a block in protein synthesis in response to nutrient deplete conditions. The regulation of translation at the level of eEF2 was shown to be important in protecting mammalian cells and *C. elegans* from the deleterious effects of nutrient deprivation (Leprivier et al., 2013).

Importantly, as described above, S6K1 can inactivate eEF2K and this can be controlled by mTOR signaling.

## Section 1.4: Effector mechanisms of pathogens that block host translation

### Section 1.4.1: Mechanisms of translation inhibition following viral infection

Intracellular microbial pathogens can have diverse effects on host cell physiology. Inhibition of host protein synthesis is increasingly being recognized as an activity that many pathogens induce. It has been well characterized and studied that viral infections can lead to a robust block in host protein synthesis and preferential translation of viral genes, a process termed host shut-off (reviewed in (Gale et al., 2000; Walsh et al., 2013). To replicate, many viruses must commandeer the host translational machinery; thus, blocking host translation and preferentially translating viral proteins likely provides an evolutionary advantage to these viruses. Strikingly, Sindbis virus (SV), encephalomyocarditis virus (ECMV), vesicular stomatitis (VSV), and influenza virus have all been shown to induce shut-off of host translation and preferential translation of viral proteins, albeit by diverse mechanisms (reviewed in (Connor and Lyles, 2005; Gale et al., 2000; Herdy et al., 2012; Walsh et al., 2013). SV-induced host shut-off has also been demonstrated *in vivo* in the brains of infected mice suggesting that host shut-off occurs in whole animal models of infection as well (Toribio and Ventoso, 2010). Many mechanisms have been described to explain how viruses shut-off host translation and each virus has a different strategy (Gale et al., 2000; Walsh et al., 2013). Polio virus and retroviruses encode proteases that directly cleave eIF4G, an important molecular scaffold for the cap-binding complex eIF4F (Walsh et al., 2013). Many RNA viruses such as EMCV, cricket paralysis virus (CrPV), VSV, Sindbis virus, and Dengue virus induce the accumulation of hypo-phosphorylated 4E-BP1, a negative regulator of eIF4E that is normally inactivated by mTOR-dependent phosphorylation (reviewed in more detail in (Walsh et al., 2013). One important host innate antiviral immune response is the regulation of the ternary complex via the phosphorylation of eIF2α by PKR (Walsh et al., 2013). PKR becomes activated up binding of dsRNA or viral replication intermediates and phosphorylates eIF2α, leading to a global block in host translation initiation (Walsh et al., 2013). As described in previous sections, eukaryotes have three other eIF2α kinases, two of which, GCN2 and PERK, have been suggested to have antiviral roles as well (Walsh et al., 2013). The phosphorylation of eIF2α by PKR, and to a lesser degree GCN2 and PERK, have put pressure on viruses to evolve ways around this host-derived blockade in translation initiation including evolution of viral RNA binding proteins to block access of PKR to dsRNA or virus-mediated dephosphorylation of eIF2α (Walsh et al., 2013). Interestingly, some viruses appear to induce phosphorylation of eIF2α suggesting that, depending on the virus, inhibition of translation by PKR can be beneficial or detrimental to the host (Walsh et al., 2013).

Other reports have described the relationship between viruses and translation inhibition in detail (Walsh et al., 2013). Of interest for this introduction is not just the interaction of viruses, which require the host translational machinery for survival, but the somewhat surprising finding that bacterial and fungal pathogens, as well as parasites, all of which have their own translational machinery, have diverse mechanisms of inducing blockade of host protein synthesis.

### Section 1.4.2: Bacterial toxins target host protein synthesis

Bacterial pathogens have developed a number of ways to modulate host translation ranging from entoxification of host cells via secreted toxins or direct injection, through bacterial secretion systems, of bacterial effector proteins that can modify host translation. *Corynebacterium diphtheriae* encodes a toxin that inhibits host cell translation, diphtheria toxin (DT) (Simon et al., 2014). Discovered in 1888 and shown to cause the characteristics of diphtheria infection, diphtheria toxin was later shown to ADP-ribosylate a modified histidine residue, termed diphthamide, in eEF2 (Simon et al., 2014). Diphtheria toxin is an AB type toxin and enters the cell via recognition of the diphtheria toxin receptor (Deng and Barbieri, 2008; Simon et al., 2014). Upon acidification of the phagosome the B subunit of DT translocates the A subunit into the host cell cytosol where it can at as an ADP-ribose transferase (Deng and Barbieri, 2008). ADP-ribosylation of eEF2 inhibits the function of eEF2 and inhibits host protein synthesis, likely through steric hindrance of eEF2, interference with mRNA positioning, or decreased ribosome stability (Deng and Barbieri, 2008; Simon et al., 2014). Exotoxin A (ExoA) from *Pseudomonas aeruginosa* and cholix toxin (ChxA) from *Vibrio cholerae* are DT-related toxins that also ADP-ribosylate eEF2 and block host protein synthesis (Simon et al., 2014). In the case of diphtheria toxin, translation inhibition is thought to kill epithelial cells and facilitate bacterial colonization in the lung; however, non-toxigenic *C. diphtheria* strains are still capable of colonizing the lung (Deng and Barbieri, 2008). Thus the role of these toxins in pathogenesis is not always clear, but they have been suggested to be important for inhibiting immune responses and increasing bacterial colonization.

**Section 1.4.3: *Legionella pneumophila* secretes effector proteins that block host protein synthesis**

Unlike *C. diphtheria*, the intracellular bacterial pathogen *Legionella pneumophila* does not encode an AB-type toxin to block translation but does induce a strong block in host protein synthesis (Barry et al., 2013; Fontana et al., 2011). *L. pneumophila* is a Gram-negative intracellular bacterial pathogen that is the causative agent of a severe pneumonia called Legionnaires' disease. The normal host of *L. pneumophila* is freshwater amoebae, but *L. pneumophila* can also infect alveolar macrophages in the lung (Copenhaver et al., 2014; Fields, 1996). *L. pneumophila* infection in the lung is a dead end for the bacteria, as the bacteria cannot spread from mammal to mammal, and thus *L. pneumophila* is unable to evolve in response to pressures from the mammalian immune system (Swanson and Hammer, 2000). In the lung, *L. pneumophila* infects alveolar macrophages through passive phagocytosis. Once in the phagosome *L. pneumophila* requires a type IV secretion system (T4SS), called the defect in organelle trafficking/intracellular multiplication (Dot/Icm) system (Berger and Isberg, 1993; Marra et al., 1992), which secretes bacterial effector proteins through the into the host cytosol (Hubber and Roy, 2010; Luo, 2012). These effectors, greater than 270 of which have been identified (Hubber and Roy, 2010; Luo, 2012), are critical for remodeling of the phagosomal membrane and establishment of the *Legionella*-containing vacuole, the specialized membrane-bound intracellular compartment in which *L. pneumophila* replicates (Hubber and Roy, 2010; Luo, 2012). The T4SS and modification of the phagosomal membrane induced by *L. pneumophila* is also required for bacterial growth in its' normal host, freshwater amoebae (Swanson and Hammer, 2000). In addition to its essential role in facilitating bacterial replication, the *L. pneumophila* T4SS is also required to induce a strong block in global host protein synthesis after *L. pneumophila* infection (Barry et al., 2013; Fontana et al., 2011). Five

*L. pneumophila* T4SS-secreted effectors have been identified that block host protein synthesis (Fontana et al., 2011). The five *L. pneumophila* effectors that block host translation include a family of three glucosyltransferases named *Legionella pneumophila* glucosyltransferase 1-3 (Lgt1-3) that glucosylate eukaryotic elongation factor-1A (eEF1A), thus inhibiting the recruitment of aminoacyl-charged tRNAs to the ribosome (Belyi et al.; Belyi et al.; Belyi et al., 2009; Belyi et al.; Hurtado-Guerrero et al., 2010; Lu et al., 2010; Tzivelekidis et al., 2011); the effector SidI, which has been shown to bind directly to eEF1A and eEF1Bγ and block translation, (Shen et al., 2009); and SidL, which is toxic to mammalian cells and is capable of inhibiting protein translation *in vitro* by an unknown mechanism (Fontana et al., 2011). However, a mutant lacking these five effectors still induces residual translation inhibition, and further screening identified two novel bacterial effectors that block host protein synthesis when over-expressed in 293T cells, Pkn5, an effector annotated to have serine/threonine kinase activity and an uncharacterized effector Lpg1489 (Barry et al., 2013). Thus, *L. pneumophila* encodes 7 secreted effectors that are all capable of inhibiting host protein synthesis (Barry et al., 2013; Fontana et al., 2011). Interestingly, infection of bone marrow derived macrophages with a strain of *L. pneumophila* lacking all 7 effectors that block host protein synthesis still induces a strong block in host translation, suggesting the existence of additional bacterial effectors or, more interestingly, pathogen-induced host stress response pathways that block host protein synthesis (Barry et al., 2013).

## Section 1.5: Pathogen-induced modulation of host stress response pathways and translation

As described above, pathogens have evolved mechanisms to block host protein synthesis directly. As will be described in more detail below, translation inhibition appears to be a novel pathogen-associated activity and has been shown to play important roles in responding to pathogens. While some pathogens directly block host protein synthesis, it has been shown that many pathogenic infections have been linked to the induction of host stress response pathways (Ivanov and Roy, 2013; Janssens et al., 2014; Lemaitre and Girardin, 2013; Mohr and Sonenberg, 2012), which could indirectly induce a block in host protein synthesis. Here I will discuss our current understanding of the interactions between host stress response pathways, induction of innate immune responses, and microbial pathogenesis. A number of studies have delved into stress responses following infection by a number of pathogens and this review will go through a number of pathogens in more detail.

### Section 1.5.1: Pattern recognition receptor signaling and host stress response pathways

TLR signaling by purified ligands has been suggested to induce host stress response pathways; however, other studies suggest that TLR signaling inhibits host stress response pathways. Thus, there is a need to further characterize the link between TLR activation and host stress response pathways (reviewed in (Janssens et al., 2014). Woo *et al.* propose a model in which TLR signaling suppresses the UPR induced by chemical activators, such as tunicamycin, by inducing dephosphorylation of eIF2Bε, which increases the GEF activity of eIF2B (Woo et al., 2009; Woo et al., 2012). Increased eIF2B activity inhibits of the up-regulation of ATF4 and CHOP, readouts of the UPR (Woo et al., 2009; Woo et al., 2012). Importantly, TLR signaling does not suppress activation of PERK or phosphorylation of eIF2α after tunicamycin and instead

regulation appears to occur after this step (Woo et al., 2009; Woo et al., 2012). The functional relevance of this finding is unclear, as Woo *et al.* show that TLR activation alone does not induce ER stress (Woo et al., 2009; Woo et al., 2012). Another study suggested that TLR signaling induces an increase in *XBP1* splicing, a downstream readout of ER stress and the unfolded protein response, in the absence of the UPR and ER stress (Martinon et al., 2010). TLR-induced splicing of *XBP1* was shown to increase XBP1 protein levels, and XBP1 was suggested to be required for full cytokine production after TLR activation (Martinon et al., 2010). In discordance with the previously mentioned studies another group has suggested TLR4 signaling induces increased levels of ATF4 and suggest that ATF4 plays a role in cytokine production after TLR4 activation (Zhang et al., 2013). Furthermore, another study has suggested that TLR4 signaling induces ER stress through a mechanism in which insufficient ER chaperones are induced after TLR signaling and this insufficiency of chaperones leads to ER stress (Coope et al., 2012). Thus, the role of PRR signaling in directly modulating host ER stress has not reached a consensus. One major concern is that all of these studies were performed in immortalized cell lines (Coope et al., 2012; Martinon et al., 2010; Woo et al., 2009; Woo et al., 2012; Zhang et al., 2013) and immortalized cell lines are known to have non-canonical stress responses as well as disregulated metabolic pathways (Benjamin et al., 2012; Wu and Zhao, 2013). Further studies are required to determine the role of TLR and other PRR signaling pathways in host stress responses in more relevant primary cell types.

### Section 1.5.2: *Pseudomonas entomophila*

*P. entomophila* is a bacterial pathogen that can orally infect and kill *Drosophila* through induction of irreversible damage to the gut (Liehl et al., 2006; Vodovar et al., 2005). *P. entomophila* infections induce a global block in host protein synthesis in the gut of infected flies that is at least partially dependent on a *P. entomophila* pore-forming toxin, Monalysin (Chakrabarti et al., 2012). *P. entomophila* infection was further shown to induce phosphorylation of eIF2α in a GCN2-dependent manner as well as inhibit the mTOR signaling cascade. Thus at least two arms of host stress response are involved in the *P. entomophila* induced translation inhibition (Chakrabarti et al., 2012). Translation inhibition caused by activation of GCN2 and inactivation of the mTOR signaling pathway were shown to be detrimental to protecting the host from *P. entomophila* infection, likely through inhibition of proper gut repair mechanisms (Chakrabarti et al., 2012).

### Section 1.5.3: *Streptococcus pyogenes*

*Streptococcus pyogenes* (Group A *Streptococcus*) is a strict human pathogen that typically infects the throat and the skin of the host and can cause a range of disease severity (Baruch et al., 2014b; Baruch et al., 2014c). Group A *Streptococcus* (GAS) is an extracellular bacterial pathogen that requires a number of virulence factors that allow the bacterium to adhere to host cells, promote immune responses, and disseminate (Baruch et al., 2014b; Baruch et al., 2014c). Two of these virulence factors are the pore forming toxins streptolysin O (SLO) and streptolysin S (SLS; (Baruch et al., 2014b; Baruch et al., 2014c). Recent papers have shown that, through an unknown mechanism, SLO and SLS induce ER stress in host cells leading to increased expression of asparagine synthetase which leads to increased release of asparagine from the host cell (Baruch et al., 2014a). Increased release of asparagine from host cells is

sensed by the bacteria and induces transcriptional responses and increased bacterial multiplication (Baruch et al., 2014a). This study proposed a model in which human pathogens manipulate host stress response pathways to gain nutrients without inflicting irreversible cell damage. Consistent with *Streptococcus pyogenes* inducing a host stress response, a previous study showed that treatment with SLO alone induces the activation GCN2, suggesting that SLO may also induce amino acid starvation in host cells (Kloft et al., 2010).

### Section 1.5.4: *Shigella flexneri*

*Shigella flexneri* is an enteropathogenic subspecies of *Escherichia coli* that is a leading cause of dysentery in developing countries (Carayol and Tran Van Nhieu, 2013a, b). Upon ingestion, *Shigella flexneri* invades colonic epithelial cells where it lyses the phagocytic vacuole and escapes to the cytosol (Carayol and Tran Van Nhieu, 2013a, b). *Shigella flexneri* replicates in the cytosol and uses actin-based motility to spread from cell to cell (Carayol and Tran Van Nhieu, 2013a, b). Infection of HeLa cells with *Shigella flexneri* induces amino acid starvation and stress responses (Tattoli et al., 2012b). Following *Shigella flexneri* infection, amino acid starvation led to hypo-phosphorylation of 4E-BP1 and ribosomal protein S6, as well as cytosolic dispersion of mTOR, all signs of decreased mTOR activity (Tattoli et al., 2012b). *Shigella flexneri* infection also increased GCN2 and eIF2α phosphorylation (Tattoli et al., 2012b). Amino acid starvation by *Shigella flexneri* was suggested to be caused by membrane damage as accumulation of NDP52, a dynamic marker of membrane damage, could be seen during *Shigella flexneri* infection and amino acid starvation could be induced by sterile membrane damage induced by digitonin (Tattoli et al., 2012b). Prolonged inhibition of mTOR was shown to correlate with increased xenophagy of *Shigella flexneri* and was suggested to negatively regulate bacterial growth (Tattoli et al., 2012b). A later study using phosphoproteomics of *Shigella flexneri* infected HeLa cells demonstrated that the mTOR signaling pathway was the most over-represented signaling pathway in the phosphoproteome of *Shigella flexneri* infected cells; however, contradictory to previous reports, this study showed that *Shigella* infection induced mTOR-dependent phosphorylation of rpS6 15 minutes post-infection (Schmutz et al., 2013). Clearly, *Shigella flexneri* infection can induce changes to host stress response pathways; however, further studies are needed to confirm the changes in mTOR activity following infection.

### Section 1.5.5: *Salmonella enterica* serovar Typhimurium

*Salmonella enterica* serovar Typhimurium is an intracellular Gram-negative bacterial human pathogen that infects the intestinal tract. *Salmonella* Typhimurium encodes two bacterial Type-III secretion systems (T3SS) one encoded by the *Salmonella* pathogenicity island 1 (SPI-1), which is required for entry into non-myeloid cells and formation of the *Salmonella* containing vacuole (SCV), and the other encoded by the SPI-2 locus, which is required for maturation of the SCV (Ramsden et al., 2007a; Ramsden et al., 2007b; Steele-Mortimer, 2008). In a SPI-1-dependent manner, *Salmonella* Typhimurium infection also induces amino acid starvation in host cells and leads to inhibition of mTOR, decreased rpS6 phosphorylation, hypo-phosphorylation of 4E-BP1, activation of GCN2 and subsequent inhibition of eIF2α via phosphorylation (Tattoli et al., 2012a; Tattoli et al., 2012b). Similar to *Shigella flexneri* infection, *Salmonella* Typhimurium induced amino acid starvation was correlated with membrane damage; however, this damage

was only temporary and amino acid levels rapidly normalized leading to normalization of mTOR activity (Tattoli et al., 2012a; Tattoli et al., 2012b). Importantly, and unlike *Shigella flexneri*, the release of mTOR inhibition protected *Salmonella* Typhimurium from autophagy and treatment of *Salmonella*-infected cells with inhibitors of mTOR reduced bacterial burden (Tattoli et al., 2012a; Tattoli et al., 2012b). Thus, these studies suggest that there can be a complicated interplay between bacterial induction of host stress response pathways and bacterial pathogenesis.

**Section 1.5.6: *Listeria monocytogenes***

*Listeria monocytogenes* is a Gram-positive facultative intracellular pathogen that can lead to severe food-borne infections (Portnoy et al., 2002). *Listeria monocytogenes* can infect nearly any adherent cell type including macrophages, dendritic cells, fibroblasts, epithelial, and endothelial cells (Portnoy et al., 2002). Upon phagocytosis by a macrophage *L. monocytogenes* uses the pore-forming toxin listeriolysin O (LLO) and two phospholipase Cs (PLCs) to escape the vacuole (Portnoy et al., 2002). *L. monocytogenes* grows in the cytosol and uses actin-based motility for cell-to-cell spread (Portnoy et al., 2002). *L. monocytogenes* infection has also been shown to induce phosphorylation of eIF2α (Pillich et al., 2012; Shrestha et al., 2012; Tattoli et al., 2013). In addition to induction of eIF2α phosphorylation, wildtype *L. monocytogenes* induces phosphorylation of the amino acid responsive kinase GCN2; again suggesting infection by *L. monocytogenes* induces amino acid starvation (Tattoli et al., 2013). Similar to *Salmonella* Typhimurium infection, *L. monocytogenes* induced a block in mTOR signaling as levels of phosphorylated S6K1 decreased immediately after infection (Tattoli et al., 2013). Also similar to *Salmonella* Typhimurium the apparent amino acid starvation, block in mTOR signaling, as well as eIF2α and GCN2 phosphorylation was transient and depended on the presence of LLO (Tattoli et al., 2013). Amino acid starvation induced by LLO was neither necessary nor sufficient to explain the capacity of *Listeria monocytogenes* to escape autophagy at late timepoints (Tattoli et al., 2013). Phosphorylation of eIF2α may also play a role in *L. monocytogenes* cell invasion as the number of intracellular bacteria recovered at 1hr post-infection from MEFs expressing a mutant eIF2α that cannot be phosphorylated, eIF2α (S51A), was five-fold higher than WT MEFs (Shrestha et al., 2012). Similar to *Listeria monocytogenes* infection, infection with *Yersinia pseudotuberculosis* also induced phosphorylation of eIF2α (Shrestha et al., 2012). Interestingly, a *Y. pseudotuberculosis* virulence factor, YopJ, appears to inhibit eIF2α phosphorylation as infection with a Δ*yopj* mutant has increased levels of phosphorylated eIF2α (Shrestha et al., 2012). Further, inhibition of the eIF2α-dependent stress response by replacing eIF2α with the non-phosphorylatable S51A eIF2α mutant may also inhibit invasion of cells by *Yersinia pseudotuberculosis* and *Chlamydia trachomatis* (Shrestha et al., 2012).

**Section 1.5.7: *Legionella pneumophila***

As described previously, *Legionella pneumophila* encodes 7 secreted effector proteins that directly block host protein synthesis; however, strains lacking all 7 of these effector proteins still induce translation inhibition in host cells (Barry et al., 2013). A recent study has suggested that in addition to the 7 effector driven block in translation, *L. pneumophila* may inhibit the mTOR signaling pathway and that this inhibition drives blockade of cap-dependent translation

(Ivanov and Roy, 2013). It was proposed that this cap-dependent block in host translation could explain some of the *L. pneumophila* induced block in host translation (Ivanov and Roy, 2013). Further, this study suggested that inhibition of mTOR by wildtype *L. pneumophila* infection results in translational biasing to production of proinflammatory cytokines (Ivanov and Roy, 2013). However, the relevance of the mTOR inhibition by WT *L. pneumophila* for bacterial pathogenesis and induction of translation inhibition remains to be seen as changes in mTOR signaling can only be detected in specific conditions (e.g. serum starvation) while the residual block in host protein synthesis seen in Δ*7 L. pneumophila* is quite robust and can be seen in nutrient replete conditions (Barry et al., 2013).

### Section 1.5.8: Modulation of host stress pathways and translation by parasites

*Leishmania* is a genus of trypanosomatid protazoa that are of significant medical and veterinary importance (Cecilio et al., 2014). These protozoa have a complex life cycle but are known to replicate inside mammalian macrophages (Cecilio et al., 2014). An important virulence factor of *Leishmania* is the surface protein GP63, which mediates parasite evasion of complement-mediated lysis by cleaving C3b. GP63 is involved in parasite binding and phagocytosis by macrophages (Cecilio et al., 2014; Gomez et al., 2009; Halle et al., 2009). Cleavage and activation of three host phosphatases: SHP-1, PTP1B, and TCPTP, is also dependent on GP63 and this activity contributes to the progression of cutaneous leshmaniasis (Cecilio et al., 2014; Gomez et al., 2009; Halle et al., 2009). GP63 has also been shown to be involved in *Leishmania major* induced blockade of global protein synthesis (Jaramillo et al., 2011). GP63 was shown to directly cleave mTOR and thus induce hypo-phosphorylation and activation of 4E-BP1 (Jaramillo et al., 2011). Interestingly, *Leishmania major* growth in macrophages depends on the upstream kinases S6K1 and S6K2 as well as 4E-BP1 and 4E-BP2 as 4E-BP1/2 double deficient or S6K1/2 double deficient macrophages harbor reduced parasite replication (Jaramillo et al., 2011). Further, animals deficient in 4E-BP1 and 4E-BP2 are protected from leishmaniasis (Jaramillo et al., 2011). Results from Jaramillo *et al.* suggest that, at least in part, the 4E-BP-dependent restriction of *Leishmania major* is due to increased immune responses to *Leishmania*, specifically, increased production of type-I interferons which induce nitric oxide production that is important for killing the parasites (Jaramillo et al., 2011). Unlike *Leishmania major* infection, another report shows that the parasite *Toxoplasma gondii* increases rpS6 phosphorylation (Wang et al., 2009). However, the mechanism of increase rpS6 phosphorylation was shown to be independent of the mTOR signaling pathway and S6K1 (Wang et al., 2009). Further, there was no clear evidence that the increased phosphorylation of rpS6 after *Toxoplasma gondii* infection had any role in changing global translation rates within infected cells (Wang et al., 2009).

## Section 1.6: Translation inhibition as a pattern of pathogenesis

Clearly there are many mechanisms by which pathogenic microbes can induce modulations of host stress response pathways and/or a block in host protein synthesis. There are a number of intriguing questions that remain in regard to the consequences of pathogen-induced blockade of host protein synthesis. First and foremost, is translation inhibition generally beneficial to the host or does it favor the pathogen? Further, given that this activity seems to be conserved among many different pathogens, including pathogens that encode their own translation machinery such as bacteria and parasites, this activity seems likely to provide an

advantage for the pathogen – but what are these advantages? The field has proposed a number of models to address these questions. It appears that pathogens and their hosts have each adapted to pathogen-induced inhibition of host protein synthesis for many reasons and translation inhibition has now clearly been shown to induce immune responses preferentially to pathogens.

**Section 1.6.1: Translation inhibition and the immune response to pathogens**

*L. pneumophila* requires a T4SS for virulence. The *L. pneumophila* T4SS is also required for the induction of many host inflammatory responses to *L. pneumophila* infection (Hubber and Roy, 2010; Luo, 2012; Shin et al., 2008). *L. pneumophila* lacking the T4SS are still recognized by Toll-like receptors (TLRs), leading to canonical NF-κB and MAPK signaling; however, only virulent *L. pneumophila* induces a number of inflammatory genes such as *Il23a* and *Csf2* (Fontana et al., 2011). Previously we showed that the induction of *Il23a and Csf2* required 5 bacterial effectors that block host protein synthesis (Fontana et al., 2011) and that *Myd88/Nod1/Nod2*$^{-/-}$ macrophages infected with a *L. pneumophila* strain lacking these effectors exhibit diminished MAP kinase activation (Fontana et al., 2012). In our previous study we found that *L. pneumophila* infection activated TLR signaling and led to the degradation of the labile inhibitor of NF-κB, IκBα (Fontana et al., 2011). IκBα degradation allowed for the translocation of NF-κB to the nucleus where it drove transcription of target genes including *Il23a*, *Csf2*, and the gene encoding IκBα, *Nfkbia*. In the absence of translation inhibition (e.g. T4SS$^{-}$ *L. pneumophila* infection) IκBα is re-synthesized and acts as a negative feedback on NF-κB signaling (Fontana et al., 2011). In the presence of T4SS$^{+}$ *L. pneumophila* induced translation inhibition IκBα is not reformed, leading to super-induction of NF-κB signaling and increased mRNA levels of *Csf2* and *Il23a* (Fontana et al., 2011). We proposed that the large increase in cytokine transcripts overcame the global block in translation induced by *L. pneumophila* and allowed for the specific production of inflammatory cytokines in response to virulent infection (Fontana et al., 2011; Fontana and Vance, 2011). In a later study, I showed, similar to IL-23 and GM-CSF, that IL-1α, which was shown to have important effects in recruiting neutrophils to the lungs of *L. pneumophila* infected mice, can also be induced by a similar mechanism that requires TLR stimulation in the presence of translation inhibition (Barry et al., 2013). These studies suggested that translation inhibition could be sensed in concert with PRR recognition to provide the contextual cue to PRR signaling that a pathogen was present and to induce inflammatory responses important for protecting the host *in vivo* (Barry et al., 2013; Fontana et al., 2011; Fontana and Vance, 2011). In addition to the effector driven block in translation (Barry et al., 2013; Fontana et al., 2011), I found that *L. pneumophila* induces translation inhibition independently of these effectors (Barry et al., 2013). A recent study suggested that *L. pneumophila* may inhibit the mTOR signaling pathway and that this inhibition drives blockade of cap-dependent translation and may explain some of the *L. pneumophila* induced block in host translation (Ivanov and Roy, 2013). Further, this study suggested that inhibition of mTOR by wildtype *L. pneumophila* infection results in translational biasing to production of proinflammatory cytokines (Ivanov and Roy, 2013). However, the relevance of the mTOR inhibition by WT *L. pneumophila* for bacterial pathogenesis and induction of translation inhibition remains to be seen as changes in mTOR signaling can only be detected when cells are serum starved while the residual block in host protein synthesis seen in Δ*7 L. pneumophila* occurs in the presence of serum and is quite robust (Barry et al., 2013).

An alternative model for IL-1α and IL-1β production was proposed by Asrat and colleagues, who suggested that translation of IL-1α and IL-1β occurred in the presence of translation inhibition induced by *L. pneumophila* because, in a MyD88-dependent manner, ribosomes preferentially bind to and translate the *Il1a* and *Il1b* transcripts (Asrat et al., 2014). The authors suggest that *Myd88*$^{-/-}$ macrophages infected with WT *L. pneumophila* induce similar *Il1a* and *Il1b* transcript levels to B6 macrophages but do not produce IL-1α and IL-1β protein (Asrat et al., 2014); the authors suggest that these findings demonstrate that MyD88 is playing a role in selectively translating these transcripts (Asrat et al., 2014). However, previous studies have shown that transcriptional induction of *Il1a* and *Il1b* transcript levels are significantly reduced in *Myd88*$^{-/-}$ macrophages infected with WT *L. pneumophila* at 4hrs post-infection (Shin et al., 2008). Thus an alternative hypothesis, consistent with the previously published model of cytokine production in the presence of translation inhibition (Barry et al., 2013; Fontana et al., 2011; Fontana and Vance, 2011), is that *Myd88*$^{-/-}$ macrophages have defects in *Il1a* and *Il1b* transcript levels and thus are less able to overcome the block in protein synthesis inhibition. Clearly, more studies are needed to look at global transcriptional and translational responses to *L. pneumophila* infection to resolve this discrepancy. Taken together, these findings suggest that the innate immune system can integrate two signals, PRR stimulation and translation inhibition, to provide the information needed to preferentially induce inflammatory responses to pathogens.

A similar host-response to translation inhibition has been described in *Pseudomonas aeruginosa* infection of the nematode *Caenorhabditis elegans* (Dunbar et al., 2012; McEwan et al., 2012). *P. aeruginosa* causes a lethal intestinal infection in *C. elegans* and recent studies show that the bacterial toxin, exotoxin A (ExoA), which ADP-ribosylates and inactivates the eukaryotic elongation factor eEF2, induces a distinct anti-microbial transcriptional response (McEwan et al., 2012). In response to *P. aeruginosa* infection or chemical inhibitors of translation elongation (e.g. cycloheximide or hygromycin), the authors demonstrated that translation inhibition could be sensed by *C. elegans* to induce increased protein levels of the transcription factor ZIP-2 (Dunbar et al., 2012; McEwan et al., 2012). ZIP-2 was then shown to be important for the upregulation of the infection response gene-1 (*irg-1*) (Dunbar et al., 2012; McEwan et al., 2012) and, at least partially, resistance to ExoA toxicity (McEwan et al., 2012). Regulation of ZIP-2 protein levels was shown to be independent of *zip-2* mRNA levels and the authors identified an upstream open reading frame (uORF) in *zip-2* mRNA that they suggest could explain the increase in ZIP-2 protein in response to translation inhibition (Dunbar et al., 2012). However, ExoA and the chemical inhibitors of translation used in these studies block host translation elongation and the classical genes regulated by uORFs, mammalian *Atf4* for example, are upregulated in response to translation initiation blockade via the phosphorylation of eIF2α (Sonenberg and Hinnebusch, 2009). Thus, the mechanism by which ZIP-2 protein is upregulated in response to inhibition of translation elongation remains unclear and more work will be necessary to elucidate this. However, these studies have clearly demonstrated that similar to *L. pneumophila* infections in mammals, *Pseudomonas aeruginosa* infection in *C. elegans* induces translation inhibition in the worm intestine that is sensed by the host to induce a protective immune response (Dunbar et al., 2012; McEwan et al., 2012).

Links between translation inhibition and the immune have also been described in *Drosophila* (Chakrabarti et al., 2012). *Pseudomonas entomophila* is a bacterial pathogen of *Drosophila* that causes a lethal infection and was shown to block host translation in a manner that is, at least partially, dependent on a pore-forming toxin called monalysin (Chakrabarti et al., 2012). Infection with *P. entomophila* induces a block in host protein synthesis through

activation of the kinase GCN2 and inactivation of the mTOR signaling pathway (Chakrabarti et al., 2012). *P. entomophila* infection induces a systemic immune response and the authors demonstrated that infection with a non-lethal pathogen *Erwinia carotovora* in the presence of chemical compounds that inhibit protein synthesis could recapitulate the systemic immune response seen after *P. entomophila* infection, as read out by the production of antimicrobial peptides in the fat body (Chakrabarti et al., 2012). The others conclude that bacterial infection in the presence of translation inhibition can lead to systemic immune responses *in vivo* (Chakrabarti et al., 2012).

A number of reports have also shown that induction of host stress response pathways by pathogens, which presumably lead to a block in host protein synthesis, may be an important mechanism to induce autophagic killing of intracellular pathogens (Tattoli et al., 2012a; Tattoli et al., 2012b; Tattoli et al., 2013). These studies suggest that translation inhibition induced directly by bacterial effectors or indirectly by pathogen-dependent host stress responses provide a contextual cue to the innate immune system alerting the cell that it is infected with a pathogen. The pathogen-induced blockade in host protein synthesis allows the infected cell to induce an appropriate inflammatory immune response specifically to pathogens. The host has clearly adapted to this broadly conserved pathogen-associated activity and appears to use this as a signal to induce an inflammatory response. This model suggests that translation inhibition induced by bacterial pathogens, at least in certain circumstances, could be beneficial to the host; if this is true, why do pathogens block host protein synthesis?

## Section 1.7: Why do pathogens block host protein synthesis?

Defining a single overarching purpose for pathogen induced translation inhibition is probably not possible as there are a broad range of pathogens that can block host translation that have likely adapted this activity for many different reasons. However, there may be a number of general reasons why pathogens might need to block host protein synthesis. It is formally possible that a pathogen may not want to induce a block in host protein synthesis. As has already been discussed, pathogen-induced translation inhibition can be sensed by the host to induce an immune response (Barry et al., 2013; Chakrabarti et al., 2012; Dunbar et al., 2012; Fontana et al., 2011; Fontana et al., 2012; McEwan et al., 2012) and thus it may be detrimental to that pathogen to block translation. However, given the broad range of pathogens that induce translation inhibition it seems likely that there is an evolutionary benefit to the pathogen for this activity. The induction of an immune response by the host in response to translation inhibition may be an adaptation by the mammalian immune system to respond to an activity that is important for pathogenesis.

### Section 1.7.1: Translation inhibition may increase nutrients required for pathogenesis

The intracellular bacterial pathogen *L. pneumophila* has long been known to harbor a number of amino acid auxotrophies and has been shown to favor the usage of amino acids as a primary carbon source (Fonseca and Swanson, 2014). A recent study definitively showed that in amoebae, the natural host for *L. pneumophila*, host amino acids are taken into the *Legionella* containing vacuole where the bacteria grow, and are incorporated into bacterial proteins (Schunder et al., 2014). It has also been shown that host amino acid transporters are required for bacterial growth in human MM6-monocytes (Wieland et al., 2005). The requirement of amino acids for *L. pneumophila* growth has been well reported and there are a number key findings

suggesting that the host amino acids are required for *L. pneumophila* growth in cells (Fonseca and Swanson, 2014). It has been suggested that *L. pneumophila* T4SS-effector protein AnkB can induce the proteosomal degradation of host proteins to increase the amount of free amino acids that *L. pneumophila* can utilize for growth (Price et al., 2011). However, the role of this effector is debated (Z-Q Luo, personal communication). While the role of AnkB in release of amino acids to *L. pneumophila* is controversial, the requirement of amino acids for *L. pneumophila* growth is established (Fonseca and Swanson, 2014). Given the need for exogenous amino acids by *L. pneumophila,* it is intriguing to hypothesize that translation inhibition induced by the pathogen increases free amino acids in the cell that it can use for replication. We favor the hypothesis that translation inhibition in mammalian cells increases energy sources for *L. pneumophila*, as this pathogen is an accidental pathogen in mammals and cannot transmit from mammal to mammal (Swanson and Hammer, 2000). Thus, *L. pneumophila* it is unlikely to have evolved translation inhibition to evade the host immune response. While this is an intriguing hypothesis, it cannot be ruled out that freshwater amoebae may have some conserved innate immune responses that *L. pneumophila* has evolved to evade.

While investigating the conditions under which the quorum-sensing locus *sil* is activated, a group discovered a novel mechanism that would explain why *Streptococcus pyogenes* might induce the UPR and translation inhibition (Baruch et al., 2014a). Interestingly, the authors found that upon adherence to the host cell, *Streptococcus* delivers streptolysin O (SLO) to the cell, triggering ER stress (Baruch et al., 2014a). ER stress induces the transcription of asparagine synthetase via the PERK-eIF2α-ATF4 pathway, which leads to the release of asparagine from the cell (Baruch et al., 2014a). Asparagine is sensed by *Streptococcus* leading to reduced transcription of SLO and increased bacterial proliferation (Baruch et al., 2014a). Consistent with this pathway being important for pathogenesis, asparaginase, a widely used chemotherapeutic agent, arrests *Streptococcus* growth in human blood and a mouse model of *Streptococcus* infection (Baruch et al., 2014a). Thus, this study suggests that modulation of the UPR and induction of translation inhibition by the pathogen *Streptococcus* may be an important way in which the bacterium regulates growth within the host and could be important for pathogenesis.

**Section 1.7.2: Translation inhibition may inhibit host immune responses**

The protozoan parasite *Leishmania major* was shown to induce a strong block in host protein synthesis by the direct cleavage of mTOR by the parasite protease GP63 (Jaramillo et al., 2011). Interestingly, deletion of *Eif4ebp1* and *Eif4eb2*, the genes that encode 4E-BP1 and 4E-BP2, inhibited *L. major* growth while deletion of *Rps6kb1* and *Rps6kb2*, the genes that encode S6K1 and S6K2, had no effect on intracellular growth, suggesting that 4E-BP1/2 mediated translation inhibition was the major driver of parasite growth (Jaramillo et al., 2011). Furthermore, in a mouse model of cutaneous leishmaniasis, animals doubly deficient in *Eif4bp1* and *Eif4bp2* showed decreased disease severity after *L. major* infection (Jaramillo et al., 2011). Interestingly, the authors were able to link the 4E-BP1/2-dependent block in host protein synthesis to inhibition of immune responses against *L. major* (Jaramillo et al., 2011). An important step in controlling cutaneous leishmaniasis progression is the confinement of the parasite to the site of infection and the popliteal draining lymph node (Laskay et al., 1995) where type I IFN and inducible nitric oxide synthase (iNOS) are locally produced to eliminate the parasite *in vivo* (Diefenbach et al., 1998; Mattner et al., 2000; Mattner et al., 2004; Stenger et al., 1994). Mice and BMDMs deficient in both *Rps6kb1* and *Rps6kb2* had increased *Ifnb* and *Nos2*

transcript levels and when treated with a type I IFN blocking antibody, double deficient macrophages were as susceptible to *L. major* infection as WT BMDMs (Jaramillo et al., 2011). Overall, this study suggests a mechanism by which the *L. major* protease GP63 cleaves mTOR, leading to increased 4E-BP1/2 activity and a block in host protein synthesis (Jaramillo et al., 2011). The block in host protein synthesis inhibits the production of type I IFNs and the subsequent induction of nitric oxide, allowing the parasite to evade the immune response and grow (Jaramillo et al., 2011).

*Pseudomonas entomophila* infection in the *Drosophila* gut was also shown to inhibit host protein synthesis (Chakrabarti et al., 2012). In the gut, *P. entomophila* infection was shown to transcriptionally induce *Diptericin*, an anti-bacterial immune response gene, but translation inhibition in the gut was shown to inhibit local production of Diptericin protein as well as to inhibit epithelial repair mechanisms (Chakrabarti et al., 2012). These findings suggest that translation inhibition induced by *P. entomophila* may be a way in which the pathogen interferes with local immune and repair responses; however, this model is complicated by the fact that translation inhibition leads to a massive systemic immune response that leads to death of the infected fly (Chakrabarti et al., 2012).

There is some evidence to suggest that ExoA from *Pseudomonas aeruginosa* may inhibit immune responses, such as the upregulation of IFNγ induced co-stimulatory molecules (Michalkiewicz et al., 1999). However, more recent studies have shown that ExoA can lead to the induction of immune responses (Dunbar et al., 2012; McEwan et al., 2012). The role of diphtheria toxin in *Corynebacterim diphtheriae* infection is also unclear as nontoxiogenic strains are equally capable of colonizing the airway (Deng and Barbieri, 2008).

## Section 1.8: Conclusion and final comments

Translation inhibition is now appreciated as a pathogen-associated activity. There is clear evidence that translation inhibition can be recognized by the host immune system to induce immune responses. We can hypothesize that pathogens may have evolved this activity to increase nutrients for the pathogen or to block immune responses. What seems clear is that translation inhibition is an activity that is important for the pathogenic lifestyle of a diverse range of pathogens, from viruses to parasites. Even more interesting, it appears that the host innate immune system has evolved to detect this activity, again suggesting that this is an important activity required for pathogens to survive. Pathogen-induced translation inhibition and pathogen-induced changes in host stress response pathways are interrelated. Normal stress responses lead to translation inhibition and any step in the host stress response pathway could potentially be important for a pathogen to survive in the host. For example pathogens have been shown to modulate stress response pathways, such as mTOR, in order to circumvent host autophagy. It is becoming increasingly clear that pathogenic infection not only induce immune responses through PRRs but also through pathogen-induced host cellular stress responses and translation inhibition. We favor a model where pathogen-associated activities, such as host translation inhibition, provide the contextual cue to PRR signaling to induce inflammatory responses preferentially to pathogens. While more studies will refine the findings described above, manipulation of host translation and stress response pathways seem to be integral parts of the immune response to pathogens and should inform the design of future treatments in the clinic.

# Chapter 2: Interleukin-1α signaling initiates the inflammatory response to virulent *Legionella pneumophila in vivo*

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## Section 2.1: Introduction

*Legionella pneumophila* is a gram-negative intracellular bacterial pathogen that is the causative agent of a severe pneumonia called Legionnaires' disease. After inhalation of aerosolized bacteria, *L. pneumophila* can infect and replicate within lung alveolar macrophages. Intracellular replication of *L. pneumophila* in macrophages *in vitro,* and virulence of *L. pneumophila* in animal models, requires a Type IV secretion system (T4SS) called the Dot/Icm system, which secretes bacterial effector proteins into the host cytosol. These effectors, greater than 270 of which have been identified (reviewed in (Luo, 2012), are believed to be critical for establishment of the *Legionella*-containing vacuole, the specialized membrane-bound intracellular compartment in which *L. pneumophila* replicates. In addition to its essential role in facilitating intracellular bacterial replication, the *L. pneumophila* T4SS is also associated with the induction of several potent innate immune responses (reviewed in (Fontana and Vance, 2011).

Legionnaires' Disease is characterized by robust infiltration of neutrophils and other immune cells into the lungs (Trisolini et al., 2004; Winn and Myerowitz, 1981; Yu et al., 2009). Mice depleted of neutrophils exhibit an increased burden of *L. pneumophila* in the lungs (LeibundGut-Landmann et al., 2011; Sporri et al., 2008; Tateda et al., 2001a; Tateda et al., 2001b). Furthermore, *in vivo* blockade of the CXCR2 chemokine receptor reduces the number of neutrophils recruited to the lungs of *L. pneumophila* infected mice and increases the lethality of *L. pneumophila* infection (Tateda et al., 2001b). Despite the clear protective role of neutrophils in *L. pneumophila* infections, it is also believed that excessive neutrophil influx may be responsible for much of the pathology associated with Legionnaires' Disease (Winn and Myerowitz, 1981; Yu et al., 2009). Thus, infected hosts require mechanisms to carefully regulate the influx of neutrophils into tissues such that sufficient neutrophils are recruited to mediate pathogen clearance without causing excessive immune pathology. Despite the central role of neutrophils in Legionnaires' Disease, the mechanisms controlling neutrophil recruitment to the lung in response to *L. pneumophila* remain poorly understood.

Previous work has established that neutrophil recruitment to the lung in response to *L. pneumophila* requires bacterial expression of the Dot/Icm T4SS (LeibundGut-Landmann et al., 2011). In addition, the interleukin-1 receptor type I (IL-1R), and its downstream signaling adaptor protein, MyD88, are also required (Archer et al., 2010; Archer et al., 2009; Archer and Roy, 2006; Hawn et al., 2006; LeibundGut-Landmann et al., 2011). Toll-like receptors (TLRs), which also utilize the MyD88 signaling adaptor, appear to only have a modest role in neutrophil recruitment to the lung (Archer et al., 2010; Archer et al., 2009; Archer and Roy, 2006; Fuse et al., 2007), suggesting that IL-1R signaling is the main pathway leading to neutrophil recruitment to the lung *in vivo*. Two related cytokines, interleukin-1α (IL-1α) and interleukin-1β (IL-1β), can both signal through the IL-1R. A previous study suggested that IL-1β is critical for neutrophil recruitment in response to *L. pneumophila* (LeibundGut-Landmann et al., 2011). It was

proposed that infected macrophages generate IL-1β that signals through the IL-1R expressed by airway epithelial cells (AECs). IL-1R signaling in AECs amplifies the initial IL-1β signal by triggering the production of chemokines, such as CXCL1 and CXCL2, which stimulate the rapid and robust recruitment of neutrophils to the lung (LeibundGut-Landmann et al., 2011). However, no study has specifically addressed a possible role for IL-1α in mediating IL-1R-dependent neutrophil recruitment *in vivo*, and consequently, the relative role of IL-1α and IL-1β in responses to *L. pneumophila* remains uncertain.

Both IL-1α and IL-1β lack classical signal peptides to target the proteins to the conventional secretory pathway, and the mechanism of their release from cells remains poorly understood. Production of IL-1β appears to require two steps. First, activation of the NF-kB transcription factor results in transcription of *Il1b* mRNA, which is then translated into pro-IL-1β protein. Release of mature IL-1β has then been shown, in most instances, to require the Caspase-1 protease, which cleaves and activates IL-1β into its biologically active form (Cerretti et al., 1992; Thornberry et al., 1992). Caspase-1 is itself activated within multiprotein complexes called 'inflammasomes' (Martinon et al., 2002; von Moltke et al., 2012a). *L. pneumophila* has been shown to stimulate IL-1β release primarily via the NAIP5/NLRC4 inflammasome that senses bacterial flagellin that is translocated into the host cell cytosol via the Dot/Icm T4SS (Amer et al., 2006; Lightfield et al., 2008; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006).

In contrast to IL-1β, IL-1α does not require proteolytic processing by Caspase-1 in order to be biologically active (Gross et al., 2012; Mosley et al., 1987). Nevertheless, in certain instances, inflammasome activation can promote the extracellular release of IL-1α, perhaps as a result of inflammasome-induced cell death (Fettelschoss et al., 2011; Gross et al., 2012; Keller et al., 2008). However, it is still unclear if the inflammasome is required for IL-1α production in response to bacterial infections *in vivo*. Similar to *Il1b*, the *Il1a* gene can be transcriptionally induced by infection, but IL-1α may also be constitutively expressed in certain cell types (Dinarello, 2009; Gross et al., 2012). Virulent (T4SS$^{+}$) *L. pneumophila* has been shown to induce IL-1α production by macrophages *in vitro* as well as in lung infections *in vivo* (Shin et al., 2008). In contrast, Δ*dotA L. pneumophila* mutants, which lack an active T4SS, do not induce IL-1α *in vitro* or *in vivo* (Shin et al., 2008). Nevertheless, the precise mechanism of IL-1α production in response to *L. pneumophila* remains unclear. Previous studies have suggested that T4SS-dependent activation of p38 and JNK MAP kinases are required to induce *Il1a* transcription (Fontana et al., 2012; Shin et al., 2008). Activation of MAP kinases by *L. pneumophila* appears to be partially due to a T4SS-dependent inhibition of host protein synthesis (Fontana et al., 2012). Five *L. pneumophila* T4SS-translocated effectors have been identified that inhibit host protein synthesis (Fontana et al., 2011), and *Myd88/Nod1/Nod2*$^{-/-}$ macrophages infected with a strain lacking these five effectors (Δ*5*) exhibit diminished MAP kinase activation and reduced *Il1a* mRNA levels as compared to wild-type-infected macrophages (Fontana et al., 2012). However, infection of WT macrophages with the Δ*5* mutant still induces normal MAP kinase activation (Fontana et al., 2012), implying that MyD88/Nod signaling can also contribute. The mechanism by which protein synthesis inhibition results in MAP kinase activation remains unknown, and moreover, it is not clear whether macrophages infected with the Δ*5 L. pneumophila* strain exhibit a defect in release of IL-1α protein.

Here we show that in response to infection with virulent *L. pneumophila in vivo*, IL-1α produced by hematopoietic cells is the dominant cytokine leading to neutrophil recruitment to the lung at early timepoints (0 to 12 hours) after infection. We find that IL-1α and IL-1β act

redundantly at later timepoints as neither *Il1a*$^{-/-}$ nor *Il1b*$^{-/-}$ mice have defects in neutrophil recruitment or bacterial clearance in the lung 24 hours post-infection. Interestingly, IL-1α is produced normally in mice lacking both Caspase-1 and Caspase-11, strongly implying that inflammasomes are not required for IL-1α production. Mice deficient in both *Casp1/11* and *Il1a* phenocopied *Il1r1*$^{-/-}$ mice, confirming that inflammasomes can compensate for a lack of IL-1α at late timepoints. Interestingly, we did not detect a defect in IL-1α production in macrophages infected with the *L. pneumophila* mutant lacking 5 bacterial effectors that block host translation (Δ*5*). Although the Δ*5* mutant had no defect in IL-1α production, we find that translation inhibition in concert with TLR activation is sufficient to induce IL-1α *in vitro* and *in vivo*. Taken together with previous studies (Fontana et al., 2011; Fontana et al., 2012; Shin et al., 2008), these results suggest that an uncharacterized pathway is responsible for IL-1α production in response to *L. pneumophila* infection *in vivo*. Our results point to a critical role for IL-1α in initiating IL-1R-dependent neutrophil recruitment and inflammatory responses *in vivo* that is complementary to the established inflammasome/IL-1β signaling axis.

## Section 2.2: Materials and Methods

### Section 2.2.1: Ethics statement

These studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee at the University of California, Berkeley.

### Section 2.2.2: Mouse strains

Except for bone marrow chimeras (see below), all mice were age matched at 6-8 weeks old. *Il1r1*$^{-/-}$ and C57BL/6 mice were purchased from Jackson Laboratories. *Casp1/11*$^{-/-}$ mice (Li et al., 1995) were a gift from A. van der Velden and M. Starnbach (Harvard Medical School, Boston, MA). *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ mice have been previously described (Horai et al., 1998). *Il1a/Casp1/11*$^{-/-}$ triple knockout mice were generated from crosses at UC Berkeley. B6.SJL-*Ptprc*$^{a/BoyAiTac}$ (CD45.1) mice were purchased from Taconic. For bone marrow chimeras, 5-6 week old mice were irradiated twice with 600 rad 4 hours apart and reconstituted with $1x10^7$ donor cells by injection into the tail vein. Chimeric mice were bled 11 weeks after irradiation and reconstitution was assessed by flow cytometry of hematopoietic cells for expression of CD45.1 and CD45.2 using anti-CD45.1-FITC (eBioscience) and anti-CD45.2-PE (eBioscience) antibodies. 12 weeks after irradiation chimeric mice were infected with *L. pneumophila*. All mice were specific pathogen free, maintained under a 12hr light-dark cycle (7 a.m. to 7 p.m.) and given a standard chow diet (Harlan irradiated laboratory animal diet) *ad libitum*.

### Section 2.2.3: In vivo experiments

Age matched mice were anesthetized with ketamine and infected intranasally with $2x10^6$ LP01 or LP01Δ*dotA* in 20µL PBS. In some experiments mice were treated intranasally with ExoA, Pam3CSK4, or both in 20µL PBS, as described before (Fontana et al., 2011). Bronchoalveolar lavage was performed by introducing 800µL of PBS into the trachea with a catheter (BD Angiocath 18g, 1.3648mm). Cells in the BAL fluid were pelleted and cell free BAL fluid was analyzed by ELISA. Total host cells in the lavage fluid were counted by staining cells with Guava Viacount (Millipore) and running samples on the Guava easyCyte Plus flow cytometer running CytoSoft5.3 software (Millipore). Lavage samples were stained with anti-Gr-1-PeCy7 and anti-Ly-6G-PE (eBioscience) and analyzed on a Beckman-Coulter FC-500 analyzer. Absolute numbers of Ly-$6G^+Gr1^+$ cells were calculated by taking the percent double positive cells determined by flow cytometry and multiplying by the total number of viable cells counted by the Guava easyCyte Plus flow cytometer. Bacterial burden in lungs was enumerated by hypotonic lysis of host cells in the lavage followed by spiral plating onto buffered charcoal yeast extract (BYCE) plates with the Autoplate 5000 spiral plating system (Spiral Biotech, Inc.). CFU/mL in BAL fluid was determined by a QCount Colony Counter (version 3.0; Advanced Instruments, Inc.). BAL fluid mass was recorded prior to processing and this mass was used to estimate the volume of recovered BAL fluid. Total CFU was then calculated by multiplying CFU/mL by the estimated volume of BAL fluid. When noted, mouse body temperature and weight were monitored after infection with LP01. Mouse body temperature was measured by

rectal probe and microtherma thermometer (Braintree Scientific). The probe was lubricated with a water-based lubricant (Astroglide) before use. Temperature and weight were measured at the same time daily.

**Section 2.2.4: Bacterial strains**

For *in vitro* experiments all *L. pneumophila* strains were derived from LP02, a streptomycin-resistant thymidine auxotroph derived from *L. pneumophila* LP01. The Δ*dotA, ΔflaA, Δ5ΔflaA* strains were generated on the LP02 background and have been described previously (Fontana et al., 2011; Fontana et al., 2012; Ren et al., 2006). Mutants lacking one or more effectors were generated from LP02 by sequential in-frame deletion using the suicide plasmid pSR47S as described (Shen et al., 2009). Sequences of primers used for constructing deletion plasmids are listed in Table 2.1. Unless otherwise noted, all strains used for *in vitro*

| Effector | Gene Annotation | Deletion Primers |
|---|---|---|
| pkn5 | Lpg0208 | Upstream F:<br>5′-CTGATGCGTATGAATGGGTG-3′,<br>Internal R:<br>5′-TTAATATGGATTGCACAAATATCAAACACCATATTC-3′,<br>Internal F:<br>5′-TTGTGCAATCCATATTAACCTCAAGGAAGGCGTTCT-3′,<br>Downstream R:<br>5′-ACGCCCTCTGTTTTCTCCTT-3′ |
| Unknown | Lpg1489 | Upstream F:<br>5′-TGGTGTGAACTTCAGTCTGACGTAG-3′,<br>Internal R:<br>5′-CTTAAAAATTTAACCTGATTTAATGATGTCTACGCATCTAACTCTCCTAACAT-3′,<br>Internal F:<br>5′-ATGTTAGGAGAGTTAGATGCGTAGACATCATTAAATCAGGTTAAATTTTTAAG-3′,<br>Downstream R:<br>5′-TTTCTCAGCAGGAAAGTGCTCC-3′ |

**Table 2.1:** Deletion primers used in this study.

infections were deficient for bacterial flagellin (*ΔflaA)* and thus non-motile. *L. pneumophila* from the Δ*flaA* background were utilized *in vitro* to avoid activation of the NAIP5/NLRC4 inflammasome (Amer et al., 2006; Lightfield et al., 2008; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). For *in vivo* experiments, we utilized *L. pneumophila* wild-type strain LP01, a non-motile streptomycin-resistant strain derived from the original Philadelphia outbreak (Berger et al., 1994). The Δ*dotA* LP01 strain has been previously described (Zuckman et al., 1999).

**Section 2.2.5: Infection and stimulation**

Bone marrow derived macrophages were plated in 24 well plates at a density of $5x10^5$ cells per well and infected at an MOI of 1-3 (as indicated) by centrifugation for 10min at 400 x*g*. In some experiments macrophages were treated with Exotoxin A (List Biological Labs), a synthetic bacterial lipopeptide (Pam3CSK4) (Invivogen), or both. After one hour of infection, media was changed. All *in vitro L. pneumophila* infections were in the absence of thymidine to

curtail bacterial replication.

**Section 2.2.6: ELISA and Cytotoxicity**

At the indicated time post-treatment, supernatants or bronchoalveolar lavage fluid were collected, cleared by centrifugation and analyzed by ELISA using paired interleukin-1a antibodies (BD Biosciences and eBioscience) or paired interleukin-1β antibodies (eBioscience and BD Bioscience). Recombinant IL-1α (eBioscience) or IL-1β (eBioscience) was used as a standard for each respective ELISA. Cytotoxicity was measured by evaluation of lactate dehydrogenase (LDH) released from cells (Decker and Lohmann-Matthes, 1988). Specific lysis was calculated as a percentage of LDH released by detergent-lysed macrophages where cells were treated with 1% TritonX-100 for 30 minutes.

**Section 2.2.7: Cell culture**

Macrophages were derived from the bone marrow of C57BL/6J mice (Jackson Laboratory). Macrophages were derived by 8 days of culture in RPMI supplemented with 10% serum, 100μM streptomycin, 100U/mL penicillin, 2mM L-glutamine and 10% supernatant from 3T3-macrophage-colony stimulating factor cells, with feeding on day 5. HEK293T cells were grown in complete media (DMEM, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine).

**Section 2.2.8: Effector library screen**

The library of 259 confirmed or putative secreted effector proteins has been previously described (Losick et al., 2010). Using the Gateway cloning system (Invitrogen) the library was cloned into a Gateway compatible murine stem cell virus (MSCV) 2.2 retroviral expression construct. We modified the MSCV 2.2 expression construct with an in-frame 6x-Myc tag upstream of the cloned effectors to accommodate for non AUG start codon usage in prokaryotes and we removed the downstream internal ribosome entry site (IRES)- green fluorescent protein (GFP). HEK293T cells were plated at $2.5x10^4$ cells per well in 96-well tissue culture plates. 24hrs after plating cells were co-transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions, with a single library clone and the TK-*Renilla* luciferase reporter construct. 24hrs after transfection cells were lysed in passive lysis buffer (Promega) for 5 min at 25°C. Cell lysates were incubated with the *Renilla* luciferase substrate coelenterazine (Biotium) and luminescence was measured on a SpectraMax L microplate reader (Molecular Devices). The relative block in translation was measured by comparing *Renilla* luminescence in cells transfected with a control bacterial protein that does not block translation.

**Section 2.2.9: Quantitative RT-PCR**

Macrophage RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA samples were treated with RQ1 DNase (Promega) prior to reverse transcription (RT) with Superscript III (Invitrogen). cDNA reactions were primed with poly(dT). Quantitative PCR was performed as described previously(Monroe et al., 2009) using a Step One Plus RT-PCR system (Applied Biosystems) with Platinum *Taq* DNA polymerase (Invitrogen)

and EvaGreen (Biotium). Transcript levels were normalized to those of *Rps17.* The following primer sequences were used: for *Il1a,* Forward 5'-ATGACCTGC AACAGGAAGTAAAA-3' and Reverse 5'-TGTGATGAGTTTTGGTGTTTCTG-3' and for *Rps17*, Forward 5'-CGCCATTATCCCCAGCAAG-3' and Reverse 5'-TGTCGGGATCCACCT CAATG-3'.

**Section 2.2.10: $^{35}$S-methionine metabolic labeling**

$5x10^5$ bone marrow derived macrophages were seeded in 24-well plates and infected with bacterial strains at an MOI of 3. 25 minutes prior to labeling, macrophages were treated with 25μg/mL chloramphenicol to inhibit bacterial translation. At 6 and 24hrs post-infection media was removed and incubated with 25μCi/mL $^{35}$S-methionine (Perkin Elmer) in RPMI without methionine supplemented with 10% serum, 2mM L-glutamine, 25μg/mL chloramphenicol, and 10% supernatant from 3T3-macrophage-colony stimulating factor cells. Cells were labeled for 1 hour, washed three times with cold PBS and then lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with 2 mM $Na_3VO_4$, 1 mM PMSF, 25 mM NaF, and 1x Roche protease inhibitor cocktail (no EDTA), pH 7.2, for 10 minutes at 4°C. Total protein levels were measured by bicinchoninic acid (BCA) assay and equal amounts of protein were mixed with SDS sample buffer (40% glycerol, 8% SDS, 2% 2-mercaptoethanol, 40 mM EDTA, 0.05% bromophenol blue and 250 mM Tris-HCl, pH 6.8), boiled for 5 min and then separated by SDS–PAGE. The gels were stained with coomassie blue to show equal protein loading, dried, and exposed to a phosphor screen and visualized using a Typhoon Trio imager (GE Healthcare)

## Section 2.3: Results:

### Section 2.3.1: *Il1r1*$^{-/-}$ mice are more susceptible to *L. pneumophila* infection

A previous report identified the type-I interleukin-1 receptor (IL-1R) as a major signaling pathway that controls the recruitment of neutrophils to the lung in response to *L. pneumophila* (LeibundGut-Landmann et al., 2011). This report proposed that IL-1β is the major ligand signaling through the IL-1R in *L. pneumophila* infections, but did not specifically address a possible role of IL-1α and also did not examine the consequences of IL-1R deficiency on host health. Before addressing the relative importance of IL-1α and IL-1β, we first set out to confirm the previously proposed role of IL-1R signaling in *L. pneumophila* infection. We infected IL-1R-deficient (*Il1r1*$^{-/-}$) mice with wild-type *L. pneumophila* and examined the mice at 12hrs, 24hrs and 48hrs post-infection. Total numbers of $Ly6G^{+}Gr1^{+}$ cells (here referred to as neutrophils) in bronchoalveolar lavage (BAL) fluid were determined by flow cytometry, and bacterial burden was measured by plating for colony forming units (CFUs). Consistent with previous reports, *Il1r1*$^{-/-}$ mice recruited reduced numbers of neutrophils to the lungs in response to *L. pneumophila*, with approximately 10-fold, 5-fold and 4-fold fewer neutrophils in *Il1r1*$^{-/-}$ mice than WT mice at 12hrs, 24hrs and 48hrs post-infection, respectively (Fig. 2.1A, B, C). Interestingly, while there are significant defects in the number of neutrophils recruited to the lungs of *Il1r1*$^{-/-}$ mice, the total number of cells in the BAL fluid of these mice does not significantly differ from WT mice (Fig. 2.1A, B, C). The similarity in overall numbers of cells in the BAL appears to be because after infection with *L. pneumophila, Il1r1*$^{-/-}$ mice harbor greater numbers of alveolar macrophages and CD45-negative/low cells that compensate for the decrease in neutrophils (Fig. 2.2). One possible explanation for this is that in WT mice, damaged or dead alveolar macrophages and CD45-negative/low cells are normally phagocytosed and thereby eliminated by neutrophils. Thus, with decreased neutrophils in *Il1r1*$^{-/-}$ mice, alveolar macrophages and CD45-negative/low cells accumulate (Fig. 2.2). In addition to decreased neutrophils, *Il1r1*$^{-/-}$ mice harbor approximately 5-fold and 17-fold higher CFU in BAL fluid over B6 controls at 24 and 48 hours post-infection, respectively (Fig. 2.1B, C). *Il1r1*$^{-/-}$ mice also have a slight increase in bacterial burden measured in BAL fluid at 12hrs post-infection, but this difference is not dramatic, presumably because *L. pneumophila* does not have enough time to appreciably replicate or be cleared by the host at this time-point (Fig. 2.1A).

Although our data confirm that IL-1R signaling is critical for neutrophil recruitment and elimination of bacteria from the lung, neutrophils are also believed to be key mediators of the immune pathology of Legionnaires' Disease. Therefore we were interested to determine whether the decreased neutrophil response in *Il1r1*$^{-/-}$ mice resulted in overall increased or decreased host health. To assay host health, we followed body temperature and weight loss in wild-type and *Il1r1*$^{-/-}$ mice (Fig. 2.1D). Although both wild-type and *Il1r1*$^{-/-}$ mice eventually recover from the infection, *Il1r1*$^{-/-}$ mice show more severe weight loss and temperature decreases than WT mice after being infected with *L. pneumophila* over a two week study (note that, in contrast to humans, mice typically exhibit a hypothermic response, rather than a fever, as a result of infection (Karp, 2012). We have shown that *Il1r1*$^{-/-}$ mice exhibit increased bacterial burden in the lung at 12, 24 and 48 hours post-infection with *L. pneumophila* (Fig. 2.1A-D). We therefore suggest that increased bacterial burden in *Il1r1*$^{-/-}$ mice over the first week of infection is likely the cause of the decreased overall health of these animals in response to *L. pneumophila* infection. However, after about a week of infection, compensatory innate and/or adaptive immune responses likely

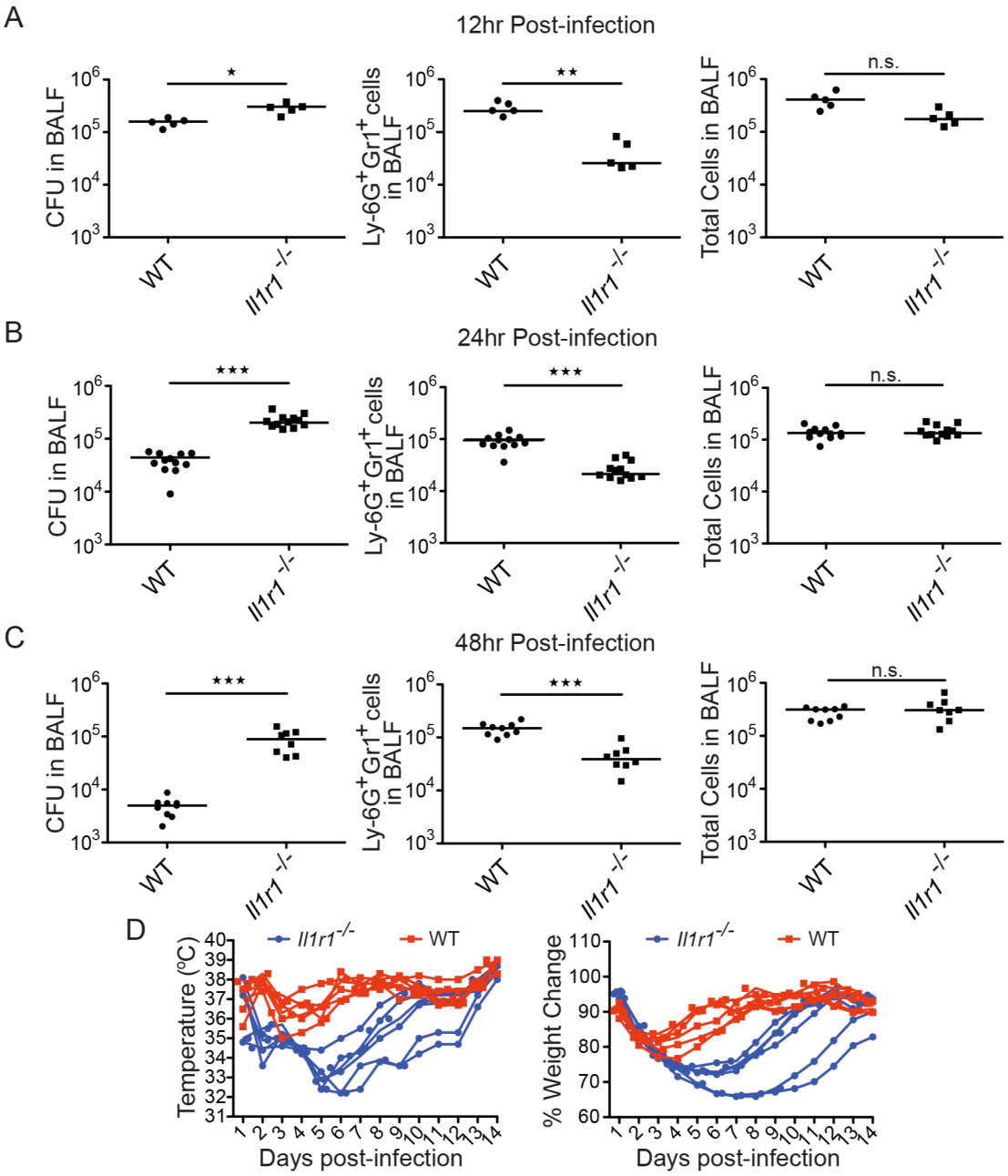


**Figure 2.1:** The IL-1 Receptor Type I is essential for control of *L. pneumophila* infection. (A-C) *Il1r1*-deficient mice were infected intranasally with $2x10^6$ *L. pneumophila* (LP01). Bronchoalveolar lavage (BAL) was performed at 12 hours (A), 24 hours (B) and 48 hours (C) post-infection. Bacterial burden in the BAL fluid was determined by plating for colony forming units. The number of Ly-$6G^+Gr1^+$ cells was determined by flow cytometry and the total number of cells in the BAL fluid as determined by Guava ViaCount assay. (D) *Il1r1*-deficient (blue circles) and wild-type B6 (red squares) mice were infected with $2x10^6$ *L. pneumophila* (LP01) and monitored daily for temperature and weight change. Percent weight change is calculated to weight at day zero. Data are representative of two (D) or three (A, B, C) experiments. (Median in A-C). *, $p<0.05$. **, $p<0.01$. ***, $p<0.005$. (Statistical analysis: Mann-Whitney U test).

control the infection. Overall, our results suggest that during the course of experimental *L. pneumophila* infection, the beneficial function of early neutrophil influx in bacterial clearance outweighs the potentially negative effects of neutrophil-mediated immune pathology. These data also establish an important role for IL-1R signaling in host health in addition to the previously established role for IL-1R signaling in neutrophil recruitment and bacterial clearance.

**Section 2.3.2: IL-1α production precedes the recruitment of neutrophils to the lung**

IL-1α and IL-1β are the only known agonists of the IL-1R. We therefore tested whether there was a correlation between IL-1α or IL-1β production and the recruitment of neutrophils to the lungs of infected mice. B6 mice were infected with wild-type *L. pneumophila* or an avirulent mutant strain of *L. pneumophila* that lacks a functional Type IV secretion system (Δ*dotA*). BAL fluid was harvested at 3, 6, 9 and 12 hours post-infection, and assessed for the presence of neutrophils, IL-1α, and IL-1β. The earliest *in vivo* Dot/Icm-dependent response was the production of IL-1α, which was first detectable at 3hrs post-infection (Fig. 2.3A). By contrast, the earliest significant production of IL-1β (above that induced by Δ*dotA*) was not until 6hrs post-infection (Fig. 2.3B), the same time that the Dot/Icm-dependent influx of neutrophils can first be detected (Fig. 2.3C). It is interesting to note that there seems to be an increase in the number of neutrophils found in the BAL fluid after infection with the Δ*dotA L. pneumophila* strain at 3hrs post-infection, although this difference is not statistically significant (Fig. 2.3C). Consistent with previous results, the Δ*dotA L. pneumophila* strain did not appreciably induce IL-1α production in the lung (Fig. 2.3A, B). Thus, while there may be a low level of Dot/Icm-independent neutrophil recruitment to the lung, this recruitment appears to be IL-1α independent and likely plays a minimal role in protecting the host from infection. Taken together, these data show that IL-1α production is largely Dot/Icm-dependent and occurs prior to the recruitment of neutrophils to the lung. Our findings suggest a role for IL-1α in the early IL-1R-dependent and Dot/Icm-dependent recruitment of neutrophils to the lungs of *L. pneumophila* infected mice.

**Section 2.3.3: IL-1α, but not IL-1β, is required for early neutrophil recruitment to the lung**

We next tested whether the loss of IL-1α or IL-1β would have an effect on neutrophil recruitment. We infected wild-type (B6), *Il1a*$^{-/-}$, *Il1b*$^{-/-}$, *Casp1/11*$^{-/-}$ and *Il1r1*$^{-/-}$ mice with wild-type *L. pneumophila* and measured neutrophil recruitment and bacterial burden at 12hrs post-infection. As expected, *Il1r1*$^{-/-}$ mice showed a strong defect in recruitment of neutrophils to the lung, while both the *Il1b*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice showed no defect in neutrophil recruitment to the lung at 12hrs post-infection, as compared to B6 mice (Fig. 2.4A). However, there was approximately a 17-fold decrease in the number of neutrophils recruited to the lung of *Il1a*$^{-/-}$ mice as compared to B6 mice (Fig. 2.4A). Importantly, *Il1a*$^{-/-}$ mice have no significant difference in the production of IL-1β in the BAL fluid of infected mice at 12hrs post-infection (Fig. 2.5A, B). These data suggest that IL-1α may be more important than IL-1β for the recruitment of neutrophils to the lung at 12hrs post-infection. Interestingly, the defect in neutrophil recruitment in *Il1a*$^{-/-}$ mice was not as pronounced as the defect seen in *Il1r1*$^{-/-}$ mice. This suggests that although IL-1β is not itself essential for neutrophil recruitment, it can partially compensate for the loss of IL-1α (addressed further below). The bacterial burden in the infected mice was very similar among all of the genotypes, likely because at 12hrs post-infection *L. pneumophila* has not had enough time to appreciably grow or be cleared by the host immune

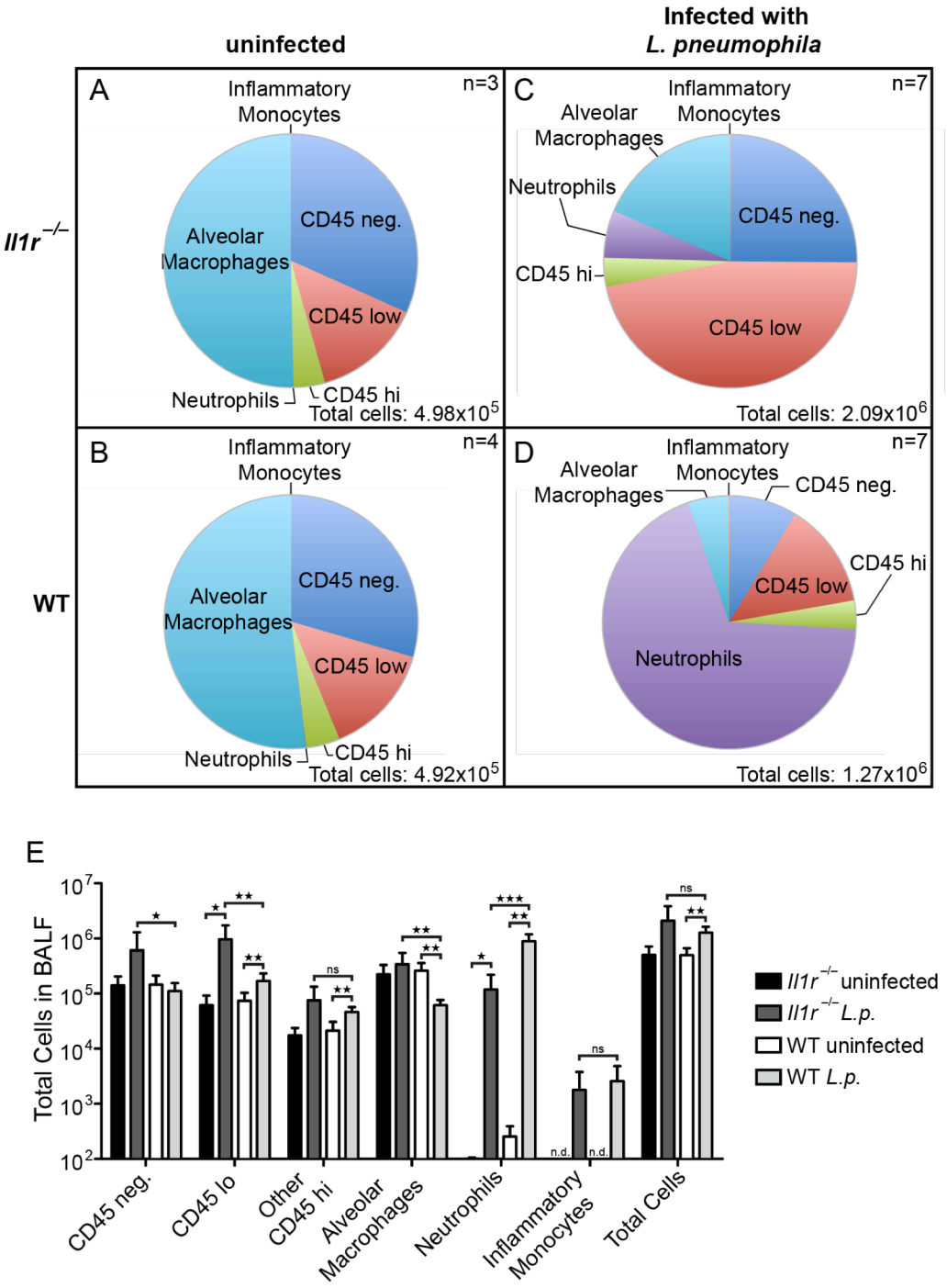


**Figure 2.2:** Cellular composition of bronchoalveolar lavage fluid from *Il1r*$^{-/-}$ and WT mice. (A-D) Cellular composition of BAL fluid from PBS treated *Il1r*$^{-/-}$ (A) and WT (B) mice or *L. pneumophila* infected *Il1r*$^{-/-}$ (C) and WT (D) mice at 12hrs post-infection (E). Quantification of cellular populations in A-D. Gating Scheme: Neutrophils: CD45$^{hi}$, CD11c$^{-}$, CD11b$^{+}$, Gr1$^{hi}$, Lyc-6C$^{lo}$. Alveolar Macrophages: CD45$^{hi}$, CD11c$^{+}$, CD11b$^{-}$, SiglecF$^{+}$. Inflammatory Monocytes: CD45$^{hi}$, CD11c$^{-}$, CD11b$^{+}$, Gr1$^{lo}$, Ly-6C$^{hi}$. Data are representative of three (A-E) experiments. (Mean ± s.d. in E). n.d., not detectable. ns, not significant. ★, $p<0.05$. ★★, $p<0.01$. ★★★, $p<0.001$. (Statistical analysis: Mann-Whitney U test).

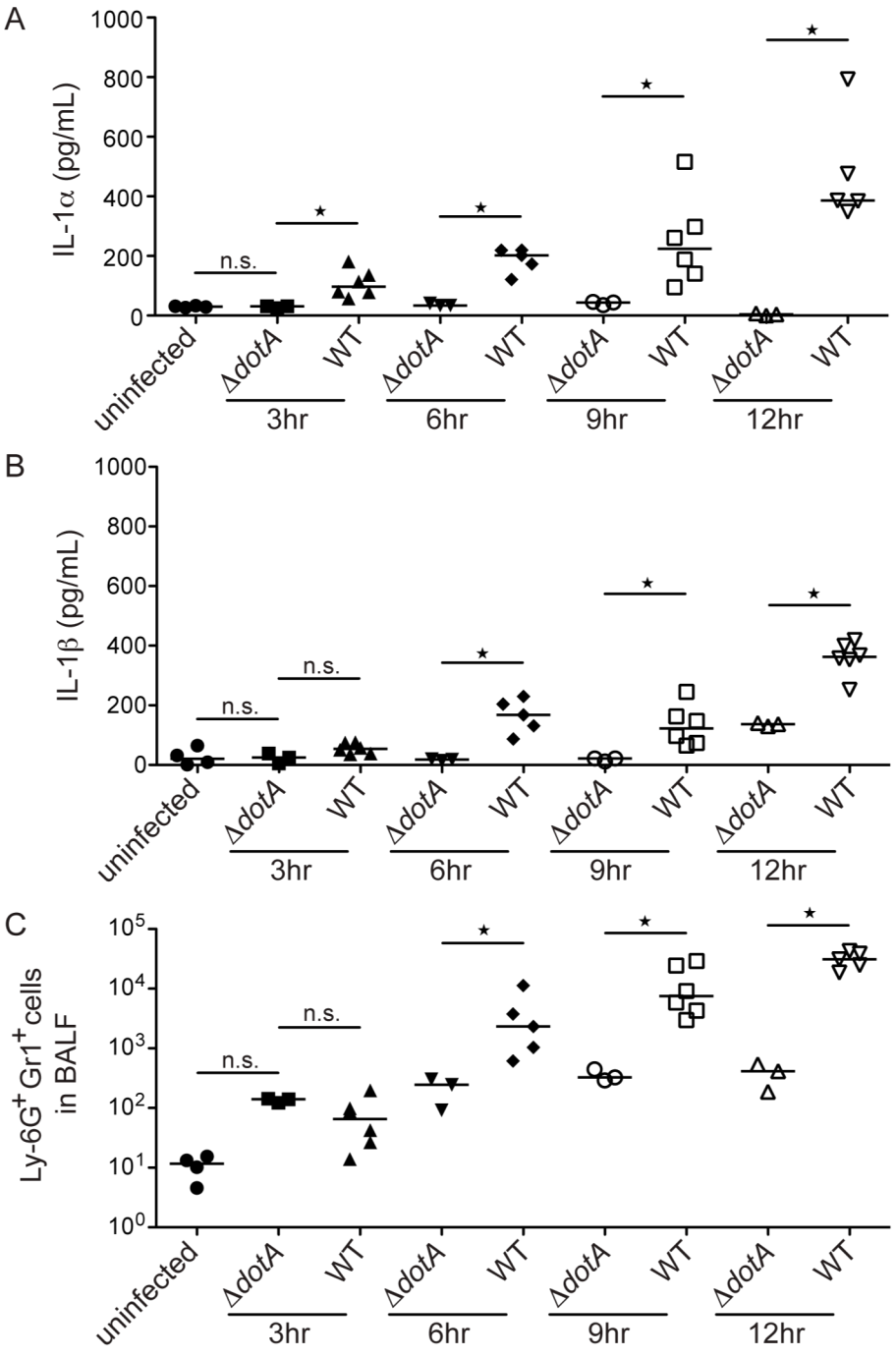


**Figure 2.3:** Dot/Icm T4SS-dependent IL-1α production precedes the recruitment of Ly-6G$^+$Gr1$^+$ cells to the lung. (A-C) B6 mice were infected intranasally with 2x10$^6$ *L. pneumophila* (WT) or a mutant lacking a functional type IV secretion system (Δ*dotA*). Bronchoalveolar lavage was performed at 3, 6, 9, or 12 hours post-infection. IL-1α (A) and IL-1β (B) levels were measured by ELISA. (C) The number of Ly-6G$^+$Gr1$^+$ positive cells in the BAL fluid was determined by flow cytometry. Data are representative of three experiments. (Median in A-C). *, $p<0.05$ (Statistical analysis: Mann-Whitney U test).

response (Fig. 2.4B). As an important control, measurement of IL-1α protein levels in the BAL fluid of infected mice demonstrated that only *Il1a*$^{-/-}$ mice had defects in production of IL-1α in response to *L. pneumophila* infection (Fig. 2.4C). The amount of IL-1α detected in the BAL fluid of *L. pneumophila* infected *Il1r1*$^{-/-}$ mice is slightly higher than WT mice, likely due to an increase in bacterial burden caused by reduced neutrophil recruitment to the lungs of these mice (Fig. 2.4C). The increase in bacterial burden in *Il1r1*$^{-/-}$ mice likely leads to more infected macrophages and thus an increase in the production of IL-1α. Additionally, the loss of the IL-1R may result in less internalization of the IL-1α protein, resulting in higher extracellular accumulation. We also note that IL-1α is produced even in *Casp1/11*$^{-/-}$ mice, indicating that in response to *L. pneumophila* IL-1α production in vivo can be independent of both Caspase-1 and Caspase-11 inflammasomes (Fig. 2.4C).

We hypothesized that the intermediate phenotype seen in the *Il1a*$^{-/-}$ mice was due to low levels of inflammasome-dependent IL-1β production that are still capable of signaling through the IL-1R. We were unable to generate *Il1a/b*$^{-/-}$ double knockout mice as these genes are located directly next to each other on the chromosome. Thus, to test whether there is redundancy between IL-1α and IL-1β, we generated *Il1a/Casp1/11*$^{-/-}$ 'triple' knockout mice (TKO). These mice are predicted to be deficient in production of IL-1α and IL-1β, as production of biologically active IL-1β is generally believed to require Caspase-1. We should note that the TKO mice are not only defective in IL-1β cytokine production, but they are also unable to undergo pyroptosis, a Caspase-1/11-dependent form of lytic cell death, which has previously been shown to evict bacteria from their intracellular niche and render them susceptible to phagocytosis and killing by neutrophils (Broz et al., 2012; Miao et al., 2010). The loss of pyroptosis could lead to an increased bacterial burden; however, at 12hrs post-infection we see very little differences in bacterial burden in the BAL fluid and thus we argue that the major defect in the TKO mice at 12hrs post-infection is the loss of IL-1β processing and release (Fig. 2.4D, E). Consistent with a defect in IL-1α and IL-1β production, we find that in response to *L. pneumophila* infection TKO mice produce almost no detectable IL-1α and very low levels of IL-1β in BAL fluid at 12 hours post-infection (Fig. 2.5C, D). Interestingly, TKO mice exhibited a large defect in neutrophil recruitment to the lung; in fact, these mice were as defective in neutrophil recruitment as *Il1r1*$^{-/-}$ mice (Fig. 2.4D). These data suggest that IL-1α is the major cytokine required to signal through the IL-1R and recruit neutrophils to the lung at 12 hours post-infection, though Casp1/11-dependent signaling through the IL-1R (presumably mediated by IL-1β) can partially compensate for the loss of IL-1α. Furthermore, Caspase-1 is usually considered to be essential for IL-1β processing (Cerretti et al., 1992; Thornberry et al., 1992), although some previous reports have suggested that IL-1β can be generated in the absence of *Casp1/11* (Guma et al., 2009; Karmakar et al., 2012). Even though *Il1a/Casp1/11*$^{-/-}$ TKO mice produced very low levels of IL-1β, TKO mice were as defective in neutrophil recruitment as *Il1r1*$^{-/-}$ mice, implying that, at least in response to *L. pneumophila,* production of biologically active IL-1β requires Caspase-1/11.

**Section 2.3.4: At late timepoints after infection, IL- 1β compensates for the loss of IL-1α**

*Il1r1*$^{-/-}$ mice exhibit reduced neutrophil recruitment that is sustained until at least 48hrs post-infection (Fig. 2.1A). We therefore tested if the loss of IL-1α would lead to a defect in neutrophil recruitment and an increase in bacterial burden at late timepoints. We infected wild-type, *Il1a*$^{+/-}$, and *Il1a*$^{-/-}$ littermates with wild-type *L. pneumophila* and compared these mice to

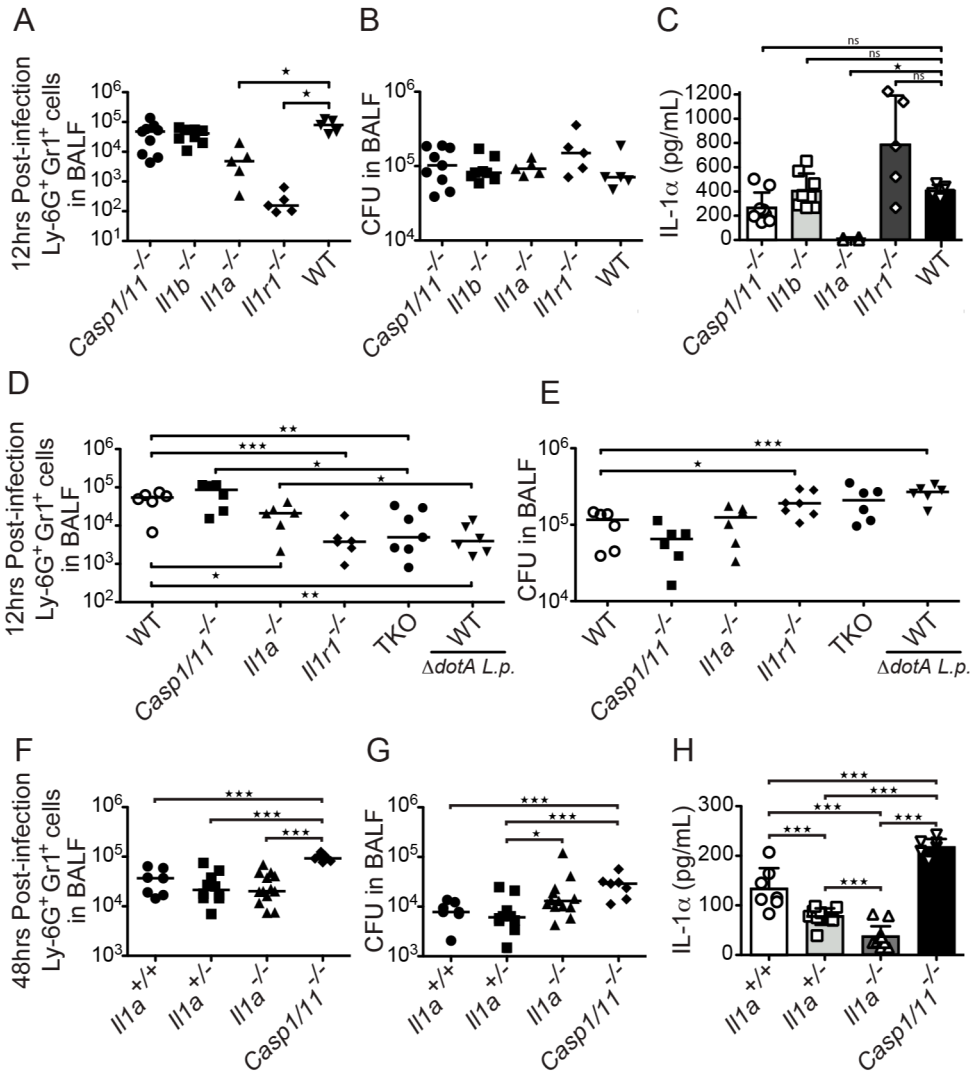


**Figure 2.4:** IL-1α is required for Ly-6G$^+$Gr1$^+$ cell recruitment to the lung in response to infection with *L. pneumophila*. (A-C) The indicated mouse strains were infected intranasally with 2x10$^6$ *L. pneumophila* (LP01). At 12h post-infection bronchoalveolar lavage (BAL) fluid was collected. (A) Ly-6G$^+$Gr1$^+$ cells in the BAL fluid were enumerated by flow cytometry. (B) Bacterial burden in the lung was determined by measuring CFU. (C) IL-1α levels were measured. (D-E) The indicated mouse strains were infected intranasally with 2x10$^6$ *L. pneumophila* (LP01) or T4SS-deficient *L. pneumophila* (LP01 Δ*dotA*) as noted. At 12h post-infection BAL fluid was harvested and Ly-6G$^+$Gr1$^+$ cell recruitment was measured (D) and bacterial burden in the lung was enumerated (E). (F-H) *Il1a*$^{-/-}$, *Il1a*$^{+/-}$, and *Il1a*$^{+/+}$ littermates were infected intranasally with 2x10$^6$ *L. pneumophila* (LP01). Non-littermate *Casp1/11*$^{-/-}$ mice were also infected with LP01. BAL fluid was collected 48h post-infection and Ly-6G$^+$Gr1$^+$ cells were quantified (F). Bacterial burden was determined (G) and IL-1α levels were measured (H). Data are representative of two (F-H) or three (A-E) experiments (Median in A, B, D-G. mean ± s.d. in C, H). The low level of apparent IL-1α protein produced in *Il1a*$^{-/-}$ mice at 48h post infection appears to be due to an unknown cross-reacting protein that produced a low signal on the ELISA assay. TKO, *Il1a/Casp1/11*$^{-/-}$ triple knockout mice. *, p<0.05. **, p<0.01. ***, p<0.005. (Statistical analysis: Mann-Whitney U test).

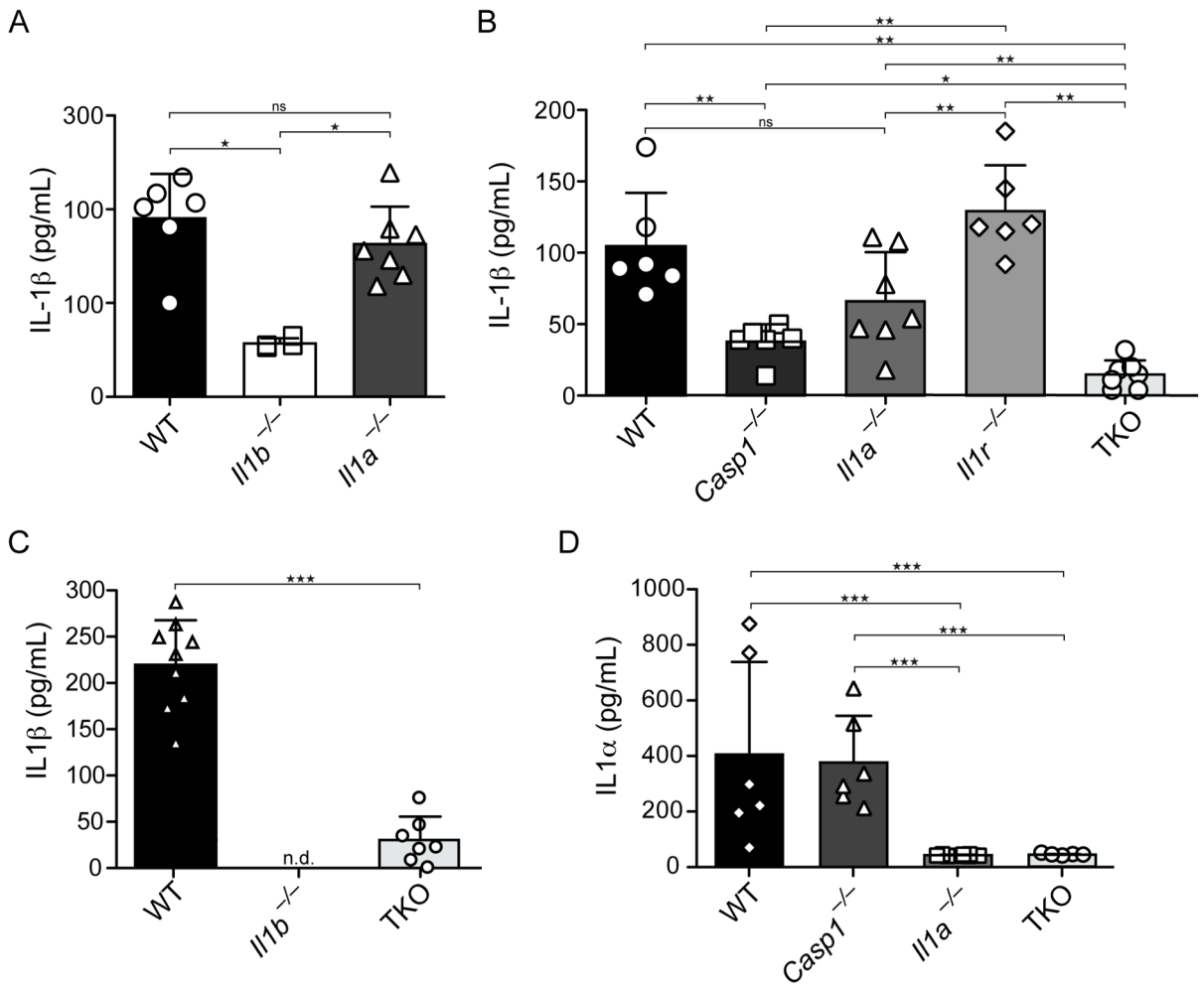


**Figure 2.5:** *Il1a*$^{-/-}$ mice have no defect in IL-1β production. (A-C) The indicated mouse strains were infected with wildtype *L. pneumophila*. 12h post-infection bronchoalveolar lavage was performed and BAL fluid was assayed for IL-1α levels by ELISA. (A) *Il1a*$^{-/-}$ mice have no defect in IL-1β production. (B) The samples shown in Figure 3D and E were assayed for IL-1β levels in the BAL fluid. (C) TKO mice produce significantly less IL-1β in response to *L. pneumophila* than wildtype mice, but still produce low levels of IL-1β. (D) The indicated mouse strains were infected with wildtype *L. pneumophila* and at 12h post-infection IL-1α levels were measured in the BAL fluid by ELISA. ★ = $p<0.05$, ★★ = $p<0.01$, ★★★ = $p<0.001$. (Statistical analysis: Mann-Whitney U test).

*Casp1/11*$^{-/-}$ mice (Fig. 2.4F-H). At 48hrs post-infection *Il1a*$^{-/-}$ mice have no defect in neutrophil recruitment to the lung and only a modest defect in control of bacterial burden (Fig. 2.4F-G). Additionally we see no defect in neutrophil recruitment by *Casp1/11*$^{-/-}$ mice at 48hrs post-infection, suggesting that both IL-1α and IL-1β are capable of signaling through the IL-1R and can compensate for the loss of each other by 48h post-infection. In fact, *Casp1/11*$^{-/-}$ mice actually appeared to exhibit increased recruitment of neutrophils to the lung (Fig. 2.4F). However, despite the increased neutrophil recruitment, *Casp1/11*$^{-/-}$ mice also exhibited increased bacterial burdens in the lung at 48h post-infection (Fig. 2.4G). As mentioned previously, this counterintuitive result is likely explained by the loss of Caspase-1/11-dependent pyroptosis,

which has previously been shown to evict bacteria from their intracellular niche and render them susceptible to phagocytosis and killing by neutrophils (Broz et al., 2012; Miao et al., 2010). Importantly, we find that *Casp1/11*$^{-/-}$ mice produce IL-1α in response to *L. pneumophila* infection and actually induce significantly more IL-1α than wild-type mice; this increase is likely due to the loss of pyroptosis and subsequent increased bacterial burden in these mice (Fig. 2.4H).

**Section 2.3.5: IL-1α is produced by cells derived from the hematopoietic lineage**

IL-1α is inducible in hematopoietic cells, but is also reported to be constitutively expressed by certain non-hematopoietic cells (Dinarello, 2009; Gross et al., 2012). We therefore wished to determine whether the rapid production of IL-1α and the ensuing neutrophil influx required IL-1α production by hematopoietic or non-hematopoietic cells. We generated bone marrow chimeras in which wild-type B6.SJL (CD45.1$^{+}$) mice were reconstituted with bone marrow from *Il1a*$^{-/-}$ (CD45.2$^{+}$) mice, and *vice-versa*. To confirm that our chimeras had been reconstituted to a high level, blood samples were collected and stained with antibodies for CD45.1 and CD45.2 that marked wild-type and *Il1a*$^{-/-}$derived hematopoietic cells, respectively (Fig. 2.6). Chimeric mice were infected with *L. pneumophila* and BAL fluid was collected 12 hours post-infection. Mice reconstituted with B6 hematopoietic cell populations produced IL-1α in response to *L. pneumophila* infection, whereas mice reconstituted with *Il1a*$^{-/-}$ bone marrow failed to produce IL-1α (Fig. 2.7A). Importantly, the production of IL-1α correlated with the recruitment of neutrophils to the lung (Fig. 2.7B). Consistent with our previous findings (Fig. 2.4) we see little difference in the total CFU found in the BAL fluid of these mice at 12h post-infection, although there was a slight increase in bacterial burden in mice that received *Il1a*$^{-/-}$

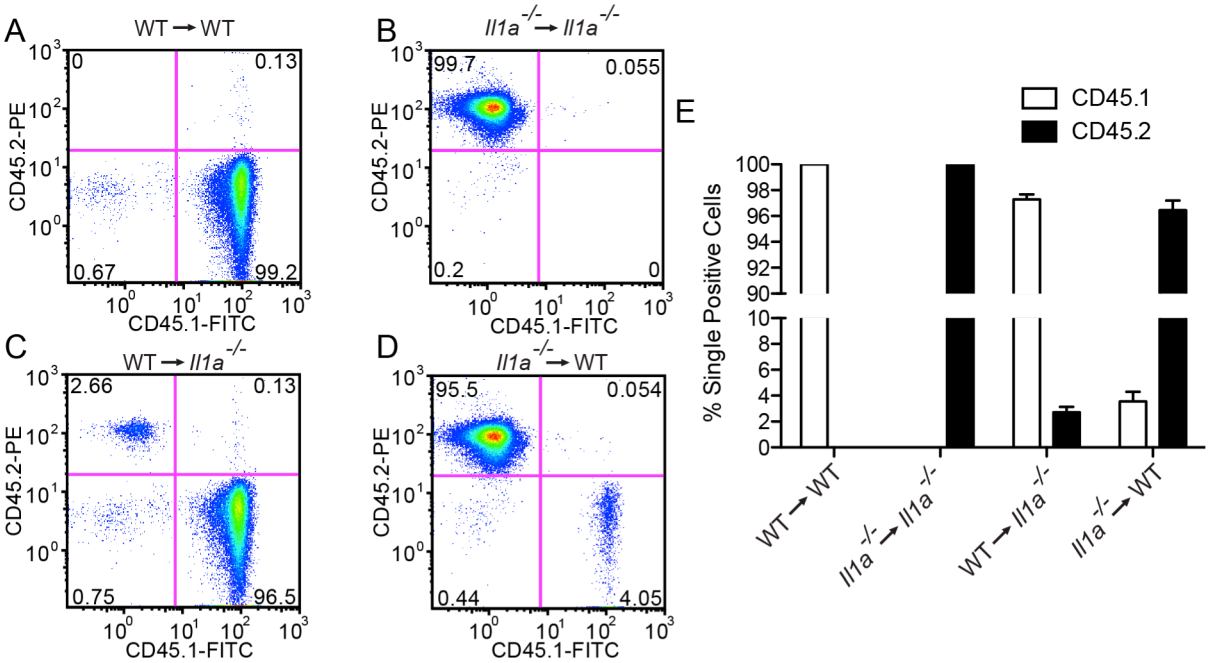


**Figure 2.6:** Quantification of CD45.1 and CD45.2 cells in peripheral blood of *Il1a* bone marrow chimeras. (A-E) 11 weeks post-irradiation blood was collected by tail vein bleeds and stained with anti-CD45.1-FITC and anti CD45.2-PE antibodies. Representative flow plots for (A) WT > WT, (B) Il1a > Il1a, (C) WT > Il1a, and (D) Il1a > WT mice are shown. (E) Average host bounce back in chimeric mice was quantified.

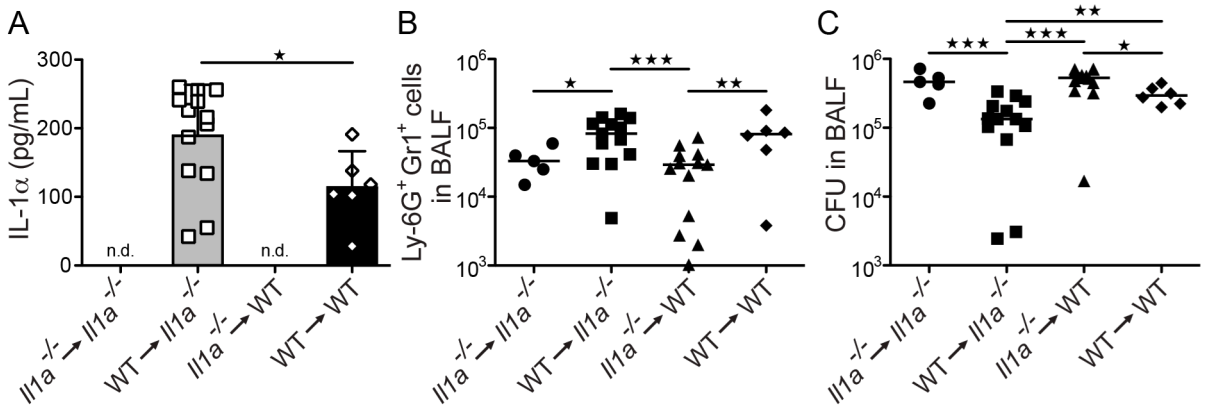


**Figure 2.7.** Hematopoietic cells are responsible for IL-1α production in response to *L. pneumophila*. (A-C) 6 week old *Il1a*$^{-/-}$ and congenically marked B6.SJL (CD45.1) mice were lethally irradiated and reconstituted with *Il1a*$^{-/-}$ (CD45.2) or B6.SJL bone marrow as indicated. After 12 weeks of recovery, chimeric mice were infected with *L. pneumophila* (LP01). Bronchoalveolar lavage (BAL) fluid was collected 12hrs post-infection. (A) IL-1α levels in BAL fluid were determined by ELISA. (B) Recruitment of Ly-6G$^{+}$Gr1$^{+}$ cells was determined by flow cytometry. (C) Bacterial burden in the lung was determined by plating BAL fluid for bacterial CFUs. Data are representative of two (A-C) experiments. (Mean ± s.d. in A. Median in B, C). n.d., not detectable. WT, B6.SJL. *, $p<0.05$. **, $p<0.01$. ***, $p<0.005$. (Statistical analysis: Mann-Whitney U test).

bone marrow (Fig. 2.7C). These chimera experiments demonstrate that hematopoietic cells in the lung, presumably macrophages that have been infected with *L. pneumophila*, are responsible for the early production of IL-1α and subsequent recruitment of neutrophils to the site of infection.

### Section 2.3.6: *L. pneumophila* lacking effectors that block host protein synthesis still induce IL-1α

Given the major role IL-1α plays in neutrophil recruitment, we next wanted to explore the molecular mechanism of IL-1α production by macrophages. We (Fontana et al., 2011) and others (Belyi et al., 2006; Belyi et al., 2008; Shen et al., 2009) previously showed that *L. pneumophila* encodes five Dot/Icm-secreted effectors that inhibit host protein synthesis. A strain lacking these five effectors (Δ*5*) was defective in the induction of a subset of inflammatory cytokines, including IL-23 and GM-CSF (Fontana et al., 2011). Moreover, Δ*5* was also defective in the transcriptional induction of the *Il1a* gene when the Toll-like receptor (TLR) and NOD-like receptor innate immune sensing pathways were severely hindered (infections of *Myd88/Nod1/Nod2*$^{-/-}$ BMDMs)(Fontana et al., 2012). The overall model emerging from our previous studies was that protein synthesis inhibition by virulent *L. pneumophila* produces a host cell stress response that leads to the production of inflammatory cytokines. Therefore, we asked whether the Δ*5 L. pneumophila* strain could still induce IL-1α protein release by wild-type BMDMs. We infected macrophages with the Δ*5 L. pneumophila* strain on the Δ*flaA* background (Δ*5*Δ*flaA*) and measured the production of IL-1α from these cells. We utilized *L. pneumophila* on the Δ*flaA* background to avoid the confounding effects of NAIP5/NLRC4 inflammasome activation by flagellin. Interestingly, we found that the Δ*5*Δ*flaA* strain still induced production of

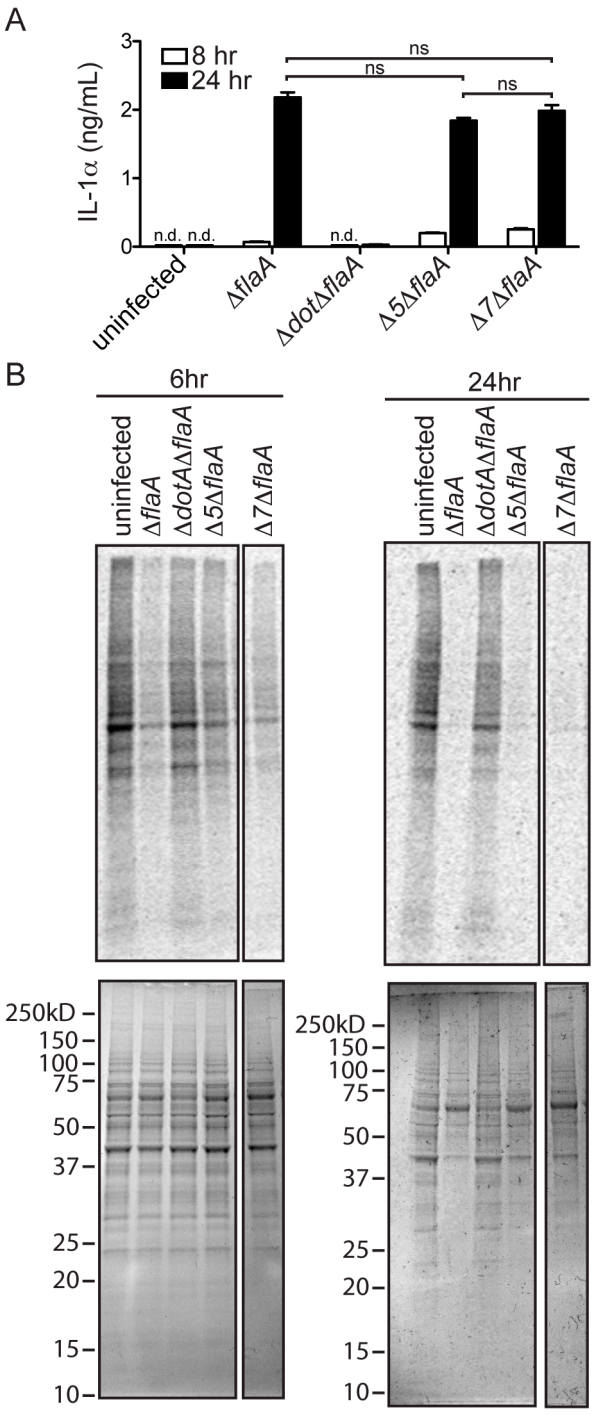


**Figure 2.8:** *L. pneumophila* mutants lacking bacterial effectors known to block translation have no defect in IL-1α production. (A) Wild-type B6 bone marrow derived macrophages were infected with the indicated strains of *L. pneumophila* (LP02) at a MOI of 1. 8hrs and 24hrs post-infection cell supernatants were collected and IL-1α levels were determined by ELISA. (B) Wild-type bone marrow derived macrophages were infected with the indicated strains of *L. pneumophila* (MOI=3) and at 6hrs (left panels) and 24hrs (right panels) post-infection cells were incubated with $^{35}$S-methionine for one hour followed by lysis in RIPA buffer. Gels were stained with coomassie blue to visualize equal loading (bottom panels) and global translation levels were determined by autoradiography (top panels). Intervening lanes on gel were removed for simplicity. Data are representative of two (B) or three (A) experiments. (Mean ± s.d. in A). n.d., not detectable. ns, not significant. (Statistical analysis: Mann-Whitney U test).

significant amount of IL-1α protein (Fig. 2.8A). This result is consistent with previous *in vivo* observations that showed that neutrophil recruitment is normal in response to the Δ*5* mutant (Fontana et al., 2012). We considered two possible explanations for the ability of the Δ*5* mutant to induce IL-1α: (1) protein synthesis inhibition is not required for IL-1α production; or (2) residual protein synthesis inhibition by the Δ*5* strain is sufficient to induce IL-1α. Consistent with the latter possibility, and with our previous work (Fontana et al., 2011), we found that the Δ*5*Δ*flaA* strain still significantly inhibited host protein synthesis in BMDMs (as measured by incorporation of $^{35}$S-methionine) as compared to infection with Δ*dotA*Δ*flaA*, which does not

block translation (Fig. 2.8B; (Fontana et al., 2011). These results raised the possibility that *L. pneumophila* might encode additional effectors that inhibit host protein synthesis. To identify these effectors we utilized a library of 259 known and putative secreted effectors (Losick et al., 2010), that we cloned into a mammalian expression vector. Each individual effector expression plasmid was co-transfected into 293T cells, along with a plasmid that constitutively expresses *Renilla* luciferase, and protein synthesis (as assessed by luminescence) was measured 24hrs after transfection (Fig. 2.9; Table 2.2). As a positive control, this screen successfully identified the five previously described effectors that are known to block host translation (Lpg0437, Lpg1368, Lpg1488, Lpg2504, and Lpg2862) (Fontana et al., 2011); Table 2.3). In addition, two other effectors that inhibit host protein synthesis were identified: Lpg0208, a Serine/Threonine Kinase, and Lpg1489, a putative effector of unknown function (Chien et al., 2004). Lpg0208 and Lpg1489 were confirmed to inhibit protein synthesis in 293T cells, as measured by reduced $^{35}$S-methionine incorporation upon overexpression of each effector (Fig. 2.10A, B). However, deletion of these two additional effectors in the Δ*5*Δ*flaA* background, to generate a strain we call Δ*7*Δ*flaA*, did not significantly affect the ability of *L. pneumophila* to inhibit host protein

| | Constructs Tested | Positive Hits | Negative Hits | % Positive Hit |
|---|---|---|---|---|
| 1° Screen | 259 | 33 | -- | 12.7 |
| Re-screen 1 | 33 | 9 | 24 | 27.3 |
| Re-screen 2 | 9 | 7 | 2 | 77.8 |

**Table 2.2:** Screening efficiencies of the effector library screen.

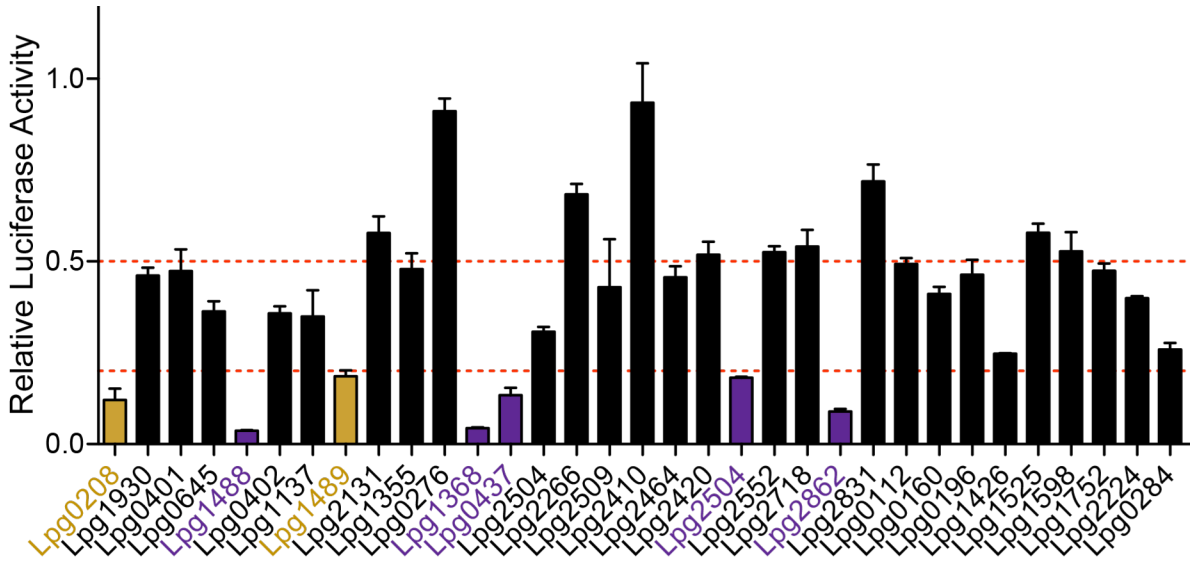


**Figure 2.9:** Results of final effector library screen. Initial hits in the effector library screen were re-screened by co-transfecting 293T cells with each individual effector and a Renilla luciferase reporter plasmid. Translation inhibition is measured by reduction in Renilla luciferase activity. Known bacterial effectors are highlighted in purple and the two novel bacterial effectors identified in this screen are shown in gold.

| Gene Number | Gene Name | Function | Target | Citation |
|---|---|---|---|---|
| Lpg0208 | pkn5 | Ser/Thr Kinase | Unknown | Chien *et al.*, 2005 |
| Lpg0437 | sidL | blocks translation | Unknown | Fontana *et al.*, 2012 |
| Lpg1368 | lgt1 | glucosyltransferase | eEF1A | Belyi *et al.*, 2008 |
| Lpg1488 | lgt3 | glucosyltransferase | eEF1A | Belyi *et al.*, 2008 |
| Lpg1489 | Hypothetical protein | unknown | unknown | Chien *et al.*, 2005 |
| Lpg2504 | sidI | binds eEF1A and eEF1Bγ | eEF1A and eEFBγ | Shen *et al.*, 2009 |
| Lpg2862 | lgt2 | glucosyltransferase | eEF1A | Belyi *et al.*, 2008 |

**Table 2.3:** Descriptions of the bacterial effectors that inhibit host translation.

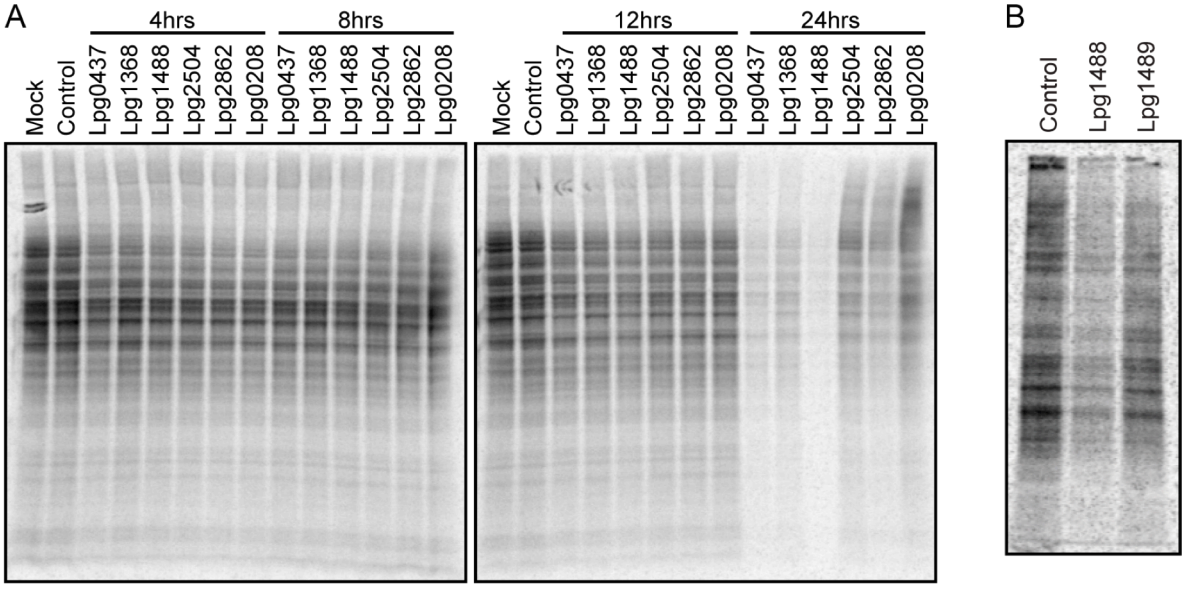


**Figure 2.10:** Novel bacterial effectors identified in effector library screen block host protein synthesis. (A) 293T cells were transfected with the indicated constructs. 4h, 8h, 12h, and 24h post-transfection cells were incubated with $^{35}$S-methionine for one hour followed by lysis in RIPA buffer. Protein levels were quantified and equal protein was loaded on the gel. (B) 293T cells were transfected with the indicated constructs and treated similar to A, except translation was assayed at 24h. Mock = no DNA transfection. Control = expression plasmid expressing β-glucuronidase gene. Lpg0208 = pkn5. Lpg1489 = hypothetical protein.

synthesis in macrophages (Fig. 2.8B). The Δ*7* strain also induced normal production of IL-1α *in vitro* (Fig. 2.8A). The residual ability of Δ*7 L. pneumophila* to inhibit host protein synthesis and/or induce IL-1α may therefore be due to additional effectors that were not present in our effector library. Alternatively, inhibition of host protein synthesis may result from the combined effects of multiple *L. pneumophila* effectors (which would not have been detected in our one-by-one effector screen), or the infection process itself.

**Section 2.3.7: Translation inhibition together with TLR activation is sufficient to induce IL-1α production *in vitro* and *in vivo***

The above results showed that induction of IL-1α by Δ*7 L. pneumophila* correlates with inhibition of host protein synthesis. We therefore wished to determine if inhibition of host protein synthesis is sufficient to cause IL-1α release *in vitro* and *in vivo*. In order to recapitulate TLR signaling that occurs during *L. pneumophila* infection, bone marrow derived macrophages (BMDMs) were treated with 10ng/mL Pam3CSK4, a TLR2 ligand. This treatment induced transient intracellular IL-1α protein (Fig. 2.11A) but did not result in significant IL-1α release (Fig. 2.11B). BMDMs were therefore additionally treated with 50ng/mL Exotoxin A (ExoA), a toxin made by *Pseudomonas aeruginosa* that blocks translation by inhibiting the activity of elongation factor 2a (reviewed in (Deng and Barbieri, 2008; Yates et al., 2006). As with TLR stimulation, ExoA treatment alone was insufficient to induce IL-1α production. However, we found that treatment of BMDMs with both Pam3CSK4 and ExoA combined to induce release of IL-1α at 24h post-infection (Fig. 2.11A, B). ExoA appeared to have two important effects that might explain its role in IL-1α release. First, in contrast to the transient induction of IL-1α induced by TLR signaling alone, additional treatment with ExoA caused the sustained presence of intracellular IL-1α protein at 24h post-treatment, similar to what is seen in *L. pneumophila* infection (Fig. 2.11A). The sustained production of IL-1α protein was associated with a prolonged elevation of *Il1a* mRNA (Fig. 2.11C). Second, ExoA caused cell death by 24hrs post-treatment (Fig. 2.11D), which may explain how intracellular accumulated IL-1α is released from these macrophages. Δ*flaA* and Δ*5*Δ*flaA L. pneumophila*-infected macrophages, which also experience a block in host protein synthesis, show sustained transcriptional induction, release IL-1α from the cell, and undergo cell death at 24h post-infection (Fig. 2.11A-D). Importantly, death of *L. pneumophila* infected cells does not appear to depend on bacterial replication because cell death and IL-1α release still occurred when bacterial replication was curtailed by removal of thymidine from the media. We speculate that inhibition of protein synthesis may be responsible for induction of host cell death. Protein synthesis inhibition and TLR stimulation also synergized to induce elevated IL-1α production *in vivo* (Fig. 2.11E). Taken together, these findings suggest that TLR activation in concert with translation inhibition can recapitulate IL-1α production and release in response to *L. pneumophila* infection and that this treatment is sufficient to induce release of IL-1α.

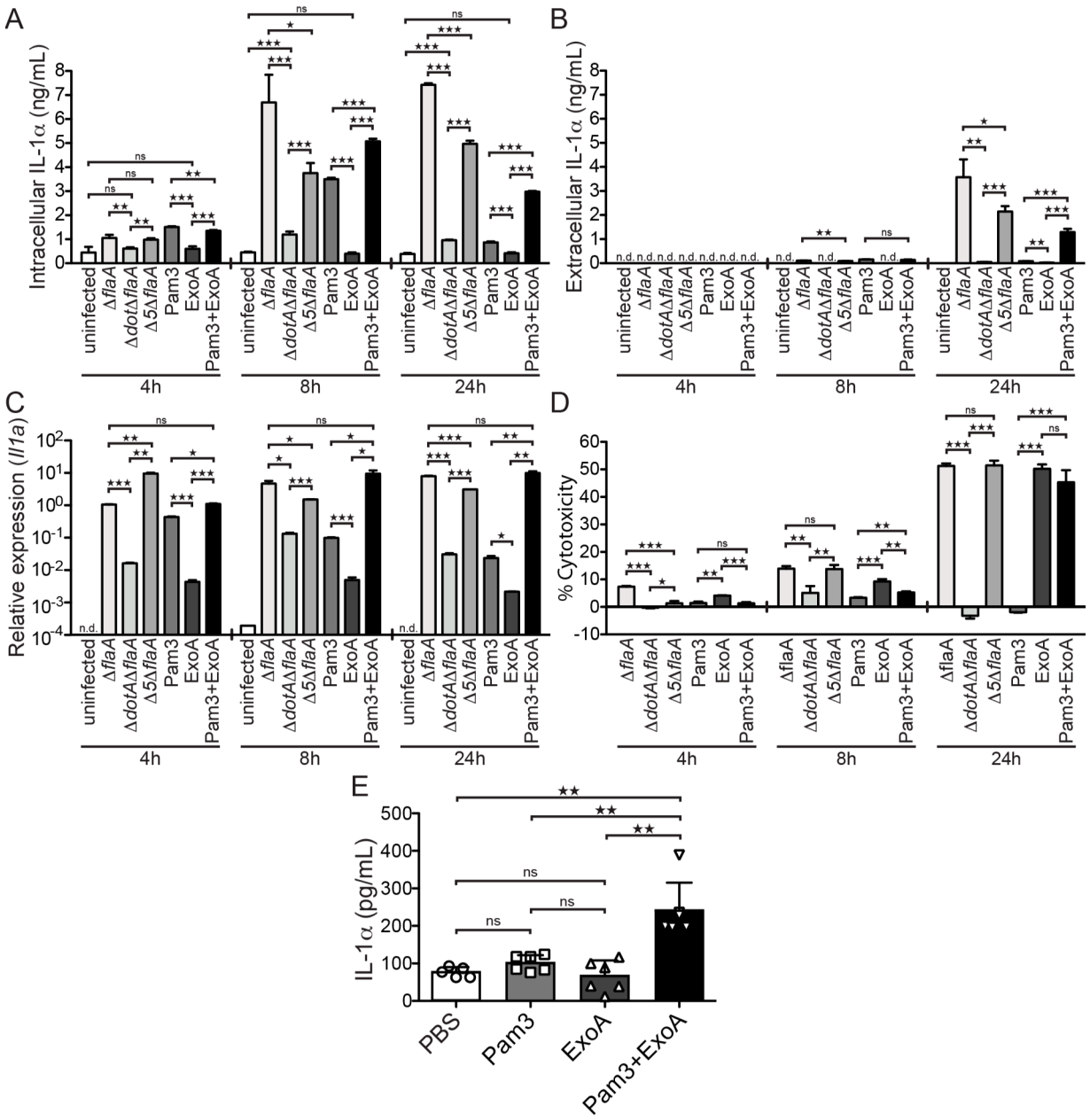


**Figure 2.11.** Translation inhibition in conjunction with TLR activation is sufficient to induce the production of IL-1α both *in vitro* and *in vivo*. (A-D) B6 BMDMs were infected with the indicated strains of *L. pneumophila* or treated with Pam3 (10ng/mL), ExoA (50ng/mL) or both Pam3 and ExoA. Samples were collected 4, 8, or 24h post-treatment. (A) Cells were lysed with RIPA buffer and intracellular IL-1α levels or (B) extracellular IL-1α levels in cell supernatants were determined. (C) *Il1a* transcript levels were assayed by qRT-PCR. (D) Cell cytotoxicity was determined by measuring release of LDH into cell supernatants. (E) B6 mice were treated intranasally with Pam3 (10μg/mouse), ExoA (2μg/mouse) or both in 20 μL of PBS. BAL fluid was collected 24h post-treatment and IL-1α levels determined by ELISA. Data are representative of three (A-E) experiments [mean ± s.d. in A-E]. *, $p<0.05$. **,$p<0.01$. ***,$p<0.001$. [Statistical analysis: Unpaired *t* test (A)-(D), Mann-Whitney *U* Test (E)]. n.d., Not detectable, n.s., not significant; Pam3, Pam3CSK4; ExoA, Exotoxin A.

## Section 2.4: Discussion

Legionnaires' disease is an inflammatory pneumonia associated with a pronounced influx of neutrophils to the lung (Winn and Myerowitz, 1981; Yu et al., 2009). The recruitment of neutrophils to the lung is important for controlling bacterial burden; however, excessive neutrophil recruitment can also be detrimental to the host and may be responsible for immune pathologies associated with Legionnaires' disease (Winn and Myerowitz, 1981; Yu et al., 2009). Thus, the host must tightly regulate the recruitment of neutrophils to the site of infection. In animal models of *L. pneumophila* infection, neutrophil recruitment has been shown to be important for protecting the host (LeibundGut-Landmann et al., 2011; Tateda et al., 2001a; Tateda et al., 2001b), yet the mechanism for this recruitment has remained unclear. A number of studies have demonstrated that MyD88 is an important host factor that protects mice from *L. pneumophila* infection (Archer et al., 2010; Archer et al., 2009; Archer and Roy, 2006; Hawn et al., 2006; LeibundGut-Landmann et al., 2011) and the IL-1R has been shown to be the critical receptor upstream of MyD88 that controls the recruitment of neutrophils to the lung in response to *L. pneumophila* infection (LeibundGut-Landmann et al., 2011). Indeed, it has been shown that IL-1R signaling is required in AECs to induce chemokines, such as CXCL1 and CXCL2, which then recruit neutrophils to the site of infection (LeibundGut-Landmann et al., 2011).

In our study, we identify the cytokine interleukin-1α (IL-1α) as a critical initiator of IL-1R-dependent neutrophil recruitment to the lungs of *L. pneumophila*-infected mice. We find that IL-1α, but not IL-1β, precedes neutrophil recruitment to the lung and we show that IL-1α is generated specifically by cells in the hematopoietic compartment (presumably infected macrophages). Given these data, we therefore propose a model by which IL-1α is produced by alveolar macrophages in response to virulent *L. pneumophila* and signals through the IL-1R on AECs, amplifying the original signal and generating chemokines, which recruit the initial wave of neutrophils to the lung. Importantly, at timepoints later than 12hrs post-infection, IL-1α and IL-1β can both signal through the IL-1R and compensate for the loss of each other. Our data suggest that IL-1α is one of the earliest cytokines produced in response to *L. pneumophila in vivo*, and thus initiates the recruitment of neutrophils and the inflammatory response to *L. pneumophila in vivo*.

Similar to *L. pneumophila*, *Streptococcus pneumoniae* leads to a severe pneumonia associated with massive influx of neutrophils. In mouse models of *S. pneumoniae* infection in the lung, *Il1a/I1b*$^{-/-}$ double knockout and *Il1b*$^{-/-}$ mice are more susceptible to disease and have decreased clearance of bacteria from the lung (Kafka et al., 2008). Moreover, *Il1r1*$^{-/-}$ mice have increased bacterial burden in the lung and decreased neutrophil recruitment to the lung (Marriott et al., 2012). Macrophage uptake of *S. pneumoniae* induces inflammasome activation and IL-1β release which can signal to epithelial cells to recruit neutrophils by releasing the chemokine CXCL8 (Marriott et al., 2012). Studies with *S. pneumoniae* suggest a model whereby activated macrophages secrete IL-1β which signals through the IL-1R of AECs thus leading to the production of chemokines, which recruit neutrophils to the site of infection (Kafka et al., 2008; Marriott et al., 2012). This proposed mechanism is similar to the mechanism that we propose for *L. pneumophila* infections, except that it appears that IL-1α, rather than IL-1β, is the dominant cytokine early during *L. pneumophila* infections. These studies with *S. pneumoniae* suggest that amplification of early responses to infection by IL-1R signaling in AECs may be a conserved immune strategy important for recruiting neutrophils in response to bacterial infections. Importantly, the role for IL-1α in *S. pneumoniae* infections remains unclear.

In addition to *S. pneumoniae*, IL-1R signaling has been shown to be important for host protection from numerous pathogens, including *Listeria monocytogenes* (Havell et al., 1992; Labow et al., 1997; Rogers et al., 1992), *Mycobacterium tuberculosis* (Guler et al., 2011; Juffermans et al., 2000; Mayer-Barber et al., 2010; Zuckman et al., 1999), *Pseudomonas aeruginosa* (Mijares et al., 2011), *Staphylococcus aureus* (Miller and Cho, 2011), *Klebsiella pneumoniae* (Cai et al., 2012) and *Candida albicans* (Bellocchio et al., 2004). In many of these infections, the mechanism by which IL-1R provides protection is not clear, and the relative roles of IL-1α and IL-1β have not been elucidated. One study that dissected the relative roles of IL-1α and IL-1β during *M. tuberculosis* infection found each cytokine played essential and non-redundant roles *in vivo* (Mayer-Barber et al., 2011). This study, along with our results showing that IL-1α is of primary importance in early responses to *L. pneumophila in vivo*, suggest it will be worthwhile to examine more carefully the relative contributions of IL-1α and IL-1β in mediating IL-1R-dependent responses to other pathogens as well.

The molecular mechanism leading to IL-1α production has remained elusive (Dinarello, 2009). This is in stark contrast to IL-1β production, where intensive effort over the past decade has defined the mechanisms leading to IL-1β release downstream of inflammasome activation (reviewed in (von Moltke et al., 2012a). Our data suggest that equal attention should be paid to the mechanisms of IL-1α production. Indeed, IL-1α has been shown to be induced in response to a number of bacterial pathogens including *L. pneumophila* (Fontana et al., 2012; Shin et al., 2008), *L. monocytogenes* (Dewamitta et al., 2010), *S. aureus* (Olaru and Jensen, 2010), and *M. tuberculosis* (Mayer-Barber et al., 2011; Mayer-Barber et al., 2010); however, the molecular mechanism of IL-1α production in response to these pathogens remains unsettled. Classic studies showed that IL-1α can be cleaved by the Calpain family of calcium dependent proteases, but IL-1α does not appear to require processing to signal through the IL-1R (Dinarello, 2009; Gross et al., 2012; Mosley et al., 1987). Some reports have suggested that IL-1α production in response to non-infectious stimuli such as toxins can involve activation of the Caspase-1 or Caspase-11 inflammasomes (Fettelschoss et al., 2011; Gross et al.; Keller et al.; Zheng et al., 2013). Additionally, a previous report suggests that at 4hrs post-infection *Casp1/11*$^{-/-}$ mice have defects in IL-1α production in response to *L. pneumophila* infection *in vivo* (Fettelschoss et al., 2011). In contrast to these reports, our data show that *Casp1/11*$^{-/-}$ mice have no defect in IL-1α production in response to *L. pneumophila* infection *in vivo*. This difference may be due to the different strains of *L. pneumophila* used in the two studies. Nevertheless, our results indicate that IL-1α and IL-1β can be produced via distinct but complementary pathways that provide alternative means to induce IL-1R signaling and immune defense *in vivo*. Given the critical importance of neutrophils in providing defense against numerous bacterial pathogens, it is perhaps to be expected that hosts would not rely on a single mechanism for activation of IL-1R signaling that could then be easily subverted or avoided.

Instead of a role for the inflammasome in IL-1α release, our data show that translation inhibition in concert with TLR stimulation is sufficient to induce IL-1α both *in vitro* and *in vivo*. Recent work from our lab and others have shown that in mice, and in *C. elegans*, translation inhibition can be sensed by the host and induce a number of immunological responses, including the production of pro-inflammatory cytokines (Dunbar et al., 2012; Fontana et al., 2011; Fontana et al., 2012; Fontana and Vance, 2011; McEwan et al., 2012). Previous research identified five *L. pneumophila* effectors that block host translation (Belyi et al., 2006; Belyi et al., 2008; Fontana et al., 2011; Shen et al., 2009). We previously found that this translation block induces a host stress response that can induce a subset of inflammatory cytokines, including IL-23 and GM-

CSF (Fontana et al., 2011; Fontana et al., 2012). Although the *L. pneumophila* Δ*5* strain lacking the five effectors is partially defective in its ability to inhibit host protein synthesis (Fontana et al., 2011) and is defective for IL-23 and GM-CSF induction, we confirmed here that cells infected with Δ*5 L. pneumophila* still experience a significant block in protein synthesis. Consistent with our finding that protein synthesis inhibition and TLR signaling is sufficient to induce IL-1α, we also find that Δ*5*-infected cells still produce IL-1α. In fact, even after identifying two novel bacterial effectors that block host translation, and generating an *L. pneumophila* mutant (Δ*7*) that lacks these effectors in addition to the original five effectors, we were still unable to abolish the Dot/Icm-dependent ability of *L. pneumophila* to inhibit protein synthesis and induce IL-1α. We propose several hypotheses to explain these results. First, there may be additional bacterial effectors in *L. pneumophila* that are not in our library of cloned effectors. Given that *L. pneumophila* is a generalist and has a multitude of natural hosts (O'Connor et al., 2011), it is possible that there is substantial additional redundancy encoded in the *L. pneumophila* genome. A second possibility is that there may not be a specific *L. pneumophila* effector that targets the host protein synthesis machinery; instead, the blockade of protein synthesis we observe may be the result of a *host* response to the infection process itself. Indeed, translation inhibition has long been recognized as a protective response during viral infections (Walsh et al., 2013), and it is now evident that numerous bacterial infections can elicit host stress pathways that affect protein synthesis, for example via phosphorylation of eukaryotic initiation factor 2α (eIF2α) (Mohr and Sonenberg, 2012). Lastly, it is possible that the ability of *L. pneumophila* to induce IL-1α is unrelated to protein synthesis inhibition. We tend not to favor this latter possibility because we found that inhibition of protein synthesis in conjunction with TLR signaling was sufficient to induce IL-1α, and moreover, it is clear that *L. pneumophila* infection results in both TLR signaling and inhibition of protein synthesis. Thus, while the mechanism of IL-1α production continues to elude the field, it seems likely that translation inhibition is at least one mechanism for IL-1α induction, even if other parallel mechanisms might also exist.

Together, our data show that IL-1α is a major cytokine responsible for the early recruitment of neutrophils to the lung in response to *L. pneumophila* infection from 0-12 hours post-infection. We propose that a dominant role for IL-1α in protection against microbial infection may hold true for other pathogens, depending on the stage and mode of infection. Although much recent work has focused on the mechanisms of IL-1β production, our work suggests that IL-1α signaling can be as important, or indeed more important, than IL-1β signaling *in vivo*. Indeed, it is probably evolutionarily advantageous for hosts to encode multiple parallel pathways to induce IL-1R signaling, given the critical role that the IL-1R appears to play in orchestrating neutrophil recruitment and other immune responses *in vivo*.

# Chapter 3: Global analysis of translation regulation following infection with intracellular bacterial pathogens

## Section 3.1: Introduction

The innate immune system is the first line of defense against pathogens. One important family of receptors in the innate immune system is the Toll-like receptors (TLRs) (Brubaker et al., 2015). TLRs recognize conserved molecular patterns of microbes and initiate a signaling cascade using the important adapter protein MyD88 (Brubaker et al., 2015; Takeda et al., 2003). It has increasingly become clear that, while integrally important for protecting the host from infection, the TLRs alone cannot explain the specific responses the innate immune system mounts toward pathogens (Fontana and Vance, 2011; Vance et al., 2009). Pathogen-associated activities (e.g. growth, cytoskeleton remodeling) have been suggested to give the contextual cue to a cell that it is infected with a pathogen (Fontana and Vance, 2011; Vance et al., 2009). TLR signaling in concert with recognition of a pathogen-associated activity induces inflammatory responses specifically to pathogens (Fontana and Vance, 2011; Vance et al., 2009). One such pathogen-associated activity that we, and others, have described is the inhibition of host protein synthesis (Barry et al., 2013; Chakrabarti et al., 2012; Dunbar et al., 2012; Fontana et al., 2011; Fontana and Vance, 2011; Lemaitre and Girardin, 2013; McEwan et al., 2012; Mohr and Sonenberg, 2012). We have shown that host inhibition of translation is only induced in response to virulent *Legionella pneumophila* and have linked this activity to the preferential production of specific cytokines (Barry et al., 2013; Fontana et al., 2011).

*Legionella pneumophila* is a gram-negative intracellular pathogen and the causative agent of a severe pneumonia called Legionnaire's Disease. The normal host of *L. pneumophila* is freshwater amoebae but it is also capable of infecting alveolar macrophages in the lung (Copenhaver et al., 2014; Fields, 1996). *L. pneumophila* virulence *in vivo* and growth in macrophages *in vitro* requires a bacterial Dot/Icm Type IV secretion system (T4SS) that injects greater than 270 effector proteins into the host cytosol (Luo, 2012). Many of the *L. pneumophila* secreted effector proteins are required for the bacteria to remodel the phagosomal membrane, turning this organelle into a replicative niche for the bacteria (Luo, 2012). Innate immune responses to *L. pneumophila* are also directly linked to the presence of a functional T4SS (Luo, 2012; Shin et al., 2008). Previously, we identified 7 bacterial effectors that block host protein synthesis (Barry et al., 2013; Fontana et al., 2011). We were further able to link translation inhibition with recognition of pathogenic infection and the production of a subset of inflammatory cytokines, including IL-23, GM-CSF and IL-1α (Barry et al., 2013; Fontana et al., 2011). We showed that translation inhibition in concert with TLR signaling leads to the massive induction of cytokine transcripts and proposed that this induction of transcript could overcome the block in host protein synthesis and allowed for preferential production of cytokines in response to pathogenic infection (Barry et al., 2013; Fontana et al., 2011). Alternatively, it has been proposed that translation of the cytokines IL-1α and IL-1β is due to MyD88-dependent preferential translation (e.g. increased ribosome binding) of the *Il1a* and *Il1b* transcripts in the cell (Asrat et al., 2014). In this alternative model the authors suggest that preferential translation of specific cytokine transcripts explained how these cytokines were produced even in the presence of *L. pneumophila* induced translation inhibition (Asrat et al., 2014). Global analyses are required to distinguish between these two models of inflammatory cytokine production in response *L. pneumophila* infection.

Interestingly, deletion of all 7 of the *L. pneumophila* effectors that block host protein synthesis has no effect on *L. pneumophila* induced translation inhibition (Barry et al., 2013). The lack of a phenotype in the Δ7 *L. pneumophila* strain suggested that translation was being blocked by another mechanism and we hypothesized there were two possibilities: first, an unidentified bacterial effector was blocking translation, or second, the host sensed the stresses of being infected by *L. pneumophila* and induced blockade of its own translation through stress response pathways. Consistent with stress response pathways blocking translation in response to *L. pneumophila* infection, a previous study suggested that *L. pneumophila* infection inhibits mTOR activity and induces translation blockade through this pathway (Ivanov and Roy, 2013). Importantly, this study utilized wildtype *L. pneumophila* and did not address the role of the 7 bacterial effectors that block translation (Ivanov and Roy, 2013).

In this study we utilized the ribosome profiling technique (Ingolia et al., 2012; Ingolia et al., 2009; Ingolia et al., 2011) and RNAseq of mRNA transcripts to track global changes in translation and mRNA levels in response to pathogenic infection. We found that *L. pneumophila* strains lacking the 7 bacterial effectors known to block host protein synthesis appear to induce residual translation inhibition at the level of translation initiation, suggesting that *L pneumophila* infection may induce host stress response pathways that in turn block host protein synthesis. Attempts to identify the stress response pathway required for this residual translation inhibition did not lead to any candidates, in contrast to previous reports (Ivanov and Roy, 2013). Undertaking global analyses of translation and mRNA levels after *L. pneumophila* infection also allowed us to address questions about the relative contributions of translational regulation and mRNA induction in controlling immune responses to pathogenic *L. pneumophila*. Previous reports have shown that after 12h LPS treatment mRNA levels explain ~87-92% of fold protein changes in BMDCs (Jovanovic et al., 2015). Consistent with this previous study, our data looking at macrophages infected with *L. pneumophila* support a model in which the vast majority of gene induction in response to pathogenic infections occurs at the level of mRNA induction. Finally, we broadened our understanding of translation inhibition induced by pathogens by demonstrating that *Listeria monocytogenes*, an intracellular bacterium with a distinct life cycle from *L. pneumophila*, can also induce host translation inhibition.

## Section 3.2: Materials and Methods

### Section 3.2.1: Ethics statement

These studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee at the University of California, Berkeley.

### Section 3.2.2: Cell culture

Macrophages were derived from the bone marrow of C57BL/6J (Jackson Laboratory), *Ifnar* $^{-/-}$ (Jackson Laboratory), *Myd88,Trif* $^{-/-}$ (G. Barton, University of California, Berkeley), and *Myd88,Nod1,Nod2* $^{-/-}$ (generated from crosses at the University of California, Berkeley) mice on the B6 background. Macrophages were derived by 8 days of culture in RPMI supplemented with 10% serum, 100μM streptomycin, 100U/mL penicillin, 2mM L-glutamine and 10% supernatant from 3T3-macrophage-colony stimulating factor cells, with feeding on day 5. HEK293T and HeLa cells were grown in complete media (DMEM, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine). Cells were re-plated 24hrs prior to infection with *L. pneumophila* or *L. monocytogenes*.

### Section 3.2.3: Bacterial strains and infections

All *L. pneumophila* strains were derived from LP02, a streptomycin-resistant thymidine auxotroph derived from *L. pneumophila* LP01. The Δ*dotA,* Δ*flaA,* Δ*5*Δ*flaA*, and Δ7Δ*flaA* strains were generated on the LP02 background and have been described previously (Barry et al., 2013; Fontana et al., 2011; Fontana et al., 2012; Ren et al., 2006). Unless otherwise noted, all strains used for *in vitro* infections were deficient for bacterial flagellin (*ΔflaA)* and thus non-motile. *L. pneumophila* from the Δ*flaA* background were utilized *in vitro* to avoid activation of the NAIP5/NLRC4 inflammasome (Amer et al., 2006; Lightfield et al., 2008; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). Bone marrow derived macrophages were plated at a density of $1.56x10^5$ cells per $cm^2$ ($1.5.x10^6$ cells per well of a 6-well plate) and infected at an MOI of 1-3 (as indicated) by centrifugation for 10min at 400 x*g*. After one hour of infection media was changed. All *in vitro L. pneumophila* infections were in the absence of thymidine to curtail bacterial replication. For *Listeria* infections, bone marrow derived macrophages were plated at a density of $1.56x10^5$ cells per $cm^2$ ($1.5.x10^6$ cells per well of a 6-well plate). WT 10403S (DP-L184) and Δ*hly* (DPL2161) *L. monocytogenes* were grown overnight, without shaking, to stationary phase ($OD_{600}$, 1.3–1.6). Overnight cultures were diluted 1:10 in BHI media and grown at 30°C to mid-log ($OD_{600}$ = 0.6) without shaking. Bacteria were then PBS-washed, resuspended in PBS at a normalized $OD_{600}$ = 1.5, and added to macrophages at a 1:100 volume:volume ratio. 0.5h post-infection macrophages were washed twice with PBS and fresh media was added. 1h post-infection gentamicin (final concentration of 50μg/mL) was added to the media to prevent extracellular bacterial growth.

### Section 3.2.4: Ribosome profiling

Ribosome profiling experiments were undertaken as previously described (Ingolia et al., 2012). Briefly, B6 bone marrow derived macrophages were plated in tissue culture treated 6-well plates or 75cm$^2$ flasks at a density of approximately 1.56x10$^5$ cells per cm$^2$ (1.5x10$^6$ macrophages per well of a 6-well plate or 1.2x10$^7$ macrophages per 75cm$^2$ flask). Macrophages were infected with the indicated strains of *L. pneumophila* at an MOI=3, or left untreated, spun at 1,200 RPM for 10 min and allowed to infect at 37°C in 5% $CO_2$ for 1hr. After 1hr incubation media was replaced and the cells were incubated for an additional 5hrs at 37°C in 5% $CO_2$. When used, harringtonine treatment was performed as previously described (Ingolia et al., 2012). Briefly, harringtonine (LKT Laboratories) was added at a final concentration of 2 μg/mL for 90 seconds at the end of the 6hr infection. 100 μg/mL of cycloheximide (Sigma) was added to freeze ribosomes after the 90-second harringtonine treatment. Following cycloheximide treatment cells were immediately lysed. For all ribosome profiling experiments cells were lysed by flash freezing and thawed in the presence of lysis buffer as previously described (Ingolia et al., 2012). Clarified lysates were split and some was used to generate ribosome footprints while some was used to isolate total RNA for RNA sequencing (described below). All RNA and DNA gel extractions were performed overnight as previously described (Ingolia et al., 2012). The Ribo-Zero Gold rRNA Removal Kit (Illumina) was used to remove rRNA from ribosome profiling samples before the dephosphorylation and linker ligation steps described by Ingolia *et al.* 2012 (Ingolia et al., 2012).

**Section 3.2.6: Generation of RNAseq libraries**

Macrophages were lysed by the methods described above following the ribosome profiling protocol (Ingolia et al., 2012). The clarified lysates were split and 300μL of lysate was mixed with 900μL of Trizol LS (Life Technologies). RNA was isolated following the manufacturer's guidelines. RNA integrity was measured utilizing the RNA Pico method on the Agilent 2100 Bioanalyzer at the University of California, Berkeley Functional Genomics Laboratory. High quality RNA with a RNA integrity number (RIN) >8 (Agilent Technologies) was submitted to the QB3-Berkeley Functional Genomics Laboratory and single read 100 base pair read length (SR100) sequencing libraries were generated. Libraries were sequenced on a HiSeq2000 System (Illumina) by the Vincent J. Coates Genomics Sequencing Laboratory at UC, Berkeley. This work used the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley, supported by NIH S10 instrumentation Grants S10RR029667 and S10RR027303.

**Section 3.2.7: Alignment of ribosome footprint sequences**

Sequences were processed as described in (Ingolia et al., 2012). Briefly, sequences were preprocessed by trimming the linker sequence from the 3' end of each sequencing read and removing the first nucleotide from the 5' end of each read. Reads were then aligned to a rRNA reference using the Bowtie short-read alignment program. All sequences aligning the rRNA reference were discarded. All non-rRNA sequencing reads were aligned using the TopHat splicing-aware short-read alignment program to a library of transcripts derived from the UCSC Known Genes data set, and those with no acceptable transcript alignment were then aligned against the genome (mm10). Perfect-match alignments were extracted and these files were used for analyses. For most analyses, footprint alignments were assigned to specific A site nucleotides by using the position and total length of each alignment, calibrated from footprints at

the beginning and the end of CDSes as previously described (Ingolia et al., 2012; Ingolia et al., 2011).

**Section 3.2.8: Alignment of RNAseq reads.**

RNA sequencing reads were preprocessed by trimming the linker sequence from the 3' end of each read and removing the first 10 nucleotides from the 5' of each read. The first 10 nucleotides at the 5' of reads had an overrepresentation of certain nucleotides. All high quality and processed sequencing reads were aligned using the TopHat splicing-aware short-read alignment program to a library of transcripts derived from the UCSC Known Genes data set, and those with no acceptable transcript alignment were then aligned against the genome (mm10). Aligned reads were then used for further analyses.

**Section 3.2.9: Footprint profile analysis**

Profiles of footprints across a transcript were constructed by generating bigWig files as previously described (Ingolia et al., 2009; Ingolia et al., 2011). These tracks were visualized using the Integrative Genomics Viewer (Broad Institute; Robinson et al., 2011; Thorvaldsdottir et al., 2013). Metagene profiles were generated as previously described (Ingolia et al., 2009; Ingolia et al., 2011).

**Section 3.2.10: Counting reads aligning to coding sequences as a measure of gene expression**

Counting of reads was performed as previously described (Ingolia et al., 2009; Ingolia et al., 2011). Reads were mapped to coding sequences and counted, excluding reads that mapped to the first 15 codons or the last 5 codons of a CDS due to accumulation of ribosomes (Ingolia et al., 2011).

**Section 3.2.11: Normalization of ribosome footprint and mRNA read counts**

Longer transcripts inherently have increased ribosome footprint read counts so ribosome footprint read counts were normalized to sequence length. This normalization generated a ribosome footprint read density (read density = read count ÷ transcript length). In order to compare across different conditions/libraries read densities of each library condition (e.g. Δ*flaA*, Δ*dotA*Δ*flaA*, or Δ*7*Δ*flaA* infected) were normalized to the sum of ribosome footprint reads of 12 mitochondrial protein-coding genes. Mitochondrial protein-coding gene read counts provide an estimate of total cells in each condition and allow for comparison among different conditions and libraries. mRNA read counts were normalized in the same manner using total mRNA read counts of the 12 mitochondrial protein-coding genes. Normalized read counts were used in subsequent analyses.

**Section 3.2.12: Analysis of ribosome footprint libraries for ribosome occupancy and T4SS-dependent gene induction**

Read counts from four independent experiments were averaged for this analysis. Average read counts were sorted and any transcript with fewer than 100 ribosome footprint and/or RNAseq reads in the Δ*flaA L. pneumophila* infected condition were removed from the dataset. This filtering left 6,407 transcripts from the 30,407 in the starting dataset. The filtered dataset was normalized as described above and used to calculate the T4SS-dependent regulation of genes (Figure 3.11) and the Δ*flaA* ribosome footprint vs. RNAseq plot (Figure 3.10A). A similar filtering strategy was used to calculate the ribosome footprint and RNAseq reads from the Δ*dotA*Δ*flaA* infected conditions. Again, average read counts from Δ*dotA*Δ*flaA*-infected conditions were sorted for transcripts with greater than 100 ribosome footprint reads. This sorting left 8,705 transcripts. The remaining transcripts were then sorted for transcripts that had greater than 100 RNAseq reads leaving 7,917 transcripts that were analyzed (Figure 3.10B).

**Section 3.2.13: MyD88-dependent gene induction analysis**

Sequences from a single experiment were used for these analyses. For analysis of Δ*flaA* infected macrophages raw ribosome footprint read counts were sorted on B6 BMDMs infected with Δ*flaA L. pneumophila* with ribosome footprint read counts ≥ 100. After this sort 6,941 RefSeq annotated transcripts, of the original 30,407 annotated sequences in the library, remained in the dataset. Any transcript in the Δ*flaA*-infected condition that had zero RNAseq reads but in the same condition had ribosome footprint reads was removed from the dataset, as this is likely noise caused by low expression levels. This filtering step left 6,930 transcripts for analysis. These normalized read counts were then used to calculate MyD88-dependent translational and transcriptional gene induction by Δ*flaA L. pneumophila* infection (MyD88-dependent induction = B6-Δ*flaA* read counts ÷ *Myd88*$^{-/-}$-Δ*flaA* read counts) (Figure 3.12A). For analysis of Δ*dotA*Δ*flaA* infected macrophages raw ribosome footprint read counts were sorted on B6 BMDMs infected with Δ*dotA*Δ*flaA L. pneumophila* with ribosome footprint read counts ≥ 100. Of the original 30,407 annotated sequences in the library, 9,421 RefSeq annotated transcripts remained in the dataset after this filtering step. As described above, any remaining transcript with zero RNA reads was removed from the dataset leaving 9,393 transcripts to be analyzed. These normalized read counts were then used to calculate the MyD88-dependent translational and transcriptional gene induction in response to Δ*dotA*Δ*flaA L. pneumophila* (MyD88-dependent induction = B6-Δ*dotA*Δ*flaA* read counts ÷ *Myd88*$^{-/-}$-Δ*dotA*Δ*flaA* read counts) (Figure 3.12B).

**Section 3.2.14: Bicistronic reporter assay**

1x10$^5$ HeLa cells were seeded in 24-well plates and transfected with 400ng of a plasmid containing SV40-*Renilla* Luciferase-CrPV-Firefly Lciferase, 200ng of a plasmid express FcRγ, and 200ng of empty pCDNA3 using Lipofectamine 2000 (Life Technologies). 24h after infection HeLa cells were infected with Δ*flaA*, Δ*dotA*Δ*flaA*, or Δ7Δ*flaA L. pneumophila*. *L. pneumophila* strains were opsonized in anti-*Legionella* antibody (1:1000 dilution; Fitzgerald Industries International Inc.) and infected at a MOI of 10. 6h post-infection cells were lysed in passive lysis buffer (Promega) for 5 min at 25°C. Cell lysates were split and half was incubated with Firefly luciferase substrate and the other half with the *Renilla* substrate coelenterazine (Biotium). Luminescence was measured on a SpectraMax L microplate reader (Molecular Devices). The ratio of cap-dependent:cap-independent translation was calculated by dividing the

RLUs measured by Renilla luciferase activity by the RLUs measured by Firefly luciferase (Ratio of cap-dependent:cap-independent translation = Renilla RLUs ÷ Firefly RLUs). All ratios were normalized to the uninfected control. Results are from one experiment.

**Section 3.2.15: $^{35}$S-methionine metabolic labeling**

$5x10^5$ bone marrow derived macrophages were seeded in 24-well plates and infected with bacterial strains at an MOI of 3. 25 minutes prior to labeling, macrophages were treated with 25μg/mL chloramphenicol to inhibit bacterial translation. At 6 and 24hrs post-infection media was removed and incubated with 25μCi/mL $^{35}$S-methionine (Perkin Elmer) in RPMI without methionine supplemented with 10% serum, 2mM L-glutamine, 25μg/mL chloramphenicol, and 10% supernatant from 3T3-macrophage-colony stimulating factor cells. Cells were labeled for 1 hour, washed three times with cold PBS and then lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with 2 mM $Na_3VO_4$, 1 mM PMSF, 25 mM NaF, and 1x Roche protease inhibitor cocktail (no EDTA), pH 7.2, for 10 minutes at 4°C. Total protein levels were measured by bicinchoninic acid (BCA) assay and equal amounts of protein were mixed with SDS sample buffer (40% glycerol, 8% SDS, 2% 2-mercaptoethanol, 40 mM EDTA, 0.05% bromophenol blue and 250 mM Tris-HCl, pH 6.8), boiled for 5 min and then separated by SDS–PAGE. The gels were stained with coomassie blue to show equal protein loading, dried, and exposed to a phosphor screen and visualized using a Typhoon Trio imager (GE Healthcare)

**Section 3.2.16: ELISAs**

At the indicated time post-treatment cell supernatants were collected and analyzed by ELISA using paired interleukin-1α antibodies (BD Biosciences and eBioscience). Recombinant IL-1α (eBioscience) was used as a standard for the ELISA.

**Section 3.2.17: Western blots and antibodies**

Antibodies used in this study: Anti-phospho-mTOR (Ser2448; clone D9C2), anti-mTOR (clone 7C10), anti-phospho-p70 S6 Kinase (Thr389; clone 108D2), anti-phospho-p70 S6 Kinase (Ser371), anti-p70 S6 Kinase (clone 49D7), anti-phospho-4E-BP1 (Thr37/46; clone 236B4), anti-phospho-4E-BP1 (Ser 65; clone 174A9), anti-4E-BP1 (clone 53H11), anti-phospho-AMPKα (Thr172; clone 40H9), anti-AMPKα (clone 23A3), anti-phospho-eEF2 (Thr56), anti-eEF2, anti-phospho-eIF2α (Ser51; clone 119A11), anti-eIF2α (clone L57A5) (Cell Signaling), and anti-β-actin (clone C4) (Santa Cruz Biotechnology). The mTOR inhibitors Torin1 (250 nM; Tocris Bioscience) and Rapamycin (100nM; Cell Signaling) were used to block mTOR activity. Lipopolysaccharide (100ng/mL; LPS O55:B5; Sigma) and Pam3CSK4 (10ng/mL; Invivogen), a synthetic bacterial lipopeptide, were used as mTOR activators. The AMPK activator AICAR (1mM; Sigma) was used as a positive control for AMPK activation. Thapsigargin (500nM; Sigma) and tunicamycin (5μg/mL; Sigma) were used as positive controls for phospho-eIF2α (Ser51). Cells were lysed in RIPA buffer supplemented with 2 mM $Na_3VO_4$, 1 mM PMSF, 25 mM NaF, and 1x Roche protease inhibitor cocktail (no EDTA), pH 7.2, for 10 minutes at 4°C. Equal amounts of total protein were separated by denaturing PAGE and transferred to Immobilon-FL PVDF membranes (Millipore). Membranes were blocked with Li-Cor Odyssey blocking buffer. Immunoblots were imaged using a Li-Cor fluorimeter. All blocking and

antibody incubation steps were performed in the presence of 25mM β-glycerophosphate, 2mM $Na_3VO_4$, and 25mM NaF to inhibit phosphatase activity.

**Section 3.2.18: Gadd34 overexpression and eIF2α phosphorylation**

HeLa cells were plated at $1x10^5$ cells per well in 24 well plates and left over night. The next day cells were transfected with the pQXCIH plasmid expressing destabilized firefly luciferase, a plasmid encoding FcRγ, empty pCDNA3, and, where indicated, a plasmid encoding Gadd34 (Plasmid 21834; AddGene, Inc.) using Lipofectamine 2000 (Life Technologies). Transfections were performed following the manufacturer's guidelines. Transfections were left overnight to allow expression of the transfected constructs. *L. pneumophila* was opsonized in anti-*Legionella* antibody (1:1000 dilution; Fitzgerald Industries International Inc.) and infected at a MOI of 10. 6h post-infection cells were lysed in passive lysis buffer (Promega) for 5 min at 25°C. Cell lysates were incubated with the Firefly luciferase substrate and luminescence was measured on a SpectraMax L microplate reader (Molecular Devices). The relative block in translation was measured by comparing Firefly luciferase luminescence in uninfected cells. Thapsigargin (500nM) was used as a control to induce phosphorylation of eIF2α and translation inhibition. Two independent transfections were used to generate the phospho-eIF2α blots and translation inhibition results.

## Section 3.3: Results:

### Section 3.3.1: Ribosome footprints of infected macrophages map to coding sequences

It is becoming increasingly clear that translation regulation is an important part of sensing and responding to pathogens (Reviewed in Lemaitre and Girardin, 2013; Mohr and Sonenberg, 2012). We, and others, have demonstrated that translation inhibition induced in host cells following infection by pathogenic bacteria can induce an inflammatory response (Barry et al., 2013; Chakrabarti et al., 2012; Dunbar et al., 2012; Fontana et al., 2011; Fontana et al., 2012; McEwan et al., 2012). We previously showed that translation inhibition in concert with TLR signaling was sufficient for the production IL-1α protein *in vitro* and *in vivo*, and we showed that IL-1α is an important cytokine that initiates the inflammatory response to *Legionella pneumophila in vivo* (Barry et al., 2013). To obtain a better understanding of the global effects that infection by bacterial pathogens has on host translation we undertook experiments using ribosome profiling (Ingolia et al., 2012; Ingolia et al., 2009; Ingolia et al., 2011) and total RNA sequencing. B6 bone marrow derived macrophages (BMDMs) were infected with Δ*flaA*, Δ*dotA*Δ*flaA*, or Δ*7*Δ*flaA Legionella pneumophila*, and at 6hrs post-infection, RNA was isolated form the infected macrophages. This time point was chosen because this was the earliest time point we could detect significant translation inhibition induced by *L. pneumophila* without non-specific cell cytotoxicity (data not shown). The RNA isolated from infected macrophages was split and part of the sample was used to isolate polysomes and prepare ribosome profiling libraries while the other part was used to generate RNAseq libraries to measure total RNA levels. *L. pneumophila* on the Δ*flaA* background were used in these studies to avoid the confounding effects of NAIP5/NLRC4 inflammasome activation by flagellin. We previously showed that *L. pneumophila* on the Δ*flaA* background has no defect in inducing translation inhibition and production of inflammatory cytokines (Barry et al., 2013). Using RNAseq and ribosome profiling we were able to identify global changes in translation (Ingolia et al., 2009; Ingolia et al., 2011) and total RNA abundance from the same conditions. Ribosome footprint reads were mapped onto the genome. As an important validation that the reads we obtained were indeed ribosome-protected fragments, we found that the vast majority of ribosome footprint reads map to the coding regions of genes (Figure 3.1A-F). A subset of genes, including *Il23a*, *Csf2*, *Dusp1*, *Actb*, *Il1a*, and *Il1b* are depicted in Figure 3.1 and show that ribosome footprints all map to the coding sequences of these genes. Consistent with previous studies that showed *Csf2* and *Il23a* expression was dependent on the *L. pneumophila* effectors that block host protein synthesis (Fontana et al., 2011), the Δ*7*Δ*flaA* ribosome footprints show reduced levels of *Csf2* and *Il23a* ribosome occupancy in our ribosome profiling libraries (Figure 3.1A-B). *Dusp1* transcript has been shown to be induced by WT *L. pneumophila* in a T4SS-dependent manner (Asrat et al., 2014). Further, DUSP1 protein levels are not similarly upregulated in response to *L. pneumophila* infection (Asrat et al., 2014). Interestingly, the ribosome profiling footprints map robustly to the *Dusp1* transcript suggesting that there may be translation of this transcript or that translation inhibition induced by *L. pneumophila* is stalling ribosomes on this transcript (Figure 3.1C). Importantly, ribosome footprints mapping to the *Actb* transcript appear to be similar among all three *L. pneumophila* strains (Figure 3.1D). *Il1a* and *Il1b* transcripts also only have ribosome footprints mapping to the CDS of these transcripts and appear to have increased ribosome footprints on these transcripts in response to Δ*flaA* and Δ*7*Δ*flaA L. pneumophila* infections. As further validation of the quality of the ribosome profiling libraries that we

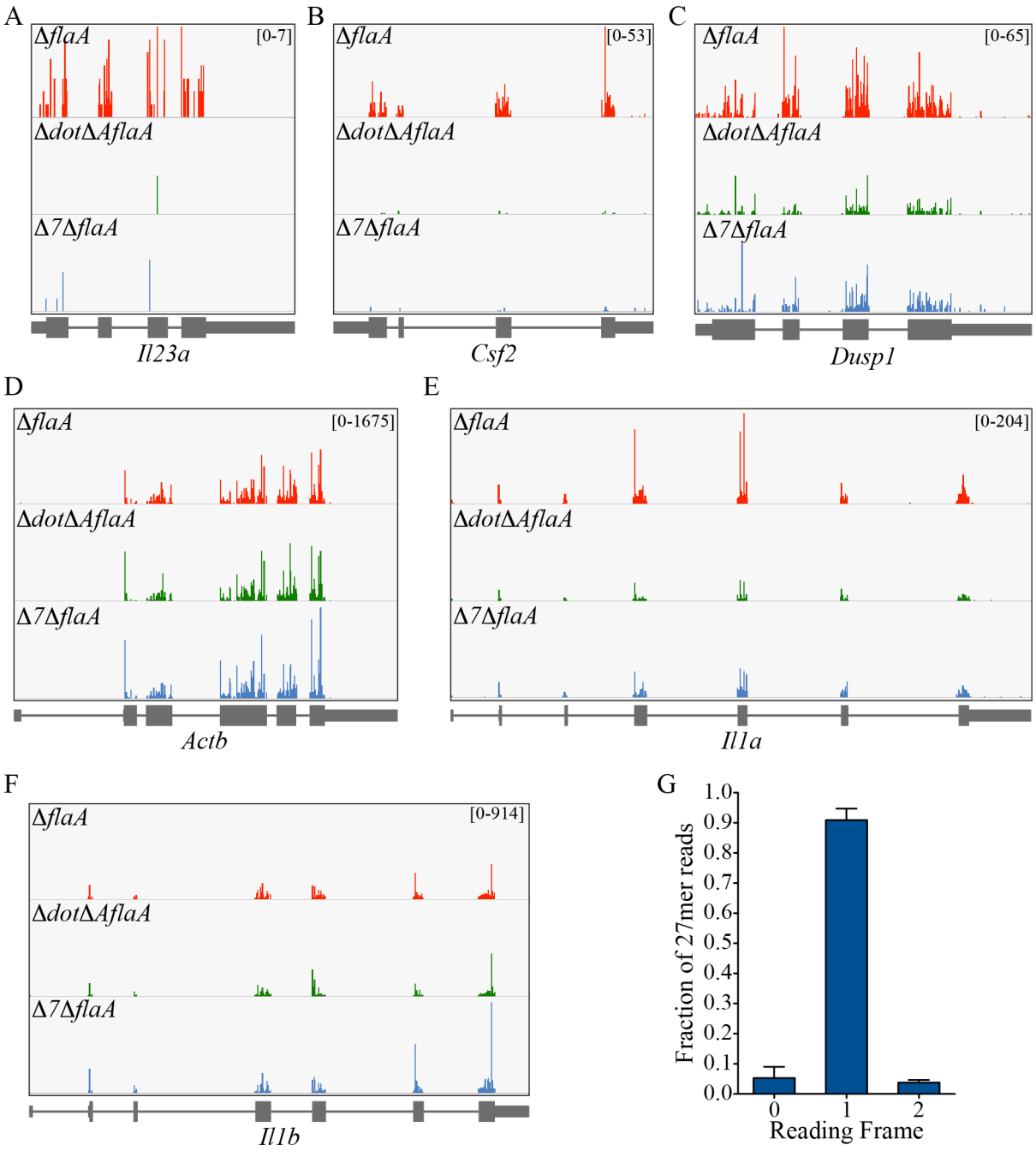


**Figure 3.1:** Ribosome footprint libraries map to ORFs and have strong reading frame bias. (A-F) Individual gene plots were generated by mapping the 5' end of ribosome footprint reads onto the genome and visualized using the Integrative Genomics Viewer (Robinson et al., 2011). Ribosome footprint profile on the *Il123a* (A), *Csf2* (B), *Dusp1* (C), *Actb* (D), *Il1a* (E), and *Il1b* (F) transcripts. (G) The mean fraction of 27mer ribosome footprint reads in each possible reading frame. The fraction of 27mers in each reading frame was averaged for all 14 libraries used in this study (G). (A-F) Reads were visualized using a median windowing function and the data range of each condition was set to be able to compare across conditions. Data range is indicated in the upper right-hand corner of each track (A-F). (G) Error bars depict standard deviation.

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generated, the most abundant ribosome footprints in the libraries generated in this study were 27-nucleotides long (average of 14 libraries = 44.8% of all footprints) and showed a strong reading frame bias (Figure 3.1G).These results suggest that the ribosome footprint libraries generated after *L. pneumophila* infection are high quality and map to the genome as expected.

### Section 3.3.2: Global translation patterns look similar in Δ*flaA* or Δ*dotA*Δ*flaA*-infected BMDMs

A benefit of ribosome profiling is the ability to examine global changes in translation. As a first step to understand the global changes in translation that occur during infection with virulent (Δ*flaA*) or avirulent (Δ*dotA*Δ*flaA*) *L. pneumophila,* we generated metagene ribosome footprint profiles for libraries generated from these two conditions. In this analysis, ribosome footprints are mapped relative to the start or stop site of the mRNA, and then the number of footprints per nucleotide position of the open reading frame are averaged. Averaging results from four independent replicates, we found that Δ*flaA* infected of BMDMs appear to have a similar ribosome distribution around the start site or the termination site of transcripts as compared to Δ*dotA*Δ*flaA* infected BMDMs (Figure 3.2A-B). It should be noted that virulent *L. pneumophila* induces a strong block in host protein synthesis (Asrat et al., 2014; Barry et al., 2013; Fontana et al., 2011), thus the finding that the global translation patterns do not differ between Δ*flaA* and Δ*dotA*Δ*flaA* infections is surprising. It is interesting to note that in both Δ*flaA* and Δ*dotA*Δ*flaA* infected BMDMs there appear to be ribosomes stalling just 5' of the stop codon, suggesting that, in BMDMs, translation termination may be a limiting step in translation (Figure 3.2B). As further validation for the quality of our ribosome profiling datasets, our metagene analyses show peaks at every three nucleotides, corresponding to the codon-to-codon shifts of the ribosome (Figure 3.2).

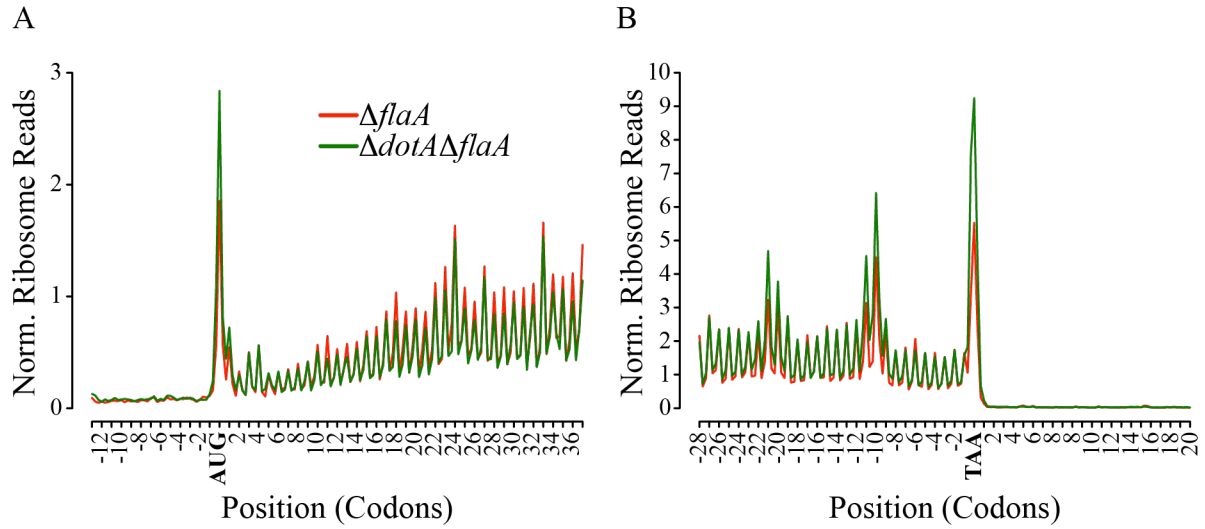


**Figure 3.2:** Metagene profiles of Δ*flaA* and Δ*dotA*Δ*flaA L. pneumophila* infected macrophages are similar. Metagene profiles of Δ*flaA* and Δ*dotA*Δ*flaA L. pneumophila* infected macrophages relative to the start site (A) and (B) stop site. Metagene profiles were generated by mapping the 5' ends of reads relative to the start (A) or stop site (B). Mapped reads were counted and normalized to the total number of mitochondrial proteins in each condition. Normalized read counts were averaged from 4 independent libraries. Red = Δ*flaA*, Green = Δ*dotA*Δ*flaA*. Each peak in the graph represents the middle nucleotide of a codon.

**Section 3.3.3: Δ7Δ*flaA L. pneumophila* infection induces a unique global translation pattern**

We previously identified 7 bacterial effectors that *L. pneumophila* secretes into the cytosol that are all capable of inducing a block in host protein synthesis (Barry et al., 2013; Fontana et al., 2011). Unfortunately, deletion of all 7 of these bacterial effectors has no effect on translation inhibition induced by *L. pneumophila* infection (Barry et al., 2013). We were interested in understanding the residual translation inhibition that is induced by the Δ7Δ*flaA L. pneumophila* strain. We thus characterized the global translation pattern after infection with this strain (Figure 3.3). The metagene analysis of Δ7Δ*flaA* infected BMDMs showed increased binding of ribosomes at the start site as compared to that seen in Δ*flaA* or Δ*dotA*Δ*flaA* infected BMDMs (Figure 3.3A). Increased ribosome accumulation at the start site of genes is could be consistent with a block in host translation initiation or the first steps of translation elongation (Ingolia et al., 2011). There were no drastic differences in the global footprint distributions around the stop site of genes with the Δ7Δ*flaA* infection, although there again appeared to be ribosome stalling at the 5' end of genes (Figure 3.3B). These data suggest that the residual translation inhibition that is induced by Δ7Δ*flaA* infection may be at the level of translation initiation.

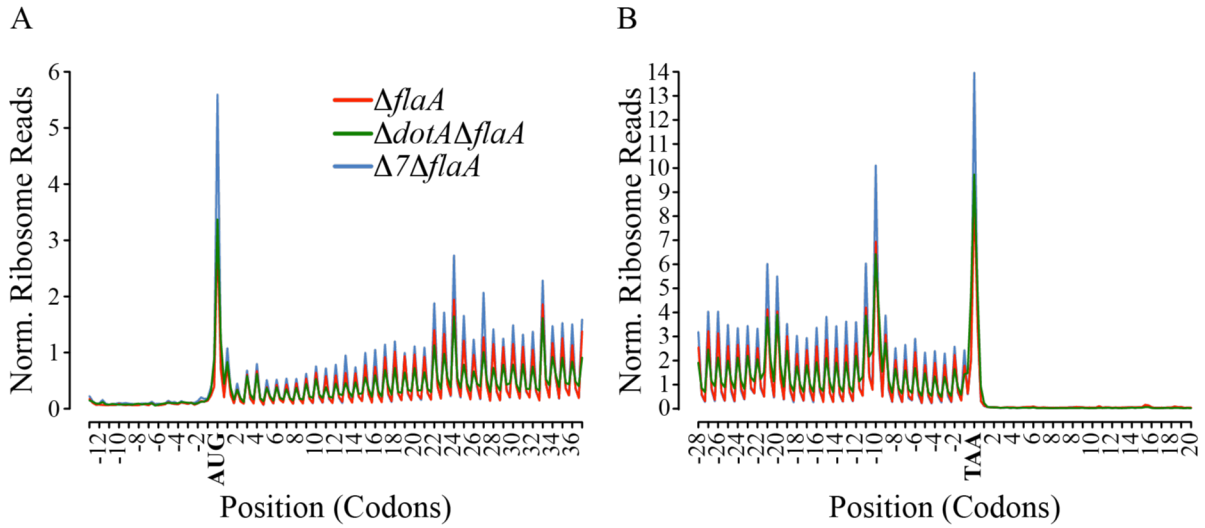


**Figure 3.3:** Δ7Δ*flaA L. pneumophila* infection may bock translation initiation. (A-B) Metagene profiles of Δ*flaA*, Δ*dotA*Δ*flaA*, and Δ7Δ*flaA* infected macrophages relative to the start (A) and stop site (B). The 5' end of reads were mapped relative to the start/stop site and counted. Mapped reads were normalized to total mitochondrial proteins in the respective condition. Metagene analysis depicts sequences from a single experiment/library. Red = Δ*flaA*, Green = Δ*dotA*Δ*flaA*, Blue = Δ7Δ*flaA L. pneumophila*.

**Section 3.3.4: Ribosome run-off experiments suggest residual translation inhibition induced by Δ7Δ*flaA L. pneumophila* infection is at translation initiation**

A number of the previously identified bacterial effectors that block host translation have been shown to directly interact or post-translationally modify host elongation factor 1A (eEF1A), suggesting that *L. pneumophila* directly blocks host translation elongation (Belyi et al., 2013; Belyi et al., 2009; Belyi et al., 2008; Shen et al., 2009; Tzivelekidis et al., 2011). We

hypothesized that the similar metagene profiles of Δ*flaA* and Δ*dotA*Δ*flaA L. pneumophila* infected BMDMs was related to a block in translation elongation induced by Δ*flaA L. pneumophila*. Inhibition of translation elongation by Δ*flaA L. pneumophila* could lead to stalled ribosomes throughout transcripts, and the pattern exhibited by these ribosomes would resemble the pattern of ribosomes produced during active translation in Δ*dotA*Δ*flaA L. pneumophila* infected BMDMs. In other words, we hypothesized that a block in translation elongation would explain why virulent and avirulent *L. pneumophila* infection leads to similar metagene profiles, even though translation is severely blocked in the former but not in the latter. To address this hypothesis, we utilized translation run-off experiments where we treated macrophages for 90 seconds with the drug harringtonine, which effectively blocks initiation by inhibiting elongation during the first rounds of peptide bond formation following subunit joining. Harringtonine has previously been shown to cause ribosomes to pile up at the start site (Fresno et al., 1977; Huang, 1975; Ingolia et al., 2012; Ingolia et al., 2011; Tscherne and Pestka, 1975). Treatment with harringtonine for 90 seconds is sufficient time for ribosomes to be preferentially cleared from the 5' end of transcripts (Ingolia et al., 2012; Ingolia et al., 2011). Cells stalled at the step of translation elongation exhibit less ribosome run-off after harringtonine treatment, and an increased number of reads at the 5' end of genes after drug treatment (Ingolia et al., 2011). By contrast, cells stalled at the step of translation initiation would likely exhibit fewer ribosomes at the start site after harringtonine treatment, as the drug blocks the formation of the first peptide bond and the majority of translation initiation control occurs before this step (Hershey et al., 2012).

We found that Δ*dotA*Δ*flaA* infected BMDMs treated with harringtonine exhibited an increase in ribosome footprints at the start site and a preferential loss of reads at the 5' end of genes, consistent with the expected effects of the drug (Figure 3.4A). Consistent with Δ*flaA L. pneumophila* inducing a block in host translation elongation, there is a marked increase in the number ribosome footprints at the 5' end of genes after harringtonine treatment as compared to the Δ*dotA*Δ*flaA L. pneumophila* infection (Figure 3.4B). Interestingly, the metagene profile of Δ*7*Δ*flaA L. pneumophila* infected BMDMs exhibited reduced ribosome footprints at the start site of genes, and clear run-off of elongating ribosomes (Figure 3.4C). The difference in 5' run-off after infection with these strains of *L. pneumophila* is even more obvious when the plots are overlayed (Figure 3.4D). These results suggest that deletion of the 7 bacterial effectors that block translation relieves the block in translation elongation induced by Δ*flaA L. pneumophila* and that the residual translation inhibition induced by Δ*7*Δ*flaA L. pneumophila* is at the level of translation initiation (Figure 3.4D). This finding is interesting because regulation of translation by the host is generally at the level of initiation (Hershey et al., 2012). Thus our results may suggest that the residual translation inhibition induced by Δ*7*Δ*flaA L. pneumophila* may be host-mediated, through the activation of host stress responses.

To confirm the ribosome profiling results we used a bicistronic reporter, driving cap-dependent translation of *Renilla* luciferase and the cricket paralysis virus (CrPV) internal ribosome entry site (IRES) driving expression of firefly luciferase from the same transcript in a manner independent of initiation factors (Pestova and Hellen, 2003) (Figure 3.4E). Inconsistent with the ribosome profiling data, 293T cells expressing the bicistronic reporter showed a loss of *Renilla* and firefly luciferase activity after Δ*flaA* and Δ*7*Δ*flaA L. pneumophila* infection (Figure 3.4E). Cap-dependent and CrPV-IRES-dependent translation would both be blocked only if *L. pneumophila* induced a block translation elongation, suggesting that translation elongation was blocked after Δ*7*Δ*flaA L. pneumophila* infection (Figure 3.4E). A similar bicistronic reporter

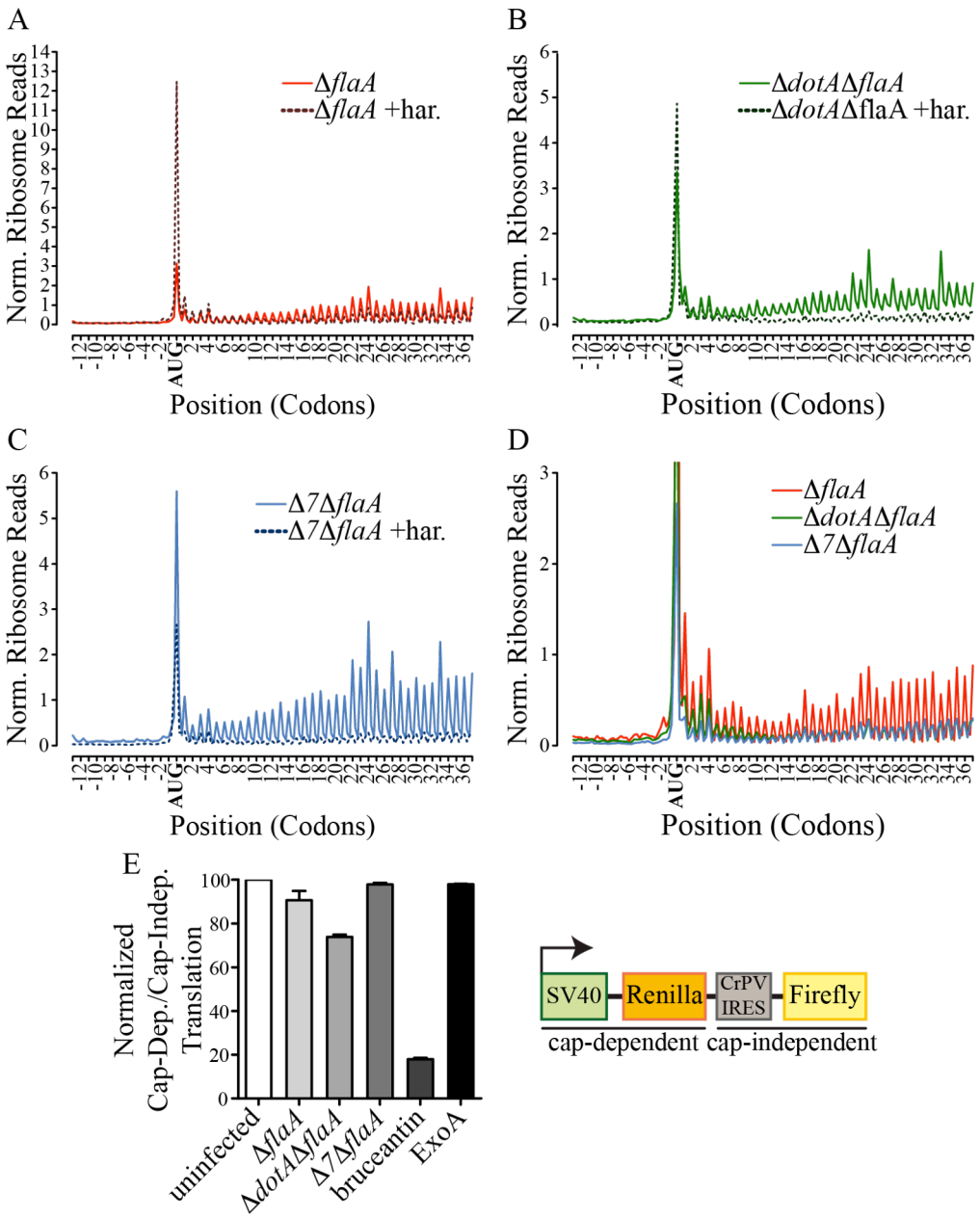


**Figure 3.4:** Ribosome run-off experiments suggest *L. pneumophila* can block translation initiation and elongation. (A-D) Metagene analysis of run-off elongation experiment. Metagene analysis of macrophages infected (MOI=3) with Δ*flaA* (A), Δ*dotA*Δ*flaA* (B), or Δ7Δ*flaA* (C) in the presence or absence of harringtonine. (D) Metagene profile of Δ*flaA*, Δ*dotA*Δ*flaA*, Δ7Δ*flaA L. pneumophila* infected macrophages (MOI=3) treated with harringtonine and scaled to show preferential loss of reads at the 5' end of genes. (E) HeLa cells were transfected with a bicistronic reporter (right panel) and a plasmid encoding FcRγ to facilitate bacterial uptake. HeLa cells were infected with the indicated strains of *L. pneumophila* (MOI=10) or treated with Bruceantin (50 nM) to block cap-dependent translation or Exotoxin A (500 ng/mL) to block both cap-dependent and cap-independent translation. Cap-dependent:cap-independent ratios were normalized to uninfected. ExoA = Exotoxin A, CrPV = Cricket paralysis virus, IRES = Internal ribosome entry site.

assay was used in a recent study to conclude that WT *L. pneumophila* and the Δ*5 L. pneumophila* mutant both block cap-dependent translation (Ivanov and Roy, 2013). WT *L. pneumophila* encodes effectors that directly block translation elongation (Belyi et al., 2013; Belyi et al., 2009; Belyi et al., 2008; Shen et al., 2009; Tzivelekidis et al., 2011) so it is unclear why this reporter did not detect a block in translation elongation; however, these data do suggest there may be some modulation of translation initiation in response to the Δ*5 L. pneumophila* strain, consistent with our ribosome profiling data after Δ*7*Δ*flaA L. pneumophila* infection (Figure 3.4C). The discrepancy between our bicistronic reporter and ribosome profiling results could be caused by differences in the cell types used for the assays, as 293T cells are not a physiological cell type infected by *L. pneumophila*. Further, 293T cells and RAW macrophages, which were used by Ivanov *et al.,* are an immortalized cell lines which are known to have abnormal host stress response pathways as well as disregulated metabolic pathways (Benjamin et al., 2012; Wu and Zhao, 2013).

While further experiments will be necessary to confirm our ribosome profiling results, it is important to note that these experiments were undertaken in physiologically relevant, primary BMDMs. Thus any effect seen here, while needing to be confirmed, is more relevant to *L. pneumophila* infection *in vivo* leading us to conclude that the residual translation inhibition induced by the Δ*7*Δ*flaA* strain of *L. pneumophila* is at the level of translation initiation. This finding is exciting as it suggests that the residual translation inhibition induced by Δ*7*Δ*flaA L. pneumophila* may not be caused by a specific bacterial effector but could be a more general host stress response to infection by an intracellular pathogen, a response that may be conserved among infections by multiple intracellular bacterial pathogens.

### Section 3.3.5: Common innate immune signaling pathways have no effect on residual *L. pneumophila*-induced translation inhibition

As TLR and the type I IFN receptor (IFNAR) signaling have been linked to translation inhibition, we investigated whether these pathways could explain the residual block in host protein synthesis induced by Δ*5*Δ*flaA* and Δ*7*Δ*flaA L. pneumophila*. $^{35}$S-methionine metabolic labeling experiments were undertaken to test if deficiencies in common innate immune signaling pathways could account for the residual translation inhibition induced by Δ*7*Δ*flaA L. pneumophila.* Infection of BMDMs deficient in MyD88 and the cytosolic NLRs, NOD1 and NOD2 with Δ*5*Δ*flaA* or Δ*7*Δ*flaA L. pneumophila* induced a block in host protein synthesis to a similar degree as in B6 BMDMs (Figure 3.5A). Similarly, Δ*7*Δ*flaA L. pneumophila* infection of BMDMs deficient in the important TLR adapter proteins MyD88 and TRIF induced a block in host protein synthesis similar to that seen in B6 BMDMs (Figure 3.5B). Lastly, Δ*flaA* or Δ*5*Δ*flaA L. pneumophila* infection of *Ifnar*-deficient BMDMs induced translation inhibition similar to that of B6 BMDMs (Figure 3.5C). In addition to looking at translation inhibition we were interested in determining if these signaling pathways played a role in IL-1α production in response to *L. pneumophila* infection. Not surprisingly, *Myd88;Nod1;Nod2* triple-deficient BMDMs and *Myd88;Trif* double-deficient BMDMs do not release IL-1α protein in response *L. pneumophila* infection (Figure 3. 5A-C). This lack of IL-1α production is likely due to a previously described loss of *Il1a* transcriptional induction in *Myd88*$^{-/-}$ macrophages (Shin et al., 2008). These data suggest that common innate immune signaling pathways that have been

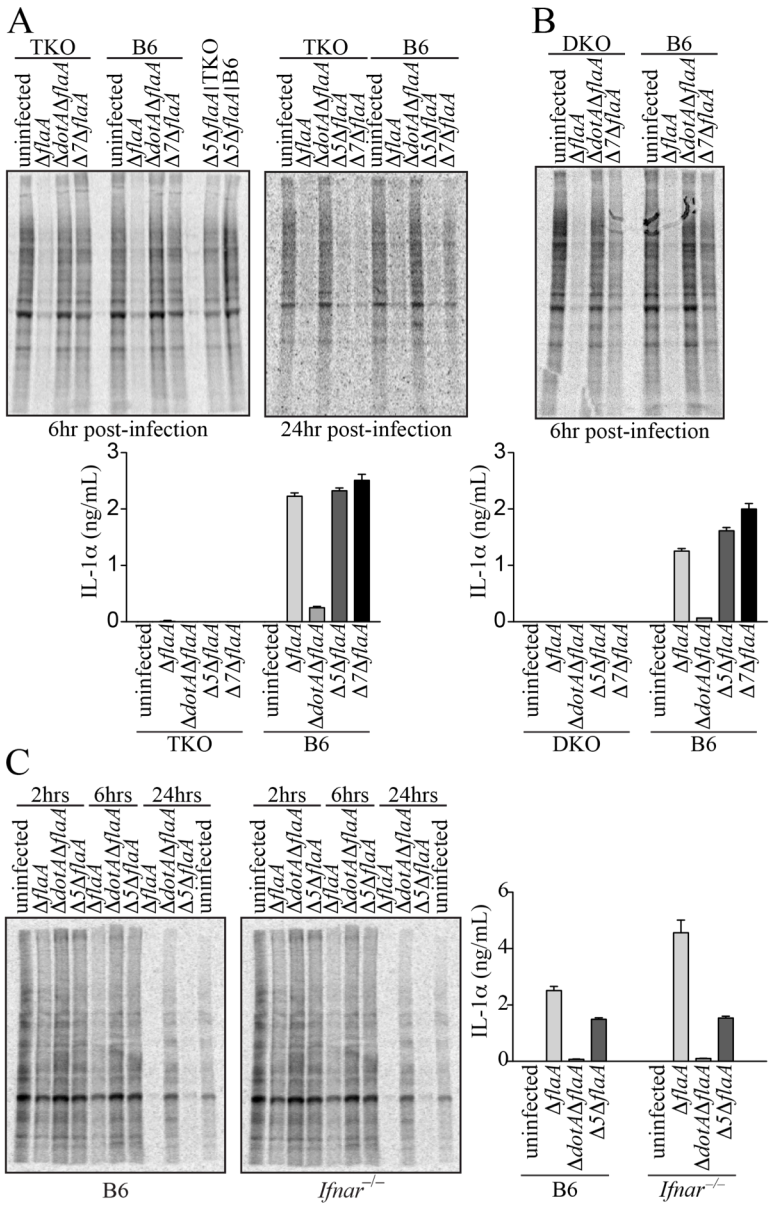


**Figure 3.5:** Common innate immune signaling pathways do not control *L. pneumophila* induced translation inhibition. (A) WT (B6) and *Myd88,Nod1,Nod2*$^{-/-}$ (TKO) BMDMs were infected with the indicated strains of *L. pneumophila* (MOI=3) and at 6hrs (left panels) and 24hrs (right panels) post-infection cells were incubated with $^{35}$S-methionine for one hour. Global translation levels were determined by autoradiography. Cell supernatants were collected at 24hrs post-infection and IL-1α levels were measured by ELISA. (B) Wild-type and *Myd88,Trif*$^{-/-}$ (DKO) BMDMs were infected with the indication strains of *L. pneumophila* (MOI=3) and at 6hrs post-infection cells were incubated with $^{35}$S-methionine as described in (A). At 24hrs post-infection extracellular IL-1α levels were determined by ELISA (lower panel). (C) WT (B6) and *Ifnar*$^{-/-}$ BMDMs were infected with the indicated strains of *L. pneumophila* (MOI=3). At 2, 6 and 24hrs post-infection cells were incubated with $^{35}$S-methionine and treated as described in (A). IL-1α levels in cell supernatants were determined at 24hrs post-infection (right panel). Data are from a single experiment.

associated with translation inhibition do not play role in *L. pneumophila* induced blockade of host protein synthesis.

### Section 3.3.6: Modulation of mTOR signaling does not explain residual *L. pneumophila*-induced translation inhibition

Ribosome profiling run-off experiments suggested that the residual translation inhibition induced by Δ*7*Δ*flaA L. pneumophila* might be at the level of translation initiation (Figure 3.4C). Given that all of the known *L. pneumophila* effectors with characterized biochemical activities target elongation factors (Belyi et al., 2013; Belyi et al., 2009; Belyi et al., 2008; Shen et al., 2009; Tzivelekidis et al., 2011), and that the vast majority of host-derived translation regulation occurs at the level of translation initiation (Hershey et al., 2012), we hypothesized that infection of macrophages by Δ*7*Δ*flaA* may induce a stress response that leads to inhibition translation initiation. This would suggest that in response to the stress of a pathogenic infection the host cell has adapted to block its' own translation. To test whether common stress response pathways were playing a role in the residual block in translation initiation induced by the Δ*7*Δ*flaA* infection we performed western blots looking for post-translational modifications of different components of common stress response pathways including the mTOR signaling pathway, eIF2α and eEF2. It should be noted that all of these experiments are done in the presence of serum. While many reports show that serum can lead to high background activation of these pathways, we can detect very strong blocks in host protein synthesis induced by *L. pneumophila* infection in the presence of serum. Thus, we reasoned that if translation inhibition is robust in the

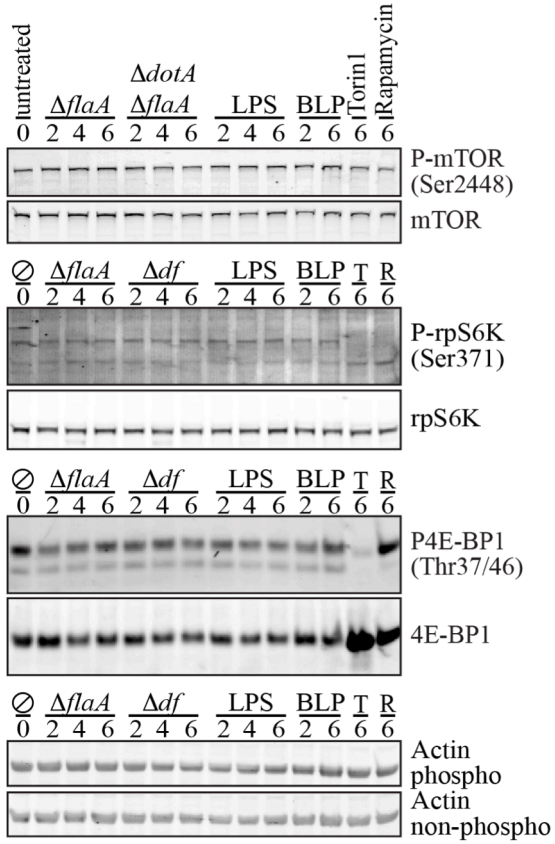


**Figure 3.6:** Components of the mTOR signaling pathway are not altered following *L. pneumophila* infection. Wild-type B6 bone marrow derived macrophages were infected with the indicated strains of *L. pneumophila* (MOI=3). At 2, 4 and 6hrs post-infection cells were lysed with RIPA buffer. Lysates were used for western blotting experiments to visualize the amount of phospho-mTOR (Ser2448), total mTOR, phospho-rpS6K (Ser371), total rpS6K, phospho-4E-BP1 (Thr37/46), total 4E-BP1 and actin after *L. pneumophila* infection. Cells were treated with Pam3CSK4 (10ng/mL) and LPS (100ng/mL) as they were previously shown to induce mTOR activity. Cells were incubated with the mTOR inhibitors Rapamycin (100nM) and Torin1 (250nM) for 6hrs. ⃠, untreated. Δ*df*, Δ*dotA*Δ*flaA*. LPS, lipopolysaccharide. BLP, bacterial lipoprotein (Pam3CSK4). T, Torin1. R, Rapamycin.

presence of serum then changes in the signaling pathway that controls this phenotype must also be detected in the presence of serum. We began by looking at post-translational modifications of components of the mTOR-signaling pathway. mTOR signaling is active in nutrient replete conditions and can combine multiple signals to control a number of downstream activities, one of which is control of translation initiation (reviewed in Efeyan et al., 2012; Kim et al., 2013; Showkat et al., 2014; Thoreen, 2013; Zoncu et al., 2011b). Active mTOR phosphorylates and inactivates 4E-BP1 and 4E-BP2, two proteins that when hypo-phosphorylated are able to bind to the initiation factor eIF4E blocking translation initiation within the cell. S6K1 is another downstream target of active mTOR and active S6K1 has been show to increase translation through a number of downstream targets including ribosomal protein S6 (rpS6), eIF4B, PDCD4, and eukaryotic elongation factor 2 kinase (eEF2K; reviewed in Showkat et al., 2014). Phosphorylation of rpS6 has been suggested to increase global translation rates; however, recent experiments replacing all of the phosphorylated serines of rpS6 with alanines had no effect on global translation, calling into question the role of S6K-dependent phosphorylation of rpS6 in translational control (Ruvinsky et al., 2005).

We were interested in understanding if mTOR signaling could explain any of the translation inhibition induced by *L. pneumophila*. We infected B6 BMDMs with Δ*flaA* or Δ*dotA*Δ*flaA L. pneumophila* (MOI=3) or treated macrophages with LPS or BLP to activate mTOR (Figure 3.6). To inhibit mTOR activity we utilized the mTOR inhibitors Torin1 or

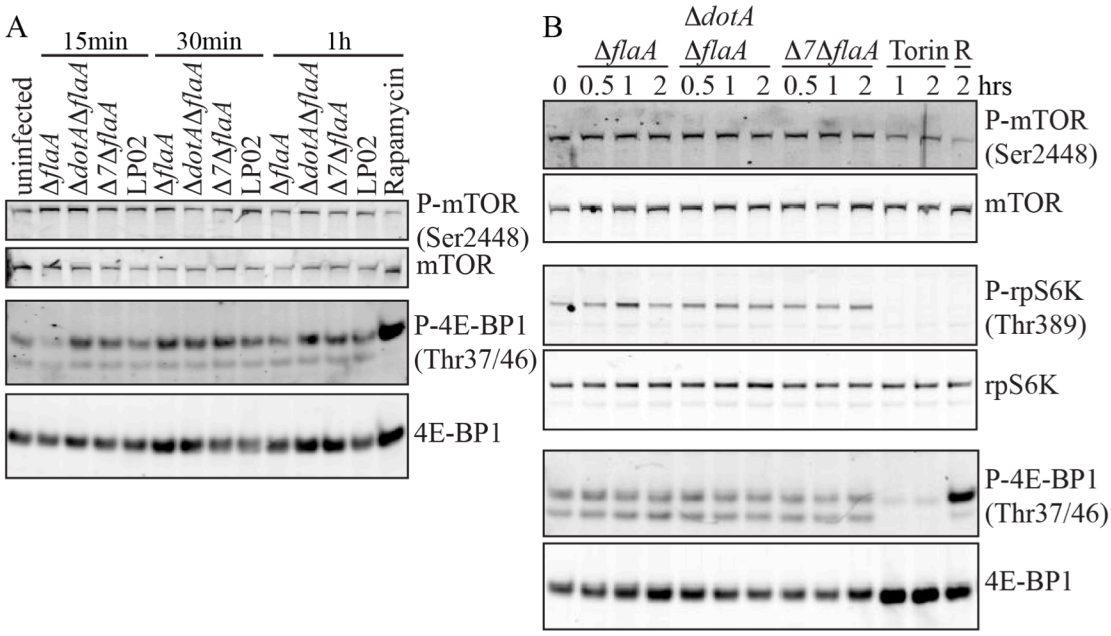


**Figure 3.7:** The mTOR signaling pathway is unaltered after Δ7Δ*flaA L. pneumophila* infection. WT BMDMs were infected with the indicated strains of *L. pneumophila*. (A) Cells were lysed with RIPA buffer at 15, 30 or 60min post-infection and western blots were performed to look at the phosphorylation state of mTOR and the mTOR target, 4E-BP1. (B) At 0.5, 1 and 2hrs post-infection lysates were harvested and blotted for the phosphorylation state of mTOR (Ser2448), rpS6K (Thr389), and 4E-BP1 (Thr37/46). Total proteins were used as loading controls in these experiments (A-B). The mTOR inhibitors Rapamycin (100 nM) and Torin1 (250 nM) were used as controls to block mTOR signaling. R, Rapamycin.

rapamycin (Figure 3.6). Lysates were harvested at 2, 4 and 6 hrs post-infection and blotted for the phosphorylation states of mTOR and the mTOR targets 4E-BP1 and S6K. In response to Δ*flaA* or Δ*dotA*Δ*flaA L. pneumophila* infection, we saw no changes in the phosphorylation state of mTOR, S6K or 4E-BP1 (Figure 3.6). Importantly, the mTOR ATP-competitive inhibitor Torin1 blocks 4E-BP1 and S6K phosphorylation (Figure 3.6). We also detected no changes in the 4E-BP1 phosphorylation state after infection with Δ*flaA*, Δ*dotA*Δ*flaA*, or Δ*7*Δ*flaA L. pneumophila* infection for a time course of infection ranging from 15 minutes to 2hrs post-infection (Figure 3.7A-B). Similarly, at early time points post-infection (15min-1hr) there were also no detectable differences in 4E-BP1 or mTOR phosphorylation state in response to WT (LP02) *L. pneumophila* infection (Figure 3.7A). It should be noted that rapamycin treatment has little to no effect on 4E-BP1 phosphorylation in any of the experiments presented here (Figure 3.6-3.7). This is to be expected as it has been previously shown that only active-site inhibitors or ATP-competitive inhibitors, such as Torin1, inhibit 4E-BP1 phosphorylation in mammalian cells (Feldman et al., 2009; Thoreen et al., 2009). Importantly, when Torin1 was used to as a control for mTOR inhibition, we see robust inhibition of 4E-BP1 phosphorylation (Figure 3.6, 3.7B). These data suggest that, at least in the presence of serum, *L. pneumophila* has a minimal effect on the mTOR signaling pathway, and further, that this pathway most likely does not explain the residual translation inhibition induced by infection with the Δ*7*Δ*flaA* strain of *L. pneumophila* (Figure 3.6 and 3.7).

### Section 3.3.7: eIF2α phosphorylation does not explain residual translation inhibition after Δ*7*Δ*flaA L. pneumophila* infection

Other important host stress response kinases are the eIF2α kinase family. The eIF2α kinases consist of four kinases, GCN2, PERK, PKR and HRI. The eIF2 α kinases are activated and phosphorylate eIF2α in response to a variety of stresses. Phospho-eIF2α acts as a competitive inhibitor for the GEF eIF2B, leading to a block in translation initiation (Reviewed in(Baird and Wek, 2012; Donnelly et al., 2013; Wek et al., 2006). Interestingly, infection of B6 BMDMs with Δ*flaA*, Δ*5*Δ*flaA*, or Δ*7*Δ*flaA* but not Δ*dotA*Δ*flaA* was found to induce a subtle but often inconsistent increase in the amount of phosphorylated eIF2α in the cell (Figure 3.8A-B). It is well established that *L. pneumophila* uses amino acids as a carbon source (Fonseca and Swanson, 2014; Schunder et al., 2014; Wieland et al., 2005) and the eIF2α kinase GCN2 (encoded by the gene *Eif2ak4*) recognizes and binds uncharged tRNAs that are present in amino acid starved conditions; tRNA binding activates the kinase and phosphorylation of eIF2α at Ser51 leading to inhibition of translation initiation (Baird and Wek, 2012; Donnelly et al., 2013; Wek et al., 2006). We hypothesized that GCN2 could recognize the stress of *L. pneumophila* infection and this signaling pathway could explain the residual translation inhibition induced by Δ*7*Δ*flaA L. pneumophila* infection. However, macrophages deficient in *Eif2ak4* had no defect in Δ*flaA* or Δ*7*Δ*flaA L. pneumophila* induced translation inhibition (Figure 3.8C).

While genetic deletion of *Eif2ak4* had no effect on *L. pneumophila* induced translation inhibition in macrophages it remained possible that one of the other three eIF2α kinases could compensate for the loss of GCN2. To confirm that phosphorylation of eIF2α by any of the eIF2α kinases could not explain the residual block in translation induced by Δ*7*Δ*flaA L. pneumophila,* we wanted to disrupt all phosphorylation of eIF2α. Mice homozygous for non-phosphorylatable eIF2α, the *Eif2a* S51A knock-in allele, die within 18hrs of birth (Scheuner et al., 2001), so we could not use BMDMs from these mice. To get around this lethality we decided to over-express

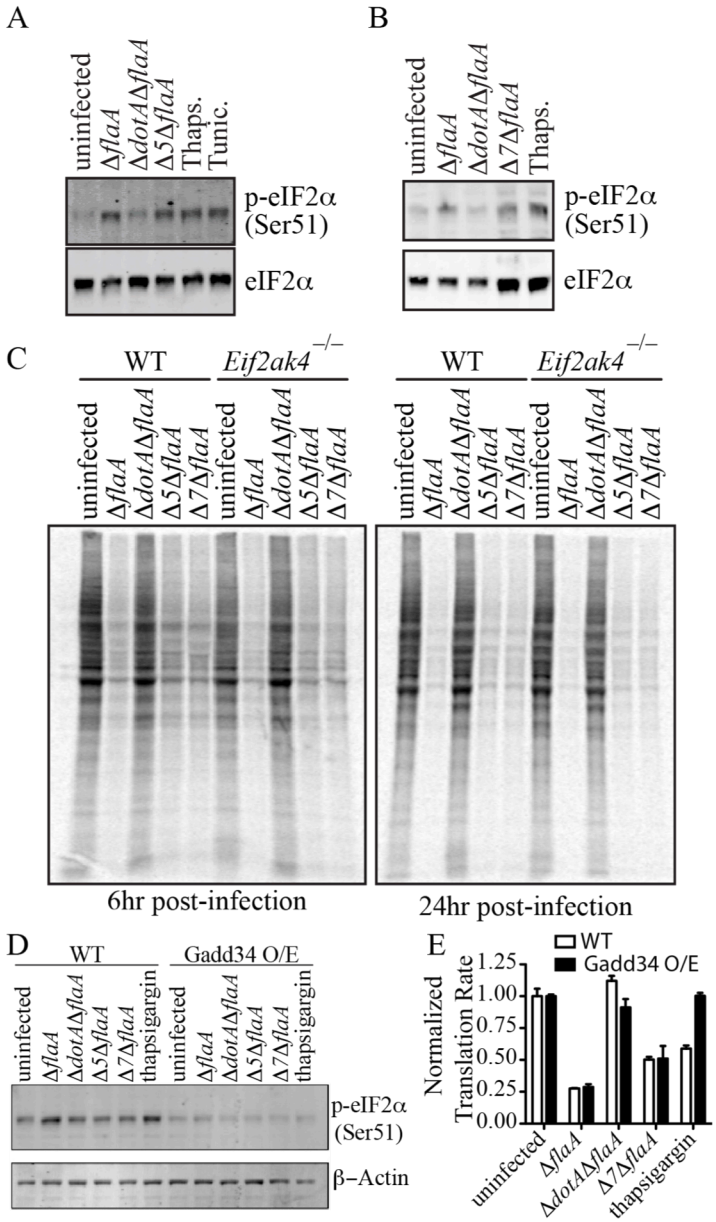


**Figure 3.8:** Phosphorylation of eIF2α does not explain the residual translation inhibition in Δ7Δ*flaA L. pneumophila* infected macrophages. (A) WT BMDMs were infected with the indicated strains of *L pneumophila* (MOI=3). 6hrs post-infection lysates were probed by western blot for phospho-eiF2α (Ser51) and total eIF2α. (B) Same as in (A) except lysates were collected at 5hrs post-infection. (C) Wild-type and *Eif2ak4*$^{-/-}$ BMDMs were infected with the indicated strains of *L. pneumophila* (MOI=3) and at the indicated times post-infection were incubated with $^{35}$S-methionine as in Figure 3.5A. (D) HeLa cells transfected with a destabilized firefly luciferase construct were infected with the indicated strains of *L. pneumophila* (MOI=10). 6hrs post-infection lysates were probed by western blot for the levels of phospho-eIF2α (Ser51) and β-actin as a loading control. (E) HeLa cells were treated the same as in (D), but were lysed in passive lysis buffer and light production by firefly luciferase was measured. D and E are independent experiments. Data are representative of two experiments (A-B). Results shown are from one experiment (C-E).

the eIF2α phosphatase, growth arrest and DNA damage gene (GADD)34 (Novoa et al., 2001), to inhibit phosphorylation of eIF2α in 293T cells. GADD34 and firefly luciferase, a readout of translation in the cells, were over-expressed in 293T cells and infected with Δ*flaA*, Δ*dotA*Δ*flaA*, Δ*5*Δ*flaA* or Δ*7*Δ*flaA L. pneumophila* (MOI=10) or treated with the ER-stress inducing drug thapsigargin (Figure 3.8D). Unfortunately, in this experiment, the Δ*5*Δ*flaA* and Δ*7*Δ*flaA* did not robustly induce eIF2α phosphorylation. Indeed, across multiple experiments, the induction of eIF2α phosphorylation by virulent strains of *L. pneumophila* (Δ*flaA*, Δ*5*Δ*flaA*, and Δ*7*Δ*flaA*) in BMDMs and 293T cells was inconsistent, suggesting that the induction of this pathway is not robustly activated by virulent *L. pneumophila* (data not shown). Nevertheless, over-expression of GADD34 in 293T cells inhibited thapsigargin and Δ*flaA L. pneumophila* induced phosphorylation of eIF2α (Figure 3.8D). Given that GADD34 over-expression inhibited eIF2α phosphorylation, we next tested if GADD34 over-expression could rescue the block in translation induced by *L. pneumophila* infection (Figure 3.8E). GADD34 over-expression rescued the block in protein synthesis induced by thapsigargin treatment, as read out by a rescue of luciferase activity, but had no effect on Δ*flaA* or Δ*7*Δ*flaA* induced translation inhibition (Figure 3.8E). Taken together, these data argue against eIF2α phosphorylation by the eIF2α kinases as playing an important role in the residual translation inhibition induced by Δ*7*Δ*flaA L. pneumophila*.

### Section 3.3.8: AMPK-signaling does not explain the residual translation inhibition induced by Δ*7*Δ*flaA L. pneumophila*

The final host stress response pathway we tested was the AMPK signaling pathway. In response to nutrient deprivation the ratio of AMP:ATP or ADP:ATP increases and activates the energy sensor AMPK (Hardie, 2011). Activated AMPK then phosphorylates and activates the eukaryotic elongation factor 2 kinase (eEF2K) leading to the phosphorylation and inactivation of the elongation factor eEF2 (Leprivier et al., 2013). Phosphorylation of eEF2 by eEF2K leads to a block in protein synthesis in response to nutrient deplete conditions. It is important to note that eEF2K can also be targeted by the mTOR target S6K1 to inactivate eEF2K (Showkat et al., 2014); thus, an interesting cross-talk occurs that regulates eEF2K activity. To test whether the AMPK/eEF2K pathway was regulating translation upon *L. pneumophila* infection, we infected B6 BMDMs with Δ*flaA*, Δ*dotA*Δ*flaA*, or Δ*7*Δ*flaA L. pneumophila* and blotted for

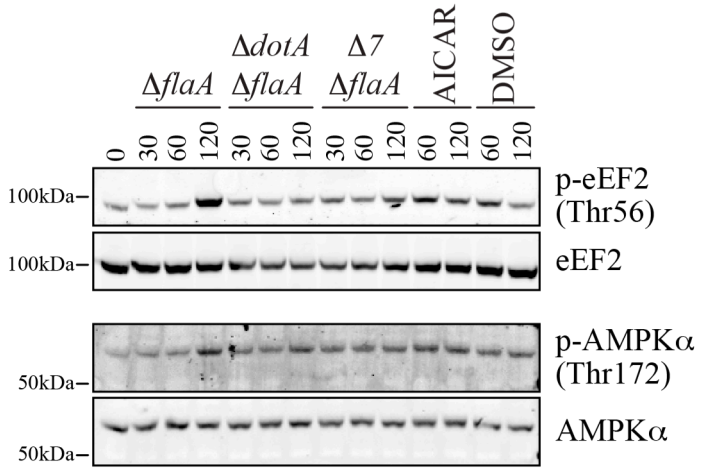


**Figure 3.9:** Virulent *L. pneumophila* may induce phosphorylation of eEF2 but this does not explain the residual translation inhibition after Δ*7*Δ*flaA L. pneumophila* infection. Wild-type B6 BMDMs were infected with the indicated strains of *L. pneumophila*. At 30, 60, and 120 min post-infection lysates were harvested. Western blot analysis was used to look at the phosphorylation of eEF2 (Thr56) and of AMPKα (Thr172). AICAR (1mM) was used a positive control.

phosphorylated AMPKα and eEF2 (Figure 3.9). We found that Δ*flaA L. pneumophila* infection induces robust phosphorylation of eEF2 and AMPKα, suggesting the virulent *L. pneumophila* may activate this pathway (Figure 3.9). Interestingly, however, the inhibition of eEF2 appears to be dependent on the 7 bacterial effectors that block host protein synthesis as Δ7Δ*flaA L. pneumophila* do not induce phosphorylation of eEF2 or AMPKα (Figure 3.9). Thus, the eEEF2/AMPK pathway cannot explain the residual translation inhibition induced by the Δ7Δ*flaA L. pneumophila* mutant. However, it is intriguing to hypothesize that predicted serine/threonine kinase Pkn5, one of the 7 bacterial effectors we identified as inducing a block in host protein synthesis upon over-expression (Barry et al., 2013), could directly phosphorylate APMKα and/or eEF2. Clearly, more experiments are needed to test this intriguing hypothesis.

In summary, our ribosome profiling data demonstrate that Δ7Δ*flaA* infection induces a block in host translation initiation (Figure 3.4C), and may suggest that a host stress response pathway could be playing a role in this residual translation inhibition. However, our attempts at identifying the stress response responsible for this blockade were unsuccessful. More studies are needed to identify the pathways required for the residual block in host protein synthesis induced by Δ7Δ*flaA L. pneumophila* infection.

### Section 3.3.9: The majority of macrophage responses to *L. pneumophila* are controlled by mRNA induction

It has been previously proposed that specific cytokines such as IL-1α and IL-1β could be preferentially translated in response to *L. pneumophila* infection (Asrat et al., 2014). In this study, the authors proposed that ribosomes could be preferentially loaded onto *Il1a* and *Il1b* transcripts in a MyD88-dependent manner, and that this preferential translation was the mechanism by which host cytokine transcripts could circumvent the global block in host protein synthesis induced by *L. pneumophila* (Asrat et al., 2014). Previously we have described a pathway in which a number of cytokines, including IL-1α, are induced in response to TLR stimulation along with translation inhibition (Barry et al., 2013; Fontana et al., 2011). We demonstrated that, somewhat paradoxically, TLR activation and translation inhibition combined to induce a massive and sustained increase in *Il1a* transcripts, that overcame the block in translation and led to the production of IL-1α protein (Barry et al., 2013); we went on to propose that this mechanism could explain how the innate immune system makes IL-1α only in response to virulent *L. pneumophila* (Barry et al., 2013). Thus two models have been proposed for how cytokine proteins are made in the presence of *L. pneumophila* induced translation inhibition: preferential translation (Asrat et al., 2014) or mRNA induction (Barry et al., 2013; Fontana et al., 2011). It is important to note that we purposely refer the increased expression of *Il1a* mRNA as mRNA induction. While we favor a model of transcriptional induction, our studies do not exclude mRNA stability in playing a role in the induction of *Il1a* mRNA and protein (Barry et al., 2013; Fontana et al., 2011).

The preferential translation hypothesis (Asrat et al., 2014) proposes that certain genes should have more ribosomes per individual mRNA transcript, or increased ribosome occupancy. To test if preferential translation occurs after *L. pneumophila* infection on a global scale we utilized our ribosome profiling datasets and analyzed the data to see if any genes had increased ribosome footprint density, more ribosome footprint reads than mRNAs reads, compared to the global landscape. Importantly, a subset of genes should be controlled by preferential translation and this should be detected in our ribosome profiling and RNAseq datasets. To undertake these

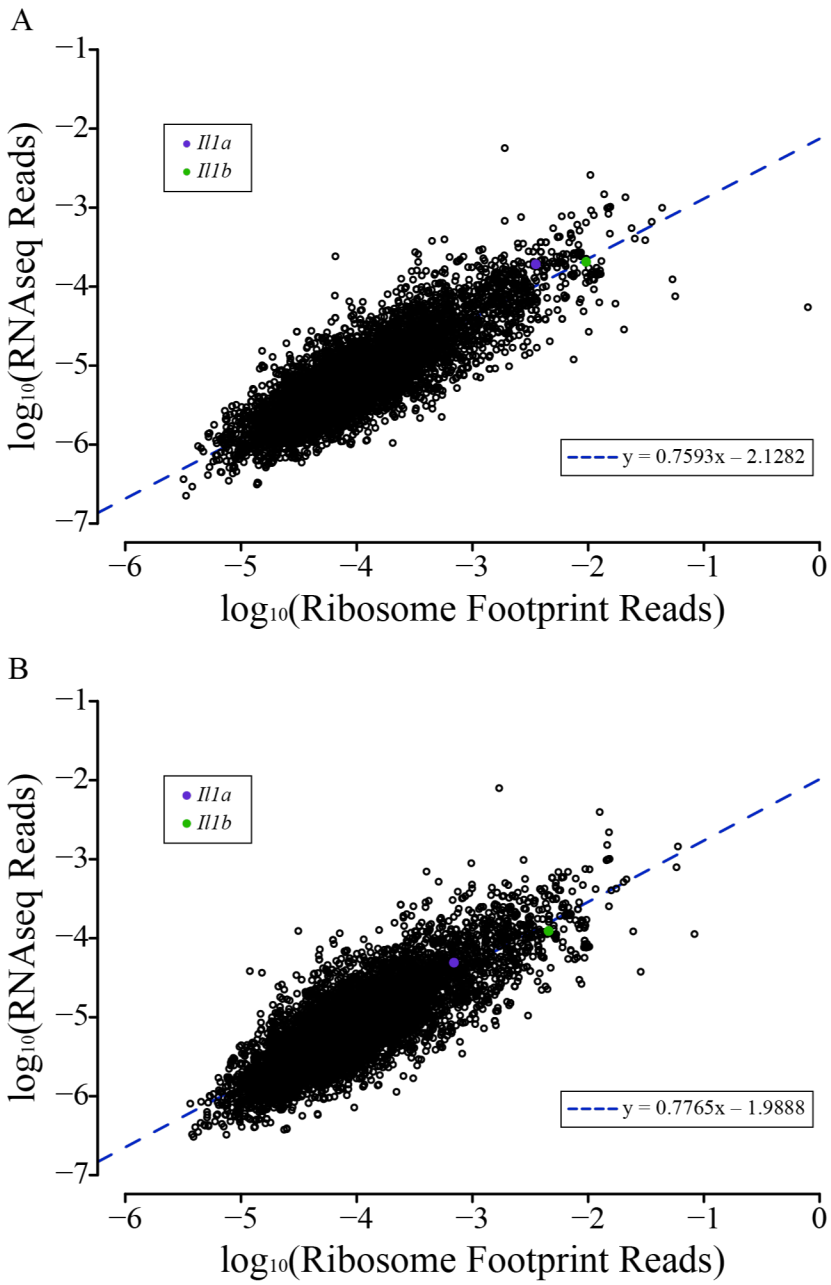


**Figure 3.10:** mRNA levels control the majority of macrophage responses to *L. pneumophila*. The 5' ends of ribosome footprint and mRNA reads were mapped to the genome and counted. Ribosome footprint and mRNA read counts were filtered for well-expressed transcripts. Ribosome footprint and RNAseq read counts were then normalized to the ribosome footprint read counts or RNAseq read counts of 12 protein coding mitochondrial genes, respectively. Normalized ribosome footprint and RNAseq reads from four independently generated libraries were averaged for both Δ*flaA* and Δ*dotA*Δ*flaA L. pneumophila* infected macrophages. The averaged ribosome footprint and RNAseq reads were plotted for Δ*flaA*-infected (A) and Δ*dotA*Δ*flaA*-infected macrophages (B). Linear regressions were calculated in the software R. Purple circles mark the *Il1a* transcript and green circles mark the *Il1b* transcript. Open circles represent an individual annotated transcript.

analyses we averaged the ribosome profiling and RNAseq reads of four independently generated libraries of B6 BMDMs infected with Δ*flaA* or Δ*dotA*Δ*flaA L. pneumophila* (MOI=3). The averaged dataset was filtered to remove any transcripts with fewer than 100 ribosome footprint and/or RNAseq read counts in the Δ*flaA* infection condition as these low count transcripts add variation to the analyses. Consistent with the majority of macrophage responses to Δ*flaA L. pneumophila* being controlled by mRNA induction, most transcripts demonstrated a linear relationship between ribosome footprint reads and RNAseq reads, suggesting that mRNA levels are the main determinant of the protein levels in infected cells (Figure 3.10A). Δ*dotA*Δ*flaA L. pneumophila* infection, which is thought to be mainly a TLR-dependent response, also induces a linear relationship between ribosome footprint reads and RNAseq reads (Figure 3.10B). Importantly, the *Il1a* and *Il1b* gene products fall perfectly on the linear regression line suggesting that, in these conditions, production of IL-1α and IL-1β protein is controlled by mRNA levels (Figure 3.10A-B).

**Section 3.3.10: T4SS-dependent macrophage responses are controlled at the level of mRNA induction**

To further demonstrate that mRNA levels control the translation of important innate immune proteins after virulent *L. pneumophila* infection, we analyzed the role of mRNA

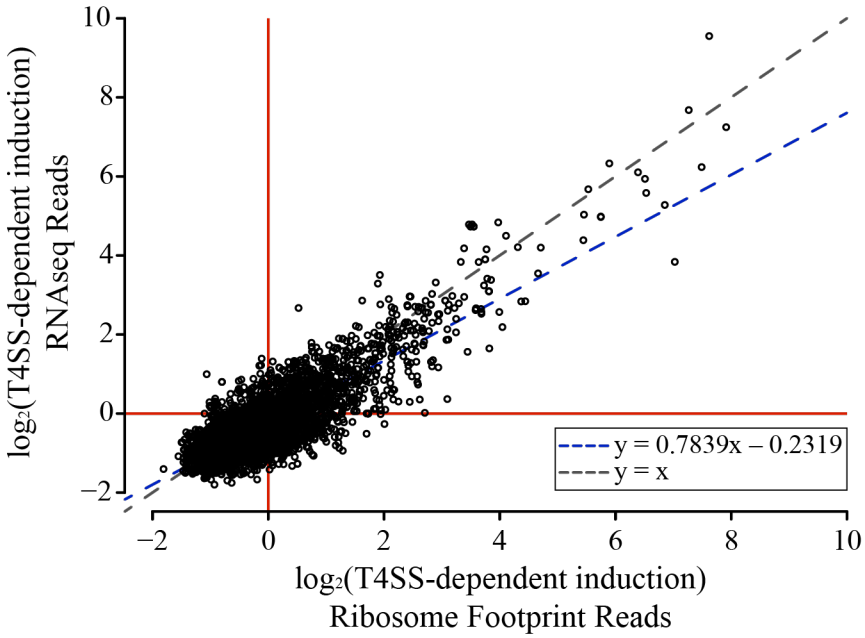


**Figure 3.11:** T4SS-dependent macrophage responses are controlled by mRNA induction. Ribosome footprint and RNAseq read counts were again sorted for well-expressed transcripts and normalized to mitochondrial protein coding genes. The ratio of ribosome footprint read counts in Δ*flaA*-infected and Δ*dotA*Δ*flaA*-infected macrophages was calculated for each transcript in the dataset. The same calculation was made for RNAseq read counts. These ratios depict the T4SS-dependent induction in macrophages and were plotted. Normalized read counts are averaged from 4 independent libraries for each condition. Grey dotted line, y = x. Blue dotted line, calculated linear regression. Linear regression calculations were made in the software R. Open circles represent an individual annotated transcript.

induction in controlling T4SS-dependent macrophage responses (Figure 3.11). T4SS-dependent mRNA induction was measured by taking the ratio of Δ*flaA* over Δ*dotA*Δ*flaA* read counts for every transcript with an average ribosome footprint and RNAseq read count greater than 100. T4SS-dependent induction was calculated for ribosome footprints in a similar fashion and these two ratios were plotted. We refer to this ratio as the T4SS-dependent induction, as any value greater than one shows increased expression that is dependent on the T4SS. T4SS-dependent transcriptional induction has nearly a 1:1 relationship with T4SS-dependent ribosome footprint read induction (Figure 3.11), suggesting that all T4SS-dependent macrophage responses can be explained by increases in mRNA levels. These data again support a model in which mRNA levels control the translation of not only IL-1α and IL-1β protein, but also the vast majority of proteins induced in response to virulent *L. pneumophila* infection.

**Section 3.3.11: MyD88-dependent signaling controls gene expression via mRNA induction**

Previous reports have suggested a role for MyD88 signaling after *L. pneumophila* infection in inducing preferential translation of specific transcripts (Asrat et al., 2014). Alternatively, we considered the possibility that following *L. pneumophila* infection, MyD88-dependent signaling increases transcript levels which overcome the block in host protein synthesis induced by *L. pneumophila* and leads to production of cytokine proteins. We undertook ribosome profiling and RNAseq experiments on B6 and *Myd88*$^{-/-}$ BMDMs infected with Δ*flaA* or Δ*dotA*Δ*flaA L. pneumophila* to determine if MyD88 controlled ribosome occupancy globally. To look at the MyD88-dependent induction of genes after Δ*flaA L. pneumophila* infection we took the ratio of ribosome footprint read counts in B6 BMDMs to *Myd88*$^{-/-}$ BDMDs and calculated a similar ratio for the RNAseq read counts. Similar to above, the ratio of B6 to *Myd88*$^{-/-}$ read counts allows for the visualization of the role of MyD88 in gene induction after *L. pneumophila* infection as a ratio greater than one suggests MyD88-dependent induction. By comparing the B6:*MyD88*$^{-/-}$ ratio of ribosome footprint reads to RNAseq reads we can determine the relative contribution of mRNA levels and translation to the total MyD88-dependent gene induction. Plotting these ratios for 6,930 filtered (read count >100) and normalized transcripts shows nearly a one to one relationship between the MyD88-dependent mRNA induction and MyD88-dependent translational induction, suggesting that the main role for MyD88 during gene induction after *L. pneumophila* infection is to induce increased mRNA levels (Figure 3.12A). Importantly, *Il1a* and *Il1b* are found to have a nearly one to one relationship between the MyD88-dependent mRNA and translational induction (Figure 3.12A). A similar, albeit less distinct, relationship can be seen in the case of Δ*dotA*Δ*flaA* infection (Figure 3.12B). The mRNA induction of genes in *Myd88*$^{-/-}$ BMDMs after Δ*dotA*Δ*flaA* infection is known to be diminished (Shin et al., 2008) and this could explain the reduced correlation between mRNA and translational induction in response Δ*dotA*Δ*flaA* infection. These data favor a model where mRNA induction of target genes overcomes the block in host protein synthesis leading to translation of cytokine genes. There is no evidence that MyD88 induces increased ribosome occupancy in response to *L. pneumophila* infection. It is important to note that these data do not rule out a mechanism by which MyD88 signaling increases ribosome processivity on specific transcripts, such as *Il1a* and *Il1b*. In this case, MyD88 signaling would not change the total number of ribosome footprints per transcript, or ribosome occupancy, but would instead increase the rate of translation by ribosomes on specific transcripts. While our data do not

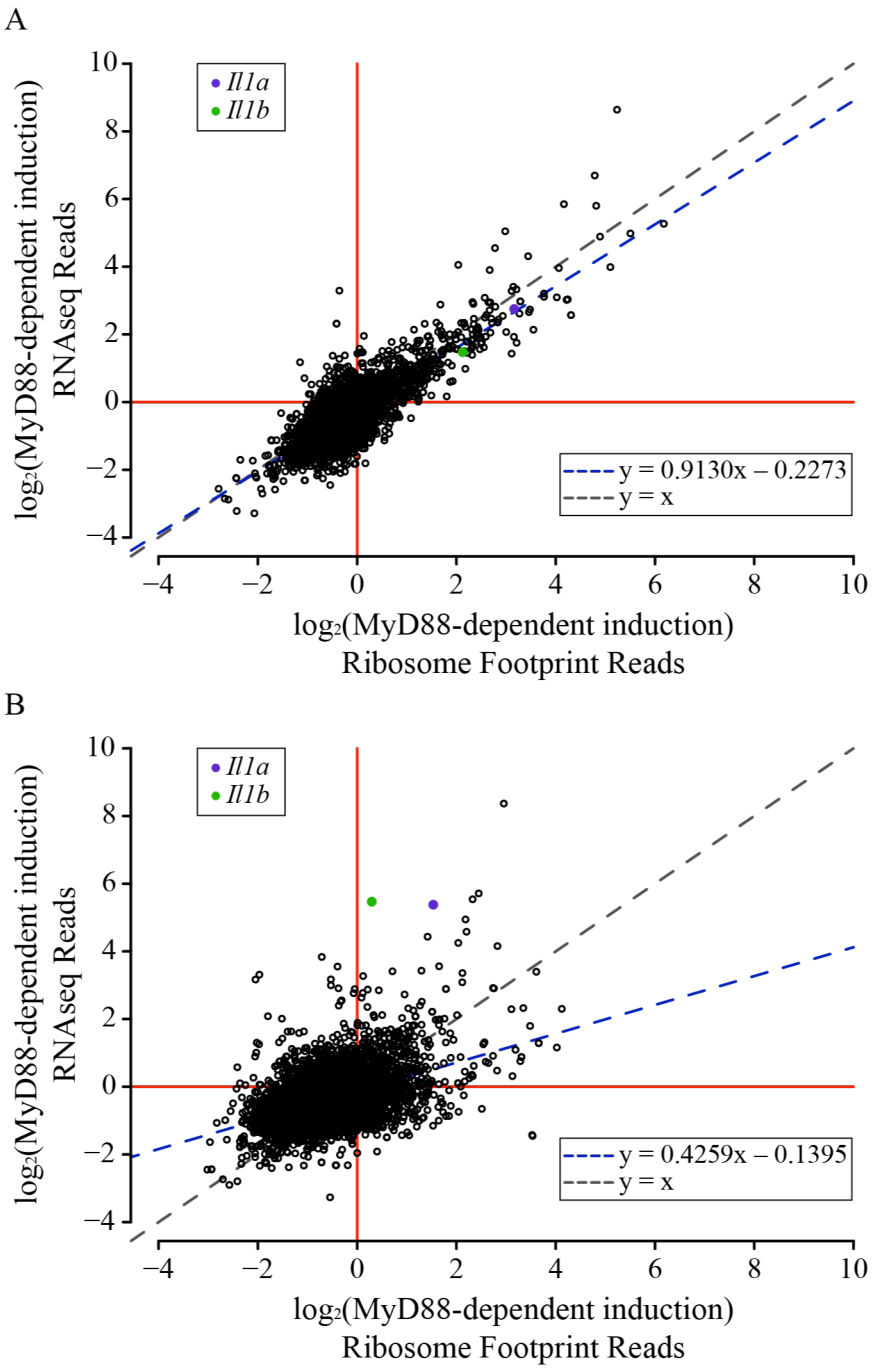


**Figure 3.12:** MyD88-dependent signaling controls gene expression at the level of mRNA induction. Ribosome footprint and RNAseq read counts were sorted for well-expressed transcripts and normalized to mitochondrial protein coding genes. The ratio of ribosome footprint read counts in WT B6 and *Myd88*$^{-/-}$ macrophages was calculated. This same ratio was calculated for RNAseq read counts. The ratio of read counts in WT:*Myd88*$^{-/-}$ macrophages elucidates the MyD88-dependent gene induction. MyD88-dependent induction was plotted for Δ*flaA* (A) and Δ*dotA*Δ*flaA* (B) *L. pneumophila* infection. Purple, *Il1a* transcript. Green, *Il1b* transcript. Open circles represent individual transcript. Grey dotted line, y = x. Blue dotted line, calculated linear regression.

address this model, we do not favor it as previous studies have demonstrated that the rate of ribosome processivity is consistent among different classes of messages (Ingolia et al., 2011).

### Section 3.3.12: A subset of host genes may have altered ribosome occupancy in response to *L. pneumophila* infection

While the vast majority of the gene induction that we see in response to virulent *L. pneumophila* infection appears to be controlled at the level of mRNA induction, there do appear to be a subset of transcripts that have higher ribosome occupancy (more ribosome footprints per mRNA) than the majority of genes (Figure 3.13A). These genes range in their annotated function but many of the genes with increased ribosome occupancy encode non-classical major histocompatibility complex proteins, histones, and some immune related genes (Table 3.1). Many of these genes show increased ribosome occupancy in response to Δ*dotA*Δ*flaA L. pneumophila* infection as well, suggesting that this may be a response common to primary BMDMs, or TLR stimulation (Figure 3.13B, Table 3.2). Further experiments analyzing uninfected BMDMs will be required to separate these two possibilities. It is interesting to note that many of the genes with higher ribosome occupancy appear to be related to host stress responses and/or normal cell homeostasis suggesting that this increased ribosome occupancy of these transcripts may be a stress response in macrophages. More research is needed to characterize functional consequences of this finding. Future experiments identifying gene products with increased ribosome occupancy in response to other intracellular bacterial pathogens will be important to determine if there a set of common stress response genes that are more efficiently translated in response to diverse intracellular pathogens. It is possible that macrophages have a normal bias to have increase ribosome occupancy on specific transcripts at steady state. However, given that the subset of genes found to have increased ribosome occupancy are immune-related may suggest this is a method to increase production of certain proteins important for immune responses.

We identified a number of transcripts that have reduced ribosome occupancy in response Δ*flaA* and/or Δ*dotA*Δ*flaA L. pneumophila* infection (Figure 3.13A-B, Table 3.3-3.4). One interesting gene product that has very low ribosome occupancy in response to both Δ*flaA* and Δ*dotA*Δ*flaA L. pneumophila* infection is *Atf4* (Table 3.3-3.4). *Atf4* is known to be upregulated in response to eIF2α phosphorylation by a mechanism involving increased translation of the *Atf4* transcript (Donnelly et al., 2013). As *Atf4* appears to have reduced ribosome occupancy in response to Δ*flaA* and Δ*dotA*Δ*flaA L. pneumophila* infection these data further support the conclusion that *L .pneumophila* does not block translation via the eIF2α pathway.

### Section 3.3.13: The intracellular pathogen *Listeria monocytogenes* induces a block in host protein synthesis

Having demonstrated a link between blockade of host protein synthesis and immune responses to *L. pneumophila* we were interested in exploring if the induction of host translation inhibition was conserved in an intracellular bacterial pathogen with a distinct life cycle. To address this question we undertook infections with the Gram-positive bacterial pathogen *Listeria* monocytogenes. After phagocytosis by macrophages, *L. monocytogenes* uses the pore-forming toxin listeriolysin O (LLO) and two phospholipase Cs (PLCs) to escape the vacuole and enter the cytosol where it replicates (Portnoy et al., 2002). B6 BMDMs were infected with a 1:100

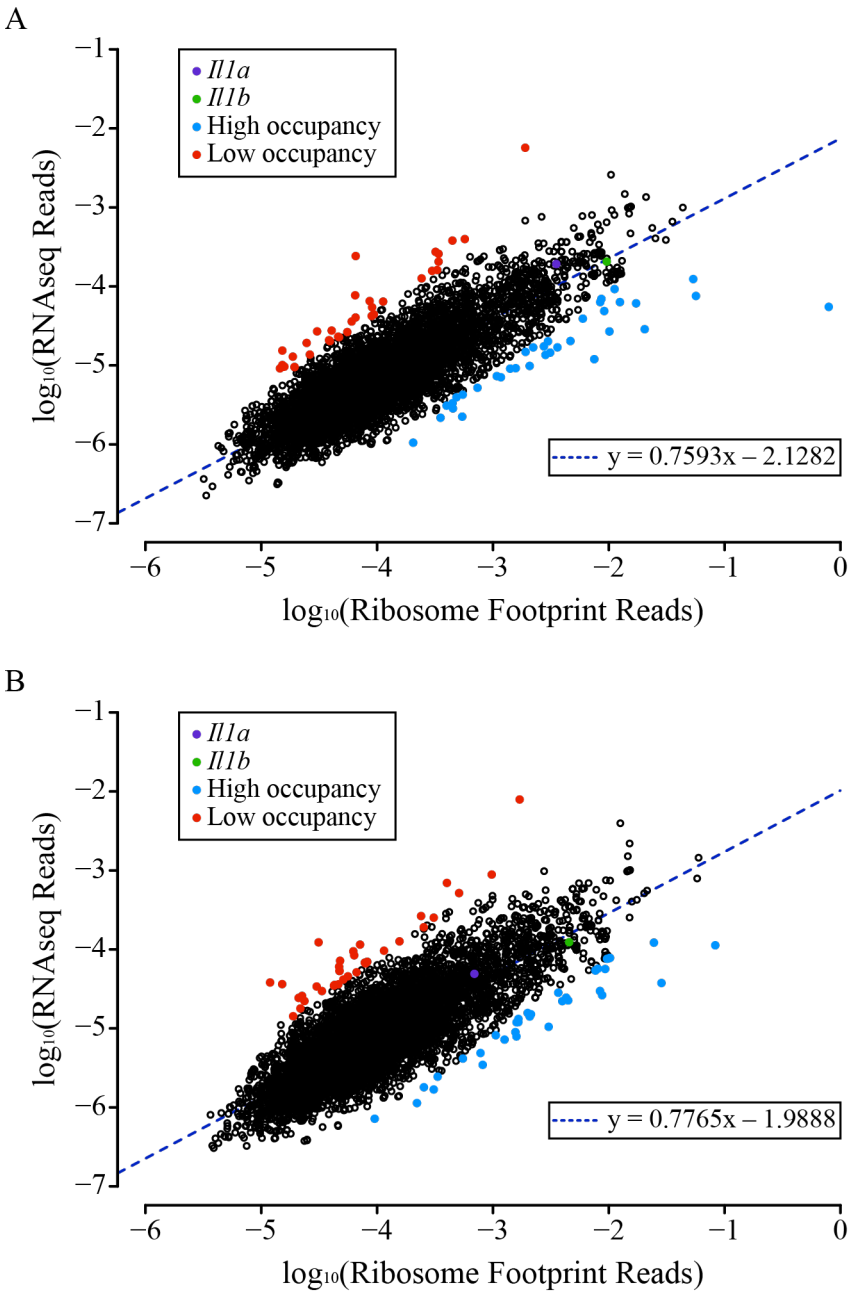


**Figure 3.13:** Some transcripts show altered ribosome occupancy following *L. pneumophila* infection. Normalized read counts from Figure 3.10 we used to calculate ribosome occupancy for each transcript in the dataset. Ribosome occupancy is defined as the ratio of ribosome footprint reads to mRNA reads (ribosome occupancy = footprint reads ÷ mRNA reads). The 35 transcripts with the highest (blue) and lowest (red) ribosome occupancy were overlayed with the global transcripts for Δ*flaA* (A) and Δ*dotA*Δ*flaA* (B) *L. pneumophila* infected macrophages. Linear regressions were calculated in the software R. Purple circles mark the *Il1a* transcript and green circles mark the *Il1b* transcript. Open circles represent an individual annotated transcript.

dilution of mid-log *L. monocytogenes* and at 1 and 3hrs post-infection incubated for 1h with $^{35}$S-methionine. Equal protein was loaded on to a denaturing gel and $^{35}$S-methionine incorporation was visualized (Figure 3.14A). Interestingly, WT *L. monocytogenes* induced a small but detectable block in global host protein synthesis that was dependent on LLO, as the Δ*hly L. monocytogenes* mutant did not block host translation (Figure 3.14A). To determine the mechanism by which *L. monocytogenes* blocks host protein synthesis we blotted for components of the mTOR signaling pathway and eIF2α as these pathways have been previously shown to be inhibited after infection in a LLO-dependent manner (Pillich et al., 2012; Shrestha et al., 2012; Tattoli et al., 2013). Consistent with previous reports WT *L. monocytogenes* induces phosphorylation of eIF2α while Δ*hly* does not (Figure 3.14B). However, we found no evidence for perturbations of the mTOR signaling pathway following *L. monocytogenes* infection as we detect no changes in the phosphorylation state of mTOR or 4E-BP1 after WT or Δ*hly L. monocytogenes* infection (Figure 3.14C). These data suggest that *L. monocytogenes*, an intracellular bacterium with a different life cycle than *L. pneumophila,* is also capable of inducing a block in host protein synthesis. In future ribosome profiling experiments it will be interesting to see if *L. monocytogenes* infection induces global changes in translation in a similar fashion to *L. pneumophila*.

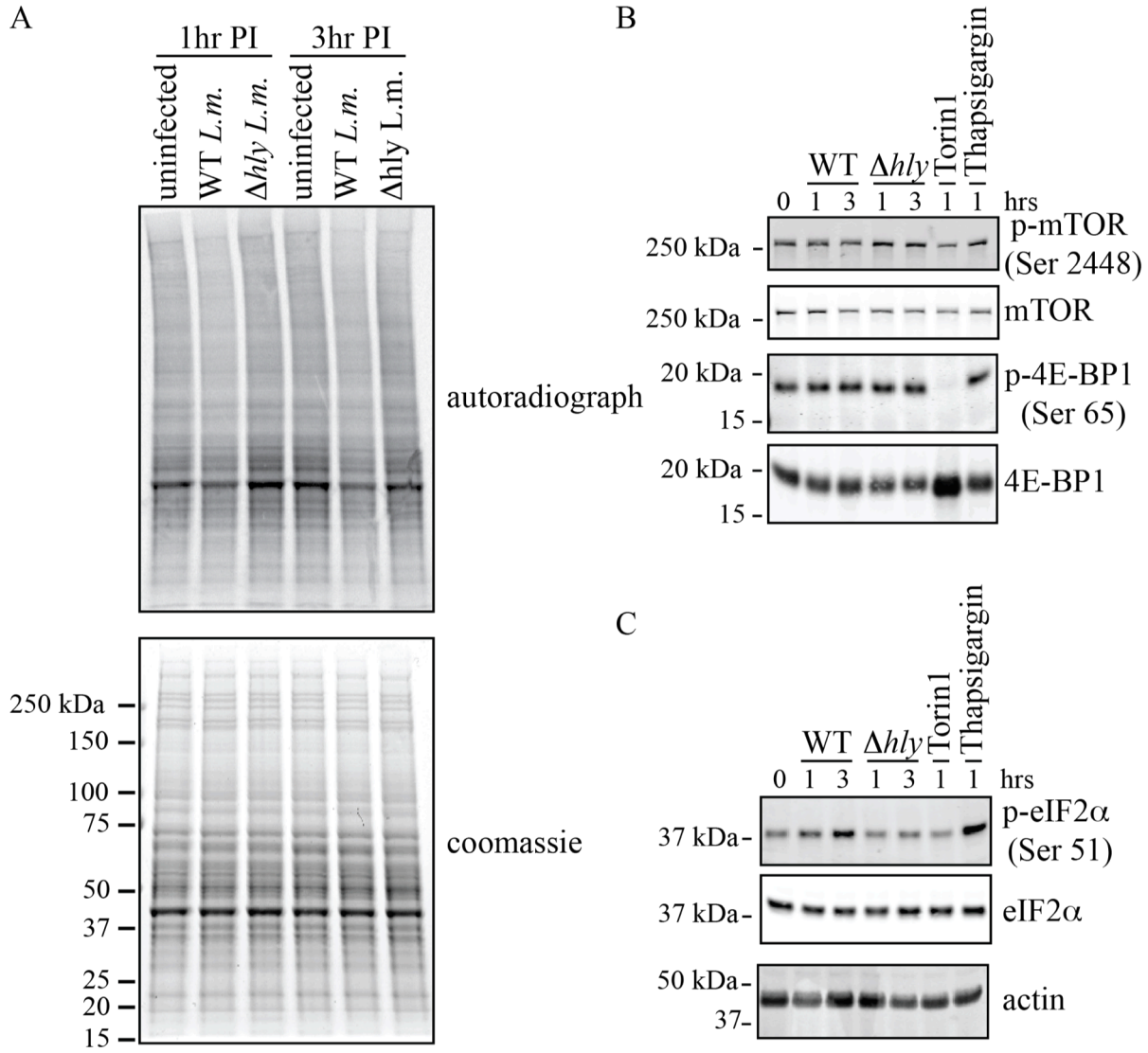


**Figure 3.14:** *Listeria monocytogenes* blocks host translation possibly through eIF2α phosphorylation. Wild-type B6 BMDMs were infected witht he indicated strains of mid-log *L. monocytogenes*. (A) 1 and 6hrs post-infection BMDMs were incubated with $^{35}$S-methionine as described in Figure 3.5A. Global translation levels were determined by autoradiography (top panel) and equal protein loading was demonstrated by coomassie staining (bottom pane). (B) At the indicated times lysates were collected and used for western blot analysis of the phosphorylation state of 4E-BP1 (Ser65) and mTOR (2448). (C) At the indicated times lysates were collected and used for western blot analysis of the phosphorylation state of eIF2α (Ser51). Total non-phosphorylated protein and β-actin was used as a loading control. (A-C) All infections were completed at the same time with the same starting bacterial cultures. (B-C) These westerns were run on the same lysates and blots. The β-actin control in C is the same for B. Torin1 (250nM) and thapsigargin (500nM) were used as positive controls.

| | Gene | RefSeq | Norm. Ribo. footprint averages | Norm. mRNA averages | Ribo/mRNA Ratio |
|---|---|---|---|---|---|
| 1 | Gm20594 | NM_001190732 | 7.92E-01 | 5.49E-05 | 14438.23 |
| 2 | **Tmsb4x** | NM_021278 | 5.65E-02 | 7.54E-05 | 749.39 |
| 3 | **Lyz1** | NM_013590 | 2.04E-02 | 2.86E-05 | 712.35 |
| 4 | Cxcl1 | NM_008176 | 7.49E-03 | 1.19E-05 | 626.59 |
| 5 | **Cxcl2** | NM_009140 | 5.36E-02 | 1.23E-04 | 433.71 |
| 6 | **Hist1h2bh** | NM_178197 | 1.01E-02 | 2.68E-05 | 377.54 |
| 7 | Cxcl3 | NM_203320 | 1.72E-02 | 6.09E-05 | 282.59 |
| 8 | **Gm5803** | NM_001165971 | 5.42E-04 | 2.24E-06 | 242.34 |
| 9 | **Rpl37a** | NM_009084 | 4.66E-03 | 2.03E-05 | 229.69 |
| 10 | **Txn1** | NM_011660 | 3.12E-03 | 1.45E-05 | 215.51 |
| 11 | **Pf4** | NM_019932 | 3.60E-03 | 1.69E-05 | 213.46 |
| 12 | **Acta1** | NM_009606 | 2.07E-03 | 9.81E-06 | 211.19 |
| 13 | **H2afj** | NM_177688 | 2.84E-03 | 1.35E-05 | 209.63 |
| 14 | **Actg1** | NM_009609 | 1.25E-02 | 6.31E-05 | 197.80 |
| 15 | **Rrbp1** | NM_133626 | 2.05E-04 | 1.05E-06 | 195.37 |
| 16 | Hist1h2bf | NM_178195 | 9.13E-03 | 4.86E-05 | 187.76 |
| 17 | **Myl12b** | NM_023402 | 1.57E-03 | 9.19E-06 | 170.95 |
| 18 | Ube2d3 | NM_025356 | 1.17E-03 | 7.05E-06 | 165.84 |
| 19 | **Sdc3** | NM_011520 | 3.52E-04 | 2.17E-06 | 162.58 |
| 20 | H2-T24 | NM_008207 | 4.51E-04 | 2.84E-06 | 158.58 |
| 21 | Cd63 | NM_007653 | 2.75E-03 | 1.74E-05 | 158.42 |
| 22 | **Myadm** | NM_016969 | 1.41E-03 | 9.07E-06 | 155.17 |
| 23 | **Lgals3** | NM_010705 | 5.97E-03 | 3.90E-05 | 153.13 |
| 24 | **Hmox1** | NM_010442 | 2.98E-03 | 2.01E-05 | 148.28 |
| 25 | Rpl29 | NM_009082 | 1.08E-03 | 7.29E-06 | 147.93 |
| 26 | Vma21 | NM_001081356 | 7.35E-04 | 5.20E-06 | 141.46 |
| 27 | Elovl1 | NM_001039175 | 4.48E-04 | 3.31E-06 | 135.07 |
| 28 | Hist1h2bm | NM_178200 | 8.43E-03 | 6.31E-05 | 133.63 |
| 29 | **Hist1h2aa** | NM_175658 | 2.22E-03 | 1.69E-05 | 131.98 |
| 30 | Sh3bgrl | NM_019989 | 1.91E-03 | 1.48E-05 | 129.33 |
| 31 | Ifi204 | NM_008329 | 5.48E-04 | 4.25E-06 | 129.10 |
| 32 | Ociad1 | NM_023429 | 3.97E-04 | 3.08E-06 | 128.80 |
| 33 | Hist1h2bj | NM_178198 | 8.62E-03 | 6.93E-05 | 124.49 |
| 34 | Pcbp1 | NM_011865 | 4.84E-04 | 3.94E-06 | 122.88 |
| 35 | **Hist1h4f** | NM_175655 | 1.12E-02 | 9.23E-05 | 121.43 |

**Table 3.1:** Ranked list of the 35 transcripts with the highest ribosome occupancy following Δ*flaA L. pneumophila* infection. Bolded genes are also found in Δ*dotA*Δ*flaA L. pneumophila* infected macrophages.

| | Gene | RefSeq | Norm. Ribo. footprint averages | Norm. mRNA averages | Ribo/mRNA Ratio |
|---|---|---|---|---|---|
| 1 | **Lyz1** | NM_013590 | 2.85E-02 | 3.75E-05 | 759.50 |
| 2 | **Tmsb4x** | NM_021278 | 8.30E-02 | 1.13E-04 | 736.91 |
| 3 | **Hist1h2bh** | NM_178197 | 8.75E-03 | 2.63E-05 | 332.70 |
| 4 | Cd52 | NM_013706 | 3.02E-03 | 1.05E-05 | 289.10 |
| 5 | **Cxcl2** | NM_009140 | 8.39E-03 | 2.97E-05 | 282.59 |
| 6 | **Gm5803** | NM_001165971 | 8.15E-04 | 3.45E-06 | 236.32 |
| 7 | **Hist1h2aa** | NM_175658 | 1.59E-03 | 7.89E-06 | 201.98 |
| 8 | **Actg1** | NM_009609 | 2.45E-02 | 1.22E-04 | 201.51 |
| 9 | **Rpl37a** | NM_009084 | 4.46E-03 | 2.26E-05 | 197.39 |
| 10 | **Rrbp1** | NM_133626 | 2.21E-04 | 1.13E-06 | 194.96 |
| 11 | Trp53 | NM_011640 | 3.08E-04 | 1.68E-06 | 183.08 |
| 12 | **Acta1** | NM_009606 | 3.96E-03 | 2.21E-05 | 179.38 |
| 13 | **Txn1** | NM_011660 | 4.28E-03 | 2.44E-05 | 175.20 |
| 14 | Rpl31 | NM_001258458 | 1.26E-03 | 7.20E-06 | 174.75 |
| 15 | Rpl35a | NM_001130485 | 1.56E-03 | 8.96E-06 | 174.22 |
| 16 | **Hist1h4f** | NM_175655 | 9.30E-03 | 5.64E-05 | 164.90 |
| 17 | Dazap2 | NM_011873 | 7.80E-04 | 4.86E-06 | 160.41 |
| 18 | **H2afj** | NM_177688 | 2.07E-03 | 1.41E-05 | 147.14 |
| 19 | **Lgals3** | NM_010705 | 7.67E-03 | 5.41E-05 | 141.82 |
| 20 | **Lgals3** | NM_016969 | 7.67E-03 | 5.41E-05 | 141.40 |
| 21 | **Myadm** | NM_001093764 | 2.13E-03 | 1.51E-05 | 141.40 |
| 22 | **Myadm** | NM_010442 | 8.04E-03 | 5.69E-05 | 141.31 |
| 23 | **Hmox1** | NM_019678 | 2.53E-04 | 1.80E-06 | 140.96 |
| 24 | Tfg | NM_010886 | 1.66E-03 | 1.20E-05 | 138.26 |
| 25 | Ndufa4 | NM_011520 | 3.33E-04 | 2.44E-06 | 136.18 |
| 26 | **Sdc3** | NM_023402 | 1.60E-03 | 1.19E-05 | 134.11 |
| 27 | **Myl12b** | NM_183208 | 9.52E-05 | 7.17E-07 | 132.78 |
| 28 | Zmiz1 | NM_008208 | 5.50E-04 | 4.14E-06 | 132.71 |
| 29 | H2-T3 | NM_178211 | 1.02E-02 | 7.80E-05 | 131.25 |
| 30 | Hist1h4k | NM_153173 | 1.00E-02 | 7.70E-05 | 130.02 |
| 31 | Hist1h4h | NM_173742 | 1.06E-03 | 8.16E-06 | 129.48 |
| 32 | Rnasek | NM_019932 | 3.66E-03 | 2.83E-05 | 129.38 |
| 33 | **Pf4** | NM_030694 | 1.99E-03 | 1.56E-05 | 127.36 |
| 34 | Ifitm2 | NM_008084 | 9.65E-03 | 7.60E-05 | 127.00 |
| 35 | Gapdh | NM_001081274 | 1.66E-03 | 1.32E-05 | 125.95 |

**Table 3.2:** Ranked list of the 35 transcripts with the highest ribosome occupancy following Δ*dotA*Δ*flaA L. pneumophila* infection. Bolded genes are also found in Δ*flaA L. pneumophila* infected macrophages.

| | Gene | RefSeq | Norm. Ribo. footprint averages | Norm. mRNA averages | Ribo/mRNA Ratio |
|---|---|---|---|---|---|
| 1 | **Atf4** | NM_009716 | 6.54E-05 | 2.42E-04 | 0.27 |
| 2 | **Ftl1** | NM_010240 | 1.90E-03 | 5.67E-03 | 0.34 |
| 3 | **Spred1** | NM_033524 | 6.47E-05 | 7.70E-05 | 0.84 |
| 4 | Ankrd28 | NM_001024604 | 1.52E-05 | 1.54E-05 | 0.99 |
| 5 | Mapk6 | NM_027418 | 3.04E-05 | 2.69E-05 | 1.13 |
| 6 | **Cflar** | NM_009805 | 3.20E-04 | 2.73E-04 | 1.17 |
| 7 | **Gpx1** | NM_008160 | 4.47E-04 | 3.79E-04 | 1.18 |
| 8 | **Dennd5a** | NM_021494 | 2.47E-05 | 1.92E-05 | 1.29 |
| 9 | **Srp14** | NM_009273 | 3.39E-04 | 2.58E-04 | 1.32 |
| 10 | Mdfic | NM_175088 | 8.63E-05 | 6.51E-05 | 1.33 |
| 11 | **Tspo** | NM_009775 | 5.72E-04 | 3.96E-04 | 1.44 |
| 12 | **Kif1c** | NM_153103 | 1.87E-05 | 1.29E-05 | 1.46 |
| 13 | Smek2 | NM_134034 | 4.06E-05 | 2.75E-05 | 1.47 |
| 14 | Setd5 | NM_028385 | 1.52E-05 | 1.01E-05 | 1.51 |
| 15 | **2610507B1** | NM_001002004 | 1.45E-05 | 9.14E-06 | 1.59 |
| 16 | Cyld | NM_001128170 | 1.54E-05 | 9.57E-06 | 1.61 |
| 17 | **Pqbp1** | NM_001252528 | 6.52E-05 | 4.03E-05 | 1.62 |
| 18 | Cyld | NM_173369 | 1.58E-05 | 9.62E-06 | 1.65 |
| 19 | **Tmem167** | NM_025335 | 3.40E-04 | 2.05E-04 | 1.65 |
| 20 | **Mtmr14** | NM_026849 | 9.06E-05 | 5.42E-05 | 1.67 |
| 21 | **Pqbp1** | NM_001252529 | 6.04E-05 | 3.58E-05 | 1.69 |
| 22 | **Aes** | NM_010347 | 1.13E-04 | 6.41E-05 | 1.76 |
| 23 | **Anapc5** | NM_001042491 | 3.85E-05 | 2.08E-05 | 1.85 |
| 24 | **Cflar** | NM_207653 | 2.98E-04 | 1.57E-04 | 1.90 |
| 25 | Gtf2h1 | NM_008186 | 2.63E-05 | 1.37E-05 | 1.92 |
| 26 | Snrpg | NM_026506 | 2.42E-04 | 1.26E-04 | 1.92 |
| 27 | **Anapc5** | NM_021505 | 3.92E-05 | 2.02E-05 | 1.94 |
| 28 | Akt1 | NM_009652 | 4.61E-05 | 2.30E-05 | 2.00 |
| 29 | Hipk3 | NM_010434 | 1.94E-05 | 9.50E-06 | 2.04 |
| 30 | Hipk3 | NM_001145824 | 1.94E-05 | 9.51E-06 | 2.04 |
| 31 | Hprt | NM_013556 | 3.31E-04 | 1.61E-04 | 2.06 |
| 32 | Akt1 | NM_001165894 | 4.70E-05 | 2.27E-05 | 2.07 |
| 33 | Golph3l | NM_001177670 | 5.53E-05 | 2.63E-05 | 2.10 |
| 34 | **Ube2j2** | NM_001039158 | 9.38E-05 | 4.41E-05 | 2.13 |
| 35 | **Ube2j2** | NM_001039157 | 8.99E-05 | 4.21E-05 | 2.14 |

**Table 3.3:** Ranked list of the 35 transcripts with the lowest ribosome occupancy following Δ*flaA L. pneumophila* infection. Bolded genes are also found in Δ*dotA*Δ*flaA L. pneumophila* infected macrophages.

| | Gene | RefSeq | Norm. Ribo. footprint averages | Norm. mRNA averages | Ribo/mRNA Ratio |
|---|---|---|---|---|---|
| 1 | **Ftl1** | NM_010240 | 1.70E-03 | 7.92E-03 | 0.21 |
| 2 | **Atf4** | NM_009716 | 3.13E-05 | 1.23E-04 | 0.25 |
| 3 | Vps54 | NM_139061 | 1.19E-05 | 3.82E-05 | 0.31 |
| 4 | Cenpb | NM_007682 | 1.52E-05 | 3.64E-05 | 0.42 |
| 5 | **Srp14** | NM_009273 | 4.01E-04 | 6.96E-04 | 0.58 |
| 6 | **Aes** | NM_010347 | 7.13E-05 | 1.15E-04 | 0.62 |
| 7 | **Pqbp1** | NM_019478 | 6.24E-05 | 9.49E-05 | 0.66 |
| 8 | Vti1b | NM_016800 | 4.80E-05 | 7.20E-05 | 0.67 |
| 9 | **Pqbp1** | NM_001252529 | 6.35E-05 | 8.43E-05 | 0.75 |
| 10 | Drap1 | NM_024176 | 4.70E-05 | 6.05E-05 | 0.78 |
| 11 | Cnot6 | NM_212484 | 2.10E-05 | 2.44E-05 | 0.86 |
| 12 | **Kif1c** | NM_153103 | 2.25E-05 | 2.59E-05 | 0.87 |
| 13 | Slc30a9 | NM_178651 | 3.01E-05 | 3.40E-05 | 0.89 |
| 14 | Amd2 | NM_007444 | 4.74E-05 | 5.35E-05 | 0.89 |
| 15 | Amd1 | NM_009665 | 4.74E-05 | 5.35E-05 | 0.89 |
| 16 | Cdc42se1 | NM_172395 | 2.40E-04 | 2.65E-04 | 0.91 |
| 17 | **Gpx1** | NM_008160 | 5.10E-04 | 5.18E-04 | 0.99 |
| 18 | Adipor1 | NM_028320 | 2.36E-05 | 2.23E-05 | 1.06 |
| 19 | **Tspo** | NM_009775 | 9.77E-04 | 8.88E-04 | 1.10 |
| 20 | Becn1 | NM_019584 | 3.34E-05 | 2.98E-05 | 1.12 |
| 21 | **Spred1** | NM_033524 | 7.84E-05 | 6.81E-05 | 1.15 |
| 22 | **Ube2j2** | NM_001039158 | 8.23E-05 | 6.98E-05 | 1.18 |
| 23 | Mpc2 | NM_027430 | 1.15E-04 | 9.66E-05 | 1.19 |
| 24 | **Ube2j2** | NM_001039157 | 7.95E-05 | 6.66E-05 | 1.19 |
| 25 | **Anapc5** | NM_001042491 | 4.30E-05 | 3.56E-05 | 1.21 |
| 26 | **Dennd5a** | NM_021494 | 2.18E-05 | 1.79E-05 | 1.22 |
| 27 | **Anapc5** | NM_021505 | 4.24E-05 | 3.49E-05 | 1.22 |
| 28 | **Tmem167** | NM_025335 | 3.09E-04 | 2.53E-04 | 1.22 |
| 29 | Unc50 | NM_026123 | 5.61E-05 | 4.57E-05 | 1.23 |
| 30 | Snf8 | NM_033568 | 5.11E-05 | 4.16E-05 | 1.23 |
| 31 | **Cflar** | NM_009805 | 1.56E-04 | 1.27E-04 | 1.23 |
| 32 | **Mtmr14** | NM_026849 | 4.63E-05 | 3.62E-05 | 1.28 |
| 33 | Mea1 | NM_010787 | 6.66E-05 | 5.15E-05 | 1.29 |
| 34 | **2610507B1** | NM_001002004 | 1.89E-05 | 1.43E-05 | 1.32 |
| 35 | Odc1 | NM_013614 | 2.53E-04 | 1.91E-04 | 1.32 |

**Table 3.3:** Ranked list of the 35 transcripts with the lowest ribosome occupancy following Δ*dotA*Δ*flaA L. pneumophila* infection. Bolded genes are also found in Δ*flaA L. pneumophila* infected macrophages.

## Section 3.4: Discussion

There has been a longstanding question, especially in the field of immunology, about what mRNA levels can inform us about overall protein levels in a cell. A number of studies have been undertaken to address the relative importance of transcriptional induction in controlling protein levels at steady state, but the results have been contentious (Breker and Schuldiner, 2014; de Sousa Abreu et al., 2009; Li et al., 2014; Maier et al., 2009; Schwanhausser et al., 2011; Vogel and Marcotte, 2012). A recent study generated an integrated experimental and computational strategy to quantitatively assess how protein levels are maintained in bone marrow derived dendritic cells (BMDCs; Jovanovic et al., 2015). This study was able to demonstrate that mRNA levels explain ~87-92% of protein changes in BMDCs after 12hr LPS treatment (Jovanovic et al., 2015). These data suggest that in response to innate immune stimulation BMDCs almost entirely control the induction of genes at the level of transcriptional induction (Jovanovic et al., 2015).

Our study examines the relative contribution of mRNA levels and translational regulation in the induction of gene products after intracellular bacterial infection. Consistent with the previous study using LPS stimulation alone (Jovanovic et al., 2015), we find that in response to infection by the intracellular pathogen *L. pneumophila* the vast majority of the changes in gene expression are dependent on mRNA induction. One caveat to this conclusion is that T4SS$^{+}$ *L. pneumophila* blocks host protein synthesis at the level of translation elongation. Therefore, our ribosome profiling data does not directly correlate with protein production as has been previously described (Ingolia et al., 2009). Previous reports have demonstrated that a number of inflammatory cytokines are induced in response to virulent *L. pneumophila* (Asrat et al., 2014; Barry et al., 2013; Fontana et al., 2011; Shin et al., 2008). For these inflammatory cytokines, including IL-1α, the majority of control of protein induction following *L. pneumophila* infection is controlled by mRNA induction. Another caveat to the analyses presented here is that due to the block in host translation elongation induced by *L. pneumophila* we are unable to rule out increased ribosome processivity in playing a role in the induction of these cytokines. However, previous studies have shown that the rate of translation remains constant across many classes of transcripts (Ingolia et al., 2011). Therefore, our study is consistent with previous reports (Jovanovic et al., 2015) and suggests that mRNA levels control the induction of proteins in response to virulent *L. pneumophila* infection.

*L. pneumophila* has been shown to block host protein synthesis (Barry et al., 2013; Fontana et al., 2011; Fontana and Vance, 2011). We previously identified 7 *L. pneumophila* effector proteins that block host protein synthesis (Barry et al., 2013; Belyi et al.; Belyi et al.; Belyi et al., 2009; Belyi et al.; Fontana et al., 2011; Hurtado-Guerrero et al., 2010; Lu et al., 2010; Tzivelekidis et al., 2011). However, a *L. pneumophila* mutant lacking all 7 of the translation blocking effectors still induces host translation inhibition (Barry et al., 2013). A previous report suggested that *L. pneumophila* could modulate the mTOR signaling pathway and this could induce a block in cap-dependent host protein synthesis (Ivanov and Roy, 2013). It therefore seemed that one explanation for the residual translation inhibition in the Δ7Δ*flaA* strain of *L. pneumophila* could be due to pathogen induced changes in host stress response pathways. Consistent with a host stress response explaining the residual translation inhibition induced by Δ7Δ*flaA L. pneumophila* our ribosome profiling analyses suggest that Δ7Δ*flaA L. pneumophila* induced translation inhibition occurs at translation initiation. Intriguingly, the majority of host translation regulation occurs at the level of translation initiation (Hershey et al., 2012).

However, our study finds no evidence for *L. pneumophila* induced changes in the mTOR pathway. We do see some evidence for increased phosphorylation of eIF2α after *L. pneumophila* infection, but inhibiting this phosphorylation by genetic deletion of *Eif2ak4* or over-expression of the eIF2α phosphatase, Gadd34, had no effect on *L. pneumophila* induced translation inhibition. Further, *Atf4*, a transcript that is preferentially translated in response to phosphorylation of eIF2α, is one of the least efficiently translated transcripts after *L. pneumophila* infection, suggesting that phosphorylation of eIF2α is not involved in *L. pneumophila* induced translation inhibition. Our data suggest that common stress response pathways cannot explain the residual block in host protein synthesis. It is interesting to hypothesize that there may be a novel stress response to *L. pneumophila* infection that is blocking translation initiation, but more studies are required to address this hypothesis. It should also be noted that our results are inconsistent with a previous study that suggested that virulent *L. pneumophila* inhibits the mTOR pathway (Ivanov and Roy, 2013). The discrepancy between the results presented here and this previous study can likely be explained by differences in experimental conditions. Ivanov *et al.* undertook all of their studies in serum-starved cells. While serum starvation is common practice to detect changes in the mTOR signaling pathway, the translation inhibition induced by *L. pneumophila* infection is robust even in the presence of serum. Therefore, the relevance of the mTOR findings in the absence of serum remain to be seen because if the changes in mTOR can only be detected in the absence of serum they do not explain the residual translation inhibition seen in the Δ*7*Δ*flaA* mutant. Taken together, these results suggest that residual translation inhibition seen in the Δ*7*Δ*flaA L. pneumophila* mutant is at the level of translation initiation and may be caused by a yet unidentified host stress response pathway or a novel bacterial effector that targets translation initiation.

Translation inhibition induced by pathogenic infection has been shown in a number of infection models to be sensed by the host and induce an inflammatory response (Barry et al., 2013; Chakrabarti et al., 2012; Dunbar et al., 2012; Fontana et al., 2011; Fontana et al., 2012; McEwan et al., 2012). We previously identified IL-1α as a key inflammatory cytokine induced preferentially in response to virulent *L. pneumophila* and linked this preferential production to the translation inhibition in concert with TLR stimulation (Barry et al., 2013). We proposed a model, in which translation inhibition induced by pathogenic infection in combination with TLR signaling induces a massive and sustained production of *Il1a* transcript that overcomes the block in translation and allows for production of IL-1α protein (Barry et al., 2013; Fontana et al., 2011). Another report suggested that instead of transcriptional induction controlling the production of IL-1α protein, IL-1α protein was regulated at the level of translation (Asrat et al., 2014). This model proposed that adapter protein MyD88 was required for preferential loading of ribosomes to *Il1a* and *Il1b* transcripts (Asrat et al., 2014). In this study we find that global analysis of gene induction, using ribosome profiling and RNAseq of macrophages infected with *L. pneumophila*, demonstrates that the vast majority of gene expression in infected macrophages occurs at the level of mRNA induction. We also find that globally, the role of MyD88 signaling in gene expression appears to be at the level of mRNA induction and not translational regulation. Again, it should be noted that *L. pneumophila* induces a block in host protein synthesis so the data presented in this study cannot rule out a role for MyD88 in increasing the processivity of ribosomes on specific transcripts. While this is a possibility, it seems unlikely to be the mechanism by which *Il1a* and *Il1b* transcripts can be translated in the presence of translation inhibition as ribosome processivity has previously been shown to be consistent among different classes of messages (Ingolia et al., 2012).

Lastly, our study shows that another unrelated intracellular bacterial pathogen, *Listeria monocytogenes*, also induces a block in host protein synthesis. This is consistent with previous studies that demonstrated that *L. monocytogenes* infection induces phosphorylation of eIF2α and GCN2 (Pillich et al., 2012; Shrestha et al., 2012; Tattoli et al., 2013). A previous report suggested that mTOR may also be inhibited after WT *L. monocytogenes* infection, but our data do not support these findings, as we see no differences in 4E-BP1 phosphorylation after WT or Δ*hly L. monocytogenes* infection. These differences may be due to differences in cell types utilized in the two studies, primary BMDMs in our study and an immortalized cell line in the other (Tattoli et al., 2013), or to differences in infection times or conditions. In any case our results suggest that two distinct intracellular pathogens can block translation and further studies looking at global translation and transcriptional regulation after *L. monocytogenes* infection will provide interesting comparisons to the host responses to *L. pneumophila*.

The results presented here further support a role for translation inhibition in allowing the innate immune system to recognize and preferentially respond to pathogens. The data presented here provide molecular details on the block in host protein synthesis induced by *L. pneumophila* globally and suggest that *L. pneumophila* can inhibit host protein synthesis both at the level of translation initiation and elongation. An interesting hypothesis that comes from the data presented here is that a still unidentified host stress response pathway could cause the *L. pneumophila* induced block in translation initiation. Given that translation inhibition can induce production of inflammatory cytokines it is intriguing to hypothesize that the host may block its' own translation as a mechanism to induce these cytokines. Clearly, further studies are required to address these speculations. Lastly, the global analyses presented here demonstrate that in response to the intracellular bacterial pathogen *L. pneumophila* mRNA levels control the induction of genes, not translational regulation. These results suggest that measuring mRNA levels may depict more relevant changes to protein levels than previously aknowldeged.

# Chapter 4: Questions and perspectives

## Section 4.1: Remaining questions

### Section 4.1.1: Does the host directly regulate translation in response to *L. pneumophila* infection?

There are two remaining questions regarding the block in host protein synthesis and induction of cytokines in response to *L. pneumophila* infection: what explains the residual block in host protein synthesis induced by the Δ*7*Δ*flaA* strain of *L. pneumophila* and if you ablate the residual block in host protein synthesis does Δ*7*Δ*flaA L. pneumophila* still induce IL-1α? To address the role of translation inhibition in IL-1α production genetically, we must first understand the mechanism of translation inhibition induced by Δ*7*Δ*flaA L. pneumophila*. The ribosome profiling data presented here suggests that the 7 effector-independent block in host translation is at the level of translation initiation and may also suggest that a host stress response pathway could be responsible for the residual blockade of host protein synthesis. Experiments with bicistronic reporters have confounded the ribosome profiling results as these reporter assays suggest that the residual block in host protein synthesis by the Δ*7*Δ*flaA* strain of *L. pneumophila* blocks translation elongation. As discussed above, there are experimental caveats with the bicistronic reporter experiments; however, further studies are required to confirm that the residual block in host protein synthesis is at the level of translation initiation. One method to confirm these results will be to utilize polysome profiling in the presence or absence of the initiation blocking drug harringtonine. If the residual block in host protein synthesis is at the level of translation initiation we expect that the Δ*7*Δ*flaA L. pneumophila* strain will have ribosome run-off after harringtonine treatment and this will be visualized by a loss of polysomes.

If the residual block in host protein synthesis is at the level of translation initiation there are two possible mechanisms for this block: first, there may be a novel bacterial effector that blocks translation initiation and second, in response to pathogenic *L. pneumophila* infection the host may block its' own translation by activating stress response pathways. To address the possibility of a novel bacterial effector blocking translation initiation I propose that a bacterial transposon mutagenesis screen should be undertaken. Our previous screen, described in Chapter 2, utilized an overexpression approach to look at the activity of individual bacterial effector proteins (Figure 2.9, Table 2.2). While this approach identified novel effectors that can block host protein synthesis, there were inherent biases in this approach. In the overexpression screen we assumed that the bacterial effectors could function as individuals and did not need to cooperate with other effectors for their activity. We also assumed that the residual block in host protein synthesis in the Δ*5*Δ*flaA L. pneumophila* strain was due to an effector protein and not a non-effector bacterial process (e.g. metabolism, growth, etc.). Thus, I propose that a transposon mutagenesis screen of *L. pneumophila* should be undertaken as an unbiased approach to identify bacterial genes that are required for the block in host protein synthesis. The ideal screen would involve infection of cells with a pooled library of Δ*7*Δ*flaA L. pneumophila* transposon mutants. 6 hrs post-infection a reporter protein should be transcriptionally induced (e.g. doxycycline inducible fluorescent protein or surface marker). The majority of Δ*7*Δ*flaA L. pneumophila* infected cells would have no or reduced production of the reporter protein as translation is inhibited at 6 hrs after *L. pneumophila* infection. Any cell that expresses the reporter protein

would either be uninfected or infected with a Δ7Δ*flaA L. pneumophila* transposon mutant that no longer induces a block in host protein synthesis. Expression of the reporter protein could be used to isolate these mutants via FACS sorting (fluorescent reporter) or MACS cell separation (surface marker) followed by lysis and plating for bacterial single colonies. This screen would allow for the identification of novel bacterial genes involved in the block in host protein synthesis in an unbiased manner.

One caveat to the bacterial transposon mutagenesis screen proposed above is that *L. pneumophila* effector proteins are highly redundant (O'Connor et al., 2011). Thus, a single transposon mutation may not block the ability of Δ7Δ*flaA L. pneumophila* to induce translation inhibition. As a complimentary approach to the bacterial transposon mutagenesis screen I propose that proteomic studies should be undertaken to measure the changes in the post-translational modification of host proteins following *L. pneumophila* infection. Much of the control of translation by host signaling pathways occurs by the phosphorylation of translation factors (Hershey et al., 2012). Whether a novel bacterial effector or a yet unidentified host stress response pathway causes the residual block in host protein synthesis by Δ7Δ*flaA L. pneumophila* infection, a proteomic approach looking at post-translational modifications of host proteins would elucidate possible signaling pathways or translation factor targets that could account for the block in translation initiation. The data presented in this thesis do not support a role for known stress response pathways in the Δ7Δ*flaA L. pneumophila* induced translation inhibition. Thus, a very exciting possibility is that the block in translation initiation seen in response to Δ7Δ*flaA L. pneumophila* infection could be due to a novel host stress response pathway. The proposed proteomic studies could identify this novel pathway and may be another example of a pathogen teaching us about eukaryotic cell biology.

### Section 4.1.2: Do other pathogens induce global changes to translation in a similar manner to *L. pneumophila*?

In chapter 3 of this thesis I present experiments that demonstrate that infection by the intracellular pathogen *Listeria monocytogenes* induces a block in host protein synthesis in BMDMs (Figure 3.14). I have also presented data from other groups that demonstrate that a number of pathogens can induce host stress responses and/or a block in host protein synthesis (Section 1.4). The ribosome profiling experiments presented in this thesis are the first such experiments undertaken in response to infection by an intracellular bacterial pathogen. In future studies it will be interesting to undertake similar global analyses on macrophages infected with *L. monocytogenes* and/or other intracellular bacterial pathogens to determine if the mechanisms of gene induction and translational regulation are similar among distinct intracellular pathogens.

## Section 4.2: Final perspectives

It is clear that the original PRR system for pathogen detection, while important, does not entirely explain how the innate immune system can distinguish pathogenic from non-pathogenic microbes. My graduate work has focused on elucidating the mechanism by which the innate immune system distinguishes pathogenic from non-pathogenic microbes and preferentially induces inflammatory responses to pathogens. The worked presented here suggests that host stress responses induced by intracellular bacterial infection as well as direct inhibition of host protein synthesis by secreted bacterial effector proteins may be an important and conserved pathogen-associated activity that provides a contextual cue to a cell that it is infected with a

pathogen. This distinction allows for a preferential inflammatory response to only pathogens. The work I have presented here suggests that sensing of pathogen-associated activities is a key step to inducing preferential immune responses to pathogens. The field of host-pathogen interactions has boomed since it was first hypothesized 26 years ago and we are beginning to find many novel and exciting mechanisms by which the host recognizes pathogens. I believe the field is now moving towards an understanding that intracellular pathogens not only activate immune signaling pathways, but that infection can induce important changes in metabolism and stress response pathways. It will be exciting to watch the novel discoveries being made as the field begins to move towards a merging of host metabolism, stress responses and immune signaling in explaining how host pathogen-interactions lead to protection of the host.

# References


Akerley, B.J., Cotter, P.A., and Miller, J.F. (1995). Ectopic expression of the flagellar regulon alters development of the Bordetella-host interaction. Cell *80*, 611-620.

Amer, A., Franchi, L., Kanneganti, T.D., Body-Malapel, M., Ozoren, N., Brady, G., Meshinchi, S., Jagirdar, R., Gewirtz, A., Akira, S.*, et al.* (2006). Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. J Biol Chem *281*, 35217-35223.

Archer, K.A., Ader, F., Kobayashi, K.S., Flavell, R.A., and Roy, C.R. (2010). Cooperation between multiple microbial pattern recognition systems is important for host protection against the intracellular pathogen Legionella pneumophila. Infect Immun *78*, 2477-2487.

Archer, K.A., Alexopoulou, L., Flavell, R.A., and Roy, C.R. (2009). Multiple MyD88-dependent responses contribute to pulmonary clearance of Legionella pneumophila. Cell Microbiol *11*, 21-36.

Archer, K.A., and Roy, C.R. (2006). MyD88-dependent responses involving toll-like receptor 2 are important for protection and clearance of Legionella pneumophila in a mouse model of Legionnaires' disease. Infect Immun *74*, 3325-3333.

Asrat, S., Dugan, A.S., and Isberg, R.R. (2014). The frustrated host response to Legionella pneumophila is bypassed by MyD88-dependent translation of pro-inflammatory cytokines. PLoS Pathog *10*, e1004229.

Baird, T.D., and Wek, R.C. (2012). Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv Nutr *3*, 307-321.

Bar-Peled, L., Schweitzer, L.D., Zoncu, R., and Sabatini, D.M. (2012). Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell *150*, 1196-1208.

Barbalat, R., Ewald, S.E., Mouchess, M.L., and Barton, G.M. (2011). Nucleic acid recognition by the innate immune system. Annu Rev Immunol *29*, 185-214.

Barry, K.C., Fontana, M.F., Portman, J.L., Dugan, A.S., and Vance, R.E. (2013). IL-1alpha signaling initiates the inflammatory response to virulent Legionella pneumophila in vivo. J Immunol *190*, 6329-6339.

Baruch, M., Belotserkovsky, I., Hertzog, B.B., Ravins, M., Dov, E., McIver, K.S., Le Breton, Y.S., Zhou, Y., Cheng, C.Y., and Hanski, E. (2014a). An extracellular bacterial pathogen modulates host metabolism to regulate its own sensing and proliferation. Cell *156*, 97-108.

Baruch, M., Hertzog, B.B., Ravins, M., Anand, A., Cheng, C.Y., Biswas, D., Tirosh, B., and Hanski, E. (2014b). Induction of endoplasmic reticulum stress and unfolded protein response constitutes a pathogenic strategy of group A streptococcus. Frontiers in cellular and infection microbiology *4*, 105.

Baruch, M., Hertzog, B.B., Ravins, M., Youting, C.C., and Hanski, E. (2014c). Group A streptococcus and host metabolism: virulence influences and potential treatments. Future microbiology *9*, 713-716.

Bellocchio, S., Montagnoli, C., Bozza, S., Gaziano, R., Rossi, G., Mambula, S.S., Vecchi, A., Mantovani, A., Levitz, S.M., and Romani, L. (2004). The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. J Immunol *172*, 3059-3069.

Belyi, Y., Jank, T., and Aktories, K. (2013). Cytotoxic glucosyltransferases of Legionella pneumophila. Current topics in microbiology and immunology *376*, 211-226.
Belyi, Y., Niggeweg, R., Opitz, B., Vogelsgesang, M., Hippenstiel, S., Wilm, M., and Aktories, K. (2006). Legionella pneumophila glucosyltransferase inhibits host elongation factor 1A. Proceedings of the National Academy of Sciences of the United States of America *103*, 16953-16958.
Belyi, Y., Stahl, M., Sovkova, I., Kaden, P., Luy, B., and Aktories, K. (2009). Region of elongation factor 1A1 involved in substrate recognition by Legionella pneumophila glucosyltransferase Lgt1: identification of Lgt1 as a retaining glucosyltransferase. J Biol Chem *284*, 20167-20174.
Belyi, Y., Tabakova, I., Stahl, M., and Aktories, K. (2008). Lgt: a family of cytotoxic glucosyltransferases produced by Legionella pneumophila. Journal of bacteriology *190*, 3026-3035.
Benjamin, D.I., Cravatt, B.F., and Nomura, D.K. (2012). Global profiling strategies for mapping dysregulated metabolic pathways in cancer. Cell metabolism *16*, 565-577.
Benko, S., Philpott, D.J., and Girardin, S.E. (2008). The microbial and danger signals that activate Nod-like receptors. Cytokine *43*, 368-373.
Berger, K.H., and Isberg, R.R. (1993). Two distinct defects in intracellular growth complemented by a single genetic locus in Legionella pneumophila. Molecular microbiology *7*, 7-19.
Berger, K.H., Merriam, J.J., and Isberg, R.R. (1994). Altered intracellular targeting properties associated with mutations in the Legionella pneumophila dotA gene. Molecular microbiology *14*, 809-822.
Black, D.S., and Bliska, J.B. (2000). The RhoGAP activity of the Yersinia pseudotuberculosis cytotoxin YopE is required for antiphagocytic function and virulence. Molecular microbiology *37*, 515-527.
Breker, M., and Schuldiner, M. (2014). The emergence of proteome-wide technologies: systematic analysis of proteins comes of age. Nature reviews Molecular cell biology *15*, 453-464.
Broz, P., Ruby, T., Belhocine, K., Bouley, D.M., Kayagaki, N., Dixit, V.M., and Monack, D.M. (2012). Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. Nature *490*, 288-291.
Brubaker, S.W., Bonham, K.S., Zanoni, I., and Kagan, J.C. (2015). Innate Immune Pattern Recognition: A Cell Biological Perspective. Annu Rev Immunol.
Buermans, H.P., and den Dunnen, J.T. (2014). Next generation sequencing technology: Advances and applications. Biochimica et biophysica acta *1842*, 1932-1941.
Cai, S., Batra, S., Wakamatsu, N., Pacher, P., and Jeyaseelan, S. (2012). NLRC4 inflammasome-mediated production of IL-1beta modulates mucosal immunity in the lung against gram-negative bacterial infection. J Immunol *188*, 5623-5635.
Carayol, N., and Tran Van Nhieu, G. (2013a). The inside story of Shigella invasion of intestinal epithelial cells. Cold Spring Harbor perspectives in medicine *3*, a016717.
Carayol, N., and Tran Van Nhieu, G. (2013b). Tips and tricks about Shigella invasion of epithelial cells. Current opinion in microbiology *16*, 32-37.
Cecilio, P., Perez-Cabezas, B., Santarem, N., Maciel, J., Rodrigues, V., and Cordeiro da Silva, A. (2014). Deception and manipulation: the arms of leishmania, a successful parasite. Frontiers in immunology *5*, 480.

Cerretti, D.P., Kozlosky, C.J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T.A., March, C.J., Kronheim, S.R., Druck, T., Cannizzaro, L.A*., et al.* (1992). Molecular cloning of the interleukin-1 beta converting enzyme. Science *256*, 97-100.

Chakrabarti, S., Liehl, P., Buchon, N., and Lemaitre, B. (2012). Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the Drosophila gut. Cell Host Microbe *12*, 60-70.

Chang, J.H., Desveaux, D., and Creason, A.L. (2014). The ABCs and 123s of bacterial secretion systems in plant pathogenesis. Annual review of phytopathology *52*, 317-345.

Chavarria-Smith, J., and Vance, R.E. (2013). Direct proteolytic cleavage of NLRP1B is necessary and sufficient for inflammasome activation by anthrax lethal factor. PLoS Pathog *9*, e1003452.

Chien, M., Morozova, I., Shi, S., Sheng, H., Chen, J., Gomez, S.M., Asamani, G., Hill, K., Nuara, J., Feder, M*., et al.* (2004). The genomic sequence of the accidental pathogen Legionella pneumophila. Science *305*, 1966-1968.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell *124*, 803-814.

Connor, J.H., and Lyles, D.S. (2005). Inhibition of host and viral translation during vesicular stomatitis virus infection. eIF2 is responsible for the inhibition of viral but not host translation. J Biol Chem *280*, 13512-13519.

Coope, A., Milanski, M., Arruda, A.P., Ignacio-Souza, L.M., Saad, M.J., Anhe, G.F., and Velloso, L.A. (2012). Chaperone insufficiency links TLR4 protein signaling to endoplasmic reticulum stress. J Biol Chem *287*, 15580-15589.

Copenhaver, A.M., Casson, C.N., Nguyen, H.T., Fung, T.C., Duda, M.M., Roy, C.R., and Shin, S. (2014). Alveolar macrophages and neutrophils are the primary reservoirs for Legionella pneumophila and mediate cytosolic surveillance of type IV secretion. Infect Immun *82*, 4325-4336.

de Sousa Abreu, R., Penalva, L.O., Marcotte, E.M., and Vogel, C. (2009). Global signatures of protein and mRNA expression levels. Molecular bioSystems *5*, 1512-1526.

Decker, T., and Lohmann-Matthes, M.L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. Journal of immunological methods *115*, 61-69.

Deng, Q., and Barbieri, J.T. (2008). Molecular mechanisms of the cytotoxicity of ADP-ribosylating toxins. Annual review of microbiology *62*, 271-288.

Deslandes, L., and Rivas, S. (2012). Catch me if you can: bacterial effectors and plant targets. Trends in plant science *17*, 644-655.

Desvaux, M., Hebraud, M., Talon, R., and Henderson, I.R. (2009). Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. Trends in microbiology *17*, 139-145.

Dever, T.E., and Green, R. (2012). The elongation, termination, and recycling phases of translation in eukaryotes. Cold Spring Harbor perspectives in biology *4*, a013706.

Dewamitta, S.R., Nomura, T., Kawamura, I., Hara, H., Tsuchiya, K., Kurenuma, T., Shen, Y., Daim, S., Yamamoto, T., Qu, H*., et al.* (2010). Listeriolysin O-dependent bacterial entry into the cytoplasm is required for calpain activation and interleukin-1 alpha secretion in macrophages infected with Listeria monocytogenes. Infect Immun *78*, 1884-1894.

Diefenbach, A., Schindler, H., Donhauser, N., Lorenz, E., Laskay, T., MacMicking, J., Rollinghoff, M., Gresser, I., and Bogdan, C. (1998). Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity *8*, 77-87.

Dinarello, C.A. (2009). Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol *27*, 519-550.

Donnelly, N., Gorman, A.M., Gupta, S., and Samali, A. (2013). The eIF2alpha kinases: their structures and functions. Cellular and molecular life sciences : CMLS *70*, 3493-3511.

Dorrello, N.V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E., and Pagano, M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science *314*, 467-471.

Dunbar, T.L., Yan, Z., Balla, K.M., Smelkinson, M.G., and Troemel, E.R. (2012). C. elegans detects pathogen-induced translational inhibition to activate immune signaling. Cell Host Microbe *11*, 375-386.

Efeyan, A., Zoncu, R., and Sabatini, D.M. (2012). Amino acids and mTORC1: from lysosomes to disease. Trends in molecular medicine *18*, 524-533.

Ewald, S.E., Chavarria-Smith, J., and Boothroyd, J.C. (2014). NLRP1 is an inflammasome sensor for Toxoplasma gondii. Infect Immun *82*, 460-468.

Ewald, S.E., Lee, B.L., Lau, L., Wickliffe, K.E., Shi, G.P., Chapman, H.A., and Barton, G.M. (2008). The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature *456*, 658-662.

Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D., and Shokat, K.M. (2009). Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS biology *7*, e38.

Ferrari, S., Bandi, H.R., Hofsteenge, J., Bussian, B.M., and Thomas, G. (1991). Mitogen-activated 70K S6 kinase. Identification of in vitro 40 S ribosomal S6 phosphorylation sites. J Biol Chem *266*, 22770-22775.

Fettelschoss, A., Kistowska, M., LeibundGut-Landmann, S., Beer, H.D., Johansen, P., Senti, G., Contassot, E., Bachmann, M.F., French, L.E., Oxenius, A.*, et al.* (2011). Inflammasome activation and IL-1beta target IL-1alpha for secretion as opposed to surface expression. Proceedings of the National Academy of Sciences of the United States of America *108*, 18055-18060.

Fields, B.S. (1996). The molecular ecology of legionellae. Trends in microbiology *4*, 286-290.

Fonseca, M.V., and Swanson, M.S. (2014). Nutrient salvaging and metabolism by the intracellular pathogen Legionella pneumophila. Frontiers in cellular and infection microbiology *4*, 12.

Fontana, M.F., Banga, S., Barry, K.C., Shen, X., Tan, Y., Luo, Z.Q., and Vance, R.E. (2011). Secreted bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent Legionella pneumophila. PLoS Pathog *7*, e1001289.

Fontana, M.F., Shin, S., and Vance, R.E. (2012). Activation of host mitogen-activated protein kinases by secreted Legionella pneumophila effectors that inhibit host protein translation. Infect Immun *80*, 3570-3575.

Fontana, M.F., and Vance, R.E. (2011). Two signal models in innate immunity. Immunol Rev *243*, 26-39.

Fresno, M., Jimenez, A., and Vazquez, D. (1977). Inhibition of translation in eukaryotic systems by harringtonine. European journal of biochemistry / FEBS *72*, 323-330.
Fuse, E.T., Tateda, K., Kikuchi, Y., Matsumoto, T., Gondaira, F., Azuma, A., Kudoh, S., Standiford, T.J., and Yamaguchi, K. (2007). Role of Toll-like receptor 2 in recognition of Legionella pneumophila in a murine pneumonia model. J Med Microbiol *56*, 305-312.
Gale, M., Jr., Tan, S.L., and Katze, M.G. (2000). Translational control of viral gene expression in eukaryotes. Microbiology and molecular biology reviews : MMBR *64*, 239-280.
Gomez, M.A., Contreras, I., Halle, M., Tremblay, M.L., McMaster, R.W., and Olivier, M. (2009). Leishmania GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. Science signaling *2*, ra58.
Gouin, E., Welch, M.D., and Cossart, P. (2005). Actin-based motility of intracellular pathogens. Current opinion in microbiology *8*, 35-45.
Gross, O., Yazdi, A.S., Thomas, C.J., Masin, M., Heinz, L.X., Guarda, G., Quadroni, M., Drexler, S.K., and Tschopp, J. (2012). Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. Immunity *36*, 388-400.
Guarner, F. (2014). Decade in review-gut microbiota: the gut microbiota era marches on. Nature reviews Gastroenterology & hepatology *11*, 647-649.
Guler, R., Parihar, S.P., Spohn, G., Johansen, P., Brombacher, F., and Bachmann, M.F. (2011). Blocking IL-1alpha but not IL-1beta increases susceptibility to chronic Mycobacterium tuberculosis infection in mice. Vaccine *29*, 1339-1346.
Guma, M., Ronacher, L., Liu-Bryan, R., Takai, S., Karin, M., and Corr, M. (2009). Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. Arthritis and rheumatism *60*, 3642-3650.
Halle, M., Gomez, M.A., Stuible, M., Shimizu, H., McMaster, W.R., Olivier, M., and Tremblay, M.L. (2009). The Leishmania surface protease GP63 cleaves multiple intracellular proteins and actively participates in p38 mitogen-activated protein kinase inactivation. J Biol Chem *284*, 6893-6908.
Hardie, D.G. (2011). AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes & development *25*, 1895-1908.
Havell, E.A., Moldawer, L.L., Helfgott, D., Kilian, P.L., and Sehgal, P.B. (1992). Type I IL-1 receptor blockade exacerbates murine listeriosis. J Immunol *148*, 1486-1492.
Hawn, T.R., Smith, K.D., Aderem, A., and Skerrett, S.J. (2006). Myeloid differentiation primary response gene (88)- and toll-like receptor 2-deficient mice are susceptible to infection with aerosolized Legionella pneumophila. J Infect Dis *193*, 1693-1702.
He, P., Shan, L., and Sheen, J. (2007). Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. Cell Microbiol *9*, 1385-1396.
Herdy, B., Jaramillo, M., Svitkin, Y.V., Rosenfeld, A.B., Kobayashi, M., Walsh, D., Alain, T., Sean, P., Robichaud, N., Topisirovic, I.*, et al.* (2012). Translational control of the activation of transcription factor NF-kappaB and production of type I interferon by phosphorylation of the translation factor eIF4E. Nat Immunol *13*, 543-550.
Hershey, J.W., Sonenberg, N., and Mathews, M.B. (2012). Principles of translational control: an overview. Cold Spring Harbor perspectives in biology *4*.

Hinnebusch, A.G., and Lorsch, J.R. (2012). The mechanism of eukaryotic translation initiation: new insights and challenges. Cold Spring Harbor perspectives in biology *4*.

Holz, M.K., Ballif, B.A., Gygi, S.P., and Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell *123*, 569-580.

Horai, R., Asano, M., Sudo, K., Kanuka, H., Suzuki, M., Nishihara, M., Takahashi, M., and Iwakura, Y. (1998). Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. The Journal of experimental medicine *187*, 1463-1475.

Huang, M.T. (1975). Harringtonine, an inhibitor of initiation of protein biosynthesis. Molecular pharmacology *11*, 511-519.

Hubber, A., and Roy, C.R. (2010). Modulation of host cell function by Legionella pneumophila type IV effectors. Annu Rev Cell Dev Biol *26*, 261-283.

Hurtado-Guerrero, R., Zusman, T., Pathak, S., Ibrahim, A.F., Shepherd, S., Prescott, A., Segal, G., and van Aalten, D.M. (2010). Molecular mechanism of elongation factor 1A inhibition by a Legionella pneumophila glycosyltransferase. The Biochemical journal *426*, 281-292.

Ingolia, N.T., Brar, G.A., Rouskin, S., McGeachy, A.M., and Weissman, J.S. (2012). The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. Nature protocols *7*, 1534-1550.

Ingolia, N.T., Ghaemmaghami, S., Newman, J.R., and Weissman, J.S. (2009). Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science *324*, 218-223.

Ingolia, N.T., Lareau, L.F., and Weissman, J.S. (2011). Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell *147*, 789-802.

Ivanov, S.S., and Roy, C.R. (2013). Pathogen signatures activate a ubiquitination pathway that modulates the function of the metabolic checkpoint kinase mTOR. Nat Immunol *14*, 1219-1228.

Jackson, R.J., Hellen, C.U., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nature reviews Molecular cell biology *11*, 113-127.

Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology *54 Pt 1*, 1-13.

Janssens, S., Pulendran, B., and Lambrecht, B.N. (2014). Emerging functions of the unfolded protein response in immunity. Nat Immunol *15*, 910-919.

Jaramillo, M., Gomez, M.A., Larsson, O., Shio, M.T., Topisirovic, I., Contreras, I., Luxenburg, R., Rosenfeld, A., Colina, R., McMaster, R.W.*, et al.* (2011). Leishmania repression of host translation through mTOR cleavage is required for parasite survival and infection. Cell Host Microbe *9*, 331-341.

Jones, C.L., Napier, B.A., Sampson, T.R., Llewellyn, A.C., Schroeder, M.R., and Weiss, D.S. (2012). Subversion of host recognition and defense systems by Francisella spp. Microbiology and molecular biology reviews : MMBR *76*, 383-404.

Jovanovic, M., Rooney, M.S., Mertins, P., Przybylski, D., Chevrier, N., Satija, R., Rodriguez, E.H., Fields, A.P., Schwartz, S., Raychowdhury, R.*, et al.* (2015). Immunogenetics.

Dynamic profiling of the protein life cycle in response to pathogens. Science *347*, 1259038.
Juffermans, N.P., Florquin, S., Camoglio, L., Verbon, A., Kolk, A.H., Speelman, P., van Deventer, S.J., and van Der Poll, T. (2000). Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. J Infect Dis *182*, 902-908.
Kafka, D., Ling, E., Feldman, G., Benharroch, D., Voronov, E., Givon-Lavi, N., Iwakura, Y., Dagan, R., Apte, R.N., and Mizrachi-Nebenzahl, Y. (2008). Contribution of IL-1 to resistance to Streptococcus pneumoniae infection. International immunology *20*, 1139-1146.
Karmakar, M., Sun, Y., Hise, A.G., Rietsch, A., and Pearlman, E. (2012). Cutting Edge: IL-1beta Processing during Pseudomonas aeruginosa Infection Is Mediated by Neutrophil Serine Proteases and Is Independent of NLRC4 and Caspase-1. J Immunol *189*, 4231-4235.
Karp, C.L. (2012). Unstressing intemperate models: how cold stress undermines mouse modeling. The Journal of experimental medicine *209*, 1069-1074.
Kaufman, R.J. (1999). Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes & development *13*, 1211-1233.
Kawasaki, T., and Kawai, T. (2014). Toll-like receptor signaling pathways. Frontiers in immunology *5*, 461.
Keller, M., Ruegg, A., Werner, S., and Beer, H.D. (2008). Active caspase-1 is a regulator of unconventional protein secretion. Cell *132*, 818-831.
Kim, S.G., Buel, G.R., and Blenis, J. (2013). Nutrient regulation of the mTOR complex 1 signaling pathway. Molecules and cells *35*, 463-473.
Kloft, N., Neukirch, C., Bobkiewicz, W., Veerachato, G., Busch, T., von Hoven, G., Boller, K., and Husmann, M. (2010). Pro-autophagic signal induction by bacterial pore-forming toxins. Medical microbiology and immunology *199*, 299-309.
Kofoed, E.M., and Vance, R.E. (2011). Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. Nature *477*, 592-595.
Kono, H., and Rock, K.L. (2008). How dying cells alert the immune system to danger. Nature reviews Immunology *8*, 279-289.
Kostic, A.D., Xavier, R.J., and Gevers, D. (2014). The microbiome in inflammatory bowel disease: current status and the future ahead. Gastroenterology *146*, 1489-1499.
Krishnamoorthy, T., Pavitt, G.D., Zhang, F., Dever, T.E., and Hinnebusch, A.G. (2001). Tight binding of the phosphorylated alpha subunit of initiation factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. Molecular and cellular biology *21*, 5018-5030.
Labow, M., Shuster, D., Zetterstrom, M., Nunes, P., Terry, R., Cullinan, E.B., Bartfai, T., Solorzano, C., Moldawer, L.L., Chizzonite, R.*, et al.* (1997). Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. J Immunol *159*, 2452-2461.
Laskay, T., Diefenbach, A., Rollinghoff, M., and Solbach, W. (1995). Early parasite containment is decisive for resistance to Leishmania major infection. European journal of immunology *25*, 2220-2227.

LeibundGut-Landmann, S., Weidner, K., Hilbi, H., and Oxenius, A. (2011). Nonhematopoietic cells are key players in innate control of bacterial airway infection. J Immunol *186*, 3130-3137.
Lemaitre, B., and Girardin, S.E. (2013). Translation inhibition and metabolic stress pathways in the host response to bacterial pathogens. Nat Rev Microbiol *11*, 365-369.
Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973-983.
Leprivier, G., Remke, M., Rotblat, B., Dubuc, A., Mateo, A.R., Kool, M., Agnihotri, S., El-Naggar, A., Yu, B., Somasekharan, S.P.*, et al.* (2013). The eEF2 kinase confers resistance to nutrient deprivation by blocking translation elongation. Cell *153*, 1064-1079.
Li, J.J., Bickel, P.J., and Biggin, M.D. (2014). System wide analyses have underestimated protein abundances and the importance of transcription in mammals. PeerJ *2*, e270.
Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J.*, et al.* (1995). Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell *80*, 401-411.
Liehl, P., Blight, M., Vodovar, N., Boccard, F., and Lemaitre, B. (2006). Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog *2*, e56.
Lightfield, K.L., Persson, J., Brubaker, S.W., Witte, C.E., von Moltke, J., Dunipace, E.A., Henry, T., Sun, Y.H., Cado, D., Dietrich, W.F.*, et al.* (2008). Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. Nat Immunol *9*, 1171-1178.
Losick, V.P., Haenssler, E., Moy, M.Y., and Isberg, R.R. (2010). LnaB: a Legionella pneumophila activator of NF-kappaB. Cell Microbiol *12*, 1083-1097.
Lu, W., Du, J., Stahl, M., Tzivelekidis, T., Belyi, Y., Gerhardt, S., Aktories, K., and Einsle, O. (2010). Structural basis of the action of glucosyltransferase Lgt1 from Legionella pneumophila. Journal of molecular biology *396*, 321-331.
Luo, Z.Q. (2012). Legionella secreted effectors and innate immune responses. Cell Microbiol *14*, 19-27.
Ma, X.M., and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. Nature reviews Molecular cell biology *10*, 307-318.
Mackey, D., and McFall, A.J. (2006). MAMPs and MIMPs: proposed classifications for inducers of innate immunity. Molecular microbiology *61*, 1365-1371.
Maier, T., Guell, M., and Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. FEBS letters *583*, 3966-3973.
Marra, A., Blander, S.J., Horwitz, M.A., and Shuman, H.A. (1992). Identification of a Legionella pneumophila locus required for intracellular multiplication in human macrophages. Proceedings of the National Academy of Sciences of the United States of America *89*, 9607-9611.
Marriott, H.M., Gascoyne, K.A., Gowda, R., Geary, I., Nicklin, M.J., Iannelli, F., Pozzi, G., Mitchell, T.J., Whyte, M.K., Sabroe, I.*, et al.* (2012). Interleukin-1beta regulates CXCL8 release and influences disease outcome in response to Streptococcus

pneumoniae, defining intercellular cooperation between pulmonary epithelial cells and macrophages. Infect Immun *80*, 1140-1149.
Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell *10*, 417-426.
Martinon, F., Chen, X., Lee, A.H., and Glimcher, L.H. (2010). TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. Nat Immunol *11*, 411-418.
Martinon, F., Mayor, A., and Tschopp, J. (2009). The inflammasomes: guardians of the body. Annu Rev Immunol *27*, 229-265.
Mattner, J., Schindler, H., Diefenbach, A., Rollinghoff, M., Gresser, I., and Bogdan, C. (2000). Regulation of type 2 nitric oxide synthase by type 1 interferons in macrophages infected with Leishmania major. European journal of immunology *30*, 2257-2267.
Mattner, J., Wandersee-Steinhauser, A., Pahl, A., Rollinghoff, M., Majeau, G.R., Hochman, P.S., and Bogdan, C. (2004). Protection against progressive leishmaniasis by IFN-beta. J Immunol *172*, 7574-7582.
Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu Rev Immunol *12*, 991-1045.
Matzinger, P. (1998). An innate sense of danger. Seminars in immunology *10*, 399-415.
Matzinger, P. (2002). The danger model: a renewed sense of self. Science *296*, 301-305.
Mayer-Barber, K.D., Andrade, B.B., Barber, D.L., Hieny, S., Feng, C.G., Caspar, P., Oland, S., Gordon, S., and Sher, A. (2011). Innate and adaptive interferons suppress IL-1alpha and IL-1beta production by distinct pulmonary myeloid subsets during Mycobacterium tuberculosis infection. Immunity *35*, 1023-1034.
Mayer-Barber, K.D., Barber, D.L., Shenderov, K., White, S.D., Wilson, M.S., Cheever, A., Kugler, D., Hieny, S., Caspar, P., Nunez, G.*, et al.* (2010). Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo. J Immunol *184*, 3326-3330.
McEwan, D.L., Kirienko, N.V., and Ausubel, F.M. (2012). Host translational inhibition by Pseudomonas aeruginosa Exotoxin A Triggers an immune response in Caenorhabditis elegans. Cell Host Microbe *11*, 364-374.
Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.
Miao, E.A., Leaf, I.A., Treuting, P.M., Mao, D.P., Dors, M., Sarkar, A., Warren, S.E., Wewers, M.D., and Aderem, A. (2010). Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. Nat Immunol *11*, 1136-1142.
Michalkiewicz, J., Stachowski, J., Barth, C., Patzer, J., Dzierzanowska, D., and Madalinski, K. (1999). Effect of Pseudomonas aeruginosa exotoxin A on IFN-gamma synthesis: expression of costimulatory molecules on monocytes and activity of NK cells. Immunology letters *69*, 359-366.
Mijares, L.A., Wangdi, T., Sokol, C., Homer, R., Medzhitov, R., and Kazmierczak, B.I. (2011). Airway epithelial MyD88 restores control of Pseudomonas aeruginosa murine infection via an IL-1-dependent pathway. J Immunol *186*, 7080-7088.
Miller, L.S., and Cho, J.S. (2011). Immunity against Staphylococcus aureus cutaneous infections. Nature reviews Immunology *11*, 505-518.

Mohr, I., and Sonenberg, N. (2012). Host translation at the nexus of infection and immunity. Cell Host Microbe *12*, 470-483.

Molofsky, A.B., Byrne, B.G., Whitfield, N.N., Madigan, C.A., Fuse, E.T., Tateda, K., and Swanson, M.S. (2006). Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection. The Journal of experimental medicine *203*, 1093-1104.

Monroe, K.M., McWhirter, S.M., and Vance, R.E. (2009). Identification of host cytosolic sensors and bacterial factors regulating the type I interferon response to Legionella pneumophila. PLoS Pathog *5*, e1000665.

Montminy, S.W., Khan, N., McGrath, S., Walkowicz, M.J., Sharp, F., Conlon, J.E., Fukase, K., Kusumoto, S., Sweet, C., Miyake, K.*, et al.* (2006). Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response. Nat Immunol *7*, 1066-1073.

Mosley, B., Urdal, D.L., Prickett, K.S., Larsen, A., Cosman, D., Conlon, P.J., Gillis, S., and Dower, S.K. (1987). The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. J Biol Chem *262*, 2941-2944.

Mouchess, M.L., Arpaia, N., Souza, G., Barbalat, R., Ewald, S.E., Lau, L., and Barton, G.M. (2011). Transmembrane mutations in Toll-like receptor 9 bypass the requirement for ectodomain proteolysis and induce fatal inflammation. Immunity *35*, 721-732.

Muthamilarasan, M., and Prasad, M. (2013). Plant innate immunity: an updated insight into defense mechanism. Journal of biosciences *38*, 433-449.

Novoa, I., Zeng, H., Harding, H.P., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. The Journal of cell biology *153*, 1011-1022.

O'Connor, T.J., Adepoju, Y., Boyd, D., and Isberg, R.R. (2011). Minimization of the Legionella pneumophila genome reveals chromosomal regions involved in host range expansion. Proceedings of the National Academy of Sciences of the United States of America *108*, 14733-14740.

Olaru, F., and Jensen, L.E. (2010). Staphylococcus aureus stimulates neutrophil targeting chemokine expression in keratinocytes through an autocrine IL-1alpha signaling loop. The Journal of investigative dermatology *130*, 1866-1876.

Patel, J.C., and Galan, J.E. (2005). Manipulation of the host actin cytoskeleton by Salmonella--all in the name of entry. Current opinion in microbiology *8*, 10-15.

Pavitt, G.D., Ramaiah, K.V., Kimball, S.R., and Hinnebusch, A.G. (1998). eIF2 independently binds two distinct eIF2B subcomplexes that catalyze and regulate guanine-nucleotide exchange. Genes & development *12*, 514-526.

Pestova, T.V., and Hellen, C.U. (2003). Translation elongation after assembly of ribosomes on the Cricket paralysis virus internal ribosomal entry site without initiation factors or initiator tRNA. Genes & development *17*, 181-186.

Pillich, H., Loose, M., Zimmer, K.P., and Chakraborty, T. (2012). Activation of the unfolded protein response by Listeria monocytogenes. Cell Microbiol *14*, 949-964.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C.*, et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085-2088.

Portnoy, D.A., Auerbuch, V., and Glomski, I.J. (2002). The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity. The Journal of cell biology *158*, 409-414.

Price, C.T., Al-Quadan, T., Santic, M., Rosenshine, I., and Abu Kwaik, Y. (2011). Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. Science *334*, 1553-1557.

Rajamuthiah, R., and Mylonakis, E. (2014). Effector triggered immunity. Virulence *5*, 697-702.

Ramsden, A.E., Holden, D.W., and Mota, L.J. (2007a). Membrane dynamics and spatial distribution of Salmonella-containing vacuoles. Trends in microbiology *15*, 516-524.

Ramsden, A.E., Mota, L.J., Munter, S., Shorte, S.L., and Holden, D.W. (2007b). The SPI-2 type III secretion system restricts motility of Salmonella-containing vacuoles. Cell Microbiol *9*, 2517-2529.

Ren, T., Zamboni, D.S., Roy, C.R., Dietrich, W.F., and Vance, R.E. (2006). Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog *2*, e18.

Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nature biotechnology *29*, 24-26.

Rogers, H.W., Sheehan, K.C., Brunt, L.M., Dower, S.K., Unanue, E.R., and Schreiber, R.D. (1992). Interleukin 1 participates in the development of anti-Listeria responses in normal and SCID mice. Proceedings of the National Academy of Sciences of the United States of America *89*, 1011-1015.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P., and Meyuhas, O. (2005). Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes & development *19*, 2199-2211.

Salomon, D., and Orth, K. (2013). What pathogens have taught us about posttranslational modifications. Cell Host Microbe *14*, 269-279.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science *320*, 1496-1501.

Scaffidi, P., Misteli, T., and Bianchi, M.E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature *418*, 191-195.

Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R.J. (2001). Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol Cell *7*, 1165-1176.

Schmutz, C., Ahrne, E., Kasper, C.A., Tschon, T., Sorg, I., Dreier, R.F., Schmidt, A., and Arrieumerlou, C. (2013). Systems-level overview of host protein phosphorylation during Shigella flexneri infection revealed by phosphoproteomics. Molecular & cellular proteomics : MCP *12*, 2952-2968.

Schunder, E., Gillmaier, N., Kutzner, E., Eisenreich, W., Herrmann, V., Lautner, M., and Heuner, K. (2014). Amino Acid Uptake and Metabolism of Legionella pneumophila Hosted by Acanthamoeba castellanii. J Biol Chem *289*, 21040-21054.

Schwanhausser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., and Selbach, M. (2011). Global quantification of mammalian gene expression control. Nature *473*, 337-342.

Shen, A., and Higgins, D.E. (2006). The MogR transcriptional repressor regulates nonhierarchal expression of flagellar motility genes and virulence in Listeria monocytogenes. PLoS Pathog *2*, e30.
Shen, X., Banga, S., Liu, Y., Xu, L., Gao, P., Shamovsky, I., Nudler, E., and Luo, Z.Q. (2009). Targeting eEF1A by a Legionella pneumophila effector leads to inhibition of protein synthesis and induction of host stress response. Cell Microbiol *11*, 911-926.
Shi, Y., Evans, J.E., and Rock, K.L. (2003). Molecular identification of a danger signal that alerts the immune system to dying cells. Nature *425*, 516-521.
Shin, S., Case, C.L., Archer, K.A., Nogueira, C.V., Kobayashi, K.S., Flavell, R.A., Roy, C.R., and Zamboni, D.S. (2008). Type IV secretion-dependent activation of host MAP kinases induces an increased proinflammatory cytokine response to Legionella pneumophila. PLoS Pathog *4*, e1000220.
Showkat, M., Beigh, M.A., and Andrabi, K.I. (2014). mTOR Signaling in Protein Translation Regulation: Implications in Cancer Genesis and Therapeutic Interventions. Molecular biology international *2014*, 686984.
Shrestha, N., Bahnan, W., Wiley, D.J., Barber, G., Fields, K.A., and Schesser, K. (2012). Eukaryotic initiation factor 2 (eIF2) signaling regulates proinflammatory cytokine expression and bacterial invasion. J Biol Chem *287*, 28738-28744.
Simon, N.C., Aktories, K., and Barbieri, J.T. (2014). Novel bacterial ADP-ribosylating toxins: structure and function. Nat Rev Microbiol *12*, 599-611.
Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell *136*, 731-745.
Sporri, R., Joller, N., Hilbi, H., and Oxenius, A. (2008). A novel role for neutrophils as critical activators of NK cells. J Immunol *181*, 7121-7130.
Steele-Mortimer, O. (2008). The Salmonella-containing vacuole: moving with the times. Current opinion in microbiology *11*, 38-45.
Stenger, S., Thuring, H., Rollinghoff, M., and Bogdan, C. (1994). Tissue expression of inducible nitric oxide synthase is closely associated with resistance to Leishmania major. The Journal of experimental medicine *180*, 783-793.
Stuart, L.M., Paquette, N., and Boyer, L. (2013). Effector-triggered versus pattern-triggered immunity: how animals sense pathogens. Nature reviews Immunology *13*, 199-206.
Sudhakar, A., Ramachandran, A., Ghosh, S., Hasnain, S.E., Kaufman, R.J., and Ramaiah, K.V. (2000). Phosphorylation of serine 51 in initiation factor 2 alpha (eIF2 alpha) promotes complex formation between eIF2 alpha(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B. Biochemistry *39*, 12929-12938.
Swanson, M.S., and Hammer, B.K. (2000). Legionella pneumophila pathogesesis: a fateful journey from amoebae to macrophages. Annual review of microbiology *54*, 567-613.
Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. Annu Rev Immunol *21*, 335-376.
Tateda, K., Moore, T.A., Deng, J.C., Newstead, M.W., Zeng, X., Matsukawa, A., Swanson, M.S., Yamaguchi, K., and Standiford, T.J. (2001a). Early recruitment of neutrophils determines subsequent T1/T2 host responses in a murine model of Legionella pneumophila pneumonia. J Immunol *166*, 3355-3361.
Tateda, K., Moore, T.A., Newstead, M.W., Tsai, W.C., Zeng, X., Deng, J.C., Chen, G., Reddy, R., Yamaguchi, K., and Standiford, T.J. (2001b). Chemokine-dependent neutrophil

recruitment in a murine model of Legionella pneumonia: potential role of neutrophils as immunoregulatory cells. Infect Immun *69*, 2017-2024.
Tattoli, I., Philpott, D.J., and Girardin, S.E. (2012a). The bacterial and cellular determinants controlling the recruitment of mTOR to the Salmonella-containing vacuole. Biology open *1*, 1215-1225.
Tattoli, I., Sorbara, M.T., Vuckovic, D., Ling, A., Soares, F., Carneiro, L.A., Yang, C., Emili, A., Philpott, D.J., and Girardin, S.E. (2012b). Amino acid starvation induced by invasive bacterial pathogens triggers an innate host defense program. Cell Host Microbe *11*, 563-575.
Tattoli, I., Sorbara, M.T., Yang, C., Tooze, S.A., Philpott, D.J., and Girardin, S.E. (2013). Listeria phospholipases subvert host autophagic defenses by stalling pre-autophagosomal structures. The EMBO journal *32*, 3066-3078.
Tenthorey, J.L., Kofoed, E.M., Daugherty, M.D., Malik, H.S., and Vance, R.E. (2014). Molecular basis for specific recognition of bacterial ligands by NAIP/NLRC4 inflammasomes. Mol Cell *54*, 17-29.
Thoreen, C.C. (2013). Many roads from mTOR to eIF4F. Biochemical Society transactions *41*, 913-916.
Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., and Gray, N.S. (2009). An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J Biol Chem *284*, 8023-8032.
Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J.*, et al.* (1992). A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature *356*, 768-774.
Thorvaldsdottir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Briefings in bioinformatics *14*, 178-192.
Toribio, R., and Ventoso, I. (2010). Inhibition of host translation by virus infection in vivo. Proceedings of the National Academy of Sciences of the United States of America *107*, 9837-9842.
Trisolini, R., Lazzari Agli, L., Cancellieri, A., Procaccio, L., Candoli, P., Alifano, M., and Patelli, M. (2004). Bronchoalveolar lavage findings in severe community-acquired pneumonia due to Legionella pneumophila serogroup 1. Respir Med *98*, 1222-1226.
Tscherne, J.S., and Pestka, S. (1975). Inhibition of protein synthesis in intact HeLa cells. Antimicrobial agents and chemotherapy *8*, 479-487.
Tseng, T.T., Tyler, B.M., and Setubal, J.C. (2009). Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC microbiology *9 Suppl 1*, S2.
Tzivelekidis, T., Jank, T., Pohl, C., Schlosser, A., Rospert, S., Knudsen, C.R., Rodnina, M.V., Belyi, Y., and Aktories, K. (2011). Aminoacyl-tRNA-charged eukaryotic elongation factor 1A is the bona fide substrate for Legionella pneumophila effector glucosyltransferases. PLoS One *6*, e29525.
van Dijk, E.L., Auger, H., Jaszczyszyn, Y., and Thermes, C. (2014). Ten years of next-generation sequencing technology. Trends in genetics : TIG *30*, 418-426.

Vance, R.E., Isberg, R.R., and Portnoy, D.A. (2009). Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. Cell Host Microbe *6*, 10-21.
Vodovar, N., Vinals, M., Liehl, P., Basset, A., Degrouard, J., Spellman, P., Boccard, F., and Lemaitre, B. (2005). Drosophila host defense after oral infection by an entomopathogenic Pseudomonas species. Proceedings of the National Academy of Sciences of the United States of America *102*, 11414-11419.
Vogel, C., and Marcotte, E.M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature reviews Genetics *13*, 227-232.
von Moltke, J., Ayres, J.S., Kofoed, E.M., Chavarria-Smith, J., and Vance, R.E. (2012a). Recognition of Bacteria by Inflammasomes. Annu Rev Immunol.
von Moltke, J., Trinidad, N.J., Moayeri, M., Kintzer, A.F., Wang, S.B., van Rooijen, N., Brown, C.R., Krantz, B.A., Leppla, S.H., Gronert, K.*, et al.* (2012b). Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. Nature *490*, 107-111.
Walsh, D., Mathews, M.B., and Mohr, I. (2013). Tinkering with translation: protein synthesis in virus-infected cells. Cold Spring Harbor perspectives in biology *5*, a012351.
Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., and Proud, C.G. (2001). Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. The EMBO journal *20*, 4370-4379.
Wang, Y., Weiss, L.M., and Orlofsky, A. (2009). Intracellular parasitism with Toxoplasma gondii stimulates mammalian-target-of-rapamycin-dependent host cell growth despite impaired signalling to S6K1 and 4E-BP1. Cell Microbiol *11*, 983-1000.
Wek, R.C., Jiang, H.Y., and Anthony, T.G. (2006). Coping with stress: eIF2 kinases and translational control. Biochemical Society transactions *34*, 7-11.
Welch, M.D., and Way, M. (2013). Arp2/3-mediated actin-based motility: a tail of pathogen abuse. Cell Host Microbe *14*, 242-255.
Wieland, H., Ullrich, S., Lang, F., and Neumeister, B. (2005). Intracellular multiplication of Legionella pneumophila depends on host cell amino acid transporter SLC1A5. Molecular microbiology *55*, 1528-1537.
Winn, W.C., Jr., and Myerowitz, R.L. (1981). The pathology of the Legionella pneumonias. A review of 74 cases and the literature. Human pathology *12*, 401-422.
Wolfgang, M.C., Jyot, J., Goodman, A.L., Ramphal, R., and Lory, S. (2004). Pseudomonas aeruginosa regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients. Proceedings of the National Academy of Sciences of the United States of America *101*, 6664-6668.
Woo, C.W., Cui, D., Arellano, J., Dorweiler, B., Harding, H., Fitzgerald, K.A., Ron, D., and Tabas, I. (2009). Adaptive suppression of the ATF4-CHOP branch of the unfolded protein response by toll-like receptor signalling. Nature cell biology *11*, 1473-1480.
Woo, C.W., Kutzler, L., Kimball, S.R., and Tabas, I. (2012). Toll-like receptor activation suppresses ER stress factor CHOP and translation inhibition through activation of eIF2B. Nature cell biology *14*, 192-200.
Wu, W., and Zhao, S. (2013). Metabolic changes in cancer: beyond the Warburg effect. Acta biochimica et biophysica Sinica *45*, 18-26.
Yang, H.S., Jansen, A.P., Komar, A.A., Zheng, X., Merrick, W.C., Costes, S., Lockett, S.J., Sonenberg, N., and Colburn, N.H. (2003). The transformation suppressor Pdcd4 is a

novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. Molecular and cellular biology *23*, 26-37.
Yates, S.P., Jorgensen, R., Andersen, G.R., and Merrill, A.R. (2006). Stealth and mimicry by deadly bacterial toxins. Trends in biochemical sciences *31*, 123-133.
Yoon, S.S., Kim, E.K., and Lee, W.J. (2015). Functional genomic and metagenomic approaches to understanding gut microbiota-animal mutualism. Current opinion in microbiology *24*, 38-46.
Yu, H., Higa, F., Koide, M., Haranaga, S., Yara, S., Tateyama, M., Li, H., and Fujita, J. (2009). Lung abscess caused by Legionella species: implication of the immune status of hosts. Intern Med *48*, 1997-2002.
Zamboni, D.S., Kobayashi, K.S., Kohlsdorf, T., Ogura, Y., Long, E.M., Vance, R.E., Kuida, K., Mariathasan, S., Dixit, V.M., Flavell, R.A.*, et al.* (2006). The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of Legionella pneumophila infection. Nat Immunol *7*, 318-325.
Zhang, C., Bai, N., Chang, A., Zhang, Z., Yin, J., Shen, W., Tian, Y., Xiang, R., and Liu, C. (2013). ATF4 is directly recruited by TLR4 signaling and positively regulates TLR4-trigged cytokine production in human monocytes. Cellular & molecular immunology *10*, 84-94.
Zheng, Y., Humphry, M., Maguire, J.J., Bennett, M.R., and Clarke, M.C. (2013). Intracellular interleukin-1 receptor 2 binding prevents cleavage and activity of interleukin-1alpha, controlling necrosis-induced sterile inflammation. Immunity *38*, 285-295.
Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., and Sabatini, D.M. (2011a). mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science *334*, 678-683.
Zoncu, R., Efeyan, A., and Sabatini, D.M. (2011b). mTOR: from growth signal integration to cancer, diabetes and ageing. Nature reviews Molecular cell biology *12*, 21-35.
Zuckman, D.M., Hung, J.B., and Roy, C.R. (1999). Pore-forming activity is not sufficient for Legionella pneumophila phagosome trafficking and intracellular growth. Molecular microbiology *32*, 990-1001.