Innate Immune Bypass of Translation Inhibition Upon Cellular Challenge by *Legionella Pneumophila*

A thesis

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ABSTRACT

Many pathogens, particularly those that require their host for survival, have devised mechanisms to subvert the host immune response in order to survive and replicate intracellularly. *Legionella pneumophila*, the causative agent of Legionnaires' disease, promotes intracellular growth by translocating proteins into its host cytosol through its type IV protein secretion machinery. At least 5 of the bacterial translocated effectors interfere with the function of host cell elongation factors, blocking translation and causing the induction of a unique host cell transcriptional profile. In addition, *L. pneumophila* also interferes with translation initiation, by preventing cap-dependent translation in host cells. We demonstrate here that protein translation inhibition by *L. pneumophila* leads to a frustrated host MAP kinase response, where genes involved in the pathway are transcribed but fail to be translated due to the bacterium-induced protein synthesis inhibition. Surprisingly, few pro-inflammatory cytokines, such as IL-1α and IL-1β, bypass this inhibition and get synthesized in the presence of *Legionella* effectors. We show that the selective synthesis of these genes requires MyD88 signaling and takes place in both infected cells that harbor bacteria and neighboring bystander cells.

We elucidate the global kinetics of blockade and identify the spectrum of genes that host cells selectively synthesize after *Legionella* significantly alters protein synthesis. Interestingly, one mechanism that the innate immune system uses to overcome pathogen-induced translation inhibition is transcript abundance, in which highly transcribed immunity-associated genes, such as those encoding pro-inflammatory cytokines and chemokines, selectively get translated under conditions of intoxication. We show that, for some genes, MyD88 plays a crucial role under such conditions by enhancing the

concentration of transcripts above a minimum threshold necessary to support selective translation. Another mechanism used to overcome translation inhibition depends on *cis*-acting mRNA elements, in which upstream open reading frames (uORFs) within 5' untraslated regions (5'UTR) facilitate translation reinitiation.

Translation inhibition is a common virulence mechanism used by a number of pathogens (e.g. Diphtheria Toxin, Shiga Toxin and *Pseudomonas* Exotoxin A). It has been a mystery how host cells mount a pathogen-specific response and clear infection under conditions in which protein synthesis is blocked by pathogens. Using *Legionella pneumophila* as a model, a bacterium that efficiently blocks the host protein translation machinery, we show that the innate immune system has devised various mechanisms to cope with translation inhibition. Our findings elucidate some of these mechanisms and offer a perspective of how host cells are able to cope with pathogen-encoded activities that disrupt normal cellular processes and initiate a successful inflammatory response.

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List of Abbreviations

PAMP: Pathogen-associated molecular pattern;

DAMP: Damage-associated molecular pattern;

LPS: Lipopolysaccharide

TLR: Toll-like Receptor;

MyD88: Myeloid differentiation primary response 88;

NOD: Nucleotide-binding oligomerization domain;

TRAF6: TNF receptor-associated factor 6;

IRAK: IL-1R-associated kinase;

TAK1: Transforming growth factor β-activated kinase 1;

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells;

NEMO: NF-κB essential modulator;

MKK: Mitogen-activated kinase kinases;

ERK: Extracellular signal-regulated kinases;

JNK: c-Jun N-terminal kinase;

AP1: Activating protein-1;

cGAS: cyclic GMP-AMP synthase;

IFN: interferon;

IPS-1: interferon beta promoter stimulator protein 1;

IRF3: interferon-regulatory factor 3;

MAVS: mitochondrial antiviralsignaling protein;

MDA-5: melanoma differentiation-association protein 5;

NLRP3: NLR family, pyrin domain containing 3;

RIG-I: retinoic acid-inducible gene-1;

STING: stimulator of IFN genes;

SCF complex: Skp, Cullin, F-box containing complex

SET domain: suppressor of variegation, enhancer of zest, and trithorax domain;

uORF: Upstream open reading frame;

5'UTR: 5' untraslated region;

3'UTR: 3' untraslated region;

CHX: cycloheximide;

RPF: Ribosome protected fragment

ExoA: *P. aeruginosa* Exotoxin A

Stx: Shiga Toxin

Chapter 1: INTRODUCTION

This chapter contains excerpts from:

Asrat, S., K.M. Davis, and R.R. Isberg, *Modulation of the Host Innate Immune and Inflammatory Response by Translocated Bacterial Proteins.* Cell Microbiol, 2015. **17**(6): p. 785-95.

Asrat, S., D.A. de Jesus, A.D. Hempstead, V. Ramabhadran, and R.R. Isberg, *Bacterial Pathogen Manipulation of Host Membrane Trafficking.* Annu Rev Cell Dev Biol, 2014. **30**: p. 79-109.

Asrat, S., A.S. Dugan, and R.R. Isberg, *The Frustrated Host Response to Legionella Pneumophila Is Bypassed by Myd88-Dependent Translation of Pro-Inflammatory Cytokines.* PLoS Pathog, 2014. **10**(7): p. e1004229.

1.1 Pattern-Recognition

The pathogen-associated molecular pattern (PAMP) hypothesis has been proposed to explain how the innate immune system recognizes foreign microbial invaders [74]. By this model, germline-encoded pattern-recognition receptors (PRRs) recognize conserved foreign ligands associated with microbes, such as nucleic acids, lipopolysaccharide (LPS), peptidoglycan or flagellin, and generate a response directed at clearing the microorganism [4, 109].

Studies in the past 20 years have classified PRRs into five families based on protein domain homology. These families include the membrane bound Toll-like receptors (TLRs), the cytosolic NOD-like receptors (NLRs), RIG-1 like receptors (RLRs), AIM-2 like receptors (ALRs) and C-type lectin receptors (CLRs) [29]. Toll-Like receptors are transmembrane glycoproteins that contain a horseshoe-like extracellular domain for ligand recognition, a single helix transmembrane domain and an intracellular TIR domain for signal transduction [26]. Currently, 10 human and 12 mouse TLRs have been described [29]. TLR4, the best-studied TLR in innate immunity, recognizes lipopolysacccharide (LPS) on the surface of Gram-negative bacteria [131]. TLR2 recognizes bacterial lipoproteins from both Gram-negative and Gram-positive bacteria while TLR5 recognizes bacterial flagellin [4].

Signaling downstream of pattern-recognition receptors typically requires a cytosolic adaptor (or set of adaptors) that facilitate protein-protein interactions. Upon detection of external stimuli, these adaptors bind to PRRs and link them to downstream signaling proteins in order to initiate a signaling cascade. Most TLRs use myeloid differentiation primary response 88 (MyD88) for signal transduction, with the exception

of TLR3, which uses TIR-domain-containing adaptor inducing IFN-β (TRIF) [4]. TLR4 is unique in that it can use both adaptors and initiate Myd88-depedent as well as TRIF-dependent signaling cascades [29].

MyD88 was first discovered in the 1990s as a protein induced during differentiation of myeloid precursor cells (hence the name) upon IL-6 treatment [98]. Its involvement in IL-1 receptor signaling was later characterized [169], followed by the elucidation of its role downstream of various TLRs [110]. Studies that came from MyD88 knockout mice showed the crucial role MyD88 plays in signaling downstream of TLR2, 4, 5, 7 and 9 [166].

Detection of microbial patterns by PRRs ultimately leads to transcription of pro-inflammatory cytokines and chemokines, largely controlled by the transcription factor NF-κB. There are multiple pathways that lead to activation of NF-κB, and signaling typically involves the addition of ubiquitin and phosphate residues to downstream adaptor proteins, first by ubiquitylation of proteins recruited to sensing molecules to form complexes, such as TNF receptor-associated factor 6 (TRAF6), RIP1, and RIP2 [82]. NF-κB is sequestered in the cytosol by its inhibitor protein (IκB). During activation, the IKK complex phosphorylates IκB, and the E3 ligase complex SCF then ubiquitylates IκB, which targets it for proteasomal degradation. Degradation of IκB releases NF-κB, which translocates into the nucleus and activates transcription of pro-inflammatory cytokines [10, 61, 159]. (Simplified pathway shown in Fig. 1.2).

1.2. Two-signal model in innate immunity ('Effector-triggered immunity')

More recently, it has become clear that pattern recognition by itself does not explain how multicellular organisms differentiate virulent pathogens from harmless commensals and mount an appropriate response. One concept that emerged is sensing of 'danger' by the immune system, in parallel to PAMP recognition, leading to a more potent and directed immune response against invading pathogens that is not seen with harmless microbes. There is compelling evidence that the host immune system can sense the presence of danger and respond to pathogen-encoded enzymatic activities that disrupt normal cellular processes. This mode of immune recognition, referred to as "effector triggered immunity" has been shown to play a significant role in pathogen clearance both in plants and mammalian cells [15, 37, 41, 46, 47, 77, 107, 108, 147, 155].

The danger signal theory, which was first proposed by Matzinger, has been well established in the context of sterile inflammation [106]. Immune mediators of 'danger' have been described, such as uric acid, high-mobility group protein B1 (HMGB1) as well as ATP and DNA, which are released by damaged cells and serve as cues that there is a threat to the host cell [84, 106]. These molecules have collectively been termed damage-associated molecular patterns (DAMPs) [84].

Danger signals in the context of pathogen infection have also been characterized in the past few years. One mode of discrimination between pathogens and commensals is the location of the microorganism and the context in which the PAMP was recognized [161]. Non-pathogenic microbes do not typically enter sterile environments of host cells, such as the cytosol, and activation of PRRs within such environments indicates the presence of a threat. Similarly, the immune response downstream of PAMP recognition

can also vary depending on the presence of a second signal. For example, PAMP recognition is sufficient to induce TLR signaling that leads to transcription of pro-inflammatory cytokines such as IL-1β. The posttranslational processing (cleavage) and secretion of this cytokine however, is dependent on the presence of a second 'danger' signal and only occurs after assembly of inflammasomes [48, 161]. Such dual control ensures an appropriate response against virulent pathogens while limiting the production of inflammatory cytokines against harmless commensals.

Another common 'pathogen pattern' that can be sensed by the host immune system is the ability to replicate upon invasion [161]. Actively growing microbes secrete molecules such as quorum-regulating autoinducers, peptidoglycan fragments and second messengers (such as c-di-GMP). Detection of these molecules, in addition to PRR activation, could lead to a more potent immune response [161]. Similarly, pore formation upon entry, disruption of the bacteria-containing vacuole or disruption of the host cytoskeleton can all serve as second signals that alert the immune system of the presence of danger [48, 161].

1.3. The cytosolic innate immune response

The host cytosol is filled with various surveillance mechanisms that allow activation of the innate immune response when pathogens or their ligands contaminate this sterile environment. One well characterized class of cytosolic surveillance receptors are NOD-like Receptors (NLRs), which activate inflammatory mediators (pro-inflammatory cytokines and chemokines) and induce formation of inflammasomes or trigger the type I interferon response [88].

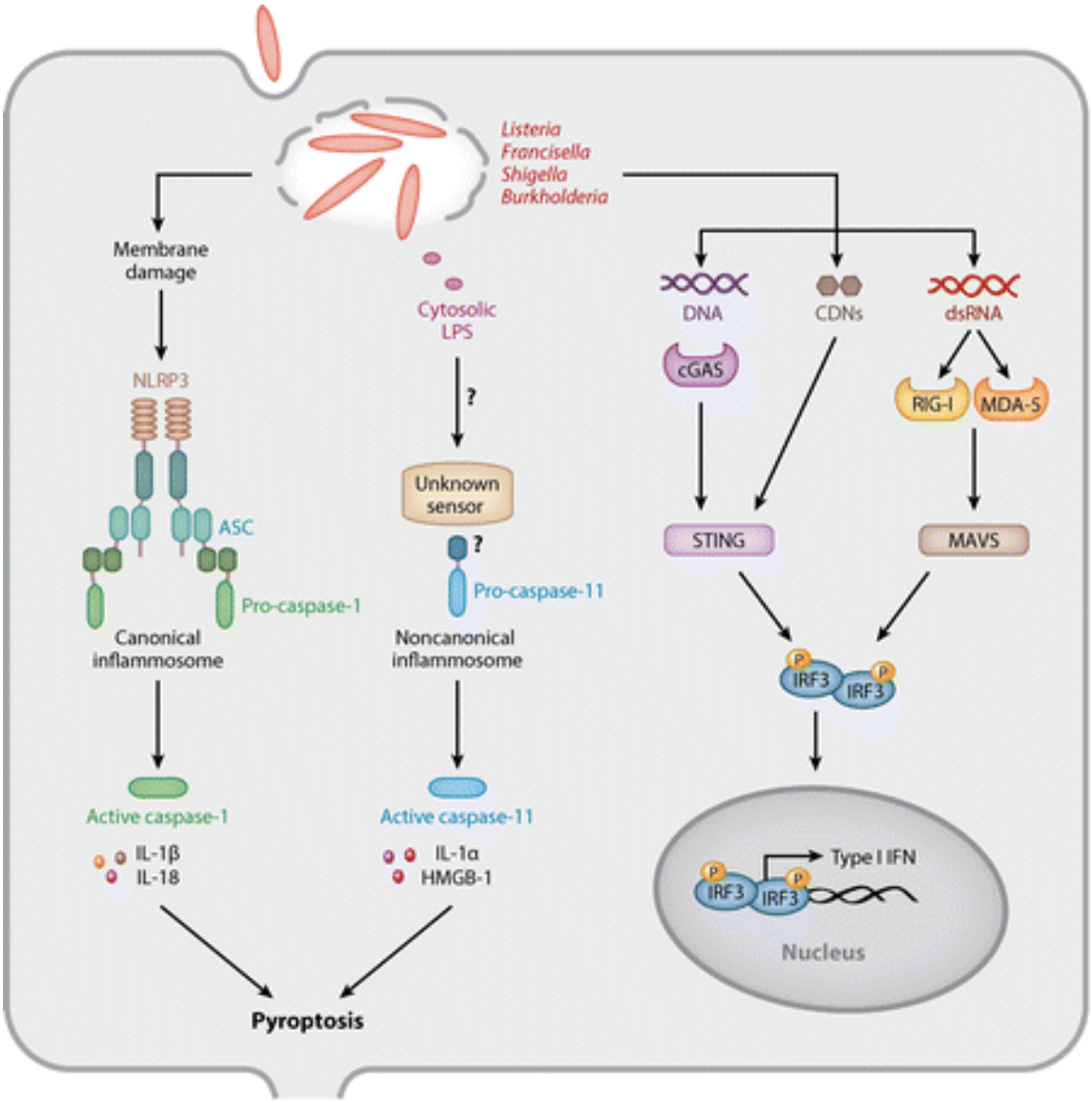
The type I interferon pathway can be activated in response to foreign RNA, DNA or cyclic-dinucleotide (CDN) contamination of the cytosol. In the case of double-stranded RNA, activation passes through the mitochondrial-associated protein MAVS**,** which receives signals from RIG-I-like receptors (RLRS) RIG-I and MDA5, helicases that directly recognize dsRNA [18] (Fig. 1.1). Cyclic-dinucleotides are recognized by the cytoplasmic protein STING, which activates a transcriptional interferon response [70]. Microbial DNA sensing also proceeds via the STING pathway, however, direct sensing of the contaminating microbial molecule occurs through the action of the cyclic GMP-AMP synthase (cGAS), a transferase that generates cyclic GMP-AMP in response to DNA binding [151].

In contrast to the interferon response, which by-and-large results in a transcriptional response, cytosolic NOD-like receptors (NLRs) are activated immediately to restrict replication of pathogens and produce inflammatory mediators. Activation of cytosolic receptors such as NLRC4, NLRP1, NLRP3 and AIM2 triggers canonical inflammasome assembly and caspase-1 activation, which is associated with IL-1β/IL-18 production and pyroptotic cell death (extensively reviewed in [164]). The first identified NLRs were Nod1 and Nod2, which recognize muramyl peptide fragments of bacterial peptidoglycan [32]. Several others have since been characterized, including sensors of flagellin (detected by NLRC4) and bacterial DNA (detected by AIM2). Membrane damage induced as a consequence of vacuole escape can also activate the NLRP3 inflammasome [111] (Fig. 1.1).

Figure 1.1: The cytosolic innate immune response.

Image published in Asrat et al., Annu Rev Cell Dev Biol. 2014 [12].

After phagocytosis, intracellular pathogens, such as *Listeria, Shigella, Francisella, and Burkholderia*, escape from their vacuole and replicate in the host cytosol. Damage of the bacterium-containing vacuole activates NLRP3, which triggers assembly of canonical inflammasomes and pyroptotic cell death. Lipopolysaccharide (LPS) from Gram-negative bacteria, which is sensed by an unknown receptor, activates caspase-11 and noncanonical inflammasomes, leading to pyroptosis. Double stranded RNA (dsRNA) that leaks to the cytosol is sensed by either MDA-5 or RIG-I, which is then recruited to the mitochondria by the adaptor protein MAVS (also called IPS-1). Bacterial DNA is sensed by cGAS, and cyclic dinucleotides (CDNs) are sensed by STING. Activation of MAVS and STING by bacterial ligands leads to phosphorylation of the transcription factor IRF3 and initiates the type I interferon response.


Listeria
Francisella
Shigella
Burkholderia
Membrane damage
NLRP3
ASC
Pro-caspase-1
Canonical inflammosome
Active caspase-1
IL-1β
IL-18
Cytosolic LPS
?
Unknown sensor
?
Pro-caspase-11
Noncanonical inflammosome
Active caspase-11
IL-1α
HMGB-1
Pyroptosis
DNA
CDNs
dsRNA
cGAS
RIG-I
MDA-5
STING
MAVS
P
IRF3
P
IRF3
Type I IFN
Nucleus

Several recent studies have shown that detection of cytosolic lipopolysacaride (LPS) from Gram-negative bacteria triggers the activation of caspase-11 (noncanonical inflammasome) independently of Toll-like receptor 4 (TLR4) [1, 57]. Caspase-11 is crucial for clearance of bacteria that escape from the vacuole, such as *Burkholderia* [1, 57] (Fig. 1.1). Caspase-11 activation and pyroptosis can also be triggered by pathogens that stay within their replication vacuole, even in the absence of canonical inflammasome activation [33].

1.4. *Legionella pneumophila* pathogenesis

The history of *Legionella pneumophila* began about 40 years ago after a large outbreak at the 1976 American Legion Convention in Philadelphia [28, 93]. The Legionnaires returning from the convention began to show severe pneumonia symptoms within two weeks after the convention, with fevers up to 107 degrees. The US center for Disease control (C.D.C.) was able to trace the disease origin to Bellevue Stratford Hotel, where the convention was held, but standard laboratory tests could not identify the pathogen that was causing this illness. Great public panic arose as the number of sick people increased the following weeks, rising up to 221 infected patients, including 34 deaths. In early 1977, after 6 months of the convention, the C.D.C scientist Dr. Joseph McDade discovered that the illness was caused by a Gram-negative rod shaped bacterium, and it was named *Legionella pneumophila* [28].

Legionella pneumophila is a pathogen for a broad range of fresh water amoebae, which provide the natural environmental niche for the microorganism and the source of exposure for humans [137, 160]. After aspiration of contaminated water sources by a

susceptible mammalian host, the bacterium is engulfed by alveolar macrophages in the lungs, the primary cellular host for the microorganism [119]. Infection with *Legionella* can result in two distinct diseases: a mild illness called Pontiac fever and a severe form of pneumonia called Legionnaires' disease.

In healthy individuals, *Legionella* can be cleared from the lungs by the innate and adaptive immune systems. Legionnaires' disease is thus typically associated with immunocompromised individuals, chronic lung patients, the elderly and smokers [123]. *Legionella* is still a health burden in the United States. The CDC estimates that about 8,000 – 18,000 people are hospitalized each year due to *Legionella* infection. Accurate data for Legionnaires' disease prevalence is lacking as it can easily be misdiagnosed in the clinic, primarily due to symptoms being similar to other pneumonic infections, as well as lack of tests that detect all *L. pneumophila* serotypes (C.D.C., [105]). In most cases, *Legionella* infection can successfully be cleared by fluoroquinolones and macrolide antibiotics [105].

1.5. *L. pneumophila* Type IV secretion system and translocated substrates

A major virulence strategy used by Gram-negative bacterial pathogens to overcome host defenses is the assembly of specialized secretion systems in the cell envelope. Secretion systems (Type I-VIII) enable bacteria to translocate proteins, DNA and anti-microbial molecules directly into recipient cells. Translocated proteins manipulate various host cell processes such as phagocytosis, host cell sensing and inflammatory signaling as well as host transcription and translation, which ultimately promote a successful bacterial infection.

Legionella pneumophila promotes intracellular growth by translocating more than 300 bacterial proteins (effectors) into its host cytosol through its type IV (Dot/Icm) protein secretion machinery [27, 65, 163]. Type IV secretion systems are large macromolecular complexes that span the inner and outer membrane of bacteria without a clear division between complex and pore forming components. T4SS are evolutionarily related to bacterial conjugation systems and may function by direct penetration of complex components into the host cell membrane [90].

Upon uptake by phagocytic cells, *Legionella* enters a replication compartment called the *Legionella* containing vacuole (LCV), where it multiplies to high numbers and eventually lyses out, releasing bacteria for another round of infection [64, 152]. The LCV does not traffic to lysosomes. Instead, it rapidly recruits mitochondria and vesicles derived from the endoplasmic reticulum (ER) through its Type IV translocated effectors [79, 152, 153]. Ribosomes have also been shown to surround the LCV as the infection progresses, creating a rough endoplasmic reticulum (RER) like compartment [156].

Legionella translocated effectors serve various purposes inside host cells, including recruitment of ER-derived membrane to the *Legionella* containing vacuole, inhibition of cell death pathways, manipulation of host ubiquitin pathway and regulation of innate and adaptive immune responses [17, 62, 66, 79, 80, 100]. Some examples of these translocated effectors, their host targets and function during intracellular growth is summarized in Table 1.

Table 1: *Legionella* pneumophila translocated effectors and their function

T4SS substrate	Target host factor/pathway	Function during infection	Ref.
LidA	Rab GTPases (Rab1, Rab6, Rab8)	Prolongs activation of Rab1 and enhances fusion of the LCV with ER-derived vesicles	[146]
PlaA	Cholesterol and phospholipids	Prevents membrane instability	[36]
RalF	Arf1	Recruitment of ER-derived vesicles to the LCV	[118]
RavZ	Atg8/LC3	Irreversibly delipidates LC3, inhibits autophagy	[35]
RidL	VPS29	Inhibits retrograde traffic	[45]
SdhA	Unknown	Maintains vacuole integrity	[36]
SidM/DrrA	PI4P, Rab1, Stx3, Stx4	LCV- and ER-derived vesicle fusion	[104, 117]
WipB	Unknown	Coordinates membrane fusion events	[124]
LegAU13/AnkB	Ubiquitin pathway	Associates with host SCF complex*, E3 ubiquitin ligase activity	[43]
Leg U1	Ubiquitin pathway	Associates with host SCF complex, E3 ubiquitin ligase activity	[132]
LicA	Ubiquitin pathway	Associates with host SCF complex	[132]
LubX	Ubiquitin pathway	Polyubiquitination of host Clk1	[89]

LegK1	IκB	Ser/Thr kinase that phosphorylates IκB and leads to NF-κB activation	[52]
LnaB	NF-κB	Induces NF-κB activation	[99]
RomA	Host transcription	SET domain trimethylates histone H3 (H3K14) to repress transcription	[136]
Lgt1/Lgt2/Lgt3	Elongation factor eEF1A and eEF1Bγ	Glycosyltransferases that modify host elongation factors	[22, 145]
SidI/SidL	Host protein translation	Interferes with translation elongation	[22, 145]

Modified from reviews Asrat et al., Annu Rev Cell Dev Biol. 2014 [12] and Asrat et al., Cell Microbiol. 2015 [11].

*The SCF complex (Skp, Cullin, F-box containing complex), is a multi-protein E3 ubiquitin ligase complex that facilitates the transfer of ubiquitin from ubiquitin-conjugation enzymes (E2) to the target substrate.

1.6. Manipulation of the innate immune response by *Legionella pneumophila*

After contact with macrophages, *Legionella pneumophila* stimulates a pathogen-specific response that is the consequence of simultaneous recognition of its PAMPs and T4SS substrate activities (damage induced by effectors) that results in a unique response to this microorganism [46]. One of the advantages of studying *L. pneumophila* is that it provides an excellent model to dissect the complex innate immune strategies used by mammalian cells. Upon recognition, the bacterium provokes signaling through various Toll-like receptors (TLRs) and cytosolic NOD-like receptors (NLRs) [147].

1.6.1. TLR response during *Legionella* infection

When macrophages come in contact with *Legionella pneumophila*, multiple TLRs are activated that are critical for restricting infection. During the early stages of infection in a mouse pneumonia model, activation of TLR2 is required for control of *L. pneumophila* growth and replication [51]. Interestingly, TLR4 deficient mice do not show any susceptibility to *Legionella* infection despite the fact that it is a Gram-negative bacterium [9, 51]. It has been demonstrated that LPS from *Legionella* is about a 1000X less potent compared to other Gram-negative pathogens in its ability to activate downstream cytokines [121]. This is primarily due to its inability to bind to CD14/TLR4 [121]. LPS from *Legionella* may instead be detected by TLR2, although this has not been directly demonstrated [51, 54]. Some groups have attributed the lack of TLR4 signaling to the unique structure of Lipid A from *Legionella* (the length of fatty acid chains and the presence of a hydrophobic core) [51]. This divergence from TLR4 recognition is interesting from an evolutionary standpoint, as *Legionella* does not face immune pressure

in its natural environment. Instead, the unique lipid A structure of *Legionella* may be beneficial for uptake or survival within freshwater amoeba.

In vitro studies have shown that flagellin from *L. pneumophila* can be recognized by TLR5 [60]. Polymorphisms in human TLR5, specifically a common stop codon mutation in the ligand-binding domain (TLR5392STOP), has been shown to increase susceptibility to Legionnaires' disease [60]. Similarly, TLR9, which detects bacterial CPG DNA, has also been implicated in the control of *Legionella* infection [122].

Consistent with the idea that TLRs are crucial for restricting *Legionella* infection, mice lacking MyD88, the cytosolic adaptor protein required for TLR signaling, are more susceptible to lethal disease, show profound defects in controlling infection and are unable to contain the bacterial load in their lungs [9, 59].

1.6.2. Manipulation of MAPKs and NF-κB by *Legionella pneumophila*

Interestingly, macrophage challenge with wild type *L. pneumophila* (Dot/Icm$^+$) triggers a unique transcriptional response in host cells compared to mutants that lack a functional type IV secretion system, supporting the model that there is a pathogen-specific response involved in innate immune recognition [46-48, 100, 147]. Microarray studies have identified many of these transcriptional targets as being genes controlled by the NF-κB and mitogen-activated protein kinase (MAPK) transcriptional regulators [100, 147], including downstream dual specificity phosphatases (*Dusp1* and *Dusp2*), stress response genes (*Hsp70*, *Gadd45a, Egr1*) and pro-inflammatory cytokines and chemokines (*Il1α, Il1β, Tnfα, Il23a, Csf1, Csf2*) [20, 46, 47, 147].

Several *Legionella* translocated substrates have been shown to modulate MAPK and NF-κB activity during infection. When overexpressed in mammalian cells, the *Legionella* eukaryotic-like Ser/Thr kinase LegK1 activates NF-κB by phosphorylating its inhibitor IκB [52]. This phosphorylation is independent of the host kinase IKK, as well as the upstream signaling proteins TRAF2/TRAF6, TAK1 and MEKK3 [52](Pathway shown in Fig. 1.2). Ectopic expression of another *Legionella* protein LnaB, also strongly induces NF-κB activation, and *L. pneumophila* mutants that lack this protein show lowered ability to activate NF-κB [99]. A small putative coiled-coil domain of LnaB is required for NF-κB activation but it is not known if LnaB directly acts on NF-κB or if the activation is the consequence of the protein being sensed by host receptors [99].

Mitogen-activated protein kinases (MAPKs) are also another component of the innate immune system that are targeted by *Legionella pneumophila*. The well-characterized MAPKs in mammalian cells include JNK, p38 and ERK, and they control a number of different physiological processes, such as cell division, apoptosis and production of inflammatory cytokines and chemokines [76]. MAPKs are activated by threonine and tyrosine phosphorylation as part of a sequential phosphorelay system, where MAPKKKs activate MAPKK, which in turn activates MAPKs [76].

Upon *Legionella* infection, host cells show transient phosphorylation of MAPK members shortly after exposure to the bacterium both in amoebae and mammalian cells [97, 147, 167]. At early time points following bacterial challenge, MAPK phosphorylation is dependent on pattern recognition (TLR-mediated response), while at later time points, JNK and p38 activation is driven by Type IV translocated effectors

[147]. At least 5 *Legionella* translocated substrates have been shown to activate the host MAPK pathway indirectly as well [47, 147]. These 5 proteins inhibit host protein translation (discussed at a later section) and this inhibition triggers phosphorylation of MAPK members [47]. Consistent with this hypothesis, chemical inhibitors of translation (cycloheximide) as well as bacterial toxins that target translation (*Pseudomonas aeruginosa* Exotoxin A) have been shown to induce phosphorylation of JNK and P38 [47].

Activation of TLRs and MAPKs upon *L. pneumophila* infection leads to robust production of pro-inflammatory cytokines a few hours post-infection [20, 46, 47, 147]. Once secreted, cytokines activate various host immune pathways and recruit immune cells to the site of infection, eventually leading to clearance of the pathogen. This response is critical for clearance of the microorganism and mouse mutants defective in these responses succumb to lethal pneumonia [8, 20].

1.6.3. Type I IFN response and inflammasome assembly during *Legionella* infection

The Type I IFN response is typically associated with antiviral immunity but its role in restricting intracellular bacteria has received more attention recently. This response is more important in the context of cytosolic bacteria, such as *Listeria monocytogenes* and *Francisella tularensis* [34], but it can also be activated when DNA or RNA from intravacuolar pathogens accesses the host cytosol.

Upon *Legionella* infection, the Type I IFN pathway is transcriptionally activated, and this mainly occurs downstream of the RIG-I/MDA5 pathway ([115], Fig. 1.1). It was

shown that this is triggered by *L. pneumophila* RNA that leaks into the cytosol via the Type IV secretion system, and the activation was independent of TLR signaling [115]. Interestingly, the Type I IFN pathway may be dispensable for controlling bacterial replication as $MAVS^{-/-}$ and IFN-receptor knockout mice do not show a difference in *L. pneumophila* burden compared to WT mice [115].

A number of other cytosolic innate immune pathways also get activated upon *Legionella* infection, including assembly of canonical and non-canonical inflammasomes [165]. Interestingly, macrophages from most inbred mouse strains are restrictive to *L. pneumophila* growth and replication with the exception of A/J mice. The genomic region responsible for this resistance was mapped to an autosomal recessive locus on chromosome 13, and ultimately to a single NLR gene, Naip5 [40, 49, 91, 114, 170]. Upon *Legionella* infection, Naip5 is activated by cytosolic flagellin that is translocated by *L. pneumophila* type IV secretion system. This in turn activates NLRC4 (Ipaf)-mediated inflammasome assembly (Naip5-NLRC-4-Caspase-1) and triggers pyroptosis [91, 114, 170]. A/J mice have a defective Naip5 allele, which makes them permissive to *Legionella* growth [101].

Type IV translocated effectors also induce flagellin-independent Caspase-11 inflammasome activation, followed by IL-1α release and pyraptotic cell death [33]. To date, there are no known *Legionella* effectors that directly inhibit or overcome inflammasome-dependent pyroptosis. This could be due to its evolution in its non-mammalian host amoebae, where there is much less selective pressure to evade this pathway.

1.6.4. Hijacking host transcription and translation

It has become increasingly evident that a number of pathogens interfere with host transcription directly by acting on RNA polymerase [103] or through manipulation of host epigenetic regulatory factors [25]. Some bacteria achieve this by encoding enzymes that modify the host chromatin, and are termed 'nucleomodulins'[25]. Various pathogens, including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Shigella flexneri* and *Helicobacter pylori* induce histone modifications and alter expression of innate immune genes. Modification of histones, specifically histone methylation, is mediated by proteins that contain SET domains (suppressor of variegation, enhancer of zest, and trithorax domain). SET domains, typically found in eukaryotic methyltransferases, consist of a 130 amino acid sequence and have enzymatic activities that methylate histone lysine residues [171]. *Chlamydia pneumonia* and *Chlamydia trachomatis* encode proteins that have SET-domains that methylate histones H2A, H3 and H4 [129].

Legionella pneumophila also uses a similar strategy to modify the host transcriptional machinery. The translocated protein LegAS4 (in *L. pneumophila* Philadelphia strain), which contains a SET domain, localizes to host nucleolus during infection [96]. The SET domain of LegAS4 is catalytically active and has been shown to specifically activate rDNA transcription [96]. Interestingly, RomA, the equivalent protein in the *L. pneumophila* Paris strain, localizes to the nucleus during infection and tri-methylates histone H3 at lysine 14 (H3K14) [136]. H3K14 is typically a site for acetylation, which is a marker for transcriptional activation. RomA induced methylation at H3K14 thus prevents this acetylation from taking place and leads to genome wide

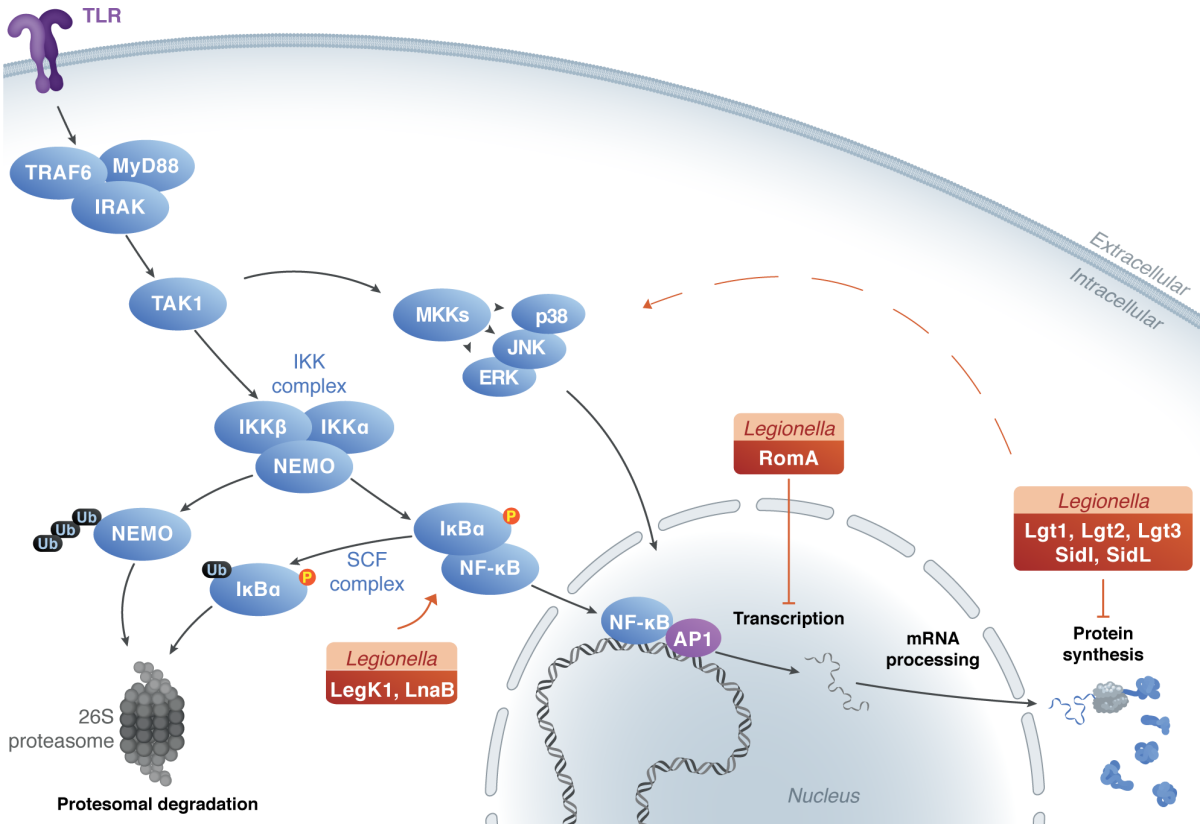
transcriptional repression. The study showed that this repression mainly affected innate immune genes, including pro-inflammatory cytokines (TNF and IL-6) and pattern-recognition receptors (TLR5 and Nalp3) [136]. This observation however, contradicts observation by other groups, who demonstrated that the presence of *Legionella* translocated proteins significantly induces the transcription of various pro-inflammatory cytokines to levels significantly higher than avirulent mutants that lack the T4SS [13, 20, 46, 147].

In addition to modifying host transcription, *Legionella* also hijacks the host translation machinery and efficiently interferes with protein synthesis. This is the result of a bacterial-driven mechanism, which targets eukaryotic elongation factors [21, 145] as well as a host-driven mechanism, which targets cap-dependent translation initiation [71]. The bacterial-driven translation inhibition is mediated by at least 5 translocated proteins that target host elongation factors eEF1A and eEF1Bγ. Three of these proteins, Lgt1, Lgt2, Lgt3, are glycosyltransferases that modify eEF1A *in vitro* and *in vivo* [46, 145], while the mechanisms of the other proteins, SidI and SidL, are not fully understood. A recent study identified two additional proteins, Lpg0208 and Lpg1489, predicted to block protein synthesis. However, deletion of the genes for these proteins in a background that lacks the 5 elongation inhibitors showed no additional effect on protein synthesis [20].

Figure 1.2: *Legionella* translocated substrates target TLR pathway, host transcription and translation.

Image published in Asrat et al., Cell Microbiol. 2015 [11].

Mammalian cells recognize *L. pneumophila* through different TLRs, resulting in the activation of NF-κB and transcription of various pro-inflammatory cytokines. Two *L. pneumophila* effectors, LegK1 and LnaB, target NF-κB while another effector RomA, targets host transcription. *L. pneumophila* also translocates five effectors that efficiently block host protein translation and this inhibition in turn leads to activation of host MAP kinases.


TLR
TRAF6
MyD88
IRAK
TAK1
MKKs
p38
JNK
ERK
IKK
complex
IKKβ
IKKα
NEMO
Ub
NEMO
IκBα
P
NF-κB
SCF
complex
IκBα
Legionella
LegK1, LnaB
26S
proteasome
Protesomal degradation
Extracellular
Intracellular
Legionella
RomA
Transcription
NF-κB
AP1
mRNA
processing
Legionella
Lgt1, Lgt2, Lgt3
SidI, SidL
Protein
synthesis
Nucleus

In addition to elongation inhibition induced by *Legionella*-translocated proteins, host cells down-regulate cap-dependent translation initiation following *Legionella* infection by targeting the mTOR pathway [71]. This mechanism of translation inhibition is not dependent on a particular *Legionella* protein but is a host response to damage, dependent on the presence of the bacterial T4SS [71]. In resting cells, the translation initiation factor eIF4E is bound by its inhibitor 4E-BP (Fig.1.3). Activation of mTOR leads to phosphorylation of 4E-BP, which frees eIF4E from its inhibitor, allowing it to associate with mRNA cap to initiate translation. Upon detection of 'pathogen-signatures', macrophages stimulate ubiquitylation and degradation of proteins in the mTOR pathway, including mTOR and AKT. During *Legionella* infection, suppression of the mTOR pathway prevents 4E-BP phosphorylation, leading to a reduction in cap-dependent translation initiation [71].

1.7. Eukaryotic protein translation

1.7.1. Translation Initiation

Initiation, the first stage of protein translation, is the most complex and highly regulated phase of the eukaryotic translation cycle that is coordinated by multiple initiation factors [3]. The process requires formation of the initiation complex (48S complex) and the joining of this complex with large ribosomal subunit (60S) (Fig. 1.3).

1. The first step, which is the formation of the initiation complex, starts with the assembly of the initiator tRNA (Met-tRNA$^{Met}_{i}$) with a GTP bound eukaryotic initiation factor eIF2 forming the ternary complex. The ternary complex then associates with small (40S) ribosomal subunit along with initiation factors eIF1,

1A, 3 and 5, forming the 43S pre-initiation complex. The eIF4F macromolecular complex is also formed in parallel, which consists of cap binding proteins eIF4E, eIF4G, as well as the RNA helicase eIF4A, which unwinds the 5' mRNA secondary structure (Fig. 1.3). [3, 128]

2. eIF4F complex binds to mRNA cap (7-methylguanosine cap) and recruits the 43S pre-initiation complex to the mRNA. The mRNA is circularized at this step by the interaction of poly(A)-binding protein (PABP) with eIF4G (not shown in Fig 1.3.) [3, 128]. Note: There are cap-independent translation initiation mechanisms that do not require this step (discussed later).
3. After association with the mRNA cap, the 43S pre-initiation complex starts to scan the 5' untranslated region (5'UTR, 5' to 3' scanning) until it locates the start codon [3, 128]
4. The recognition of the start codon triggers the formation of 48S initiation complex and eIF5-mediated conversion of eIF2 to its GDP bound state, stabilizing the conformation of the initiation complex with the mRNA and arresting scanning.
5. Initiation factor eIF5B, in association with eIF1A, facilitates the joining of the large ribosome subunit (60S), forming the 80S ribosome. [3, 128]
6. This is followed by the release of all the initiation factors and elongation begins by the 80S ribosomal complex (the Met-tRNA^{Met} is placed on the P site of ribosomes prior to elongation) [128].

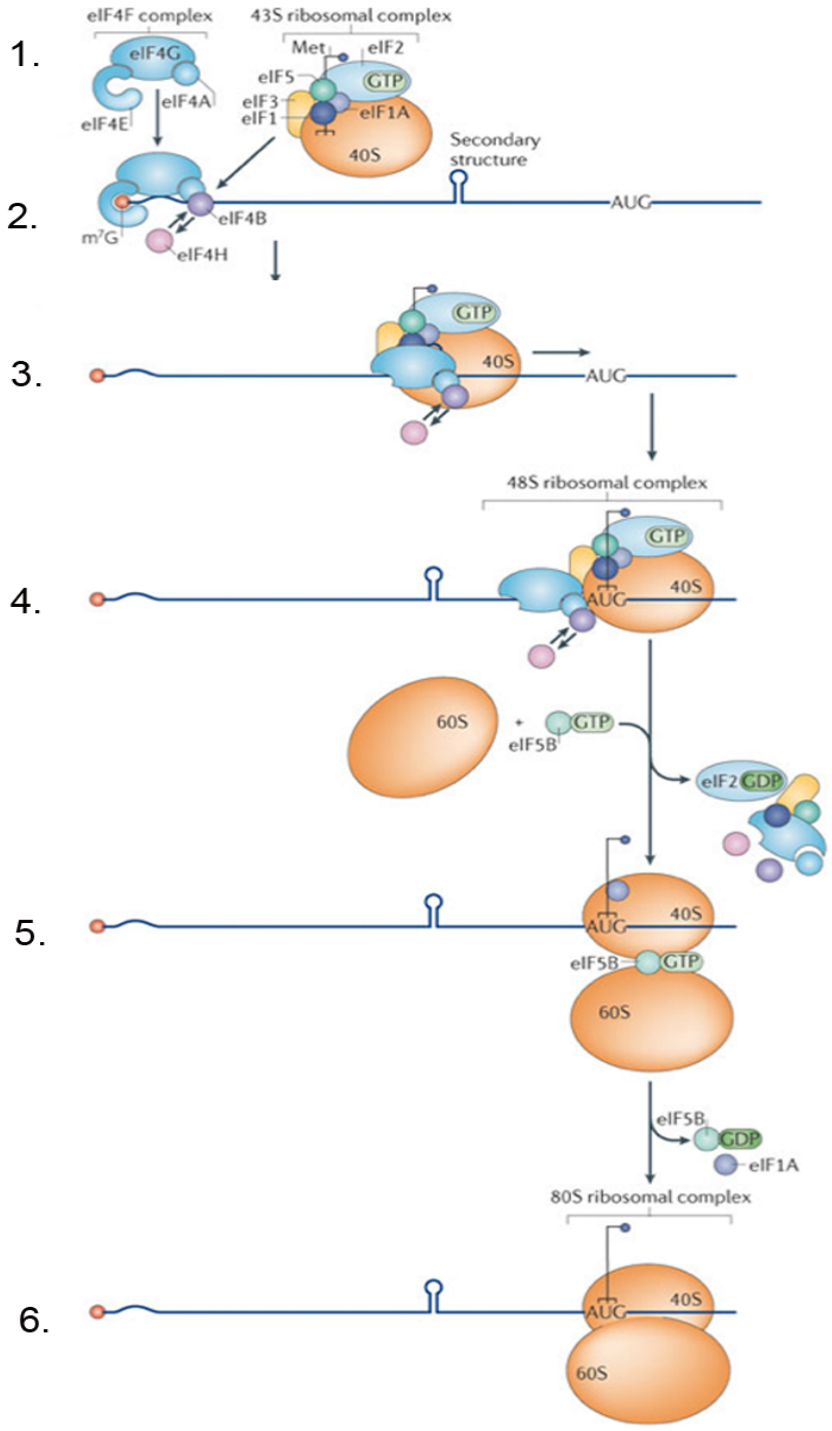


Figure 1.3: Eukaryotic Translation Initiation.

Modified (with permission) from Parsyan, A., et al., mRNA helicases: the tacticians of translational control. Nat Rev Mol Cell Biol, 2011 [128].

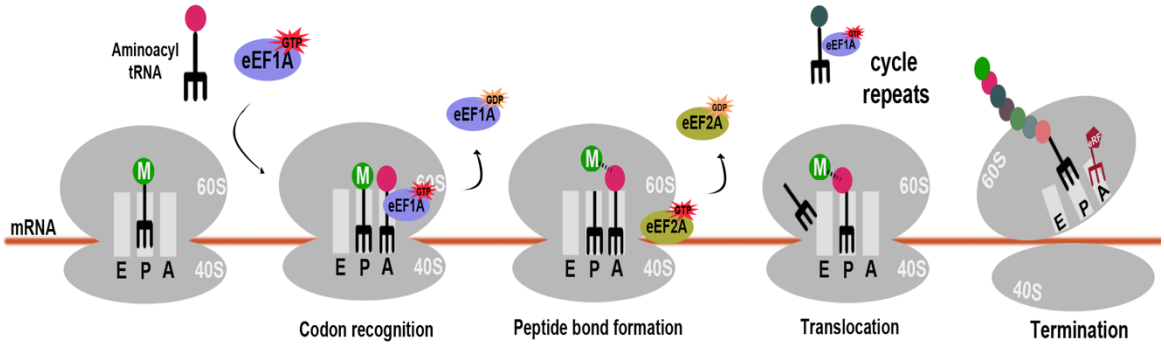
1.7.2. Translation elongation

Eukaryotic translation elongation is a much simpler process compared to initiation. The basic principle of elongation is conserved across biological kingdoms and involves a coordinated addition of amino acids to a nascent polypeptide chain. The amino acid that is added is specified by the codon sequence in the mRNA that is getting translated. In eukaryotes, there are two main translation elongation factors that facilitate this process (Summarized in Fig. 1.4).

1. The first step of elongation is facilitated by a GTP bound elongation factor eEF-1, which brings an aminoacyl-tRNA (aa-tRNA) to the A site of the assembled 80S ribosome [39, 81].
2. The anticodon of the incoming aa-tRNA will be matched against the codon of the mRNA. If the sequences do not match, the aa-tRNAs is thrown out and replaced by another aa-tRNA until a match is located.
3. When the correct aa-tRNA is detected in the A-site, there is an immediate peptide bond formation between the methionine (during the first round of elongation) or growing polypeptide chain (during subsequent rounds of elongation) that is in the P-site. This step is catalyzed by the ribosome itself, and leaves an empty tRNA sitting in the P-site while the peptidyl-tRNA is in the A-site (Fig.1.4) [39, 81].

Figure 1.4: Eukaryotic Translation Elongation cycle.

GTP bound (red star) Elongation factor 1A (eEF1A, purple) brings aminoacyl tRNA to the A site of 80S ribosomes. Codon recognition is immediately followed by peptide bond formation and hydrolysis of eEF1A-GTP to eEF1A-GDP (orange star), which is then released. Hydrolysis of GTP bound elongation factor 2A (eEF2A, yellow) facilities ribosome conformational changes that translocate peptidyl-tRNA to the P-site. The cycle repeats until the ribosome comes to a stop codon, which is detected by eukaryotic release factors (red tRNA).


Aminoacyl tRNA
eEF1A
GTP
mRNA
60S
40S
E P A
M
eEF1A
GDP
eEF2A
cycle repeats
Codon recognition
Peptide bond formation
Translocation
Termination

4. The ribosome undergoes a conformational change that moves the empty tRNA to the E-site from where it is released, and the peptidyl-tRNA into the P-site. In eukaryotes, this translocation is facilitated by GTP bound elongation factor eEF-2, which is hydrolyzed to eEF2-GDP upon translocation (Fig 1.4). After the peptidyl-tRNA is translocated to the P-site, the next codon will be available for the incoming aa-tRNA and the cycle repeats [39, 81].
5. Translation is terminated when the ribosome comes to an in-frame stop-codon in the A-site. Stop codons do not have a corresponding tRNA, but instead can be recognized by eukaryotic release factors that facilitate the cleavage of the ester bond between the peptidyl-tRNA and the polypeptide chain. This releases the newly synthesized protein, as well as the release factors from the ribosome (Fig. 1.4) [39, 81].

1.8. Alternative mechanisms of translation

Translation initiation, which is the rate-limiting step of protein synthesis, is highly regulated by different *cis*-acting elements that are present in the 5' UTR of the mRNA. These elements include Kozak sequences that determine the efficiency of start codon recognition, upstream open reading frames (uORFs) that control the translation efficiency of the main ORF, internal ribosome entry sites (IRESs) that mediate cap-independent translation initiation and secondary structures that can prevent ribosome binding/scanning [19, 31, 67].

For a successful initiation to occur, the 43S pre-initiation complex has to efficiently recognize the start codon as it scans down the 5'UTR (Fig. 1.3). The strength of this recognition depends on the nucleotide surrounding the start codon, which is

referred to as the Kozak consensus sequence: (GCC)GCCA/GCC**AUG**G [85, 86]. The critical nucleotides (underlined) that determine the strength of recognition are at positions -3 and +4 [86]. If a ribosome encounters a weaker Kozak sequence, it can either read the start codon or pass it and initiate translation at a downstream start codon, a process termed 'leaky scanning' [87].

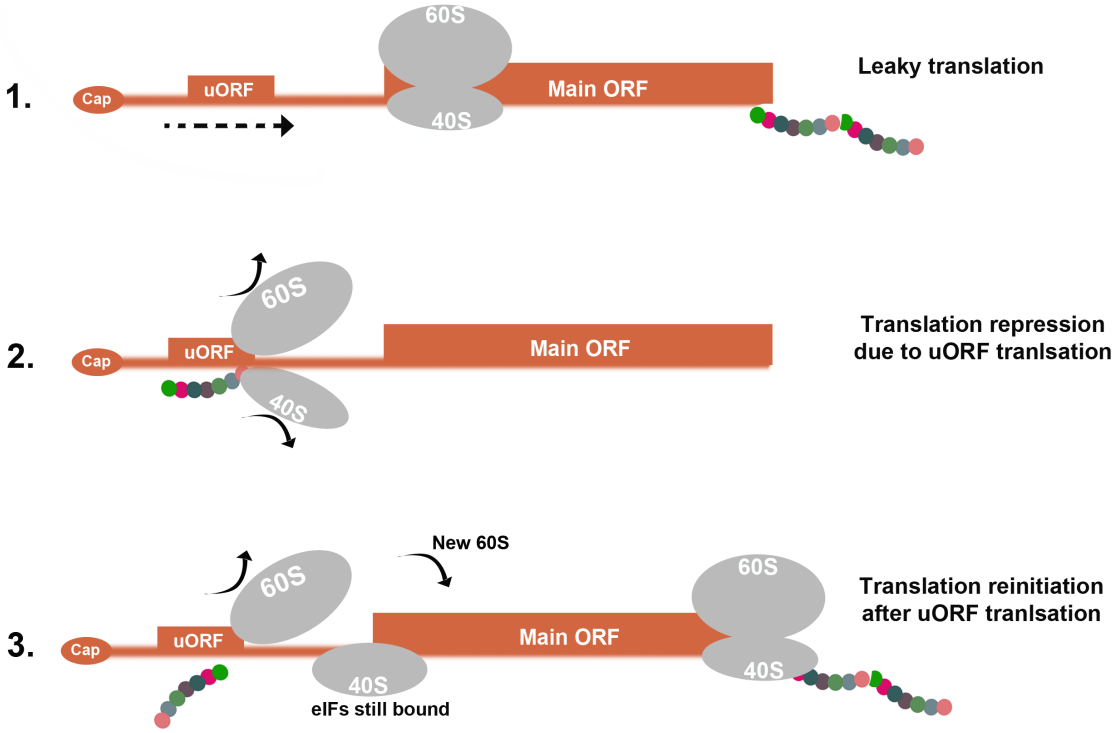
Upstream open reading frames (uORFs) are crucial translation regulatory factors that have traditionally been considered as *cis*-acting mRNA elements that reduce protein expression by limiting association of ribosomes with the main open reading frame [19]. uORFs do not typically impact mRNA levels but can lead to a significant reduction of protein translation, in some cases by 30-80 fold [19]. Studies that were done in yeast, and more recently in mammalian cells, have elucidated the diverse function of uORFs during translation in addition to reduction of protein expression [7, 19, 94].

It is estimated that about 38% of uORFs present in humans are evolutionarily conserved in mice (*M. musculus*) and rats (*R. norvegicus*) [67]. Although their function in translation regulation is well recognized, the exact mechanism by which they do so still remains to be worked out.

For a uORF to alter translation initiation, its start codon and surrounding Kozak sequences have to first be recognized by the translation pre-initiation complex.

Figure 1.5: uORF and eukaryotic translation initiation.

Mechanisms by which uORFs regulate protein translation. 1) Leaky translation: the ribosome (grey) skips the uORF (thinner orange box) and translates the main ORF (thicker, orange box). 2) The ribosome dissociates after translating the uORF, which leads to repression of the main ORF. 3) The ribosome translates the uORF, large subunit (60S) is dissociated while small subunit (40S), together with initiation factors, remains bound and continues scanning down the leader sequence. Translation is reinitiated upon detection of a start codon at the main open reading frame.


1.
60S
Cap
uORF
Main ORF
40S
Leaky translation
2.
60S
Cap
uORF
Main ORF
40S
Translation repression
due to uORF tranlsation
3.
New 60S
60S
Cap
uORF
Main ORF
60S
40S
40S
eIFs still bound
Translation reinitiation
after uORF tranlsation

Recognition of a uORF can be followed by various outcomes: 1) Leaky translation, where the ribosome passes the uORF and starts translating the main coding sequence; 2) The ribosome translates the uORF and gets released (a small peptide will be translated from the uORF under these conditions) (Fig 1.5); 3) The ribosome starts to translate the uORF and stalls during elongation or termination, leading to accumulation of more ribosomes (not shown in fig. 1.5); and 4) The ribosome can translate the uORF and continue scanning to the main ORF, leading to translation reinitiation [19].

1.8.1.Regulation of translation initiation under stress conditions

Under conditions of severe stress such as starvation, UV damage, ER stress or viral infection, host cells down-regulate there own translation by phosphorylating the α subunit of eIF2 at Ser-51, which prevents assembly of the initiation complex [150]. Some mRNAs, particularly in viruses, bypass this inhibition by using internal ribosome entry sites (IRESs) that allow them to directly recruit the 40S ribosomal subunit [150].

IRESs, first discovered in picornaviruses, are long mRNA elements that form stable secondary structures, which recruit and bind 40S ribosomes as well as translation initiation factors eIF3 and eIF5 [130]. These initiation factors can associate with Met-tRNA$^{Met}_{i}$, which allows initiation to proceed within infected cells where there is inactivation of eIF2 [130]. In some viral RNAs, IRESs have been shown to perform functions of host initiation factors, such as placing the initiator methionine tRNA on the P-site and guiding conformational changes of both 40S and 60S ribosomal subunits [150], bypassing the need for multiple host factors in order to initiate protein synthesis. IRES elements can also be found in some mammalian mRNAs, and have been shown to regulate translation when cap-depending translation initiation is impaired [83].

Another mode of translation regulation, more commonly seen in eukaryotic cells, happens in mRNAs that contain upstream open reading frames (uORFs). Reinitiation of translation due to uORFs plays a crucial role in allowing selective translation of stress-response genes when protein synthesis is inhibited [7, 19].

Translation reinitiation is a relatively inefficient process as it depends on the retention of multiple initiation factors on the 40S ribosome and reacquisition of a new initiator Met-tRNA after termination at the upstream open reading frame. It also depends on the length and translation efficiency of the uORF (higher chance of reinitiation after a ribosome translates shorter uORFs), distance of the uORF from the main ORF, Kozak sequence around the uORF and secondary structures within the leader sequence (5'UTR) [19].

mRNAs that evade global translation inhibition due to uORFs have now been reported in yeast, mice and humans. A well-studied example in mammalian cells is the transcription factor ATF4, which contains two uORFs in its 5'UTR [94]. Under normal conditions, the first uORF gets translated, resulting in a small peptide, and the large ribosomal subunit dissociates while the small subunit remains associated with mRNA and continues to scan to the second uORF. The ternary complex (eIF2 + Met-tRNA$^{Met}_{i}$) is recruited again by the small subunit at the second uORF, which overlaps with the main coding sequence (not in the same reading frame) [19]. Under conditions of translation inhibition, for example due to ER stress that leads to phosphorylation of eIF2, the translation of the second uORF is less likely due to limitations of the ternary complex, which in turn leads to increased reinitiation at the main ATF4 coding sequence [19].

Similarly, the well-studied yeast transcription factor GCN4 contains 4 uORFs in its 5'UTR that control its translation depending on the nutritional status of the cell [19, 116]. The first uORF is always translated regardless of nutritional conditions, while uORFs 2-4, which are predicted to have weak ribosome recognition sequences, are only translated when there is excess ternary complex available [116]. Translation of these uORFs under non-stress inducing conditions leads to low efficiency translation of the main ORF. Upon amino acid starvation, when there are limited initiation factors available, ribosomes will take longer to re-aquire a new ternary complex. uORF 1 is translated under such conditions but ribosomes will scan past uORF 2-4, allowing enough time to acquire a new ternary complex by the time they reach the main ORF. This allows selective translation of GCN4 under starvation conditions [116]. An intriguing question that remains is how uORF1 gets translated under such conditions and promote translation reinitiation at the main coding sequence. The 5' sequence upstream of uORF1 has been shown to interact with the N-terminal domain of initiation factor eIF3 [154]. This region of uORF1, termed 5' enhancer, contains reinitiation-promoting elements that form secondary structures ('double-circle hairpin and long bulged stem') that are predicted to facilitate translation past uORF1 [56, 154]. The 3' region of uORF1 on the other hand, is proposed to be AU-rich (60% AU content in the case of GCN4), to allow scanning to proceed after translation of this uORF [56]. The contribution of 3' nucleotide content for reinitiation is controversial, as it cannot explain how AU rich uORF2 and uORF3 control scanning [56]. However, the exact molecular details of reinitiation are still a great mystery.

A more recent study also showed that selective translation of stress-response genes is crucial for maintaining homeostasis when translation initiation is inhibited by chemical treatment (sodium arsenate treatment, which inhibits initiation factor eIF2-α) [7]. Under such conditions, it was demonstrated that mammalian cells favor selective translation of stress response regulators that contain upstream open reading frames in their 5' UTR [7]. The study confirmed that the 5' UTR of these resistant mRNAs was sufficient to allow bypass of translation inhibition when put upstream of a reporter gene, and mutations in their uORF sequences abolished selective translation when protein synthesis was inhibited [7].

Various other studies have highlighted the importance of such translation regulation by uORFs in mammalian cells [30]. Polymorphisms/mutations that alter these sequences have been linked to various human malignancies and metabolic disorders as they influence the protein expression levels of thousands of mammalian genes [19, 31].

1.8.2. Inhibition of translation elongation by bacterial toxins

Various bacterial pathogens target the highly conserved host elongation machinery as a virulence strategy. This is an effective strategy as there are only few host elongation factors and no alternative mechanisms to elongate polypeptide chains apart from the canonical pathway. Toxins such as *P. aeruginosa* Exotoxin A or *Corynebacterium diphtheriae* encoded diphtheria toxins modify elongation factor 2 (EF2) of eukaryotic cells by ADP-ribosylation [20, 46]. Shiga toxin (Stx) from *Escherichia coli* and Ricin toxin from castor beans on the other hand, are both ribosome-inactivating cytotoxic enzymes that specifically cleave *N*-glycosididc bonds [127]. These toxins inactivate 60S ribosomal subunit by cleaving a single adenine residue from 28S

ribosomal RNA (28S is the structural RNA of 60S subunit) [42]. The enzymatic cleavage prevents the EF-1A-aminoacyl-tRNA complex from binding to ribosomes [42]. In addition, Ricin has been reported to prevent binding of EF-2-GTP to ribosomes [127], leading to inhibition of translation elongation.

Interestingly, selective translation of stress response genes has been observed in the model organism *C. elegans* upon infection with *Pseudomonas aeruginosa* [108]. Inhibition of elongation factor 2 (EF2) in *C. elegans* intestinal cells by *Pseudomonas* ExoA leads to the selective translation of ZIP-2, which is required for activation of defense pathways and pathogen clearance [41]. Similarly, treatment with Shiga toxin or Ricin induces the ribotoxic stress response, where specific modifications to ribosomes leads to activation of the MAPK pathway [69]. There is rapid activation of JNK and P38 upon exposure to these toxins that leads to expression of pro-inflammatory cytokines and chemokines [69]. It has been reported that human monocytes treated with Shiga toxin respond by upregulating transcription of DUSPs, pro-inflammatory cytokines, such as TNF-α and IL-1β, as well as chemokines, such as IL-8 and MIP-1 [58, 134]. Co-stimulation of cells with Shiga toxin and LPS leads to translation and secretion of some of these pro-inflammatory cytokines and chemokines, albeit to a lesser extent than LPS treatment alone [58, 134]

It is clear that translation elongation-inhibiting toxins activate a strong innate immune and stress response pathways in host cells, including ribotoxic stress, the unfolded protein response and NF-κB dependent transcription and translation of inflammatory mediators. The mechanism by which our immune system senses such mode

of translation inhibition and initiates a successful response however remains to be determined.

1.9. Significance

Infection with *Legionella pneumophila* leads to a dramatic reduction in host protein translation. It is currently not clear how host cells are able to mount an inflammatory response when protein synthesis is blocked by *Legionella* both at the initiation and elongation stages [100, 147]. It is likely that pattern-recognition plays a role under conditions of intoxication, but the mechanism by which this is regulated is also unclear.

A strong pro-inflammatory cytokine response is crucial for clearance of *Legionella pneumophila* [20, 147]. The importance of cytokines can be seen in IL-1α, IL-12, IFN-γ and TNF knockout mouse strains that show increased susceptibility to *L. pneumophila* infection [20, 50]. Moreover, patients treated with TNF-α blockers are at high risk of developing severe Legionnaires' disease [95]. Given the key role that this innate immune response plays in clearance of *L. pneumophila*, we examined how cytokines and other immune mediators are synthesized under conditions in which the bacterium effectively blocks the host protein translation machinery.

We find that although protein synthesis inhibitors induce the transcriptional response and block the translation of most genes, the innate immune system has devised unique mechanisms to evade this blockade and initiate a successful inflammatory response.

Chapter 2: MATERIALS AND METHODS

2.1. Ethics Statement

This study was carried out in accordance with the recommendation in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee of Tufts University approved all animal procedures. Our approved protocol number is B2013-18. The animal work, which is limited to the isolation of macrophages, does not involve any procedures of infections of live animals.

2.2. Bacteria and culturing media

L. pneumophila strains Lp02 (referred to as WT) and Lp03 (referred to as *dotA3*) are streptomycin-resistant restriction-defective thymidine auxotrophs derived from *L. pneumophila* Philadelphia-1 (Lp01) (Table 1; [24]). The Δ5 and Δ5.Δ*flaA* strains were kindly provided by Zhao-Qing Luo (Purdue University) [46, 47, 132]. *ΔflaA*-GFP^{+}, *dotA3ΔflaA*-GFP^{+}, Δ5.Δ*flaA*-GFP^{+} carry GFP on an isopropyl-β-D-thiogalactopyranoside (IPTG)–inducible, Cm resistant plasmid (Table 1; [98, 144]). Solid medium containing buffered charcoal yeast extract (BCYE) and ACES-buffered yeast extract (AYE) broth culture medium supplemented with 100μg/mL thymidine were used to maintain *L. pneumophila* strains [44, 149]. Strains containing the pGFP plasmid were maintained on BCYE plates containing 100μg/mL thymidine and 5μg/mL chloramphenicol and grown in AYE containing 100μg/mL thymidine, 5μg/mL chloramphenicol and 1mM IPTG [98].

2.3. Eukaryotic cell culture

Bone marrow-derived macrophages (BMDMs) were isolated from the femurs of mice and allowed to proliferate as described [44, 149]. C57BL/6 *myd88*$^{-/-}$ femurs were

kindly provided by Tanja Petnicki-Ocwieja in the laboratory of Linden Hu (Tufts Medical Center). BMDMs were differentiated for 7 days in RPMI containing 30% L-cell supernatant, 10% FBS, 2mM L-glutamine and 1x Pen Strep (100U/mL penicillin, 100μg/mL streptomycin). Cells were lifted and either re-plated for experiments or quick-frozen for later use in FBS and 10% DMSO.

U937 cells (ATCC) were grown in RPMI supplemented with 10% FBS and 1 mM L- glutamine. For differentiation, cells were treated with 10 ng/ml 12-tetradecanoyl phorbol 13-acetate (TPA) for 48 hrs. For *L. pneumophila* infections, U937 cells were plated in fresh media without TPA and infections were carried out 12-16 hours after plating.

Table 2: Plasmids and strains used in this study

Plasmids/Strains	Genotype/relevant characteristics	Reference
pGFP	oriRSF101*cm*^{R}*ptac::*GFP^{+}	[98, 144]
pΔflaA	pSR47S*ΔflaA*	[37]
WT	Lp02, Philadelphia-1, *thyArpsLhsdR*	[24]
dotA3	Lp02 *dotA03* (Lp03)	[24]
ΔflaA	Lp02*ΔflaA* (allelic exchange using p*ΔflaA*)	[132]
dotA.ΔflaA	Lp03*ΔflaA* (allelic exchange using p*ΔflaA*)	[37]
Δ5	Missing 5 IDTS required for host protein translation inhibition	[46]
Δ5.ΔflaA	Lp02 background	[47]
ΔflaA-GFP	*ΔflaA* containing pGFP	This study
dotA.ΔflaA-GFP	*dotA3.ΔflaA* containing pGFP	This study
Δ5.Δ*flaA*-GFP	Δ5.Δ*flaA* containing pGFP	This study

2.4. Immunoblotting

To evaluate protein expression in host cells, C57BL/6 bone marrow-derived macrophages were plated in medium supplemented with 200 μg/mL of thymidine. Cells were challenged with *L. pneumophila* at the desired MOI, subjected to centrifugation at 1000 x g for 5 min and incubated at 37^{o} for the noted time periods. Lysates were collected using 2X SDS Laemmli sample buffer (0.125 M Tric-Cl pH 6.8, 4 % SDS, 20% glycerol, 10% beta-mercaptoethanol, 0.01 % bromophenol blue). Proteins were electroblotted to PVDF membranes, blocked in milk and analyzed by immunoprobing.

For phospho-specific antibodies, blots were washed of all milk and incubated overnight with 1:1000 phospho-p38 or phospho-JNK (Cell Signaling) in 5% BSA in phosphate buffered saline (PBS). Rabbit anti-DUSP1 (MKP1 V-15, Santa Cruz) and mouse anti-tubulin (sigma) antibodies were diluted to 1:200 and 1:7,500, respectively, in 5% milk in TBST. Goat anti-IL-1α and Goat anti-IL-1β antibodies (R&D systems, AF-400-NA & AF-401-NA) were diluted to 1:500 in 5% milk in TBST.

2.5. Heat Killed Yersinia, LPS, Pam3CSK4, 4EGI-1 treatment

For preparation of Heat-Killed Yersinia, wild type *Y. pseudotuberculosis* strains were grown overnight at 26^{o} in Luria-Bertani (LB) broth. Overnight cultures were heat killed at 60^{o} for 30-60min and aliquots were frozen at -80^{o} until use.

Macrophages were stimulated with HKY (MOI=50), LPS (Sigma, 0.1μg/mL or 1μg/mL) and Pam3CSK4 (Invitrogen, 2μg/mL), poly(I:C) (InvivoGen, 50μg/mL) for the desired time points. Cells were washed 3x with PBS and lysed in 2X SDS Laemmli

sample buffer. For initiation inhibition, macrophages were treated with either 25μM or 50μM of 4EGI-1 for the desired time points.

2.6. Quantitative RT-PCR

RNA was extracted from mammalian cells using RNAeasy kit (Qiagen). To determine the amount of a particular transcript, a one step, RNA-to-C_t kit (Applied Biosystems) was used according to manufacturer's instructions. Primers used for transcript analysis were as follows: human *Dusp1* (5' TTTGAGGGTCACTACCAG and 3' CCGCTTCGTAGTAGAG), mouse *Dusp1* (5'GGATATGAAGCGTTTTCGGCT and 3' ACGGACTGTCACGTCTTAGG), mouse *Il1α* (5'GCACCTTACACCTACCAGAGT and 3' TGCAGGTCATTTAACCAAGTGG), mouse *Il1β* (5' GCAACTGTTCCTGAACTCAACT and 3' ATCTTTTGGGGTCCGTCAACT), mouse *Tnf-α (5'* GCACCACCATCAAGGACTCAA and 3' GCTTAAGTGACCTCGGAGCT), mouse 18S ribosomal RNA (5' CGCCGCTAGAGGTGAAATTCT and 3' GCTTTCGTAAACGGTTCTTCA).

2.7. Determination of secreted cytokines

Macrophages were plated in 24 well tissue culture plates (2.5 x 10^5 per well) and challenged with *L. pneumophila* for either 6hrs or 24hrs. Supernatants were collected from each sample and 50μL was used for ELISA. Mouse IL-1α and IL-1β Platinum ELISA (eBiosciences) was used to measure cytokine levels according to the manufacturers manual.

2.8. Intracellular cytokine staining

Intracellular cytokine staining was performed as described before [5] with modifications. Differentiated macrophages (~1x10^7cells/plate) were washed and challenged with *L. pneumophila* GFP^+ strains in RPMI containing 200μg/mL thymidine, 5μg/mL Chloramphenicol and 1mM IPTG. For measuring TNF production, infections were carried out at MOI-3 and MOI-10 for 9hrs followed by Golgiplug incubation (BD Bioscience, 1μL/mL) for additional 5hrs. For IL-1α and IL-1β, infections were carried out for 6hrs. Cells were harvested with 10mL cold PBS, washed twice with FACS buffer (PBS+0.5%BSA+0.05%NaN), incubated with Fc Block (clone 2.4G2) for 20 minutes and fixed with 2% paraformaldehyde overnight at 4^o. Macrophage were permeabilized with Perm/Wash buffer (BD Bioscience) on ice for 20 minutes and stained with Alexa Fluor 647-conjugated anti-mouse TNF (BioLegend, clone ALF 161), phycoerythrin(PE)-conjugated anti-mouse IL-1α (BioLegend, clone MP6-XT22), PE-conjugated anti-mouse IL-1β (eBioscience, clone NJTEN3), Rabbit anti-DUSP1 (MKP1 V-15, Santa Cruz) and Goat anti-Rabbit Cy5 (Invitrogen) for 40 minutes. Stained cells were analyzed by BD LSR II flow cytometer.

2.9. Translation, pulse labeling and quantification.

To detect whole cell translation during defined timepoints, C57BL/6 bone marrow-derived macrophages were challenged with *L. pneumophila*-GFP^+ at MOI-10 for 2 hrs, followed by labeling cells at various timepoints with 50μM of L-azidohomoalanine (AHA) (Invitrogen) added to the culture medium. Cells were incubated for 1 hr (time course experiments) or for 4hrs (IL-1α co-staining experiments) to allow incorporation of AHA into growing polypeptide chains. At the end of each incubation period, cells were

washed with PBS, fixed with 4% paraformaldehyde for 15-20 min and left overnight in PBS. Incorporation of AHA was monitored by a Biotin- or APC-conjugated phosphine reagent (Pierce). For flow cytometry, fixed cells were blocked with 1x BSA/PBS for 30min at RT and 100μM of APC-phosphine was added. Cells were incubated at 37^{o} for 2-3hrs and excess dye was removed by washing with 0.5% Tween-20/PBS. For IL-1α co-staining, PE conjugated anti-mouse IL-1α (BioLegend, clone MP6-XT22) was added to cells on ice for 30min. Cells were analyzed by BD LSR II flow cytometer or by BD FACScalibur.

2.10. Puromycin incorporation (SunSET assay) and ELISA

SunSET assay was used to determine the kinetics of protein translation over time [138] with modifications. Macrophages were plated in 6 well plates and infected with Δ*flaA* for the desired time points. 10μg/mL of puromycin (Sigma) was added to cells for either 15min or 1hr. Macrophages were washed and lysed with IP Wash/Lysis buffer (Pierce) in the presence of protease inhibitors (Roche). Lysates were incubated on ice for 20min, centrifuged at 13,000 rpm for 5min and the supernatants were used for ELISA. The protein concentration in each sample was determined by Bradford assay.

ELISA plates were prepared by coating 96 well Nunc MaxiSorp plates with the desired antibody. Polyclonal Goat anti-IL-1α and IL-1β (R&D systems), Rabbit anti-DUSP-1 (Santa Cruz) and Rabbit anti-RhoGDI (Santa Cruz) antibodies were diluted in Carbonate/Bicarbonate buffer (PH=9.6) to 10μg/mL and 100μL was used per well. Plates were incubated overnight at 4°. The following day, plates were brought to room temperature and blocked with 0.5% BSA/PBS for 1hr. Cell lysates that have incorporated

puromycin were incubated on the ELISA plates for 2hrs, washed with 0.05% Tween-20/PBS three times and incubated with monoclonal mouse-anti-Puromycin (12D10, Millipore) for 1hr. Unbound antibody was washed exhaustively with 0.05% Tween-20/PBS and plates were incubated with Donkey anti-mouse-HRP secondary antibody. Unbound antibody was washed again with Tween-20/PBS four times and 100μL of HRP substrate (TMB solution) was added to each well for 5-10mins. The reaction was stopped by adding 100μL of stop-solution (2N H_2SO_4), and absorbance was measured at 450 nm.

2.11. Identification of newly synthesized proteins by click chemistry and mass spectrometry

C57BL/6 WT and MyD88$^{-/-}$ bone marrow-derived macrophages were incubated in methionine free media (RPMI no Met, Invitrogen) and challenged with *L. pneumophila* Δ*flaA*-GFP^{+} at MOI=15 for 4 hrs. 100μM of L-azidohomoalanine (AHA) was added to the media and cells were incubated for 2hrs, washed with regular RPMI (with methionine) and collected in cold PBS. Cells were then sorted (~5x10^{6} GFP+ macrophages) and flash frozen in liquid nitrogen.

Cells were thawed in lysis buffer (1% SDS in 50mM Tris-HCL, pH 8, 1x protease inhibitors), treated with Benzonase and incubated on ice for 15-30min. Lysates were vortexed for 5min and clarified by centrifugation at 16,000g at 4^{o} for 5min. Supernatants were transferred to a clean tube and used for click reactions.

For pulling down newly synthesized proteins, ClickIT protein enrichment kit (Invitrogen) was used according to the manufacturers instructions. For setting up click

reaction (covalent linkage between azidohomoalanine (AHA) and alkyne), 400 µL lysates and 500µL of 2X catalyst solution were added to the alkyne resin slurry and rotated at room temperature for 20hrs (18-20hrs recommended for on bead click reaction). Samples were centrifuged for 1min at 1000g and supernatants were removed.

To reduce and alkylate bead-bound proteins, the resin was washed with 900µL of ddH_20 (to prevent clumping due to residual lysis buffer and SDS wash buffer) and 500µL SDS wash buffer/ 10µL of 1M DTT was added to each sample. Samples were vortexed briefly, incubated at 70^o for 15minutes and cooled down to room temperature for another 15mins. 500µL of iodoacetamide solution (3.7mg of iodoacetamide dissolved in 500µL SDS wash buffer) was added per enrichment reaction and samples were incubated in the dark for 30 minutes. The resin was washed multiple times with SDS wash buffer and 8M urea/20% acetonitrile for stringent removal of non-specifically bound proteins (12+ wash steps following the protocol provided in Invitrogen Click enrichment kit).

For on bead-trypsin digest, 500µL of digestion buffer was first added to each sample (100mM Tris, 2mM CaCl2, 10% acetonitrile), resin was transferred to a clean tube, centrifuged for 5min at 1000g and ~400µL of digestion buffer was removed leaving 100µL buffer for trypsin digest. 5µL of 0.1µg/µL trypsin was added to the resin, vortexed briefly and incubated at 37^o overnight.

To stop the reaction, samples were washed with water and acidified with 2µL of TFA. Digested peptides were identified by LC-MS/MS at Taplin Mass Spec institute. For peptide identification, we included dynamic modification parameters on methionine residues to specifically identify peptides that show mass gain over methionine due to AHA incorporation. If a methionine residue was replaced by the analog, a mass gain of

107 AMU is expected whereas in the absence of such replacement, the mass of methionine residue would stay the same. We accounted for both possibilities during peptide identification.

For data analysis, we excluded proteins that had less than 2 valid peptides per locus and accounted for unspecific binding to the alkyne resin (association to the alkyne resin without direct covalent attachement to the alkyl group) by excluding peptides that came up in the negative control (No AHA control- generated from cells that were not incubated with the methionine analog but treated similarly during alkyne ligation and mass spec analysis). We also cross-referenced the remaining hits to CRAPome database (Contaminant Repository for Affinity Purification, [109]) in order to remove previously identified mass spec contaminants.

2.12. RNA-Seq

WT and MyD88$^{-/-}$ BMDMs were infected with *L. pneumophila*-Δ*flaA*-GFP at MOI=15 and cells harboring bacteria were sorted by flow cytometry. RNA was extracted from sorted cells, DNase treated (Turbo DNA-free kit, Invitrogen) and used for generating RNA-Seq library using TruSeq stranded total RNA library prep kit (Illumina). cDNA fragments from the library were sequenced by Illumina HiSeq 2000 (150bp, single end reads).

Data analysis was performed on Galaxy server hosted by Tufts University Core Facility Genomics (TUCF) [52]. Sequenced reads were aligned to mouse reference genome (mm9) using TopHat [153], which uses Bowtie as an alignment engine. For determining gene expression levels, mapped reads were counted by Cufflinks [154].

Reads per Kilobase of Transcript per Million mapped reads (RPKM) values determined by Cufflinks were used to compare genes expression levels across samples.

2.13. Ribosome profiling

ARTseq Ribosome profiling library kit (50picenter) was used for generating ribosome profiling library with major modifications. C57BL/6 BMDMs were infected with *L. pneumophila* Δ*flaA* at MOI=15 for 6hrs or left uninfected. Cells were lifted with cold PBS, washed once with cold PBS containing 100µg/mL cycloheximide (CHX) and spun down at 1000rpm for 3min. Pelleted cells were resuspended in 1mL lysis buffer (1X Mammalian Polysome Buffer, 1% Triton X-100, 1mM DTT, 100 µg/mL CHX and 1X protease inhibitors). Samples were passed through 25G needle 5-6 times, incubated on ice for 10min with periodic inversions and spun down at maximum speed (16,000g at 4^{o}). The supernatant was collected in sterile cold 1.5mL eppendorf tubes and flash frozen in liquid nitrogen. Samples were frozen in -80 until fractionation.

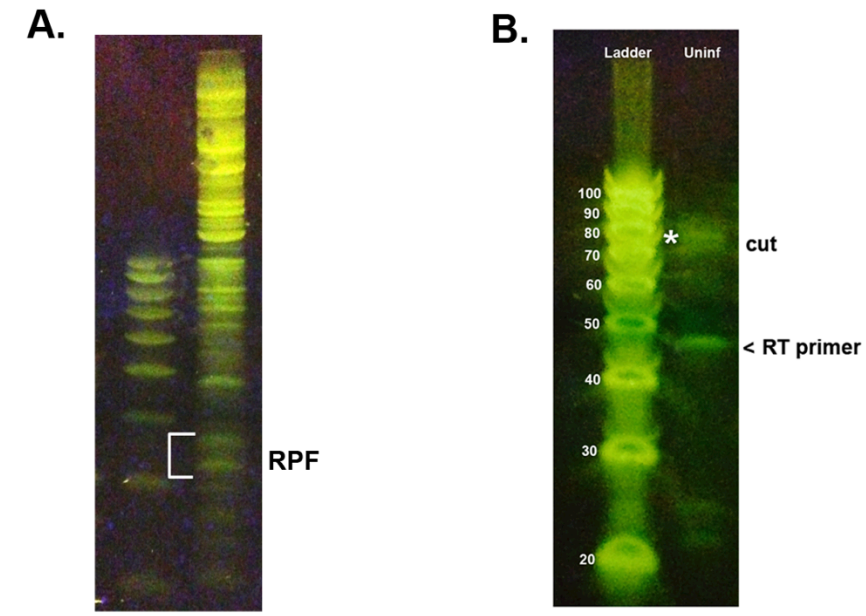
For generating ribosome-protected fragments, samples were treated with 1,000 U of Rnase T1and incubated at room temperature for 1hr. The reaction was stopped by adding SUPERase In Rnase inhibitor (Invitrogen), samples were slowly loaded onto sucrose gradients (10-50% wt/vol) and subjected to centrifugation at 35,000 RPM for 3hrs at 4^{o} in a SW41 rotor. Gradients were fractionated using Brandel gradient fractionation system coupled with Biorad UV absorbance detector and monosome fractions were collected (for digested samples).

Collected monosome fractions were concentrated to ~50µL using 100 kDa Amicon filters and footprints were extracted by hot Acid-Phenol:Chloroform, pH 4.5

(Invitrogen) followed by ethanol precipitation using 0.3M Sodium Acetate (pH 5.5) and Glycoblue. RNA pellets were solubilized in 10μL ddH_2O and analyzed on 15% Urea-polyacrylamide gel (180V for 70min). The bands between ~24-36 nt were extracted from the gel and RNA was isolated overnight following Epicenter Ribosome profiling (ARTseq) protocol. End repair, 3' adaptor ligation, reverse transcription, PAGE purification and circularization of cDNA, PCR amplification and gel purification were all performed as described by the ARTseq manual (expect for RNA precipitation steps, which were all done using ethanol precipitation).

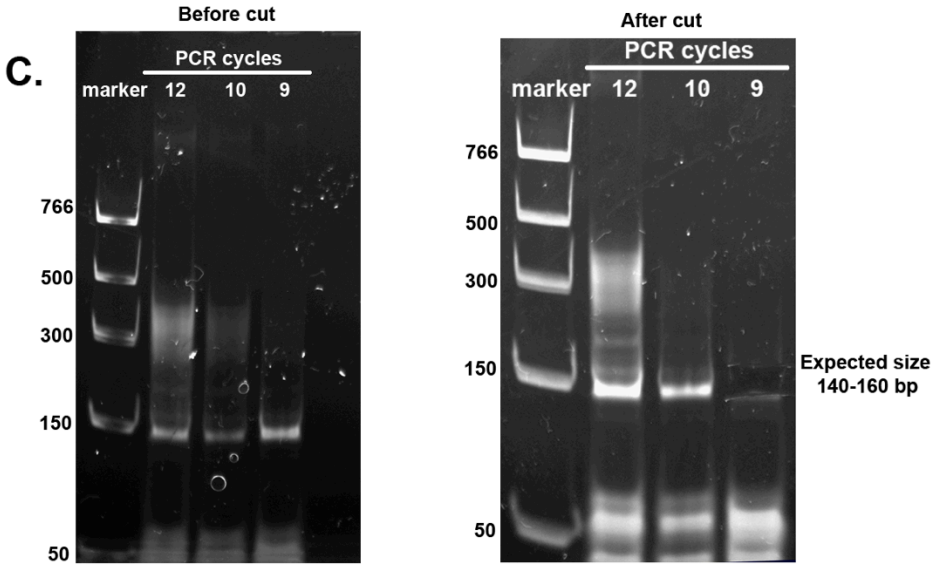
Figure 2.1: Ribosome profiling gels from library preparation.

A) After RNA extraction from purified monosomes, samples were run on 15% Urea-polyacrylamide gel to purify ribosome-protected fragment (26-34bp fragments extracted from gel). B) After reverse transcription, samples were run on 10% Urea-polyacrylamide gel and 70-80 bp cDNA was extracted. C) For optimization of the final PCR amplification step, we looked at products from 9, 10 and 12 PCR cycles run on 8% Natvie-PAGE. Over-amplification (10 and 12 cycles) led to appearance of higher, smeared amplicons. Library prepared from 9 PCR cycles was extracted and used for Illumina sequencing.



15% Urea-Polyacrylamide gel after RPF extraction

10% Urea-Polyacrylamide gel after RT



8% Native PAGE -TBE gel after library prep.

Chapter 3: RESULTS

The frustrated host response to Legionella pneumophila is bypassed by MyD88-dependent translation of pro-inflammatory cytokines

The results described in this chapter here have been published previously in Asrat, S., A.S. Dugan, and R.R. Isberg, *The frustrated host response to Legionella pneumophila is bypassed by MyD88-dependent translation of pro-inflammatory cytokines*. PloS Pathog, 2014. 10(7): p. e1004229.

Experiments 3.1, 3.2 and 3.3 were performed by Aisling Dugan.

3.1. *L. pneumophila* translation inhibitors induce a frustrated MAPK response.

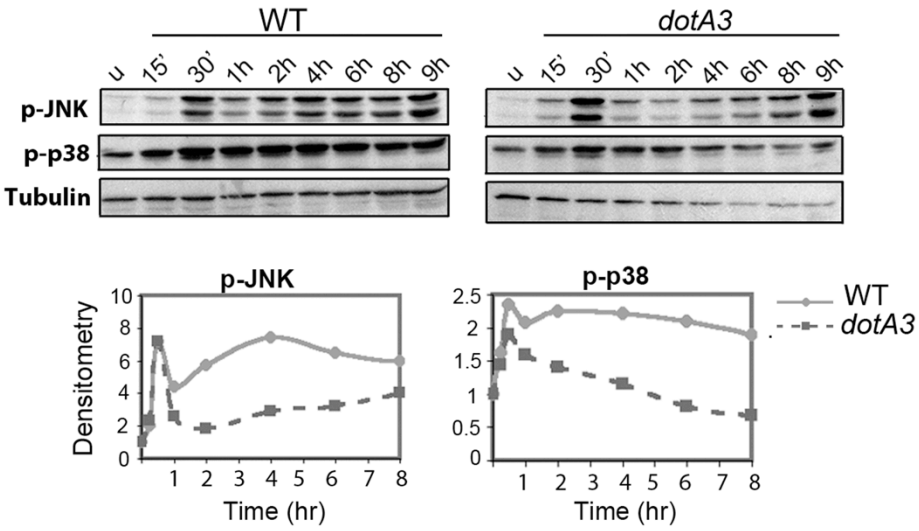
Infection with *Legionella pneumophila* leads to robust induction of innate immune genes, including as pro-inflammatory cytokines, chemokines and MAPK regulators [95, 98]. We hypothesized that *Legionella*-induced inflammatory gene transcription will be largely inconsequential, as the transcribed genes cannot be translated to proteins due to the bacterium-derived translation inhibitors. To test this hypothesis, we examined mammalian host protein synthesis predicted to be downstream of MAPK activation following exposure to *L. pneumophila.*

Bone marrow-derived macrophages challenged with wild type *L. pneumophila* showed phosphorylation of MAPK members shortly after exposure to the bacterium, with the activation kinetics being almost identical to previous observations ([95, 143, 164]; 3.1). In the first hour after *L. pneumophila* challenge, activation of JNK and P38 was independent of the Icm/Dot system, consistent with phosphorylation being the result of Tlr engagement [143]. A second wave of activation was observed beginning at two hours after challenge. This was dependent on the presence of the *L. pneumophila* type IV secretion system, as the levels of MAPK phosphorylation decayed when macrophages were challenged with the *dotA3* mutant that lacks the Icm/Dot system (Fig. 3.1). This two-wave activation, reflecting an early Tlr-dependent and a later *L. pneumophila*-specific response, mirrors our previous observations with NF-KB activation [97].

Figure 3.1: *L. pneumophila* induces an early TLR-dependent and a later Icm/Dot-dependent activation of MAPK members.

Experiments performed by Aisling Dugan [13].

A/J macrophages were infected with wild type *L. pneumophila* or *dotA3* mutant for indicated time points. Cell lysates were blotted for phosphorylated forms of JNK (p-JNK) and p38 (p-p38). Lower graphs show densitometry of p-JNK and p-p38 normalized to tubulin. Data are representative of at least three independent experiments.



Cultured macrophages challenged with wild type *L. pneumophila* transcriptionally activate a number of dual specificity phosphatase (DUSP) genes, dependent on an intact Icm/Dot system [95, 98]. Bone marrow-derived macrophages challenged with wild type *Legionella* for 4hrs showed significant induction of the *Dusp1* transcript compared to *dotA3* infection (Fig.3.2). The transcriptional induction was much higher (over 60 fold) in U937 human monocytes, the cell lines in which *dusp1* induction in response to wild type *Legionella* was first characterized (Fig. 3.3) [98]. This transcriptional response, however, was not accompanied by translation either in bone marrow-derived macrophages (Fig.3.2) or U937 cells (Fig 3.3) as DUSP-1 protein levels remained unchanged over the course of infection. Our inability to observe enhanced protein synthesis was not due to limitations with our detection system, because we observed a robust increase in DUSP-1 protein levels in response to the addition of LPS (Fig. 1C).

Presumably the multiple translocated substrates that inhibit translation elongation [46] frustrate the transcriptional response, preventing translation of induced genes. To test this hypothesis, we challenged cells with an *L. pneumophila* mutant missing five of the translation inhibitors (Δ*5*) to determine if the absence of these proteins could allow translation to proceed. Instead, we observed no transcriptional activation of the *Dusp1* gene in response to this mutant (Fig3.3) Therefore, induction of genes in the MAPK pathway and frustration of the response are tightly coupled.

Figure 3.2: *L. pneumophila* translation inhibitors induce a frustrated MAPK response.

Experiments performed by Aisling Dugan [13].

(A) *Dusp1* transcript levels in A/J macrophages infected with wild type or *dotA3 L. pneumophila* for 4hrs. Transcript levels were normalized *to* 18S ribosomal RNA (18S) and graphed as a fold increase over uninfected controls. (B) Immunoblot analysis of DUSP-1 protein levels in A/J macrophages challenged with wild type or *dotA3* strains and (C) treated with LPS (0.1μg/mL or 1μg/mL) for indicated time points. Data are representative of at least three independent experiments.

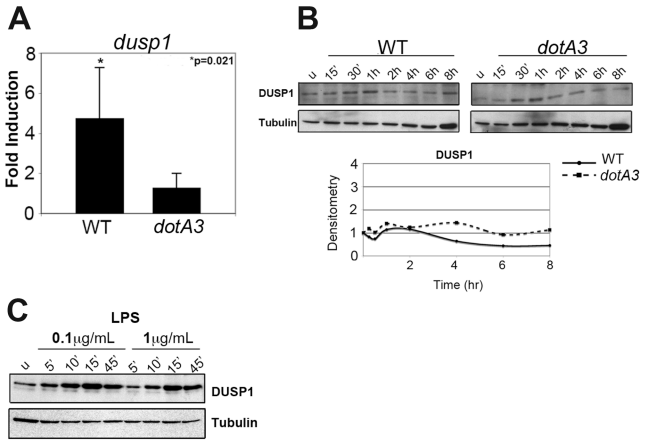
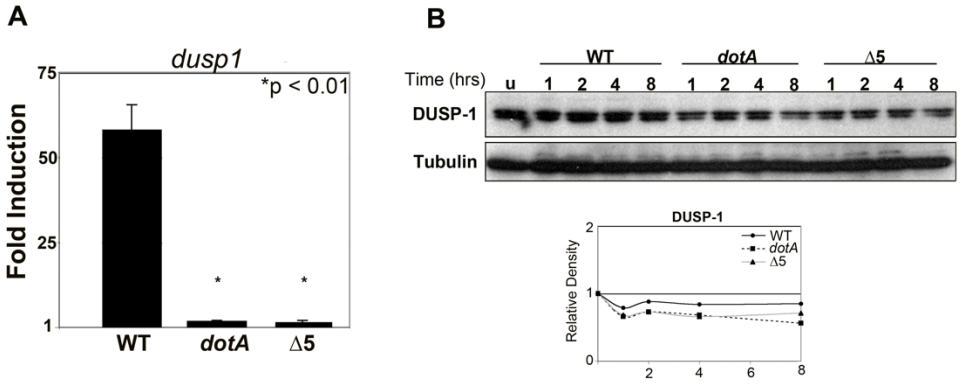


Figure 3.3: Δ5 strains fail to induce transcription of DUSP-1 in U937 human monocytes.

Experiments performed by Aisling Dugan [13].

(A) U937 cells were challenged with wild type *L. pneumophila, dotA* or Δ5 mutants for 4hrs and RNA was isolated from cells. *Dusp1* transcript levels were normalized to the housekeeping genes hydroxymethylbilane synthase (HMBS) and graphed as a fold increase over uninfected controls. (B) A time course analysis of DUSP1 protein levels in U937 cells infected with wild type, *dotA* or Δ5 strain of *L. pneumophila.*



3.2. A subset of cytokine transcripts are selectively translated following *L. pneumophila* infection.

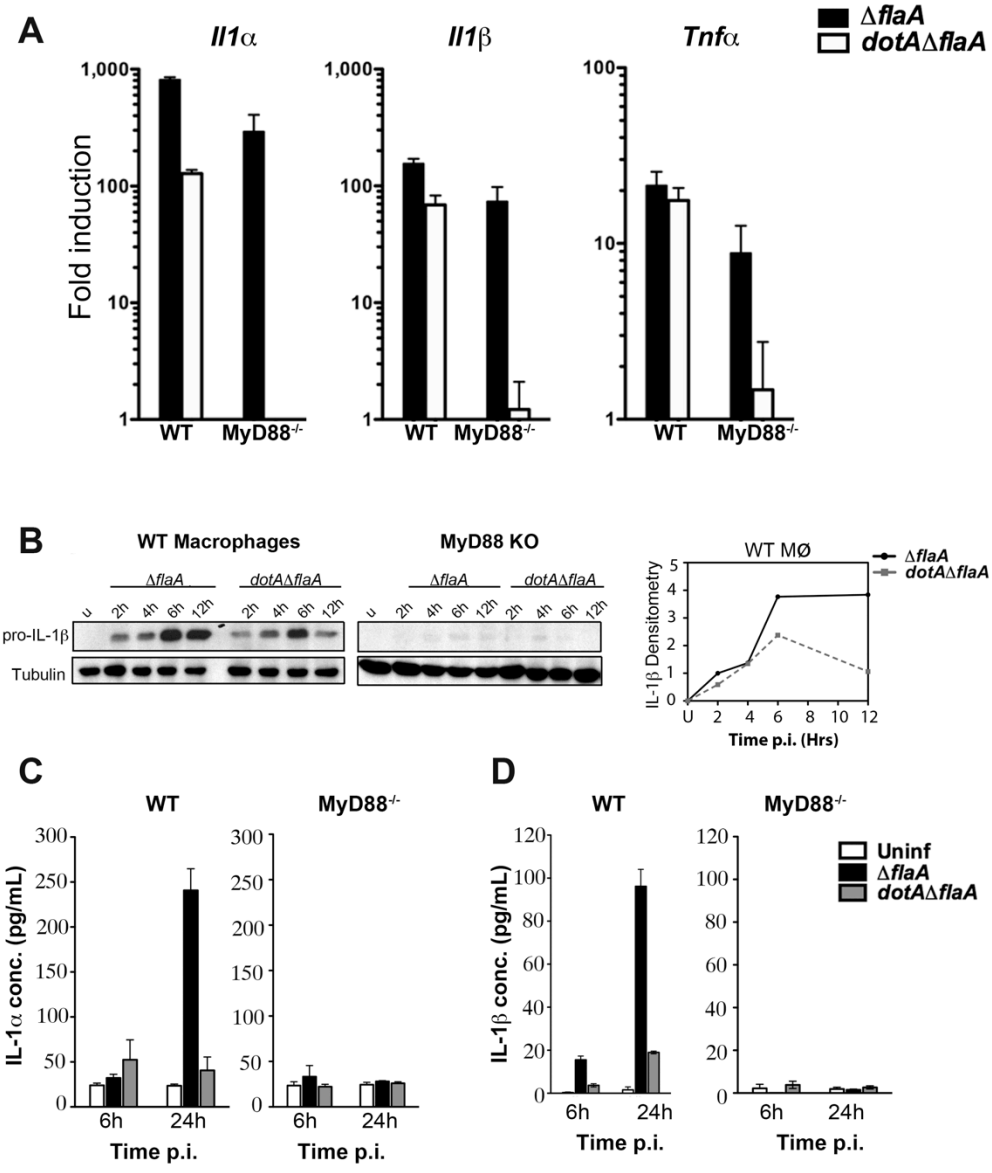
We then asked if the response leading to transcriptional up-regulation of pro-inflammatory cytokine genes was similarly affected by translational inhibition. To understand how cytokines are regulated in response to *L. pneumophila*, we measured the transcription and translation of selected pro-inflammatory cytokines in C57/Bl6 (B6) macrophages following *L. pneumophila* challenge. Flagellin deficient (*ΔflaA*) mutants were used in these experiments to avoid Caspase 1-dependent cell death downstream from NAIP5/NLRC4 recognition of flagellin by B6 macrophages [40, 132, 166].

Infection of bone-marrow macrophages with virulent (Dot^+) *L. pneumophila* induced *Il1α*, *Il1β* and *Tnfα* transcripts by 6hrs post infection (Fig.3.4 A). As previously reported, the cytokine response to Dot^+ was comprised of MyD88-dependent signaling that is layered on top of MyD88-independent, effector mediated signaling (Fig 3.4, black bars) [46]. The response to the avirulent, *dotA3* mutants on the other hand, was mostly dependent on MyD88 signaling (Fig.3.4 A , white bars) [143].

To determine if the transcribed cytokine mRNAs were efficiently translated and secreted during infection, WT and $MyD88^{-/-}$ macrophages were challenged with *L. pneumophila* and cytokine protein levels were measured by western blot and ELISA. Contrary to what we saw for DUSP-1, challenge with *L. pneumophila* Dot^+ led to a significant increase in cell-associated pro-IL-1β levels after 4hrs of infection (Fig. 3.4 B).

Figure 3.4: A subset of transcripts can bypass translation inhibition exerted by *L. pneumophila* effectors.

(A) C57BL/6 wild type and MyD88$^{-/-}$ macrophages were infected with indicated *L. pneumophila* strains at MOI-15. Cytokine and DUSP transcripts were analyzed at 6hrs post infection by qRT-PCR. Results shown are pooled from at least four independent experiments and represent the mean fold induction and SEM of samples relative to uninfected controls. (B) Immunoblot analysis of IL-1β precursor in WT and MyD88$^{-/-}$ macrophages infected with virulent *L. pneumophila* (*ΔflaA*) or avirulent mutant *dotAΔflaA* for indicated time points. Graphs on the right show densitometry of IL-1β normalized to tubulin. (C and D) WT and MyD88$^{-/-}$ macrophages were challenged with indicated *L. pneumophila* strains for 6 and 24 hrs at MOI-15 and cytokine levels were measured in culture supernatants by ELISA. Data represent mean and SEM of samples from 3 independent experiments.


A
Il1α
Il1β
Tnfα
ΔflaA
dotAΔflaA
Fold induction
1,000
100
10
1
WT
MyD88-/-
B
WT Macrophages
MyD88 KO
ΔflaA
dotAΔflaA
u
2h
4h
6h
12h
pro-IL-1β
Tubulin
WT MØ
IL-1β Densitometry
Time p.i. (Hrs)
C
D
IL-1α conc. (pg/mL)
IL-1β conc. (pg/mL)
6h
24h
Time p.i.
Uninf

IL-1α and IL-1β mature forms could also be detected in culture supernatants after 24 hrs (Fig. 3.4 C & D). Interestingly, challenge of macrophages with *L. pneumophila dotA3* mutants accumulated pro-IL-1β transiently, with steady state levels reduced by 12 hr post infection (Fig. 3.4 B densitometry), but this was not sufficient to induce the release of mature IL-1β. This is consistent with the hypothesis that in addition to TLR signaling, Icm/Dot translocated substrates are required for persistent pro-inflammatory cytokine activation and secretion [48].

Surprisingly, cytokine translation was severely diminished in MyD88$^{-/-}$ macrophages in response to wild type *L. pneumophila* (Fig. 2B) despite the presence of large amounts of transcripts (Fig. 3.4 A). Therefore, a MyD88-dependent signal appears necessary to bypass the translation block induced in response to wild type *L. pneumophila.*

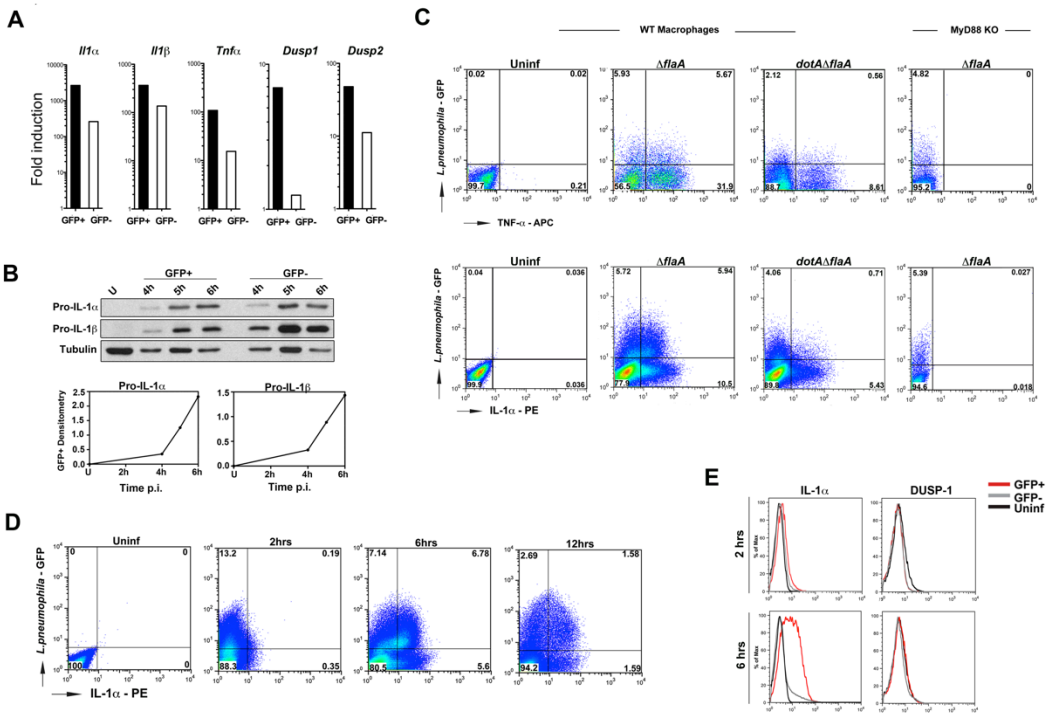
3.3. Intracellular cytokine accumulates in infected, translation-blocked macrophages.

There is no clear model for why the presence of MyD88 allowed bypass of the translation block. Engagement of MyD88 on the host cell surface may lead to selective bypass of translation inhibition on a subset of transcripts. Alternatively, translation of cytokine transcripts could largely occur in neighboring uninfected cells that have not been directly injected with *L. pneumophila* translocated proteins, but which have been activated by bacterial fragments liberated by infected cells. We therefore asked if the observed cytokine translation was derived from neighboring bystander cells.

B6 macrophages were challenged with *L. pneumophila*-GFP strains and macrophages harboring bacteria were sorted from uninfected bystanders. Cytokine transcripts (Fig.3.5 A) and protein levels (Fig. 3.5 B) were measured in each population by qRT-PCR and Western blots. To ensure accumulation of TNF-a protein, cells were treated with GolgiPlug (Brefeldin A; Materials and Methods) to prevent secretion of this cytokine. No such treatment was necessary for IL-1α and IL-1β, which accumulate as precursors via an alternate secretion pathway [136].

Relative to cells that had never been exposed to bacteria, challenge of macrophages with *L. pneumophila* resulted in high levels of *Il1α*, *Il1β*, *Tnfα* and *Dusp2* transcripts in both infected (GFP^+) and neighboring uninfected (GFP^-) populations by 4hrs post infection, (Fig.3.5 A). Depending on the cytokine, the amount of transcription in the bystander cells varied from 10% -30% of that observed in the cells harboring bacteria. *Dusp1* on the other hand, was mainly transcribed in GFP^+ cells (Fig. 3.5 A). More importantly, despite the presence of the translocated protein synthesis inhibitors, macrophages harboring bacteria were able to produce high levels of pro-IL-1α and pro-IL-1β (Fig. 3.5 B, GFP^+ cells). The kinetics of pro-IL-1α and pro-IL-1β production in infected cells indicated that there was enhanced accumulation of these proteins between 4-6hrs post-infection (Fig. 3.5 B, bottom panel). We will show that during this time window, *L. pneumophila* translation inhibitors effectively block most protein synthesis in infected cells (below, Fig. 3.9B).

Figure 3.5: Cytokines are produced from both infected and bystander macrophages. (A) B6 macrophages were challenged with *ΔflaA*-GFP at MOI-15 for 4hrs and sorted by Flow Cytometry. Cytokine and *Dusp* transcripts were measured in both GFP^+ and GFP^- population by qRT-PCR. (B) B6 macrophages were infected with *ΔflaA*-GFP at MOI-15 for indicated times and levels of IL-1β were measured by Western blot. Bottom graphs indicate densitometry of IL-1β in GFP^+ cells normalized to tubulin. (C) WT and MyD88 knockout acrophages were infected with indicated *L. pneumophila* strains at MOI-10 and intracellular cytokine levels were measured by flow cytometry. Top panels show TNF levels at 14 hrs post infection. To prevent secretion of TNF, cells were treated with Golgiplug (Brefeldin A) for 5hrs before samples were collected. Bottom panels show intracellular IL-1α levels at 6hrs post infection. Data shown are representative of at least 4 independent experiments. (D) Time course analysis of intracellular IL-1α levels in *ΔflaA*-GFP infected macrophages and (E) Comparison of intracellular IL-1α and DUSP1 levels in B6 macrophages infected with *ΔflaA*-GFP for 2 and 6hrs. Infected and uninfected (bystander) cells were gated based on GFP signal and protein levels were compared to control macrophages that were left untreated. Red lines indicate GFP^+ population; grey lines indicate GFP^- population and black lines show untreated macrophages.


A
Il1α
Il1β
Tnfα
Dusp1
Dusp2
Fold induction
GFP+ GFP-
B
GFP+
GFP-
U
4h
5h
6h
Pro-IL-1α
Pro-IL-1β
Tubulin
GFP+ Densitometry
Time p.i.
C
WT Macrophages
MyD88 KO
Uninf
ΔflaA
dotAΔflaA
L.pneumophila - GFP
TNF-α - APC
IL-1α - PE
D
2hrs
6hrs
12hrs
E
IL-1α
DUSP-1
2 hrs
6 hrs
GFP+
GFP-
Uninf

The presence of persistent cytokine synthesis in infected cells was confirmed by intracellular cytokine staining. B6 WT and MyD88$^{-/-}$ macrophages were challenged with *L. pneumophila*-GFP strains and intracellular cytokine levels were measured by flow cytometry. TNF-α was produced by both macrophages bearing bacteria (GFP^{+}) and bystander cells (GFP^{-}) after challenge with *L. pneumophila* Δ*fla*, while the *dot*Δ*fla* strain induced much lower levels of this cytokine (Fig. 3.5 C, top panel 2nd and 3rd boxes). Cells harboring bacteria (GFP^{+}) were a significant source of IL-1α (Fig. 3.5 C, bottom panel, 2nd box). Approximately 50% of the cells harboring bacteria showed detectable accumulation of IL-1α (Fig. 3.5 C, bottom panels, 2nd box), while bacteria were associated with approximately 33% of the IL-1α -producing cells. Consistent with Fig. 3.4 B, translation of IL-1α and TNF-α were both dependent on MyD88 signaling, and accumulation of IL-1α in infected cells was dependent on the presence of the Icm/Dot translocator (Fig. 3.5 C, two rightmost boxes in each panel).

Time course analysis of intracellular IL-1α levels using flow cytometry confirmed that the highest level of IL-1α accumulation occurred between 2-6hrs post infection in the GFP^{+} population (Fig. 3D and 3E). During this time period, the number of cells bearing bacteria that accumulated IL-1α increased from 1% of this population to approximately 50% (Fig. 3.5 D). DUSP-1 protein levels on the other hand, remain unchanged between 2-6hrs (Fig. 3.5 E).

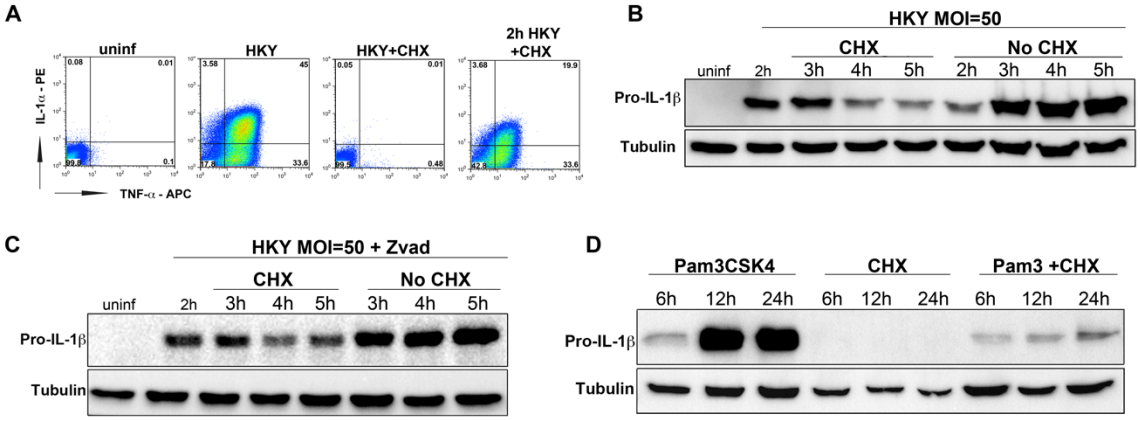
3.4. Cytokine response to chemical inhibitors of protein translation

The two signals that are received by mammalian cells during *L. pneumophila* infection (1st signal from TLR activation and 2nd signal from protein translation inhibition) synergize to induce the full cytokine response against the bacterium. It was previously reported that pharmacological inhibitors of host protein translation induce transcription of various stress response genes and cytokines such as IL-6, IL-23, IL-α and IL-1β [20, 46]. We wanted to confirm that translation and secretion of these cytokines could always bypass translation inhibition using the protein synthesis inhibitor cycloheximide (CHX). CHX interferes with protein translation elongation by binding to the E-site of the 60S ribosomal subunit and preventing tRNA translocation [139] .

Macrophages were treated with heat-killed *Yersinia* (HKY) to induce TLR signaling, together with 10μg/mL of cycloheximide. Addition of the chemical inhibitor at the same time as HKY led to a complete inhibition of TNF-α and IL-1α production in bone-marrow macrophages (Fig. 3.6 A). Contrary to what we observed during *L. pneumophila* infection, addition of CHX dampened the signal received from TLR stimulation (Fig. 3.6 A,B). Surprisingly, even at low concentrations of CHX that permit significant levels of protein translation, CHX was still able to inhibit IL-1β translation (Fig 3.6 B). To rule out the possibility that the reduction in IL-1β levels during CHX treatment was due to cell death, we lowered the CHX dose to 0.5 μg/mL (inhibits less than 50% of total host protein synthesis) and also incubated the cells with the apoptotic inhibitor Z-VAD-FMK (pan-caspase inhibitor). CHX was still able to inhibit IL-1β under these conditions, although it was clear that increased survival of cells was accompanied by higher accumulation of HKY-induced cytokine (Fig. 3.6 C).

Figure 3.6: The elongation inhibitor cycloheximide blocks cytokine translation independent of cell death.

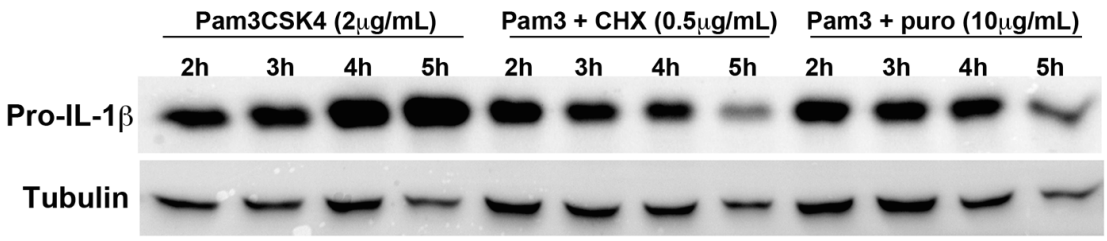
(A) B6 macrophages were either left untreated (1st box), treated with heat killed *Yersinia* at MOI=50 to induce cytokine expression (2nd box), treated with HKY MOI=50 and 10μg/mL cycloheximide (CHX) (3rd box) or treated with HKY MOI=50 for 2hrs followed by addition of 10μg/mL CHX (4th box). X-axis represents intracellular TNF-α levels and Y-axis represents intracellular IL-1α levels. (B) Macrophages were pre-stimulated with heat killed *Yersinia* at MOI=50 for 2hrs. Cells were then treated with either 1μg/mL cycloheximide or left untreated (HKY alone). IL-1β protein levels were measured by Western blot at the indicated time points. (C) B6 macrophages were pre-stimulated with heat killed *Yersinia* at MOI=50 in the presence of Pan-Caspase inhibitor (Z-VAD-FMK) for 2hrs and treated with either 0.5 μg/mL cycloheximide or left untreated. IL-1β protein levels were measured by Western blot for the next 3hrs. (D) Macrophages were treated with 2μg/mL Pam3CSK4 for 2 hrs in the presence of Z-VAD-FMK. CHX (0.5 μg/mL) was added to cells at 2hrs post Pam3CSK4 treatment and IL-1β protein levels were measured at 6, 12 or 24 hrs. Data is representative of at least 3 independent experiments.


A
uninf
HKY
HKY+CHX
2h HKY +CHX
IL-1α - PE
TNF-α - APC
B
HKY MOI=50
CHX
No CHX
uninf 2h 3h 4h 5h 2h 3h 4h 5h
Pro-IL-1β
Tubulin
C
HKY MOI=50 + Zvad
CHX
No CHX
uninf 2h 3h 4h 5h 3h 4h 5h
Pro-IL-1β
Tubulin
D
Pam3CSK4
CHX
Pam3 +CHX
6h 12h 24h 6h 12h 24h 6h 12h 24h
Pro-IL-1β
Tubulin

MyD88-dependent stimulation of mouse macrophages in response to *L. pneumophila* primarily occurs via Toll-like receptor 2 (TLR2) [9]. A recent report was able to reconstruct the cytokine induction seen during *L. pneumophila* infection by using the synthetic TLR-2 ligand Pam3CSK4 in combination with Exotoxin A (Exo A), a toxin from *Pseudomonas aeruginosa* that interferes with translation elongation [20]. Based on this observation, we wanted to determine if specific activation of TLR2 is what leads to cytokine translation in the presence of protein synthesis inhibitors. Macrophages were treated with the TLR2 agonist Pam3CSK4 and pro-IL-1β levels were measured in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). Drug treatment after addition of the TLR2 agonist led to a large reduction in pro-IL-1β levels (Fig. 3.6 D). We also observed a failure to hyperstimulate pro-IL-1b in the presence of another protein elongation inhibitor, puromycin (Fig. 3.7). Similar results were obtained when macrophages were stimulated with another TLR2 agonist lipoteichoic acid (LTA), or a TLR4 agonist LPS, followed by addition of CHX (data not shown). This indicates that the selective synthesis of cytokines may result from host cells sensing a specific mode of protein synthesis inhibition. It is also possible that the selective synthesis of pro-inflammatory cytokines is triggered by a block in translation initiation [71] instead of translation elongation, which would explain why the elongation inhibitors cycloheximide or puromycin were not able to induce the response.

Figure 3.7: Both cycloheximide and puromycin block IL-1β hyperstimulation.

Macrophages were treated with 2μg/mL Pam3CSK4 for 2 hrs. Cells were then treated with either cycloheximide (0.5 μg/mL) or puromycin for additional 3hrs and pro- IL-1β protein levels were measured by westernblot.

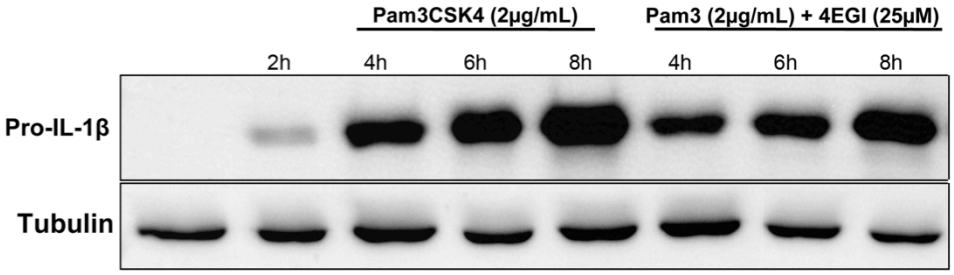


To determine if pro-inflammatory cytokines can be produced when translation initiation is blocked, we treated macrophages with 4EGI-1, a compound that blocks eIF4E:eIF4G interaction and prevent cap-dependent translation inhibition [110]. Accordingly, C56BL/6 BMDMs were stimulated with Pam3CSK4 for 2hrs and treated with 4EGI-1 for the next 6hrs. Unlike what was seen for cycloheximide and puromycin, treatment with the initiation inhibitor 4EGI did not block pro-IL-1β production (Fig. 3.8), suggesting that this cytokine is resistant to translation initiation inhibition.

Taken together, our results suggest that pro-inflammatory cytokines such as IL-1α and IL-1β are susceptible to elongation inhibition, but can be produced under condition were cap-dependent translation initiation is blocked.

Figure 3.8: Treatment of BMDMs with the initiation inhibitor 4EGI-1 does not block IL-1β production.

C57BL/6 macrophages were treated with 2μg/mL Pam3CSK4 for 2 hrs. Cells were then treated with either left untreated or incubated with 25μM of 4EGI-1 for additional 6hrs and pro- IL-1β protein levels were measured by westernblot.



3.5 Translation of IL-1 takes place in the presence of highly depressed host cell translation.

We considered two possible explanations for how cytokines were getting translated after protein synthesis was inhibited by *Legionella*: (1) translation inhibition by *L. pneumophila* is not efficient, allowing most of the cytokine to be synthesized prior to a complete block; or (2) the host preferentially translates a subset of genes after protein synthesis is shutdown by pathogens.

To distinguish between these possibilities, relative levels of protein synthesis were measured in bone-marrow macrophages at various times following *L. pneumophila* challenge, using an immunofluorescence readout. B6 macrophages were challenged with *L. pneumophila flaA*$^{-}$-GFP at MOI = 10 for 2hrs and translation of proteins was measured by incorporation of a methionine analog (L-azidohomoalanine, AHA) for an additional 4hrs. Incorporated AHA was detected by reaction with a fluorescent-labeled phosphine reagent (phosphine-APC), which covalently links to the azido-functional group on AHA (Staudinger ligation reaction) [2].

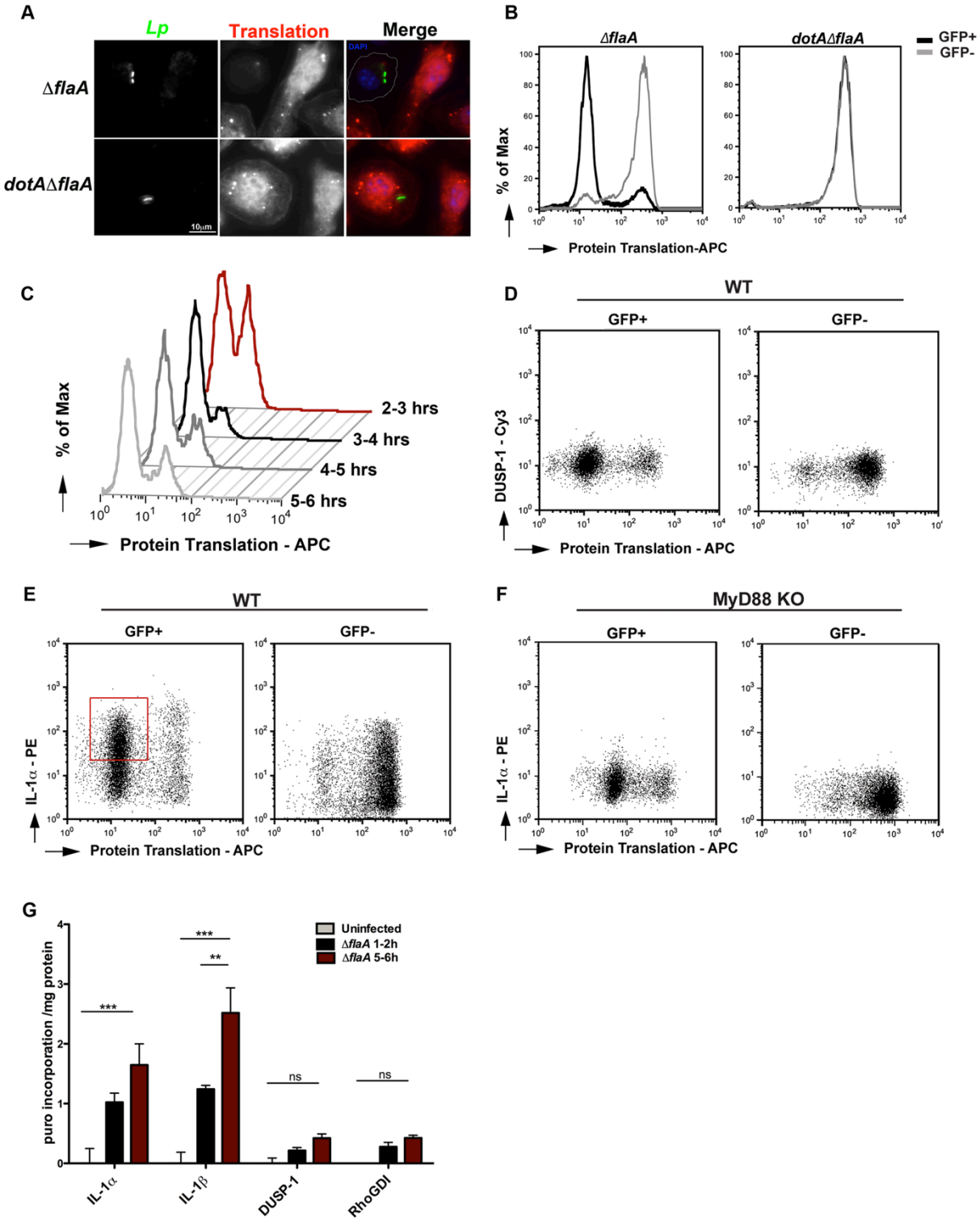
Protein translation was significantly inhibited in macrophages harboring *L. pneumophila* (Δ*flaA*) compared to bacteria lacking the Icm/Dot system (Fig. 3.9A). Furthermore, in macrophage cultures incubated with *L. pneumophila*, the macrophages harboring *L. pneumophila* showed selective protein synthesis interference, while the majority of the uninfected cells showed efficient incorporation of the amino acid analog (Fig. 5B; compare GFP^{+} to GFP^{-} population). To determine the time point at which the protein synthesis inhibitors fully shut down global protein translation, pulse-chase experiments were performed in which the methionine analog (AHA) was added for 1hr

intervals starting at 2hrs post infection (Fig. 3.9 C). Between 2-3hrs post-infection, approximately 40% of the cells harboring *L. pneumophila* were found in the population that has high levels of protein synthesis. Between 3-4 hrs post infection, we observed a major shift where almost 90% of cells harboring Δ*flaA* were found in the population having highly depressed protein synthesis. Later time points showed no further blockage in translation, perhaps reflecting the fact that there is a small fraction of wild type bacteria that fail to form replication compartments [135]. This population is predicted to show no significant translocation via the Icm/Dot system and should fail to inhibit protein synthesis (seen in ~15% of the macrophage population).

To determine if translation-blocked cells could still produce cytokines, macrophage monolayers were challenged with *L. pneumophila*-GFP$^+$ and protein synthesis was monitored by addition of AHA. The cells were then probed for IL-1α accumulation by immunofluorescence and flow analysis. In the infected GFP$^+$ population of macrophages, the majority of cells that accumulated IL-1α show evidence of an almost complete shutdown of protein translation (Fig. 3.9 E; red box). In the absence of MyD88 signaling, both the infected and uninfected populations showed little IL-1α accumulation (Fig. 3.9 F). In contrast to IL-1α, there was no DUSP-1 accumulation after *L. pneumophila* challenge of macrophages (Fig. 3.9 D). This confirmed our main hypothesis that there is translation of selected cytokine genes when protein synthesis is inhibited by *L. pneumophila* and this bypass requires MyD88 signaling.

Figure 3.9: Translation of IL-1 takes place in the presence of *L. pneumophila* elongation inhibitors.

(A) B6 macrophages were infected with indicated *L. pneumophila* strains at MOI=10 for 2hrs and a methionine analog, L-azidohomoalanine (AHA, 50μM) was incorporated into newly synthesized proteins for 4 hrs. Cells were fixed, permeabilized and the incorporated analog was detected by an APC conjugated phosphine and fluorescence microscopy. Nuclei were stained with Hoechst 33342 (blue) (B) Macrophages were infected with indicated *L. pneumophila* strains at MOI=10 for 2hrs and L-azidohomoalanine (AHA, 50μM) was added to cells for additional 4 hrs. Protein translation was quantified in infected macrophages (GFP^+) and uninfected bystanders (GFP^-) by staining with APC-labeled phosphine and flow cytometry. Experiment was performed three times. (C) B6 macrophages were infected with Dot^+ *L. pneumophila* for 2hrs and AHA was added for 1hr intervals. Cells were fixed after each time point and protein translation was quantified by APC-conjugated phosphine and flow cytometry. Translation of DUSP1 (D) or IL-1α (E) was monitored in wild type macrophages infected with Δ*flaA* for 6hrs by co-staining AHA with Cy3 conjugated DUSP1 antibody or PE-conjugated IL-1α antibody. (F) Translation of IL-1α in MyD88-deficient macrophages was determined by co-staining AHA with PE-conjugated IL-1α antibody. Dot plots show GFP^+ (left column) and GFP^- (right column) gated cells. (G) Kinetics of IL-1α, IL-1β, DUSP-1 and RhoGDI translation was quantified in Dot+ infected macrophages using puromycin incorporation. B6 macrophages were infected with Dot+ *L. pneumophila* and 10μg/mL of puromycin was added either between 1-2hrs or between 5-6 hrs post infection. Cells were washed, lysed and incubated on plates coated with anti-IL-1α, IL-1β, DUSP-1 and RhoGDI antibodies. Incorporation of puromycin in the indicated samples was monitored by anti-puromycin antibody and HRP-conjugated secondary antibody. Data represent absorbance at 450 normalized to total protein levels. Values for uninfected controls were subtracted from each sample to determine the increase in puromycin incorporation upon infection. Each bar represents mean and SEM of triplicate samples.


A
Lp
Translation
Merge
ΔflaA
dotAΔflaA
DAPI
10μm
B
ΔflaA
dotAΔflaA
GFP+
GFP-
% of Max
Protein Translation-APC
C
% of Max
2-3 hrs
3-4 hrs
4-5 hrs
5-6 hrs
Protein Translation - APC
D
WT
GFP+
GFP-
DUSP-1 - Cy3
Protein Translation - APC
E
WT
GFP+
GFP-
IL-1α - PE
Protein Translation - APC
F
MyD88 KO
GFP+
GFP-
IL-1α - PE
Protein Translation - APC
G
puro incorporation /mg protein
Uninfected
ΔflaA 1-2h
ΔflaA 5-6h
***
***
**
ns
ns
IL-1α
IL-1β
DUSP-1
RhoGDI

3.6. The increase in IL-1α and IL-1β protein levels is due to enhanced protein synthesis

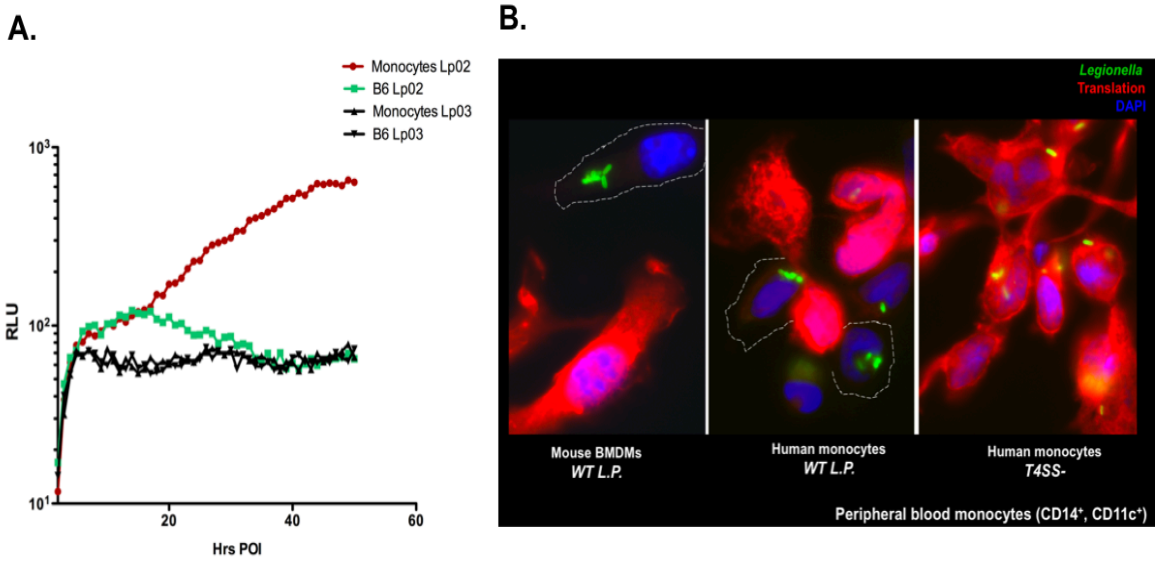
We have shown that pro-IL-1α and pro-IL-1β accumulate in cells that harbor *L. pneumophila* between 4-6hrs post infection (Fig. 3.5 B), despite a significant block in protein translation (Fig. 3.9C & E). To confirm that the accumulation we observe in infected cells was due to newly synthesized cytokines over the course of infection, we took advantage of another protein translation assay, SunSET. This assay uses puromycin incorporation into growing polypeptide chains to monitor active protein synthesis [138]. We modified this assay to measure the amount of puromycin incorporated into our protein of interest during a 1-hour pulse period. Accordingly, macrophages were challenged with *L. pneumophila* for increasing lengths of time, cells were labeled for one hour with 10μg/mL of puromycin, lysed, and individual proteins were immobilized in assay wells using specific antibodies (Materials and Methods). An ELISA was then used to determine the amount of puromycin incorporated in the immobilized proteins (Fig. 3.9 G). Consistent with our intracellular cytokine staining and Western blot data (Fig. 3.5B &E), the highest levels of IL-1α and IL-1β synthesis were detected between 5-6 hrs post-infection. On the other hand, no significant puromycin incorporation was detected for DUSP-1 and RhoGDI proteins, confirming that there is selective synthesis of few genes after *L. pneumophila* challenge.

3.7. Pro-inflammatory cytokines bypass translation inhibition during *Legionella* infection of primary human monocytes.

To determine if our observation holds true during challenge of human cells, we investigated the pro-and anti-immune cytokine response in healthy donor human monocytes that have been challenged with *Legionella pneumophila*. Purified peripheral blood human monocytes ($CD14^+$, $CD11c^+$) were challenged with either WT *Legionella* (*Lp02*, *Fla*+ strains) or mutants that lack the T4SS (*Lp03*). Flagellin-dependent Naip5 restriction was not functional in human cells and WT *Legionella* grew robustly, unlike what was observed for challenge of C57BL/6 BMDMs (Figure 3.10 A). Based on AHA incorporation and visual inspection by fluorescence microscopy, translation inhibition by *L. pneumophila* was indistinguishable in B6 macrophages and $CD14^+$ human monocytes (Figure 3.10 B).

Figure 3.10: *Legionella* challenge of human monocytes.

(A) Human monocytes or the restrictive C57Bl/6 macrophages were challenged with either WT *Legionella* (Lp02 Lux^{+}) or a mutant that lacks the T4SS (*dotA3*Lux^{+}) and relative growth was measured in human monocytes and C57BL/6 macrophages. Growth is reported as relative luminescence units (RLU). (B) Human monocytes and C57Bl/6 macrophages were chalenged with *Legionella* and protein translation was measured by incorporation of a methionine analog AHA (Materials and Methods) followed by reaction with a fluorescent derivative, staining with DAPI and detection using fluorescence microscopy.

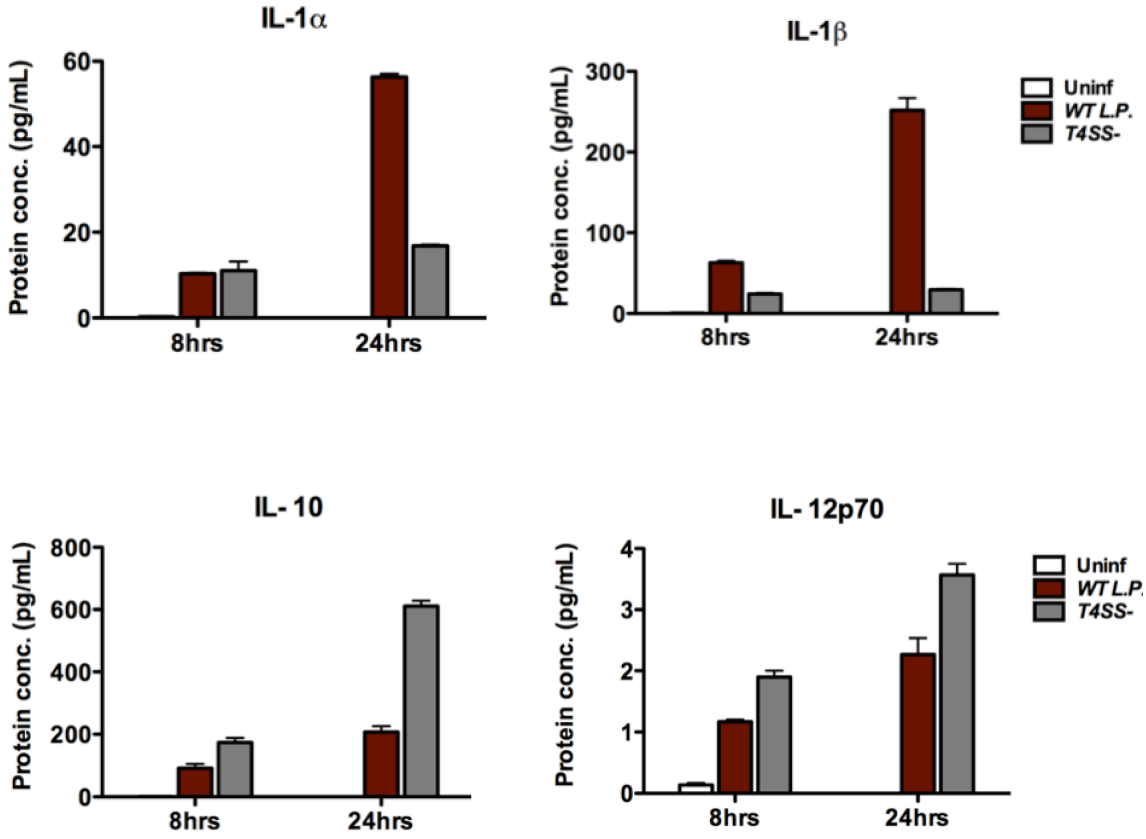


To dissect the innate immune responses that occur in humans during *Legionella* infection, we measured pro- and anti-inflammatory cytokines in monocytes that have been infected with *Legionella* at various time points using multiplex cytokine arrays.

Consistent with what is seen during mouse infections, pro-inflammatory cytokines are able to bypass *Legionella*-induced translation inhibition during human monocyte infection. Anti-inflammatory cytokines on the other hand, are susceptible to this translation inhibition (Fig. 3.11).

Figure 3.11: Infection with WT *Legionella* leads to selective translation of pro-inflammatory cytokines and inhibition of anti-inflammatory cytokines during human monocyte infection.

Secreted Il-1α and Il-1β (top panel) or secreted IL-10 and IL-12p70 (bottom panel) from human monocytes challenged with either virulent *L. pneumophila* strains (*WT L.p* - red bars) or avirulent *dotA3* mutants (*T4SS*- grey bars). Cira Multiplex immunoassay platform was used to determine cytokine levels.


IL-1α
Protein conc. (pg/mL)
60
40
20
0
8hrs
24hrs
IL-1β
Protein conc. (pg/mL)
300
200
100
0
8hrs
24hrs
Uninf
WT L.P.
T4SS-
IL- 10
Protein conc. (pg/mL)
800
600
400
200
0
8hrs
24hrs
IL- 12p70
Protein conc. (pg/mL)
4
3
2
1
0
8hrs
24hrs
Uninf
WT L.P.
T4SS-

3.8. Translation inhibition acts in concert with TLR signaling to generate the full cytokine response.

To determine the role that *L. pneumophila* translation inhibitors play in modulating host cytokine synthesis, we used a mutant that lacks the 5 Icm/Dot translocated substrates known to block host protein synthesis (Δ*5* mutant) [46]. The level of protein synthesis was first measured using AHA incorporation (Fig. 3.13 A, B) and puromycin incorporation (Fig. 3.12) in macrophages challenged with Δ*5*Δ*flaA*-GFP^{+}.

Compared to Dot^{+} strain (Fig. 3.12 and 3.13 B), there was an increase in active protein translation in cells that were challenged with the Δ*5* mutant, although the cells showed lower levels of protein synthesis than cells associated with the Dot^{-} strain (Fig. 3.13 B) or the uninfected population (Fig. 3.13 A; compare GFP^{+} to GFP^{-} populations). This is consistent with the hypothesis that in addition to Icm/Dot translocated substrates that act on translation elongation, virulent *L. pneumophila* also blocks translation initiation [71].

Figure 3.12: Δ5 mutants show partial inhibition of protein synthesis.

B6 macrophages were challenged with *L. pneumophila* Δ*flaA, dotA*Δ*flaA* and Δ*5*Δ*flaA* for 5hrs and 10μg/mL of puromycin was added between 5-6 hrs post infection. Cells were fixed, permeabilized and incorporated puromycin was detected by anti-puromycin antibody (12D10) and fluorescence microscopy.

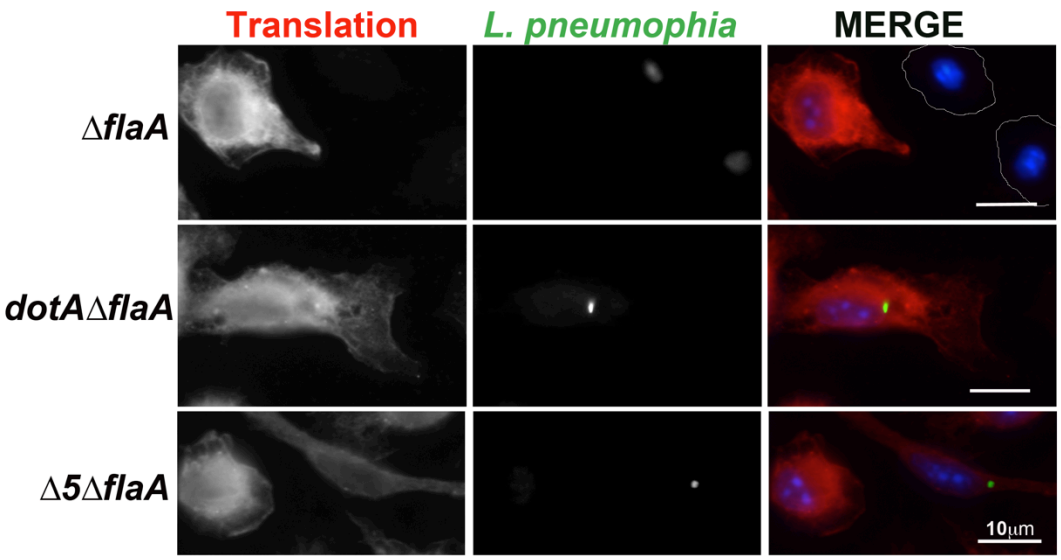
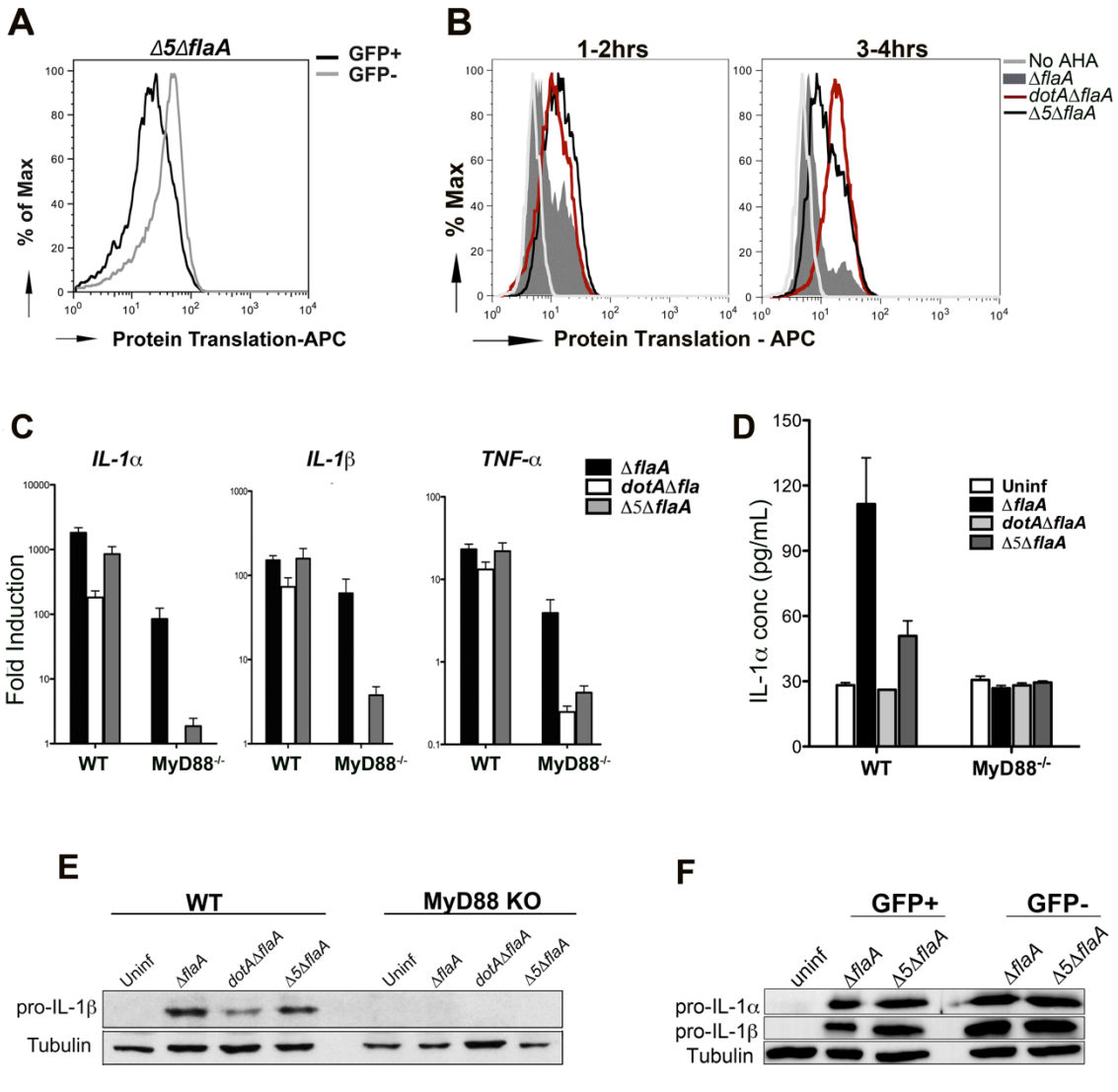


Figure 3.13: Production of the pro-inflammatory cytokines IL-1α and IL-1β is independent of the five translocated protein synthesis inhibitors.

(A) B6 macrophages were challenged with *L. pneumophila* Δ5Δ*fla*-GFP^{+} and protein translation was measured between 2-6hrs post-inoculation by incorporation of the methionine analog AHA. Translation (incorporation of AHA) was compared between infected cells (GFP^{+}, black line) and uninfected bystanders (GFP^{-}, grey line). (B) B6 macrophages were infected with GFP expressing Dot^{+}, Dot^{-} and Δ5 strains of *L. pneumophila* and protein synthesis inhibition was compared between these strains by incorporation of AHA between 1-2hrs (left graph) or 3-4hrs (right graph). Graphs show translation in cells harboring *L. pneumophila* (GFP^{+}). Cells incubated in the absence of the methionine analog (No AHA) were used as a negative control to show baseline staining. (C) WT and MyD88$^{-/-}$ macrophages were challenged with the indicated strains at MOI-15 and cytokine transcripts were analyzed at 6hrs post infection by qRT-PCR. Data represent the mean fold induction and SEM of samples relative to controls incubated in the absence of bacteria. (D) ELISA measurement of IL-1α secretion from WT and MyD88$^{-/-}$ macrophages incubated with the indicated strains for 24 hrs. Data represent mean and SEM of triplicate samples. (E) Immunoblot analysis of pro-IL-1β in WT and MyD88$^{-/-}$ macrophages challenged with the indicated *L. pneumophila* strains for 6hrs. (F) B6 macrophages were challenged with *ΔflaA*-GFP or *Δ5Δfla* at MOI-15 for 6hrs and sorted by Flow Cytometry. Pro-IL-1β levels were measured in both GFP^{+} and GFP^{-} population by Western blot.


A
Δ5ΔflaA
GFP+
GFP-
% of Max
Protein Translation-APC
B
1-2hrs
3-4hrs
No AHA
ΔflaA
dotAΔflaA
Δ5ΔflaA
% Max
Protein Translation - APC
C
IL-1α
IL-1β
TNF-α
ΔflaA
dotAΔfla
Δ5ΔflaA
Fold Induction
WT
MyD88-/-
D
Uninf
ΔflaA
dotAΔflaA
Δ5ΔflaA
IL-1α conc (pg/mL)
WT
MyD88-/-
E
WT
MyD88 KO
Uninf
ΔflaA
dotAΔflaA
Δ5ΔflaA
pro-IL-1β
Tubulin
F
GFP+
GFP-
uninf
ΔflaA
Δ5ΔflaA
pro-IL-1α
pro-IL-1β
Tubulin

It had been previously reported that in the absence of most known pathways of pattern recognition (MyD88$^{-/-}$ Nod1$^{-/-}$ Nod2$^{-/-}$ macrophages), the cytokine transcriptional response to *L. pneumophila* was primarily due to the presence of the translocated protein synthesis inhibitors [46, 47]. Using macrophages that are only defective for MyD88, this dependence on the translation inhibitors could be clearly observed for the *Il1α*, *Il1β* and *Tnfα* transcripts (Fig. 3.13 C, MyD88$^{-/-}$, gray vs. black bars). Consistent with our previous data, the transcriptional response in MyD88 knockout macrophages was unproductive, with no evidence that these highly induced transcripts are translated (Fig. 3.13 E).

In the case of wild type macrophages, challenge with *L. pneumophila* Dot^{+} and Δ*5* strains induced comparable levels of MyD88-dependent cytokine transcription and translation (Fig. 3.13 C and E), and this could be observed in macrophages that were sorted by flow cytometry, as well (Fig. 3.13 F). This result is in contrast with macrophages lacking MyD88 signaling, in which it was clear that there was protein synthesis inhibitor-dependent induction of cytokine transcripts (Fig. 3.13 C), but this induction produced no apparent cytokine translation products. Interestingly, unlike what we saw for *dotA* mutants, challenge with Δ*5* was still able to induce secretion of mature IL-1α at 24 hrs after inoculation [20], even though it was to a lesser extent than wild type (Fig. 3.13 D).

Therefore, although the protein synthesis inhibitors are responsible for the transcriptional response that occurs in the absence of pattern recognition receptors, the

production of cytokine proteins associated with fully virulent strains is not dependent on these translocated substrates.

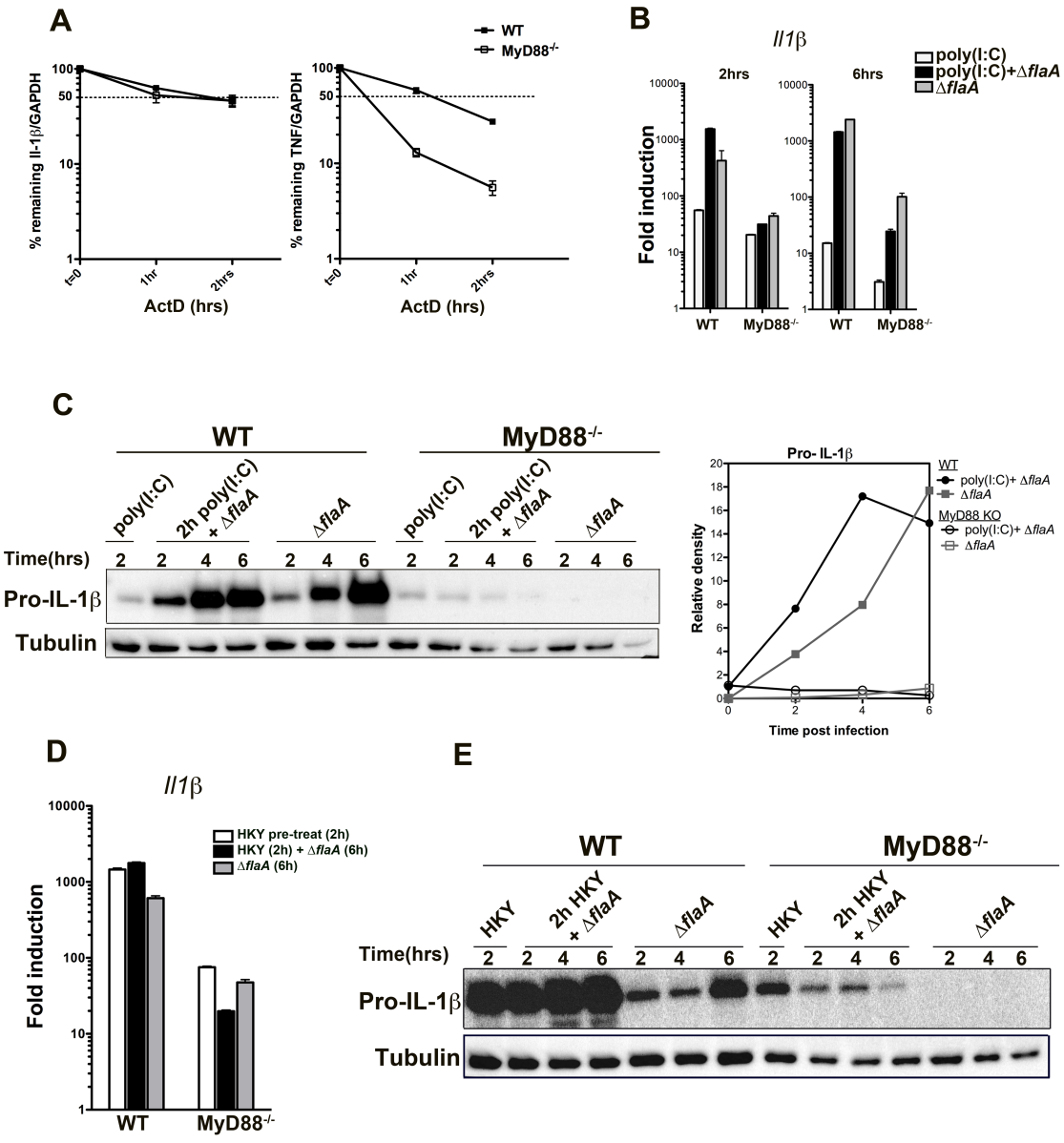
3.9. MyD88-mediated bypass of translation inhibition of the *il1b* gene is independent of mRNA stability.

Cytokine expression is regulated at various stages, including transcription, post-transcriptional processing, translation and secretion. One of the main regulatory steps for IL-1 and TNF production is their transcript stability, which is controlled by their AU-rich elements (ARE) in their 3'-noncoding regions and by various ARE-binding proteins [99, 130].

To determine if lack of cytokine translation in MyD88$^{-/-}$ macrophages was due to mRNA instability, the half-life of *Il1β* and *Tnf* transcripts were compared in WT and MyD88$^{-/-}$ macrophages after *L. pneumophila* challenge. Macrophages were first incubated with *L. pneumophila* for 2.5hrs, actinomycin D was added to the medium to block further transcription, and the amount of transcript remaining was measured by qRT-PCR at various time points after the addition of the drug. *Tnfα* mRNA was highly unstable in the absence of MyD88 when compared to MyD88$^{+/+}$ macrophages (Fig. 3.14 A). However, *Il1β* transcripts were relatively stable in the absence of MyD88, and the amount of mRNA remaining after 2 hrs was similar to control macrophages (Fig. 3.14 A). This indicates that the translation inhibition bypass of *Il1β* was independent of mRNA stability (Fig. 3.14). In the case of *tnf*, however, transcript stabilization via a MyD88-dependent signal may play a role in bypassing translation inhibition.

Figure 3.14: Stability of Il-1β mRNA or pre-activation of the NF-kB pathway is not sufficient to induce translation bypass in MyD88$^{-/-}$ macrophages.

(A)WT and MyD88 deficient bone-marrow macrophages were challenged with *L.pneumophila* Δ*flaA* for 2.5hrs and transcription was blocked by addition of 10μg/mL Actinomycin D. The percentage of remaining *Il1β* and *Tnfα* transcripts was measured by qRT-PCR after 1 or 2hrs post actinomycin D treatment. Data represent mean and SEM of samples relative to GAPDH. (B) WT and MyD88$^{-/-}$ macrophages were pre-treated with 50μg/mL poly(I:C) for 2hrs to induce NF-kB activation, after which cells were either challenged with Dot+ *Legionella* or left untreated (poly(I:C) only). Macrophages that were not pre-stimulated with poly(I:C) but were challenged with Dot+ were included for comparison. RNA was collected after 2 and 6hrs of infection and *Il1β* transcript levels were analyzed by qRT-PCR. (C) B6 WT and MyD88-deficient macrophages were pre-treated with poly(I:C) for 2hrs or left untreated. Cells were then challenged with *L. pneumophila* Dot^{+} for additional 2, 4 and 6hrs and production of pro-IL-1β was examined by Western blot. Densitometry of the blot is shown on the right. (D) WT and MyD88$^{-/-}$ macrophages were pre-treated with HKY (MOI=100) for 2hrs or left untreated after which cells were challenged with *L. pneumophila* Dot^{+}. Induction of *Il1β* transcript was determined after 6hrs of infection. (E) Pro-IL-1β synthesis was also examined in these cells after 2, 4 and 6hrs of challenge.


A
WT
MyD88-/-
% remaining Il-1β/GAPDH
% remaining TNF/GAPDH
t=0
1hr
2hrs
ActD (hrs)
B
Il1β
2hrs
6hrs
poly(I:C)
poly(I:C)+ΔflaA
ΔflaA
Fold induction
WT
MyD88-/-
C
WT
MyD88-/-
poly(I:C)
2h poly(I:C) + ΔflaA
ΔflaA
Time(hrs)
Pro-IL-1β
Tubulin
Pro- IL-1β
WT
poly(I:C)+ ΔflaA
ΔflaA
MyD88 KO
poly(I:C)+ ΔflaA
ΔflaA
Relative density
Time post infection
D
Il1β
HKY pre-treat (2h)
HKY (2h) + ΔflaA (6h)
ΔflaA (6h)
Fold induction
WT
MyD88-/-
E
WT
MyD88-/-
HKY
2h HKY + ΔflaA
ΔflaA
Time(hrs)
Pro-IL-1β
Tubulin

3.10. Pre-activation of NF-kB in MyD88-deficient macrophages is not sufficient to bypass translation inhibition.

Another possible explanation for why few pro-inflammatory cytokines, such as IL-1α and IL-1β, bypass translation inhibition could be mRNA abundance. If this were the case, we hypothesized that amplifying cytokine transcripts in MyD88 knockout macrophages, using ligands that induce NF-κB activation independent of MyD88 signaling, would be able to rescue the translation defect. Accordingly, BMDMs were pre-activated with the TLR3 agonist, poly(I:C), which signals through TRIF. We also used heat-killed *Yersinia pseudotuberculosis* (HKY) to activate NF-κB via TLR4 as well as cytosolic-pattern recognition receptors.

2hr pre-stimulation with 50μg/mL poly(I:C) was sufficient to trigger IL-1β transcription (Fig. 3.14 B, left graph, white bars) both in wild type and MyD88$^{-/-}$ macrophages. Pre-stimulation with poly (I:C) followed by *L. pneumophila* infection increased *Il1β* transcription initially (after 2hrs of infection) in wild type macrophages compared to cells that were untreated (Fig. 3.14 B, compare black bars with grey bars). Surprisingly, this was reversed by 6hrs post infection and cells that were pre-treated with poly(I:C) down regulated their *Il1β* transcription (Fig. 3.14 B, right graph). This was more pronounced in MyD88$^{-/-}$ macrophages (Fig. 3.14 B, compare black and grey bars). We observed a very similar phenomenon when cells were pre-treated with heat-killed *Yersinia* followed by *Legionella* infection (Fig. 3.14 D).

Similarly, we tried various cytosolic ligands to stimulate NF-κB in MyD88 deficient macrophages. We observed a similar phenomenon where transcription of pro-

inflammatory cytokines initially increased, but was rapidly reduced upon re-challenge with *Legionella pneumophila* (data not shown). Since we were unable to push the transcription levels of cytokines in MyD88$^{-/-}$ macrophages to a level that was comparable to WT, in the next chapter, alternative approaches will be used to test if transcript abundance controls bypass of translation inhibition during *Legionella* infection.

Chapter 4: *Two strategies for bypass of translation inhibition during Legionella infection*

4.1. Bypass of translation inhibition and transcript abundance

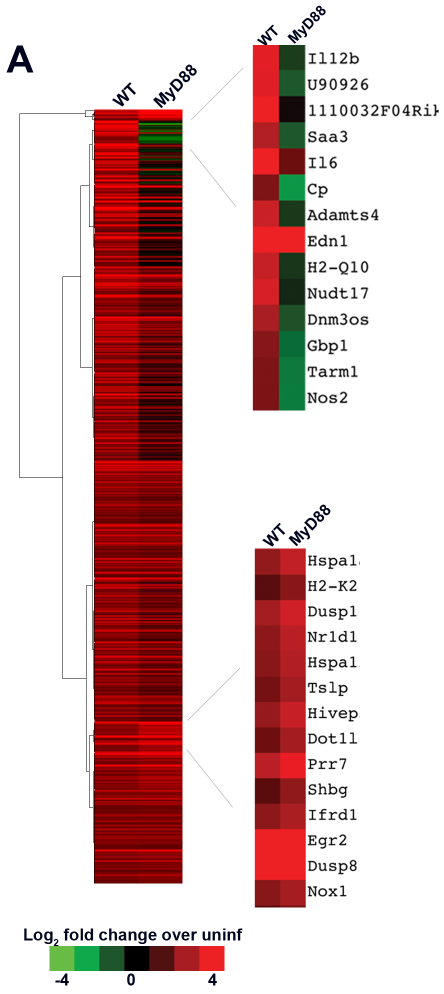
*Note: All the experiments described in this section were performed with Δ*flaA *L. pneumophila strains to avoid flagellin-dependent Naip5 restriction.*

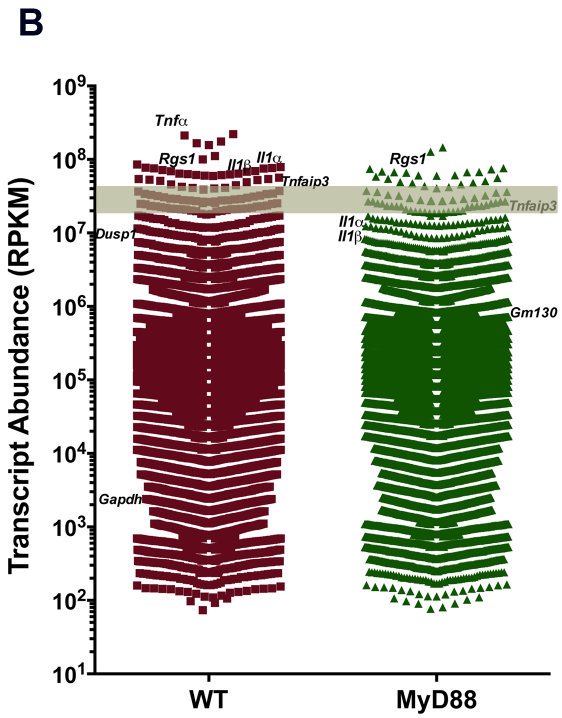
We have shown that a subset of pro-inflammatory cytokines, such as *Il1α* and *Il1β,* bypass translation inhibition during *Legionella* infection [13]. This selective translation of pro-inflammatory cytokines was dependent on MyD88 signaling and occurred after *L. pneumophila* significantly blocked protein synthesis. It was clear that macrophages that directly harbored bacteria were able to synthesize these proteins but the mechanism by which cytokines resisted translation inhibition or how MyD88 contributed to selective translation was not elucidated.

A potential mechanism for selective translation could be relative transcript abundance upon infection. In other words, genes that bypass translation inhibition could be most abundantly expressed in a cell under conditions of intoxication, which would favor association with the small remaining pool of functional ribosomes. To determine if transcript abundance facilitates bypass of translation inhibition, we took a global approach and used RNA sequencing (RNAseq) to measure transcript levels in WT and MyD88$^{-/-}$ macrophages challenged with *L. pneumophila*. C57BL/6 BMDMs were challenged with *L. pneumophila*-GFP. for 6hrs and cells harboring bacteria were sorted by flow cytometry. RNA was extracted from sorted cells, DNase treated and used for RNAseq library preparation. Sequenced reads were aligned to mouse reference genome mm9 (TopHat alignment [157]) and used for determining gene expression levels in C57BL/6 (WT) and congenic MyD88$^{-/-}$ macrophages [158] .

Figure 4.1: Fold induction and absolute transcript abundance of host cell genes in WT and MyD88$^{-/-}$ macrophages upon *Legionella* challenge.

(A) Differential expression of genes in WT and MyD88$^{-/-}$ macrophages infected with *L. pneumophila* relative to controls incubated in absence of bacteria. Sorted cells harboring *L. pneumophila* were compared to the control cultures, RNAseq was performed on the two populations, and the analysis is displayed here. Pearson hierarchical cluster analysis of 800 genes in WT and MyD88$^{-/-}$ macrophages is shown. Note: The subset shown in this figure only contains genes that were induced more than 2.5 fold during WT macrophage infection. Genes that are upregulated are shown in red, downregulated genes are shown in green and equally expressed genes are shown in black. Data shown are log2 transformed fold induction values from two biological replicates. B) Absolute transcript abundance determined from Reads Per Kilobase of exon per million mapped reads (RPKM values) in macrophages harboring bacteria from the C57Bl/6 WT (brown squares-WT) or MyD88$^{-/-}$ harboring macrophages (green traiangles-MyD88). Each square/triangle represents a single gene (total of 22,006 genes in each group). Yellow shade shows our estimated threshold (graph in logarithmic scale). Genes with lower expression than noted by the bottom line are predicted to unlikely be expressed, while those showing higher expression than the top line are likely expressed. The experiment was done in biological duplicates and the data here are representative of the two.


A
WT
MyD88
Il12b
U90926
1110032F04Rik
Saa3
Il6
Cp
Adamts4
Edn1
H2-Q10
Nudt17
Dnm3os
Gbp1
Tarm1
Nos2
Hspa1
H2-K2
Dusp1
Nr1d1
Hspa1
Tslp
Hivep
Dot1l
Prr7
Shbg
Ifrd1
Egr2
Dusp8
Nox1
Log2 fold change over uninf
-4
0
4


B
Transcript Abundance (RPKM)
Tnfα
Rgs1
Il1β
Il1α
Tnfaip3
Dusp1
Gapdh
Gm130
WT
MyD88

We first looked at the differential expression of genes in WT and MyD88$^{-/-}$ macrophages upon *Legionella* challenge. Consistent with what has previously been reported [46, 100], infection with *Legionella* significantly induced transcription of pro-inflammatory cytokines (*Tnf, Il1α, Il6*) chemokines (*Cxcl1, Cxcl2, Ccl3*) MAPK genes (*Dusp2, Dusp 8*) and NF-κB regulators (*IκB, Tnfaip3*) in WT macrophages. As expected, the overall induction of genes associated with the innate immune response was more prominent in WT macrophages compared to MyD88 knockouts (Fig 4.1 A).

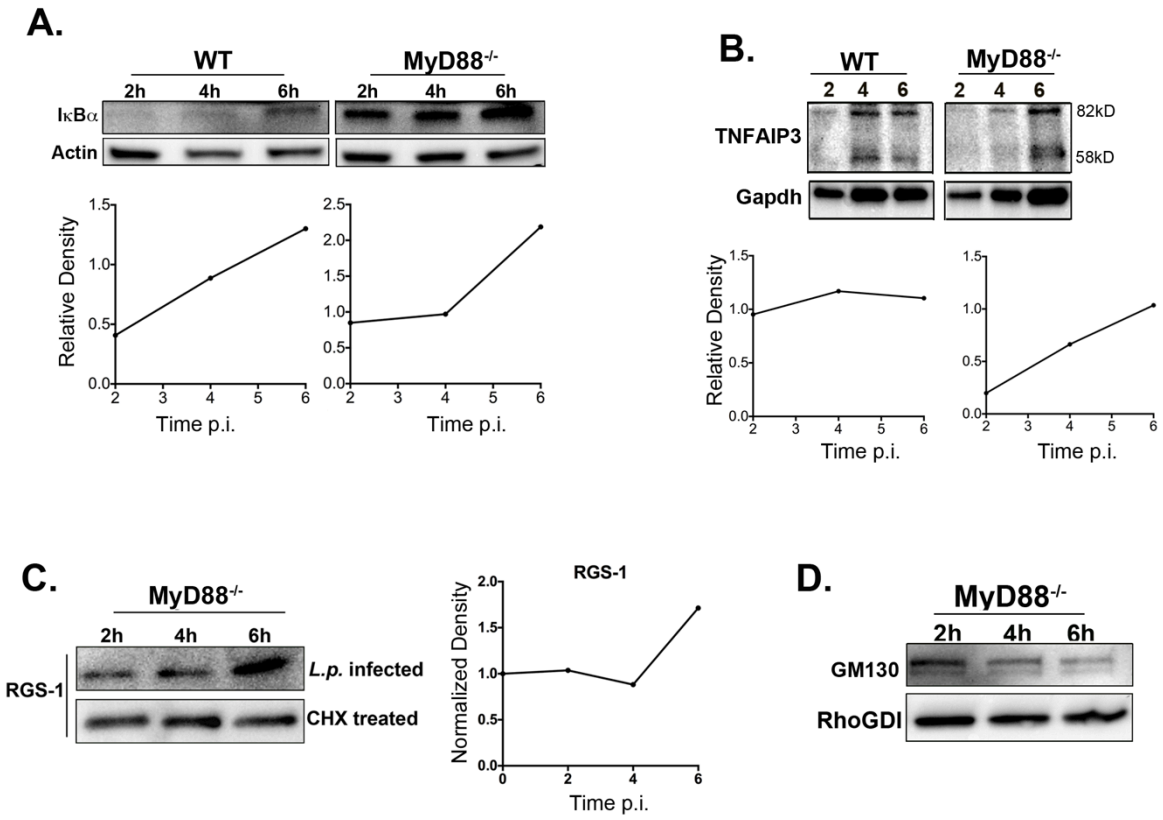
Interestingly, a few genes associated with the innate immune response, such as the pro-inflammatory cytokine interleukin 12b (*Il12b*), nitric oxide synthase 2 (*Nos2),* and interferon-inducible guanylate-binding protein 1 (*Gbp1*) showed significant upregulation in WT macrophages but a downregulation in MyD88 deficient macrophages after challenge with *L. pneumophila* (Fig 4.1 A, top panel). On the other hand, genes encoding inhibitors of NF-κB signaling pathway, such as *Nfkbia* and *Tnfaip3*, were highly induced in MyD88$^{-/-}$ macrophages. Dual specificity phosphatases *Dusp1* and *Dusp8,* which downregulate the MAPK pathway, were induced more in MyD88 knockout macrophages compared to WT (Fig 4.1 A, bottom panel). To test our hypothesis that transcript abundance allows bypass of translation inhibition, we measured absolute transcript levels in both WT and MyD88 deficient macrophages by calculating the number of aligned reads per kilobase of exon per million mapped reads (RPKM values) (Fig 4.1 B). Genes encoding pro-inflammatory cytokines (*Tnf* and *Il1*) and chemokines (*Cxcl2*, *Ccl2*, *Ccl4*) were the most abundantly expressed genes in WT macrophages upon challenge with *L. pneumophila* (Fig 4.1 B, top red squares). These expression levels were 10^4-10^5 X compared to housekeeping genes (for e.g. 10^5 > *Gapdh*), indicating that macrophages

would be completely overwhelmed with pro-inflammatory cytokine and chemokine transcripts upon *L. pneumophila* infection. We and other groups have previously shown that these transcripts bypass translation inhibition in cells harboring *L. pneumophila*, while less abundant transcripts, such as the anti-inflammatory cytokine *Il10* and MAPK regulator *dusp1*, were blocked for translation[13, 72].

Based on the expression levels (RPKM values) of genes that have previously been shown to bypass translation inhibition (*Tnf, Il1α, Il1β*) and genes that are unable to bypass translation inhibition (*Dusp1, Il10, Hsp90*), we estimated an abundance threshold (Fig 4.1 B, grey box). We hypothesized that if transcript abundance facilitates bypass of translation inhibition, highly expressed genes that are above this estimated threshold should be able to get selectively synthesized during *Legionella* infection, both in WT and MyD88$^{-/-}$ macrophages. To test this hypothesis, BMDMs were challenged with *L. pneumophila* GFP^{+} for 2, 4 and 6hrs, cells that harbor bacteria were sorted by flow cytometry and protein levels were determined in sorted cells by Western blot analysis (Fig 4.2). In parallel, protein levels were also determined in cells that were treated with cycloheximide for similar time points to differentiate between *de novo* protein synthesis and protein stability.

Figure 4.2. Abundant transcripts bypass translation inhibition in WT and MyD88$^{-/-}$ macrophages after protein synthesis is inhibited by *L. pneumophila*.

Macrophages were infected with *L. pneumophila*-GFP (Δ*flaA*) at MOI=15 for indicated time points and sorted by flow cytometry. Immunoblot analysis of (A) IκBα and (B) TNFAIP3 in WT and MyD88$^{-/-}$ infected/sorted cells. Bottom graphs show densitometry of IκBα and TNFAIP3 (82kDa band) in sorted cells normalized to Actin and Gapdh respectively. (C) RGS-1 protein levels in MyD88$^{-/-}$ macrophages challenged with *L. pneumophila* and sorted 2, 4 and 6hrs later (top lane). In parallel, cells were treated with 1μg/mL cycloheximide (CHX) (bottom lane). The graph on the right shows densitometry of RGS-1 in infected macrophages normalized to CHX treated cells. (D) Immunoblot analysis of GM130 in MyD88$^{-/-}$ macrophages infected with *Legionella pneumophila* for 2, 4 and 6hrs.


A.
WT
MyD88-/-
2h
4h
6h
2h
4h
6h
IκBα
Actin
Relative Density
Time p.i.
Time p.i.
B.
WT
MyD88-/-
2
4
6
2
4
6
TNFAIP3
82kD
58kD
Gapdh
Relative Density
Time p.i.
Time p.i.
C.
MyD88-/-
2h
4h
6h
RGS-1
L.p. infected
CHX treated
RGS-1
Normalized Density
Time p.i.
D.
MyD88-/-
2h
4h
6h
GM130
RhoGDI

Consistent with our hypothesis, transcripts that were above the estimated threshold in WT and MyD88$^{-/-}$ macrophages, such as IκBα and TNFAIP3, were translated in cells harboring *L. pneumophila* (Fig. 4.2 A&B). In resting macrophages, IκBα is bound to NF-κB and is relatively stable. Upon *Legionella* infection, it has been reported that IκB is degraded rapidly and was is unable to get re-synthesized within the first 2 hrs of infection due to *Legionella*-derived elongation inhibitors, which in turn results in persistent activation of NF-κB [46]. Our results indicated that at later time points (after 4-6hrs post infection), IκBα recovers from this block, and translation inhibition is bypassed, allowing synthesis in cells that harbor *L. pneumophila* (Fig. 4.2 A). Tumor Necrosis Factor Alpha-Induced Protein-3 (TNFAIP3), which is another negative regulator of NF-κB signaling, also showed a similar pattern and was able to get synthesized between 2-6hrs post infection both in WT and MyD88$^{-/-}$ macrophages (Fig. 4.2 B).

We have shown previously that the translation of pro-inflammatory cytokines required MyD88 signaling. Although *L. pneumophila* infection causes a large induction of cytokine transcription in MyD88 knockout macrophages [13], these levels were still significantly lower than what is seen when pattern recognition is intact ([13], Fig 4.1 B). The abundantly transcribed genes IκBα and TNFAIP3 were translated in MyD88$^{-/-}$ macrophages upon *Legionella* challenge, indicating that the role of MyD88 is to amplify transcription levels above a the threshold necessary to allow translation to occur.

Consistent with this hypothesis, the regulator of G-protein signaling family (RGS-1), which is expressed above the estimated threshold in MyD88$^{-/-}$ macrophages, was translated between 4-6hrs post-infection (Fig 4.2 C), whereas GM-130, which falls below the threshold, was not translated during this time period in the MyD88$^{-/-}$ background (Fig.

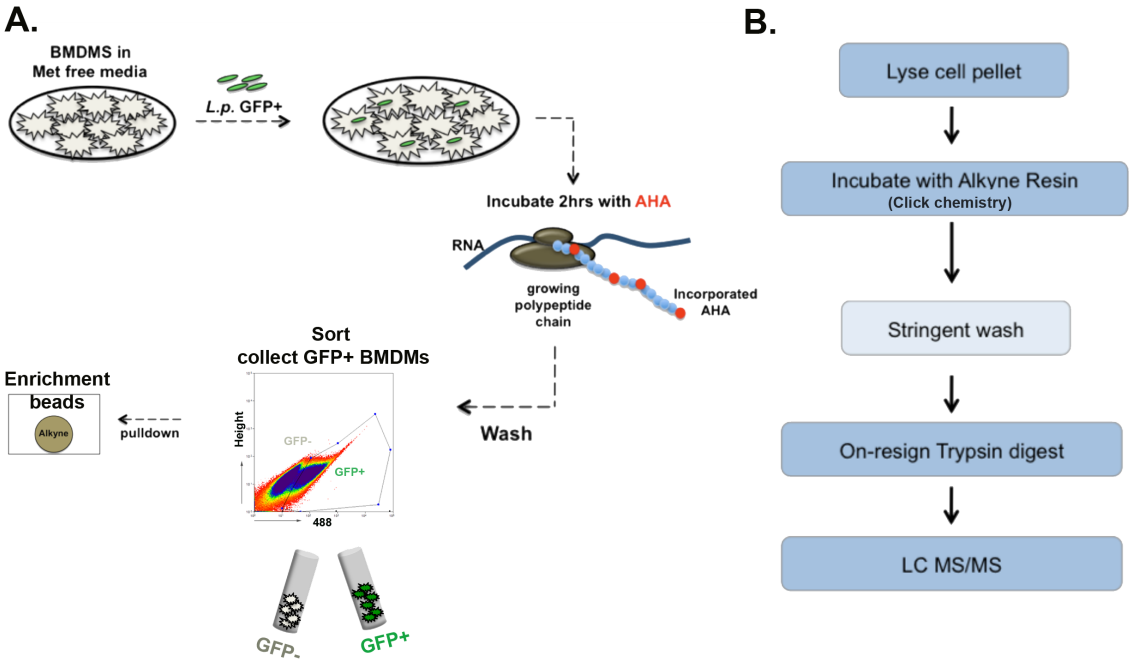
4.2 D). We accounted for the stability (half-life) of RGS-1 by normalizing protein levels during *Legionella* infection to CHX treatment and determined that there is significant *de novo* protein synthesis that takes place within MyD88$^{-/-}$ infected cells between 4-6hrs post infection (Fig 4.2 C, right graph). Based on these findings, we conclude that transcript abundance is one of the determinants for bypass of translation inhibition during *Legionella* infection. Our previous results, indicating a dependence on MyD88 for bypass of translation inhibition, reflected the fact that the cytokine transcripts we analyzed were expressed in MyD88$^{-/-}$ macrophages at levels below the threshold necessary to allow translation in cells harboring *L pneumophila*. Transcripts above this threshold, even in MyD88$^{-/-}$ macrophages, were translated 4-6 hrs post-inoculation.

4.2 Identification of proteins that are selectively translated in response to *L. pneumophila* interference of protein synthesis

To investigate the spectrum of host proteins translated while cells are harboring *L. pneumophila*, we took advantage of click chemistry to identify proteins synthesized during defined time periods post-inoculation both in WT and MyD88 KO macrophages. We had previously used this method with alkyne-biotin probes coupled to streptavidin beads. However, eluting proteins from streptavidin beads, analyzing samples on SDS-PAGE and extracting/digesting peptides from gels was not optimal for peptide identification. To avoid these steps, we labeled newly synthesized proteins, by introducing the methionine analog azidohomoalanine, (AHA) four hours after inoculation, allowing incorporation to proceed for 2 hours.

Figure 4.3: Workflow for identification of newly synthesized proteins using azido-alkyne ligation and mass spectrometry.

(A) WT and MyD88$^{-/-}$ macrophages were starved for methionine for 1hr (in Met-free RPMI media) and challenged with *L.pneumophila*-GFP for 4hrs. Infected cells were washed and 100μM azidohomoalanine (AHA) was added for the next 2 hrs (Materials and Methods). Cells harboring bacteria were sorted by flow cytometry, extracts made and the newly translated proteins were covalently linked to alkyne derivitized beads using Clickit chemistry. As a control, cells were incubated in the absence of AHA and subjected to the same reaction with the alkyne beads. B) Linkage protocol: Cells were lysed in the presence of protease inhibitors and AHA-tagged proteins were covalently attached to alkyne-coated beads by click chemistry. Labeled proteins that were linked to the alkyne beads were washed multiple times with buffers containing increasing concentrations of detergent. On-resin trypsin digest was performed overnight and peptides were identified by mass spectrometry (Materials and Methods).


A.
BMDMS in
Met free media
L.p. GFP+
Incubate 2hrs with AHA
RNA
growing
polypeptide
chain
Incorporated
AHA
Wash
Sort
collect GFP+ BMDMs
Height
GFP-
GFP+
488
Enrichment
beads
Alkyne
pulldown
GFP-
GFP+
B.
Lyse cell pellet
Incubate with Alkyne Resin
(Click chemistry)
Stringent wash
On-resign Trypsin digest
LC MS/MS

To identify the fraction of proteins synthesized during this time period, lysates were made, and extracts were incubated with alkyne-derivitized beads, resulting in covalent linkage of the newly synthesized proteins to the beads (alkyne enrichment beads). To identify the pulse-labeled proteins, on-bead trypsin digestion was performed, and the AHA-incorporated peptides were identified by mass spectrometry (Fig. 4.3).

Using the peptide analysis generated by mass spectrometry, a strategy was developed to filter out false positive hits during peptide identification and data analysis. We accounted for mass gain over methionine residues due to AHA incorporation (Materials and Methods). Peptides that were detected in the negative control (No AHA control) were considered as unspecific binding to the alkyne resin and were removed from the analysis. Proteins that had less than 2 unique peptides per locus were also excluded (Table 4). To further control for mass spec contaminants, we cross referenced the identified proteins to CRAPome database [112] and removed proteins that came up in any of these experiments (Table 5). We also identified a number of proteins that were encoded by mitochondria, such as MTCO2 (also called COX2, mitochondrially encoded cytochrome c oxidase II). Mitochondria have their own ribosomes (55S mitoribosomes) that are from a prokaryotic origin and work independently of cytosolic or ER associated ribosomes[55]. Since *Legionella* will not target translation by these ribosomes, mitochondrially encoded proteins were excluded from our analysis.

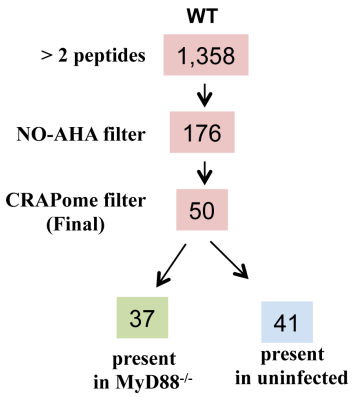
It is important to note that identification of newly synthesized proteins using this method has major limitation. It depends on the number of methionine residues within the protein, overall size of the protein and efficiency of the AHA incorporation during translation. Due to these limitations, a number of chemokines and cytokines that have

few methionine residues were not picked up in our mass spec. For example, Cxcl2, one of the most abundantly transcribed genes in cells during *L. pneumophila* incubations, only contains one methionine (initiator Met). Similarly, TNF-α and IL-6, each with 5 methionine residues, only had 1 unique peptide identified by mass spec and did not pass the cutoff criteria.

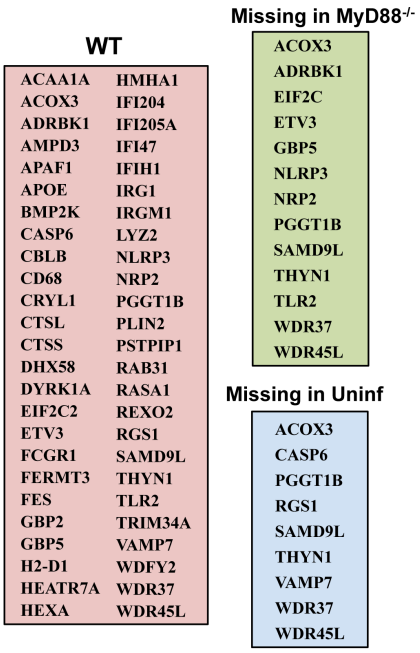
Figure 4.4. Data analysis and results from mass spectrometry.

(A) Strategy to filter out false positive hits during peptide identification and data analysis in WT macrophages. 1) Proteins that had less than 2 peptides per locus were removed from the analysis, resulting in 1,358 proteins (shown in Table 4). 2) Peptides that were detected in the negative (NO-AHA) control were considered as unspecific binding to the alkyne resin and were removed from the analysis, resulting in 176 proteins (shown in Table 5). 3) Common mass spec contaminants identified in CRAPome database [112] were removed, resulting in 50 final proteins in WT macrophages. Out of these 50 proteins identified, 37 were also translated in MyD88$^{-/-}$ macrophages infected with *L. pneumophila* and 41 proteins were translated in WT uninfected macrophages (B) List of 50 proteins identified in WT (red box). Out of this list, 13 proteins were not present in infected MyD88$^{-/-}$ macrophages (green box), and 9 were not present in WT uninfected macrophages (blue box). (B) Pathways that were overrepresented in the final output are shown as percentage of total (INTERFEROME and DAVID database[38, 141].

A)



B)



C)

Functional annotation

	WT (% total)
Type I IFN inducible	44
Nucleotide binding	30
Innate immune defense	22
Regulation of apoptosis	16
GTP binding	10
WD-repeat proteins	8

Based on these criteria, we narrowed down the pool to 50 unique proteins in WT macrophages. Out of these 50 proteins identified, 37 were also translated in MyD88 KO macrophages infected with *L. pneumophila* for 6hrs (Fig. 4.4 B). Interestingly, the pattern-recognition receptors TLR2 and NLRP3 were not able to get synthesized in MyD88$^{-/-}$ macrophages post *Legionella* infection. All 13 proteins that were not present in infected MyD88$^{-/-}$ macrophages are shown in Fig 4.4 B (green box). Similarly, 9 proteins were not actively synthesized in WT resting macrophages (not detected in Uninfected BMDMs) (Fig.4.4 B, blue box).

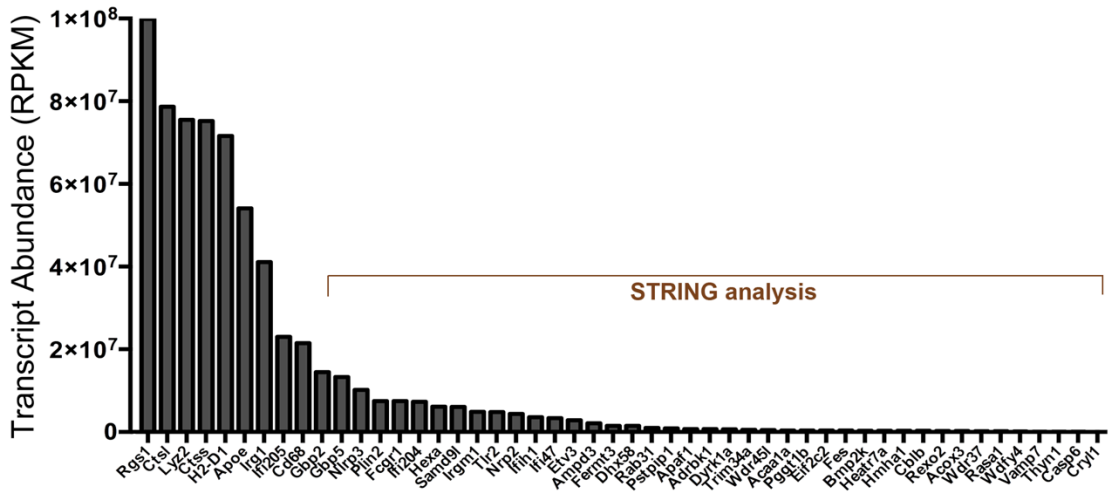
Functional analysis of the 50 identified proteins in WT macrophages indicated that there was an enrichment of IFN inducible proteins [141] (discussed in section 4.3), innate immune response proteins, nucleotide and GTP binging proteins, and regulators of apoptosis in the population (Fig. 4.4 C) (DAVID database [38]).

To determine if all these identified proteins bypassed translation inhibition due to their transcript abundance, their transcription levels were determined from our RNAseq analysis (Fig 4.4 A). Interestingly, looking at the overall transcription levels of the proteins that bypassed translation inhibition, we observed that transcript abundance by itself did not entirely explain the selective translation that occurred in response to *L. pneumophila*. About 20% of the proteins identified by mass spectrometry had transcription levels that were above the estimated threshold (*Rgs1, Lyz2, Irg1, Ifi205, H2-D1, CtsS, CtsL, Cd68* and *Apoe*). The remaining genes, however, were not highly transcribed, and their transcription levels were well below the estimated threshold (Fig 4.4 A), even though they were translated in cells harboring *L. pneumophila*.

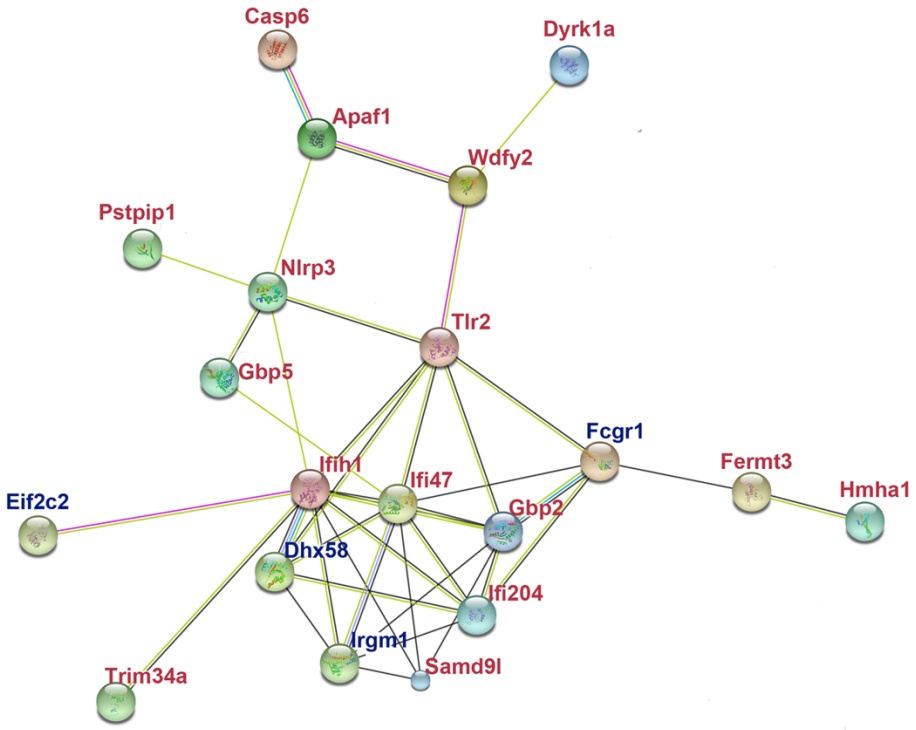
Figure 4.5: Transcript abundance and functional interaction network of proteins that were selectively translated during *L. pneumonia* challenge.

(A) Transcript abundance of 50 proteins that were selectively translated in WT macrophages following *L. pneumophila* challenge. The graph shown here is the transcript abundance of these proteins determined from RNAseq. (B) Functional interaction network (STRING database analysis [75]) for proteins that had transcript abundance below the estimated threshold. ~20 proteins were annotated as part of the same network and were type I IFN inducible. Red indicates uORF dependent translation initiation and blue indicates canonical initiation (discussed later).

A.


Transcript Abundance (RPKM)
1×10^8
8×10^7
6×10^7
4×10^7
2×10^7
0
STRING analysis
Rgs1 Ctsl Lyz2 Ctss H2-D1 Apoe Irg1 Ifi205 Cd68 Gbp2 Gbp5 Nlrp3 Plin2 Fcgr1 Ifi204 Hexa Samd9l Irgm1 Tlr2 Nrp2 Ifih1 Ifi47 Etv3 Ampd3 Fermt3 Dhx58 Rab31 Pstpip1 Apaf1 Adrbk1 Dyrk1a Trim34a Wdr45l Acaa1a Pggt1b Eif2c2 Fes Bmp2k Heatr7a Hmha1 Cblb Rexo2 Acox3 Wdfy37 Rasa1 Wdfy4 Vamp7 Tlyn1 Casp6 Cryl1

B.


Casp6
Dyrk1a
Apaf1
Wdfy2
Pstpip1
Nlrp3
Tlr2
Gbp5
Fcgr1
Ifih1
Ifi47
Fermt3
Hmha1
Eif2c2
Gbp2
Dhx58
Ifi204
Irgm1
Samd9l
Trim34a

4.3. Alternative mechanisms for bypass of translation inhibition

To further investigate how the remaining 41 proteins bypassed translation inhibition, we looked for common regulatory elements, similarities between mRNA secondary structures or AU reach (ARE) elements within 3'UTRs that regulate mRNA stability. Although some of these proteins had such regulatory elements, we were not able to detect major similarities/patterns among all the identified proteins that could explain the bypass. By focusing on the 41 proteins which had transcription levels below the threshold established as being sufficient to allow translation, we performed STRING interaction analysis [75], which revealed that a significant fraction of these proteins were associated with immune- and stress-response (Fig 4.5 B). About half of the identified proteins had been previously characterized as type I IFN inducible, and were associated with anti-viral immunity.

It has been shown in previous studies that under conditions of severe stress such as starvation, UV damage or viral infection, mammalian cells down-regulate their own protein synthesis by altering the levels of protein translation initiation [149]. Under such conditions, stress response genes are preferentially translated by a mechanism that involves initiation at an upstream open reading frame (uORFs). We hypothesized that the presence of uORFs or other alternative translation initiation mechanisms could explain the selective translation of some genes during *Legionella*-induced translation arrest.

To determine if uORFs or other *cis*-acting elements regulated translation re-initiation during *L. pneumophila* infection, we generated genome-wide maps of protein translation using ribosome profiling [68]. Libraries from ribosome-protected fragments were prepared from C57BL/6 BMDMs that were either infected with *L. pneumophila* for

6hrs or left untreated (Fig. 4.6). In this case, we could not specifically focus on macrophages harboring *L. pneumophila*, as the time taken to perform a sort resulted in extremely poor yields of ribosome-protected fragments. Therefore, our analysis was restricted to analyzing the nature of transcripts before stimulation by bacteria, compared to the bulk behavior of macrophages harboring bacteria and bystanders combined. Infection of macrophage monolayers with *L. pneumophila*-Δ*flaA* at MOI=15 for 6hrs only resulted in 20-30% infectivity and the majority of the population remained uninfected. However, since bystanders also respond to infection (previous chapter, Fig 3.5), we could use *L pneumophila* incubations as a readout for immune activation.

Examination of individual mRNA profiles of the 41 proteins that bypassed translation inhibition revealed that the majority of these proteins contained extensively translated upstream open reading frames (uORFs) within their 5'UTR. We found that ~80% of the genes that were resistant to *L. pneumophila*-induced translation inhibition contained extensively translated uORFs in their 5'UTR. This is a significant enrichment in uORF containing mRNA compared to the overall mammalian transcriptome, which is predicated to have ~ 35% uORF containing mRNAs [67].

Figure 4.6: Sucrose density gradient profile of uninfected and *L.p.* infected macrophages.

Top panel shows polysome profile of uninfected (left) and *L. pneumophila* treated (right) macrophages prior to nuclease digest. Note: The analysis was done in unsorted cells (about 20% infectivity), which results in more polysomes in the *L. pneumophila* treated samples due to bystander activation. Bottom panel shows monosome fractions from uninfected and *L.p.* treated BMDM digested with ribonuclease to generate ribosome-protected fragments.

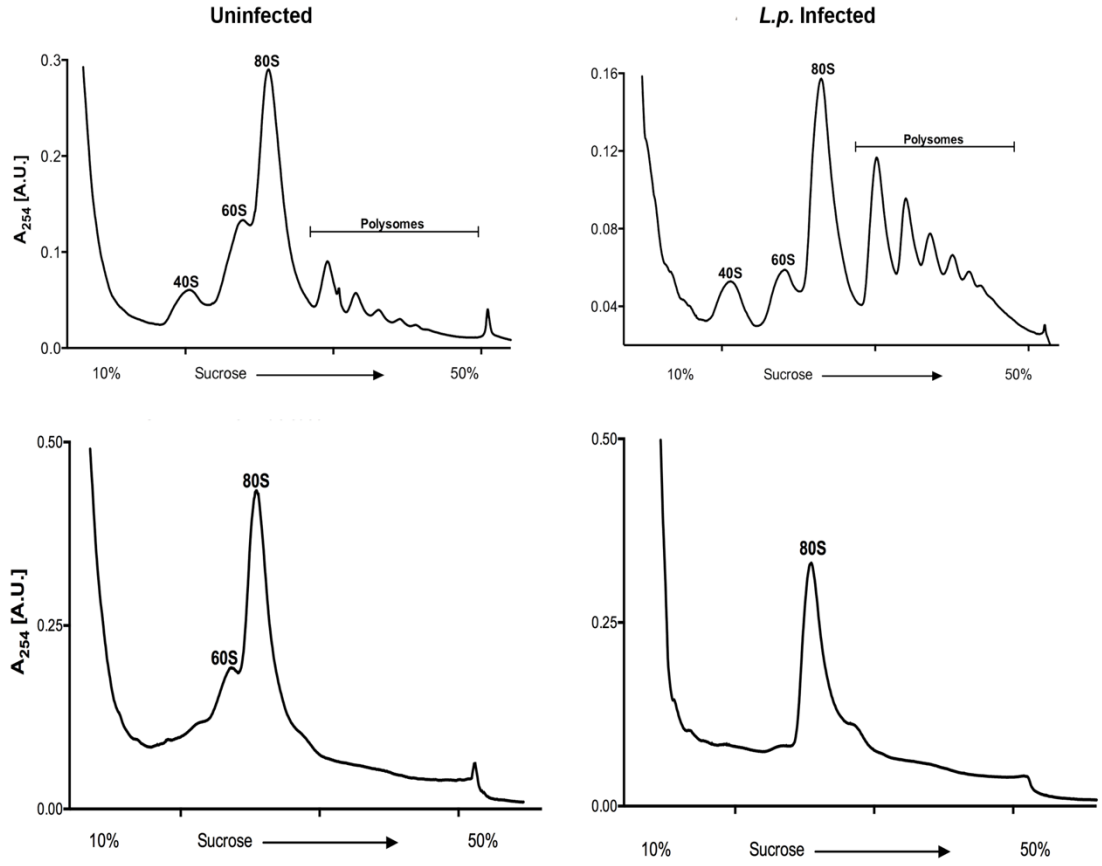

Uninfected
L.p. Infected
A254 [A.U.]
0.3
0.2
0.1
0.0
0.16
0.12
0.08
0.04
0.50
0.25
0.00
40S
60S
80S
Polysomes
10%
Sucrose
50%

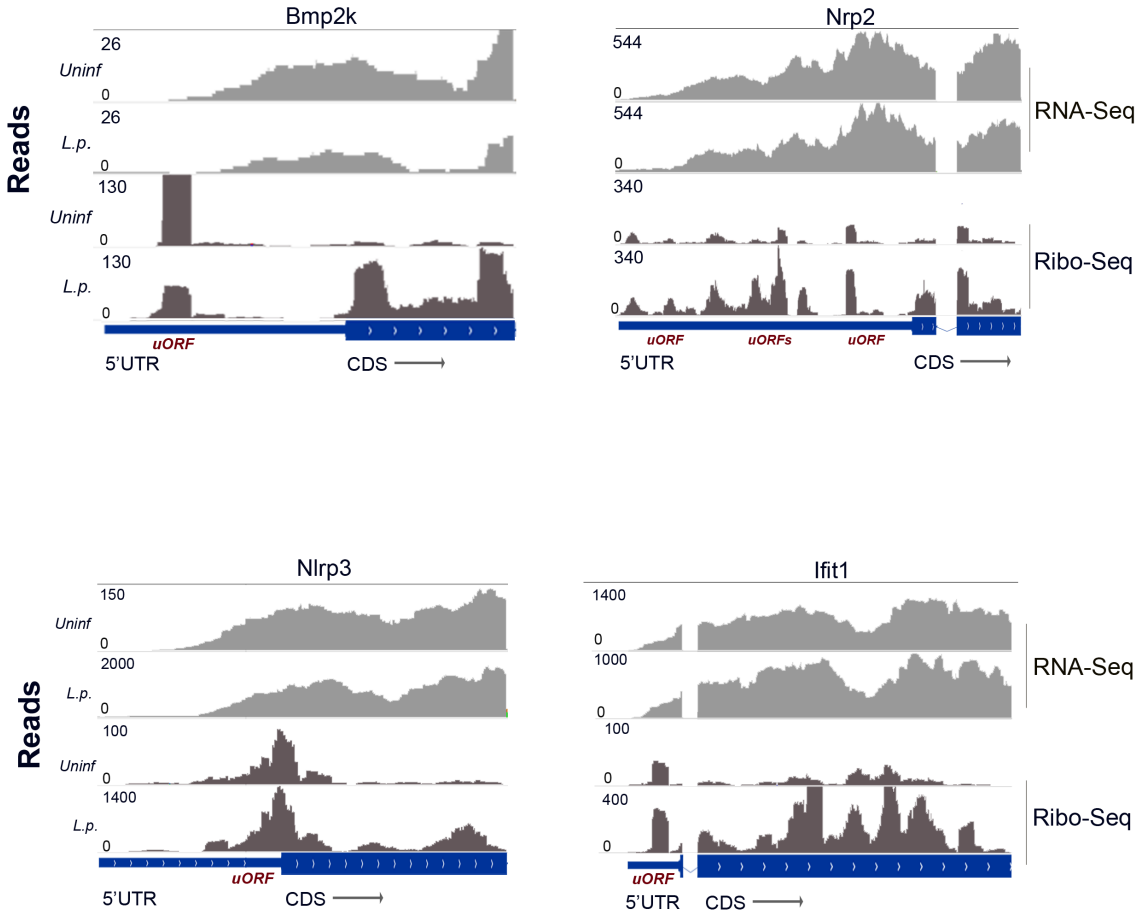
Table 3: Proteins that bypass translation inhibition during *Legionella* infection

Gene symbol	Full name	Translation initiation
FERMT3	fermitin family homolog 3	uORF
HEATR7A	HEAT repeat containing 7A	uORF
PLIN2	adipose differentiation related protein	uORF
SAMD9L	sterile alpha motif domain containing 9-like	uORF
GBP2	guanylate binding protein 2	uORF/Abundant
RAB31	RAB31, member RAS oncogene family	uORF, Truncation
HMHA1	histocompatibility (minor) HA-1	uORF, extension
PSTPIP1	proline-serine-threonine phosphatase-interacting protein 1	uORF, extension
REXO2	REX2, RNA exonuclease 2 homolog	uORF, truncation, internal out of frame [30]
NLRP3	NLR family, pyrin domain containing 3	uORF
IFI47	interferon gamma inducible protein 47	uORF
IFIH1	interferon induced with helicase C domain 1	uORF (MDA5 human analog)
IFI204	interferon activated gene 204	uORF (IFI16 human analog)
CASP6	caspase 6	uORF
APAF1	apoptotic peptidase activating factor 1	uORF
ACAA1A	acetyl-Coenzyme A acyltransferase 1A	uORF
ACOX3	acyl-Coenzyme A oxidase 3, pristanoyl	uORF
ADRBK1	adrenergic receptor kinase, beta 1	uORF
AMPD3	adenosine monophosphate deaminase 3	uORF
BMP2K	predicted gene 4521; BMP2 inducible kinase	uORF
CBLB	Casitas B-lineage lymphoma b	uORF

DYRK1A	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a	uORF
ETV3	ets variant gene 3	uORF
GBP5	guanylate binding protein 5	uORF
NRP2	neuropilin 2	uORF
RASA1	RAS p21 protein activator 1	uORF
TLR2	toll-like receptor 2	uORF
VAMP7	predicted gene 14338; vesicle-associated membrane protein 7	uORF
WDFY2	WD repeat and FYVE domain containing 2	uORF
WDR45L	Wdr45 like; predicted gene 6305	uORF
TRIM34A	Tripartite motif containing 34	uORF
THYN1	thymocyte nuclear protein 1	uORF/Extension [30]
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	uORF
IRGM1	immunity-related GTPase family M member 1	uORF
EIF2C2	eukaryotic translation initiation factor 2C, 2	uORF
HEXA	hexosaminidase A	uORF
WDR37	WD repeat domain 37	uORF
Gene symbol	Full name	Translation initiation
CRYL1	crystallin, lambda 1	canonical
FES	feline sarcoma oncogene	canonical/ Truncation [30]
FCGR1	Fc receptor, IgG, high affinity I	canonical
PGGT1B	protein geranylgeranyltransferase type I, beta subunit	canonical

Figure 4.7: Genes that are resistant to translation inhibition contain upstream open reading frames (uORFs) in their 5'UTR.

RNAseq reads (top 2 graphs for each gene) and ribosome-protected fragments (bottom 2 for each gene) were mapped to mouse reference genome (mm9). Alignments shown here are for 5'UTR (thinner blue bars) and the first one or two exons (thick blue bars) of Bmp2k, Nrp2, Nlrp3 and Ifit1, four genes that are efficiently translated upon *Legionella* challenge.


Bmp2k
Nrp2
Nlrp3
Ifit1
Reads
Uninf
L.p.
RNA-Seq
Ribo-Seq
uORF
uORFs
5'UTR
CDS

In some cases, these uORFs were efficiently translated in resting cells, but there was weak translation of the downstream coding sequence, consistent with the idea that these uORFs were preventing ribosome association with the main coding sequence (CDS) (Figure 4.7, Uninf, Ribo-Seq). Upon stimulation, this pattern was reversed, and robust translation could be detected at the main open reading frames (Fig 4.7, *L.p* treated samples, Ribo-Seq). For Bmp2k for example, there was a 30-fold increase in ribosome occupancy at the downstream coding sequence upon stimulation, which was independent of transcription (Fig 4.7 first panel, compare RNA-Seq and Ribo-Seq patterns between Uninfected and *L.p* treated BMDMs). Similar patterns were also observd for Nrp2, Nlrp3 and Ifit1 (Fig. 4.7). Such pattern of ribosome association indicates that there is translation re-initiation of these genes at the main coding sequence upon stimulation.

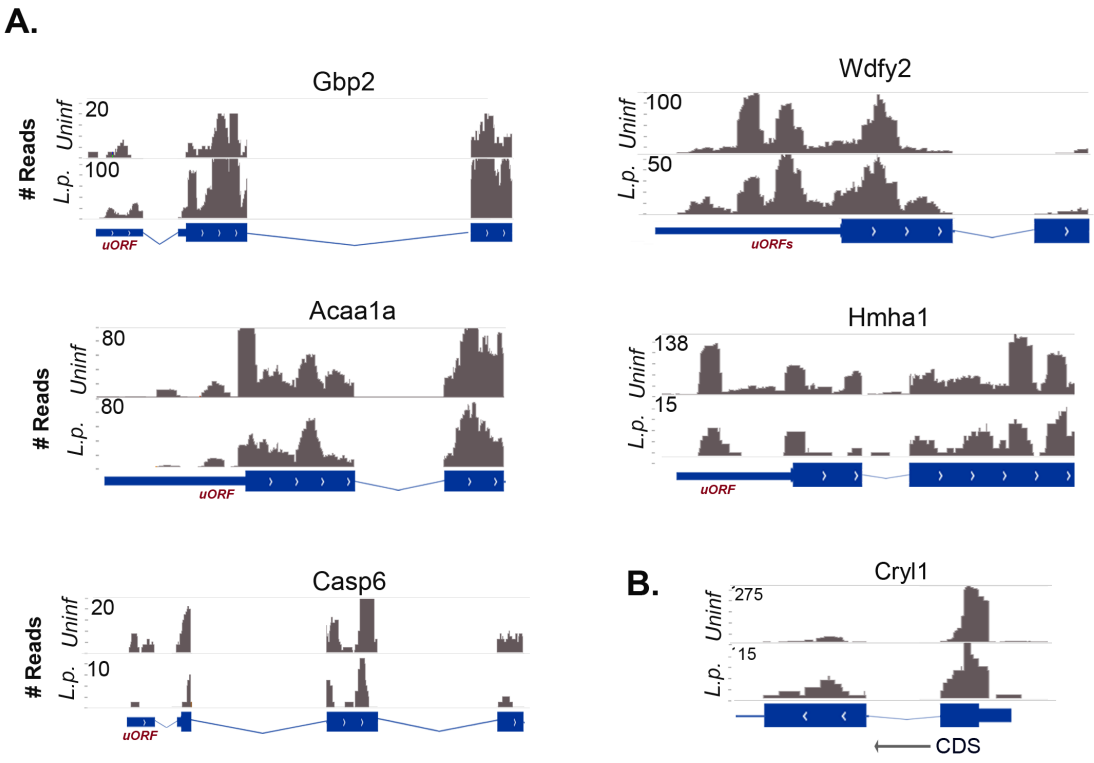
In other cases, such as guanylate binding protein 2 (Gbp2), acetyl-Coenzyme A acyltransferase 1A (Acaa1a) and Caspase 6 (Casp6), uORFs in the 5'UTR were not efficiently translated but there was an increase in ribosome association at the downstream open reading frame (Fig. 4.8 A, left panel), potentially indicating leaky ribosome scanning (refer to introduction for mechanisms of leaky translation).

A recent study has shown that a number of factors determine bypass of translation inhibition during a chemically induced translation arrest (sodium arsenate treatment), including distance of the uORF from the main ORF, length and structure of the 5'UTR and nucleotide context surrounding the uORF start codon (Kozak sequence) [7]. We were not able to determine such common factors among genes that bypassed translation inhibition during *L. pneumophila* infection (Fig 4.8 A). There was variation between the

number of uORFs observed in the 5'UTR, the distance of the uORFs from the cap and the presence/length of introns within the uORFs (Fig. 4.8 A). Although it is clear that these uORFs are playing a role in translation regulation during *L. pneumophila* infection, additional work is needed to elucidate the exact mechanism by which they facilitate bypass of translation inhibition. Interestingly, 4 proteins that bypassed translation inhibition did not have any uORFs or other *cis*-acting elements in their 5'UTR and initiation occurred at the canonical translation start site (Fig. 4.8 B). The mechanism that allows selective synthesis of these genes remains to be determined.

Figure 4.8: Ribosome profiling revealed alternative translation initiation mechanisms in most transcripts that bypass translation inhibition.

(A) A significant portion of genes that bypassed translation inhibition have uORFs in their 5'UTR. The ribosome association pattern in the 5'UTR and first two exons is shown here for a subset of these genes (B) Few transcripts that bypassed translation inhibition did not have any uORFs or other regulatory elements in their 5'UTR. The ribosome association pattern to one of these genes, Cryl, is shown here as an example. Note: The direction of translation for Cryl1 is reversed (shown by the arrow below).


A.
Gbp2
Reads
Uninf
L.p.
20
100
uORF
Wdfy2
100
50
uORFs
Acaa1a
80
80
uORF
Hmha1
138
15
uORF
Casp6
20
10
uORF
B.
Cryl1
275
15
CDS

Based on these findings, we hypothesize that the innate immune system has devised at least two distinct mechanisms to evade pathogen-induced translation inhibition. The first mechanism is dependent on transcript abundance, in which highly transcribed innate immune genes associate with remaining functional ribosomes despite a pathogen-induced translation block (Fig 4.9A). For example, lysozyme 2 (Lyz2) and regulator of G-protein signaling 1 (Rgs1), which are some of the most abundantly expressed genes upon *Legionella* infection, bypass translation inhibition due to their mRNA abundance. These transcripts do not contain any *cis*-acting regulatory elements (Fig 4.9 A), and their transcript abundance was sufficient to allow bypass.

A second mechanism for bypass of translation inhibition is seen in a number of Type I IFN-inducible innate immune genes, particularly genes that are part of the antiviral innate immunity. These genes, such as guanylate binding proteins 2 and 5 (Gbp2, Gbp5), Interferon inducible protein 47 (Ifi47), interferon induced with helicase C domain 1 (Ifih1, MDA5) and CD2 binding protein (Pstpip1), are resistant to translation inhibition due to alternative translation reinitiation mechanisms (uORFs) (Fig 4.9 B).

Figure 4.9: Two mechanisms are used for bypass of translation inhibition during *L. pneumophila* infection; Transcript abundance and uORF-mediated translation reinitiation.

(A) Patterns of translation for abundantly transcribed genes, Il1β, Rgs1 and Lyz2. (B). Initiation and elongation patterns for the innate immune genes, Gbp5, Ifi47 and Pstpip1. The cartoon on the right shows uORF-dependent translation reinitiation mechanism.

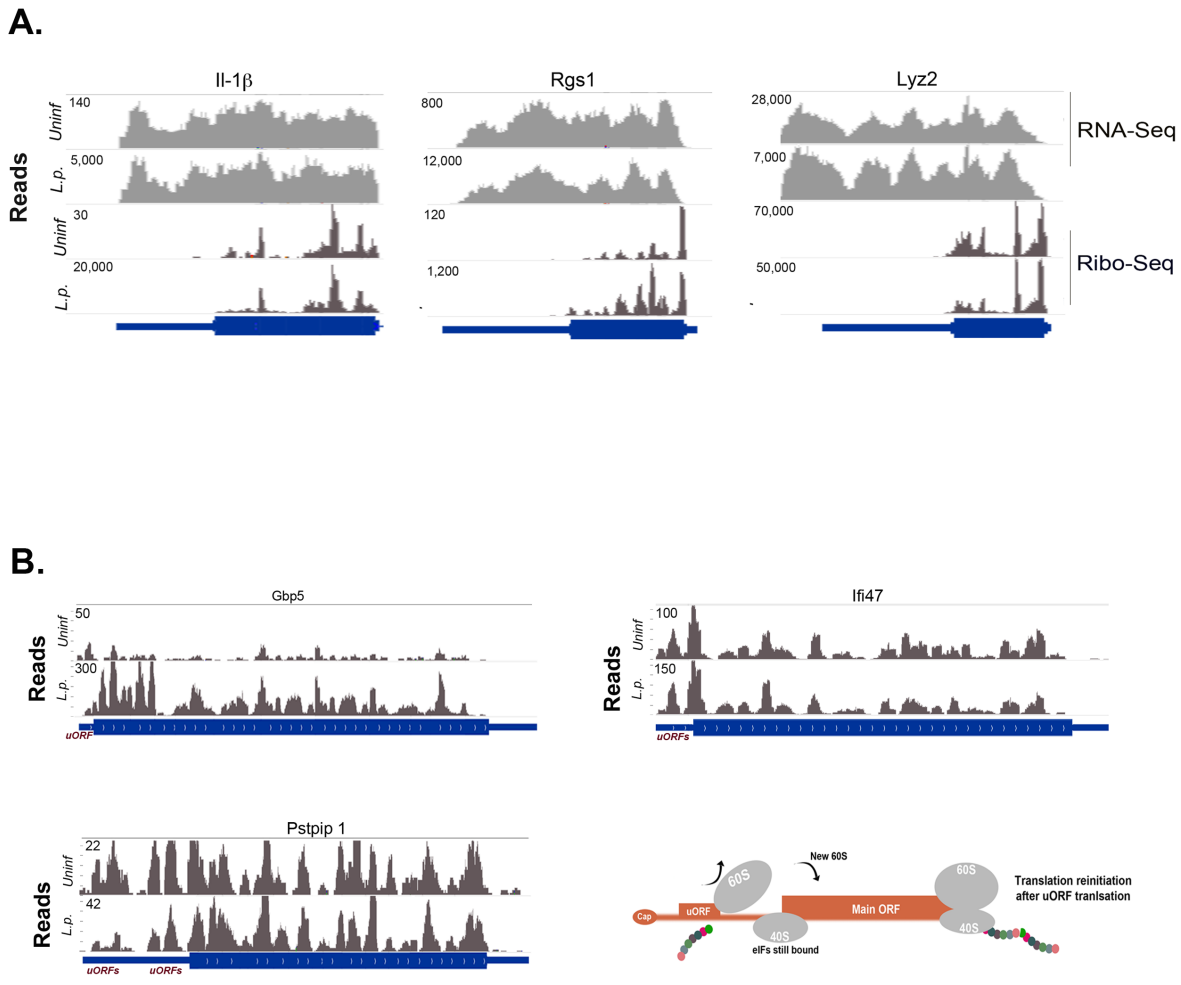


Table 4: Proteins that had more than 2 peptides per locus in WT BMDMs

Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity
Ahnak	61	66	6.79E+04	Uap1l1	12	14	6.83E+04	Hspd1	8	8	4.71E+04
Flna	54	64	9.73E+04	Fermt3	12	13	8.83E+04	Ifi204	8	8	8.73E+04
Iqgap1	48	65	1.14E+05	Fasn	12	13	2.96E+04	Ndufs1	8	8	7.73E+04
Tln1	42	48	5.87E+04	Atp1a1	12	12	8.39E+04	Tes	8	8	1.62E+05
Cltc	37	59	1.30E+05	Cct8	12	12	1.15E+05	Clic1	8	8	8.06E+04
Lcp1	35	49	1.54E+05	Rab7a	11	19	1.71E+05	Slc25a3	8	8	2.28E+05
Rnf213	33	34	4.20E+04	Akr1a1	11	18	3.19E+05	Stt3a	8	8	7.58E+04
Plec	31	33	4.29E+04	Sts	11	14	1.21E+05	Hist1h4a	7	16	8.01E+05
Dync1h1	23	23	4.91E+04	Prep	11	14	4.23E+04	Hnrnpa3	7	13	3.20E+05
Eef2	22	37	9.57E+04	Gsn	11	13	1.52E+05	Dab2	7	12	6.49E+04
Pgk1	22	35	1.78E+05	Ranbp2	11	12	4.78E+04	Ctsz	7	11	3.73E+05
Actn1	22	28	9.47E+04	Stat1	11	12	6.77E+04	Cfl1	7	10	4.43E+05
Lmna	22	24	8.02E+04	Pdia4	11	12	9.27E+04	Rps16	7	10	1.06E+05
Copa	22	24	9.80E+04	Ifit3	11	12	7.46E+04	Npepps	7	10	3.14E+04
Pdia3	21	30	1.88E+05	Atp5a1	11	12	1.20E+05	Acat1	7	9	4.97E+04
Cyfip1	20	22	1.23E+05	Myof	11	12	5.41E+04	Arhgdia	7	9	7.10E+04
Myh9	20	21	8.08E+04	Tgm2	11	11	1.22E+05	Erp29	7	9	5.23E+04
Hspa8	19	30	1.25E+05	Hspa4	11	11	8.48E+04	Lims1	7	9	1.44E+05
Dhx15	19	27	8.48E+04	Atic	11	11	5.20E+04	Rhog	7	9	6.55E+04
Myo1e	19	19	6.82E+04	Rps3	11	11	6.70E+04	Hnrnpf	7	9	1.72E+05
Tubb2a	18	35	9.93E+04	Hadha	11	11	8.34E+04	Anxa5	7	9	2.20E+05
Pkm	18	31	7.70E+05	Hnrnpa2b1	10	20	5.40E+05	Glb1	7	9	7.51E+04
Prpf8	18	20	3.77E+04	Fabp5	10	18	6.41E+04	Atp6v0d1	7	9	9.39E+04
Vim	17	25	4.06E+05	Pfn1	10	17	3.82E+05	Mapk3	7	9	7.29E+04
Serpinb6	17	20	1.12E+05	Vat1	10	14	7.68E+04	Plaa	7	8	3.15E+04
Tpp2	17	20	5.04E+04	Ctsa	10	14	2.19E+05	Dnajc13	7	8	3.98E+04
Hspa5	16	23	9.95E+04	D1Pas1	10	14	8.92E+04	FAM120A	7	8	6.30E+04
Cap1	16	23	1.78E+05	Gstm1	10	14	1.05E+05	Ppp2ca	7	8	2.23E+04
Gdi2	16	23	1.67E+05	Snx5	10	13	1.81E+05	Selenbp1	7	8	5.43E+04
Anxa1	16	21	2.51E+05	Hnrnpk	10	13	1.16E+05	Pdha1	7	8	8.97E+04
G6pdx	16	20	8.78E+04	P4hb	10	12	9.21E+04	Ifit2	7	8	9.25E+04
Vwa5a	16	19	8.90E+04	Pcbp1	10	12	8.70E+04	Vdac1	7	8	1.78E+05
Aco2	16	19	1.21E+05	Gpi	10	12	1.27E+05	Oas3	7	8	3.72E+04
Ehd4	16	18	9.97E+04	Ywhae	10	12	8.01E+04	Rars	7	8	4.16E+04
Atp6v1a	16	18	1.01E+05	Hnrnpul1	10	12	4.48E+04	Etfdh	7	8	8.13E+04
Dhx9	16	18	9.73E+04	Cat	10	11	8.66E+04	Rplp0	7	8	6.59E+04
Nono	16	18	1.01E+05	Mocos	10	11	4.84E+04	Nars	7	8	7.80E+04
Acta2	15	123	6.79E+05	Ogdh	10	11	1.07E+05	Anxa7	7	8	6.60E+04
Ctsb	15	67	7.58E+05	Blvra	10	11	9.87E+04	Prdx5	7	8	1.11E+05
Grn	15	22	1.22E+05	Gns	10	11	1.08E+05	Park7	7	8	8.58E+04
Cndp2	15	20	1.80E+05	Stip1	10	11	7.00E+04	Pxn	7	8	4.85E+04
Got2	15	20	1.42E+05	Hk3	10	10	1.27E+05	Nsun2	7	8	3.09E+04
Actn4	15	18	8.12E+04	Sri	10	10	1.63E+05	Man2c1	7	8	4.18E+04
Gnb2l1	15	18	2.55E+05	Rnpep	10	10	8.08E+04	Hmgb1	7	7	5.37E+04
Eif4a1	15	16	1.18E+05	Esd	9	20	4.44E+05	Dars	7	7	5.32E+04
Mrc1	15	16	9.86E+04	Actr3	9	13	1.18E+05	Psmc2	7	7	3.50E+04
Ppia	14	39	5.54E+05	Pdlim5	9	12	7.06E+04	Acsl1	7	7	4.78E+04
Prdx1	14	36	8.48E+05	Gdi1	9	12	7.88E+04	Pcna	7	7	7.68E+04
Eno1	14	26	2.27E+05	Psap	9	12	2.83E+05	Snd1	7	7	4.59E+04
Aldh2	14	25	1.83E+05	Fus	9	12	6.73E+04	Got1	7	7	5.11E+04
Pepd	14	24	9.86E+04	Arpc2	9	12	1.16E+05	Ifit1	7	7	1.08E+05
Hnrnpl	14	22	1.98E+05	Aldh9a1	9	11	8.01E+04	Thop1	7	7	4.61E+04
Lasp1	14	21	1.60E+05	Myo1c	9	11	2.69E+04	Metap1	7	7	9.14E+04
F13a1	14	19	9.21E+04	Atg7	9	10	4.26E+04	Snrnp200	7	7	3.98E+04
Uba1	14	17	8.49E+04	Eprs	9	10	3.85E+04	Hnrnpc	7	7	4.99E+04
Sdha	14	17	1.46E+05	Anxa3	9	10	9.75E+04	Pitrm1	7	7	4.22E+04
Tkt	14	17	1.03E+05	Naglu	9	10	4.80E+04	Dock8	7	7	4.27E+04
Hnrnpul2	14	16	7.16E+04	Hsp90aa1	9	10	1.33E+05	Lars	7	7	4.14E+04
Glud1	14	16	9.05E+04	Asah1	9	10	7.72E+04	Pa2g4	7	7	5.13E+04
Ddx17	14	16	1.04E+05	Xpo1	9	10	3.84E+04	Man2b1	7	7	4.45E+04
Vcp	14	16	5.21E+04	Rnh1	9	9	1.06E+05	Uggt1	7	7	3.19E+04
Pdcd6ip	14	15	7.51E+04	Myl6	9	9	1.90E+05	Psmd13	7	7	6.90E+04
Gvin1	14	15	4.47E+04	Usp9x	9	9	3.40E+04	Capn2	7	7	7.85E+04
Hexb	14	15	1.15E+05	Ap1b1	9	9	7.13E+04	Cct6a	7	7	3.81E+04
Ganab	14	14	1.10E+05	Cct3	9	9	7.26E+04	Hexa	7	7	7.80E+04
Myo1g	14	14	6.66E+04	Copb2	9	9	7.14E+04	Cox5b	6	14	1.21E+05
Ptpn6	13	21	7.22E+04	Ap2a2	9	9	4.50E+04	Capg	6	11	2.99E+05
Sdhb	13	20	1.89E+05	Slfn5	9	9	8.48E+04	Anxa2	6	10	1.78E+05
Tuba1a	13	20	2.56E+05	Tpi1	8	16	1.41E+05	Ctsc	6	10	3.08E+06
Hnrnpu	13	18	1.69E+05	Ckb	8	13	9.20E+04	Srsf1	6	10	5.32E+04
Pafah1b1	13	18	1.06E+05	Vps35	8	12	7.58E+04	Arpc1b	6	10	3.81E+05
Wdr1	13	18	1.84E+05	Lpxn	8	12	2.11E+05	Rpsa	6	9	7.28E+04
Ddx5	13	17	8.64E+04	Ctsd	8	12	5.56E+05	Atp6v1b2	6	9	1.59E+05
Akr1b1	13	17	7.88E+04	Hsp90ab1	8	11	9.92E+04	Dpysl2	6	9	1.40E+05
Atp5b	13	16	7.68E+04	Arf1	8	11	8.12E+04	Fhl3	6	9	2.11E+05
Xdh	13	16	7.37E+04	Sgpl1	8	11	7.23E+04	Sod2	6	9	1.35E+05
Snx2	13	16	9.88E+04	Myo1f	8	11	5.03E+04	Hist1h3a	6	9	1.88E+06
Nckap1l	13	16	6.59E+04	Psme1	8	10	6.05E+04	Trim25	6	8	4.42E+04
Coro1a	13	16	1.70E+05	Hk2	8	10	4.58E+04	Rpl23	6	8	3.67E+04
Pabpc1	13	15	8.61E+04	Capn1	8	10	4.00E+04	Gatm	6	8	5.40E+04
Idh2	13	15	6.88E+04	Capzb	8	10	1.19E+05	Pnp	6	8	1.52E+05
Khsrp	13	15	7.02E+04	Coro1c	8	10	1.17E+05	Por	6	8	6.28E+04
Gapdh	12	30	8.86E+05	Hprt1	8	10	2.63E+04	Qki	6	8	1.46E+04
Pgd	12	23	2.89E+05	Taldo1	8	9	1.27E+05		6	8	6.14E+04
Ldha	12	19	5.89E+05	Pdhb	8	9	6.86E+04	Nme1	6	8	1.75E+05
Tagln2	12	18	8.08E+04	Coro2a	8	9	3.90E+04	Calr	6	8	1.37E+05
Mdh2	12	17	1.34E+05	Rbm14	8	9	6.67E+04	Lgals1	6	7	2.50E+05
Aldoa	12	17	5.80E+05	Rps4x	8	9	6.43E+04	Kctd12	6	7	5.97E+04
Eef1a1	12	17	8.86E+05	Dock2	8	9	4.26E+04	Hmox1	6	7	6.56E+04
Actr2	12	16	1.22E+05	Manba	8	9	7.70E+04	Psmb1	6	7	1.65E+05
Samhd1	12	16	1.20E+05	Mapk1	8	9	9.67E+04	Acadl	6	7	8.43E+04
Msn	12	16	1.34E+05	Rps3a	8	9	1.54E+05	Fh	6	7	3.29E+04
Idh1	12	15	6.13E+04	Ilk	8	9	3.83E+04	Tufm	6	7	4.59E+04
Ezr	12	15	7.22E+04	Hspa9	8	8	5.22E+04	Trim28	6	7	3.67E+04
Hsp90b1	12	14	1.10E+05	Itgb2	8	8	5.67E+04	Ppib	6	7	8.81E+04
Cmpk2	12	14	1.40E+05	Anxa4	8	8	8.39E+04	Tcirg1	6	7	6.49E+04

Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity
Ahnak	61	66	6.79E+04	Uap1l1	12	14	6.83E+04	Hspd1	8	8	4.71E+04
Flna	54	64	9.73E+04	Fermt3	12	13	8.83E+04	Ifi204	8	8	8.73E+04
Iqgap1	48	65	1.14E+05	Fasn	12	13	2.96E+04	Ndufs1	8	8	7.73E+04
Tln1	42	48	5.87E+04	Atp1a1	12	12	8.39E+04	Tes	8	8	1.62E+05
Cltc	37	59	1.30E+05	Cct8	12	12	1.15E+05	Clic1	8	8	8.06E+04
Lcp1	35	49	1.54E+05	Rab7a	11	19	1.71E+05	Slc25a3	8	8	2.28E+05
Rnf213	33	34	4.20E+04	Akr1a1	11	18	3.19E+05	Stt3a	8	8	7.58E+04
Plec	31	33	4.29E+04	Sts	11	14	1.21E+05	Hist1h4a	7	16	8.01E+05
Dync1h1	23	23	4.91E+04	Prep	11	14	4.23E+04	Hnrnpa3	7	13	3.20E+05
Eef2	22	37	9.57E+04	Gsn	11	13	1.52E+05	Dab2	7	12	6.49E+04
Pgk1	22	35	1.78E+05	Ranbp2	11	12	4.78E+04	Ctsz	7	11	3.73E+05
Actn1	22	28	9.47E+04	Stat1	11	12	6.77E+04	Cfl1	7	10	4.43E+05
Lmna	22	24	8.02E+04	Pdia4	11	12	9.27E+04	Rps16	7	10	1.06E+05
Copa	22	24	9.80E+04	Ifit3	11	12	7.46E+04	Npepps	7	10	3.14E+04
Pdia3	21	30	1.88E+05	Atp5a1	11	12	1.20E+05	Acat1	7	9	4.97E+04
Cyfip1	20	22	1.23E+05	Myof	11	12	5.41E+04	Arhgdia	7	9	7.10E+04
Myh9	20	21	8.08E+04	Tgm2	11	11	1.22E+05	Erp29	7	9	5.23E+04
Hspa8	19	30	1.25E+05	Hspa4	11	11	8.48E+04	Lims1	7	9	1.44E+05
Dhx15	19	27	8.48E+04	Atic	11	11	5.20E+04	Rhog	7	9	6.55E+04
Myo1e	19	19	6.82E+04	Rps3	11	11	6.70E+04	Hnrnpf	7	9	1.72E+05
Tubb2a	18	35	9.93E+04	Hadha	11	11	8.34E+04	Anxa5	7	9	2.20E+05
Pkm	18	31	7.70E+05	Hnrnpa2b1	10	20	5.40E+05	Glb1	7	9	7.51E+04
Prpf8	18	20	3.77E+04	Fabp5	10	18	6.41E+04	Atp6v0d1	7	9	9.39E+04
Vim	17	25	4.06E+05	Pfn1	10	17	3.82E+05	Mapk3	7	9	7.29E+04
Serpinb6	17	20	1.12E+05	Vat1	10	14	7.68E+04	Plaa	7	8	3.15E+04
Tpp2	17	20	5.04E+04	Ctsa	10	14	2.19E+05	Dnajc13	7	8	3.98E+04
Hspa5	16	23	9.95E+04	D1Pas1	10	14	8.92E+04	FAM120A	7	8	6.30E+04
Cap1	16	23	1.78E+05	Gstm1	10	14	1.05E+05	Ppp2ca	7	8	2.23E+04
Gdi2	16	23	1.67E+05	Snx5	10	13	1.81E+05	Selenbp1	7	8	5.43E+04
Anxa1	16	21	2.51E+05	Hnrnpk	10	13	1.16E+05	Pdha1	7	8	8.97E+04
G6pdx	16	20	8.78E+04	P4hb	10	12	9.21E+04	Ifit2	7	8	9.25E+04
Vwa5a	16	19	8.90E+04	Pcbp1	10	12	8.70E+04	Vdac1	7	8	1.78E+05
Aco2	16	19	1.21E+05	Gpi	10	12	1.27E+05	Oas3	7	8	3.72E+04
Ehd4	16	18	9.97E+04	Ywhae	10	12	8.01E+04	Rars	7	8	4.16E+04
Atp6v1a	16	18	1.01E+05	Hnrnpul1	10	12	4.48E+04	Etfdh	7	8	8.13E+04
Dhx9	16	18	9.73E+04	Cat	10	11	8.66E+04	Rplp0	7	8	6.59E+04
Nono	16	18	1.01E+05	Mocos	10	11	4.84E+04	Nars	7	8	7.80E+04
Acta2	15	123	6.79E+05	Ogdh	10	11	1.07E+05	Anxa7	7	8	6.60E+04
Ctsb	15	67	7.58E+05	Blvra	10	11	9.87E+04	Prdx5	7	8	1.11E+05
Grn	15	22	1.22E+05	Gns	10	11	1.08E+05	Park7	7	8	8.58E+04
Cndp2	15	20	1.80E+05	Stip1	10	11	7.00E+04	Pxn	7	8	4.85E+04
Got2	15	20	1.42E+05	Hk3	10	10	1.27E+05	Nsun2	7	8	3.09E+04
Actn4	15	18	8.12E+04	Sri	10	10	1.63E+05	Man2c1	7	8	4.18E+04
Gnb2l1	15	18	2.55E+05	Rnpep	10	10	8.08E+04	Hmgb1	7	7	5.37E+04
Eif4a1	15	16	1.18E+05	Esd	9	20	4.44E+05	Dars	7	7	5.32E+04
Mrc1	15	16	9.86E+04	Actr3	9	13	1.18E+05	Psmc2	7	7	3.50E+04
Ppia	14	39	5.54E+05	Pdlim5	9	12	7.06E+04	Acsl1	7	7	4.78E+04
Prdx1	14	36	8.48E+05	Gdi1	9	12	7.88E+04	Pcna	7	7	7.68E+04
Eno1	14	26	2.27E+05	Psap	9	12	2.83E+05	Snd1	7	7	4.59E+04
Aldh2	14	25	1.83E+05	Fus	9	12	6.73E+04	Got1	7	7	5.11E+04
Pepd	14	24	9.86E+04	Arpc2	9	12	1.16E+05	Ifit1	7	7	1.08E+05
Hnrnpl	14	22	1.98E+05	Aldh9a1	9	11	8.01E+04	Thop1	7	7	4.61E+04
Lasp1	14	21	1.60E+05	Myo1c	9	11	2.69E+04	Metap1	7	7	9.14E+04
F13a1	14	19	9.21E+04	Atg7	9	10	4.26E+04	Snrnp200	7	7	3.98E+04
Uba1	14	17	8.49E+04	Eprs	9	10	3.85E+04	Hnrnpc	7	7	4.99E+04
Sdha	14	17	1.46E+05	Anxa3	9	10	9.75E+04	Pitrm1	7	7	4.22E+04
Tkt	14	17	1.03E+05	Naglu	9	10	4.80E+04	Dock8	7	7	4.27E+04
Hnrnpul2	14	16	7.16E+04	Hsp90aa1	9	10	1.33E+05	Lars	7	7	4.14E+04
Glud1	14	16	9.05E+04	Asah1	9	10	7.72E+04	Pa2g4	7	7	5.13E+04
Ddx17	14	16	1.04E+05	Xpo1	9	10	3.84E+04	Man2b1	7	7	4.45E+04
Vcp	14	16	5.21E+04	Rnh1	9	9	1.06E+05	Uggt1	7	7	3.19E+04
Pdcd6ip	14	15	7.51E+04	Myl6	9	9	1.90E+05	Psmd13	7	7	6.90E+04
Gvin1	14	15	4.47E+04	Usp9x	9	9	3.40E+04	Capn2	7	7	7.85E+04
Hexb	14	15	1.15E+05	Ap1b1	9	9	7.13E+04	Cct6a	7	7	3.81E+04
Ganab	14	14	1.10E+05	Cct3	9	9	7.26E+04	Hexa	7	7	7.80E+04
Myo1g	14	14	6.66E+04	Copb2	9	9	7.14E+04	Cox5b	6	14	1.21E+05
Ptpn6	13	21	7.22E+04	Ap2a2	9	9	4.50E+04	Capg	6	11	2.99E+05
Sdhb	13	20	1.89E+05	Slfn5	9	9	8.48E+04	Anxa2	6	10	1.78E+05
Tuba1a	13	20	2.56E+05	Tpi1	8	16	1.41E+05	Ctsc	6	10	3.08E+06
Hnrnpu	13	18	1.69E+05	Ckb	8	13	9.20E+04	Srsf1	6	10	5.32E+04
Pafah1b1	13	18	1.06E+05	Vps35	8	12	7.58E+04	Arpc1b	6	10	3.81E+05
Wdr1	13	18	1.84E+05	Lpxn	8	12	2.11E+05	Rpsa	6	9	7.28E+04
Ddx5	13	17	8.64E+04	Ctsd	8	12	5.56E+05	Atp6v1b2	6	9	1.59E+05
Akr1b1	13	17	7.88E+04	Hsp90ab1	8	11	9.92E+04	Dpysl2	6	9	1.40E+05
Atp5b	13	16	7.68E+04	Arf1	8	11	8.12E+04	Fhl3	6	9	2.11E+05
Xdh	13	16	7.37E+04	Sgpl1	8	11	7.23E+04	Sod2	6	9	1.35E+05
Snx2	13	16	9.88E+04	Myo1f	8	11	5.03E+04	Hist1h3a	6	9	1.88E+06
Nckap1l	13	16	6.59E+04	Psme1	8	10	6.05E+04	Trim25	6	8	4.42E+04
Coro1a	13	16	1.70E+05	Hk2	8	10	4.58E+04	Rpl23	6	8	3.67E+04
Pabpc1	13	15	8.61E+04	Capn1	8	10	4.00E+04	Gatm	6	8	5.40E+04
Idh2	13	15	6.88E+04	Capzb	8	10	1.19E+05	Pnp	6	8	1.52E+05
Khsrp	13	15	7.02E+04	Coro1c	8	10	1.17E+05	Por	6	8	6.28E+04
Gapdh	12	30	8.86E+05	Hprt1	8	10	2.63E+04	Qki	6	8	1.46E+04
Pgd	12	23	2.89E+05	Taldo1	8	9	1.27E+05		6	8	6.14E+04
Ldha	12	19	5.89E+05	Pdhb	8	9	6.86E+04	Nme1	6	8	1.75E+05
Tagln2	12	18	8.08E+04	Coro2a	8	9	3.90E+04	Calr	6	8	1.37E+05
Mdh2	12	17	1.34E+05	Rbm14	8	9	6.67E+04	Lgals1	6	7	2.50E+05
Aldoa	12	17	5.80E+05	Rps4x	8	9	6.43E+04	Kctd12	6	7	5.97E+04
Eef1a1	12	17	8.86E+05	Dock2	8	9	4.26E+04	Hmox1	6	7	6.56E+04
Actr2	12	16	1.22E+05	Manba	8	9	7.70E+04	Psmb1	6	7	1.65E+05
Samhd1	12	16	1.20E+05	Mapk1	8	9	9.67E+04	Acadl	6	7	8.43E+04
Msn	12	16	1.34E+05	Rps3a	8	9	1.54E+05	Fh	6	7	3.29E+04
Idh1	12	15	6.13E+04	Ilk	8	9	3.83E+04	Tufm	6	7	4.59E+04
Ezr	12	15	7.22E+04	Hspa9	8	8	5.22E+04	Trim28	6	7	3.67E+04
Hsp90b1	12	14	1.10E+05	Itgb2	8	8	5.67E+04	Ppib	6	7	8.81E+04
Cmpk2	12	14	1.40E+05	Anxa4	8	8	8.39E+04	Tcirg1	6	7	6.49E+04

Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity
Plcg2	6	7	2.40E+04	Hcls1	5	5	6.52E+04	Usp5	4	5	2.00E+04
Fubp1	6	7	9.13E+04	P4ha1	5	5	4.18E+04	Vdac3	4	5	2.26E+05
Sec23b	6	7	3.45E+04	2-Sep	5	5	5.86E+04		4	5	2.46E+04
St13	6	7	1.22E+05	Uqcrc1	5	5	9.23E+04	Psma6	4	5	1.19E+05
Eif2s1	6	7	7.08E+04	Acads	5	5	5.90E+04	Vcpip1	4	5	3.96E+04
Psmd2	6	7	3.46E+04	Nfkb2	5	5	5.27E+04	Sec24b	4	5	3.21E+04
Cs	6	7	6.95E+04	Irgm1	5	5	6.30E+04	Abhd12	4	5	8.41E+04
Hnrnpm	6	7	4.48E+04	Eif2a	5	5	5.69E+04	Dync1i2	4	5	7.77E+04
Ahcy	6	7	6.12E+04	Dnpep	5	5	4.27E+04	Psmc5	4	5	3.60E+04
Ipo5	6	7	2.35E+04	Pccb	5	5	1.65E+04	Gda	4	5	2.39E+04
Aco1	6	7	5.81E+04	Cct2	5	5	4.59E+04	Brox	4	5	1.47E+05
Blvrb	6	7	3.75E+05	Mvp	5	5	5.33E+04	Erap1	4	5	3.36E+04
Iqgap2	6	7	3.96E+04	Ppa2	5	5	2.64E+04	Myl12b	4	5	6.02E+04
Gnpda1	6	7	9.23E+04	Rps21	5	5	2.61E+05	Tomm70a	4	5	4.37E+04
Npc2	6	7	2.47E+05	Psma4	5	5	8.34E+04	Tnfaip3	4	5	4.49E+04
Slc25a4	6	7	1.29E+05	Cpd	5	5	3.90E+04	Prmt5	4	5	3.89E+04
Eif3a	6	7	2.21E+04	Pygb	5	5	5.58E+04	Lpp	4	5	2.57E+04
Pgm1	6	6	6.74E+04	Atp6v1c1	5	5	4.56E+04	Rtn4	4	5	8.35E+04
Vars	6	6	5.17E+04		5	5	3.76E+04	Ddx21	4	5	2.65E+04
Nufip2	6	6	1.90E+05	Aldoc	5	5	5.30E+04	Nans	4	5	9.74E+04
Lta4h	6	6	6.41E+04	Ivd	5	5	1.08E+05	Vps39	4	5	9.25E+03
Bmp2k	6	6	5.20E+04	Man2a1	5	5	3.05E+04	Lipa	4	5	7.47E+04
Ehd1	6	6	6.14E+04	Ddost	5	5	8.45E+04	Rpl27	4	5	1.74E+05
Anxa11	6	6	7.17E+04	Apaf1	5	5	4.93E+04	Ptbp1	4	5	1.55E+05
Hnrnph1	6	6	1.25E+05	Rragc	5	5	3.85E+04	Snx3	4	5	7.98E+04
Ankrd44	6	6	3.47E+04	D10Wsu52e	5	5	5.53E+04	Etfa	4	4	4.31E+04
Hk1	6	6	7.60E+04	Anxa6	5	5	4.61E+04	Eea1	4	4	4.09E+04
Cpeb4	6	6	2.94E+04	Ppa1	5	5	4.64E+04	Eci1	4	4	3.61E+04
Rpn1	6	6	6.39E+04	Mta2	5	5	3.05E+04	Inpp5d	4	4	3.58E+04
Snx9	6	6	4.70E+04	Sacm1l	5	5	8.35E+04	Dnm2	4	4	4.87E+04
Eif3c	6	6	3.28E+04	Dhx58	5	5	2.94E+04	Eif3l	4	4	3.67E+04
Bub3	6	6	1.37E+05	Malt1	5	5	3.53E+04	Taok3	4	4	3.90E+04
Phgdh	6	6	7.05E+04	Hadhb	5	5	1.00E+05	Ndufv2	4	4	3.54E+04
Coro7	6	6	3.54E+04	Camk2d	5	5	4.66E+04	Apoe	4	4	8.91E+04
Znfx1	6	6	4.72E+04	Rpl4	5	5	4.51E+04	Ndufs7	4	4	6.41E+04
Rpl13	6	6	1.54E+05	Psmd1	5	5	4.67E+04	Slc25a11	4	4	3.97E+04
Coro1b	6	6	7.10E+04	Mpeg1	5	5	6.51E+04	Mthfd1	4	4	3.52E+04
Lgals3bp	6	6	4.02E+04	Sec16a	5	5	3.62E+04	Rab2a	4	4	4.35E+04
Gbe1	6	6	1.95E+04	Ap2m1	5	5	3.14E+04	Stom	4	4	3.46E+04
Copb1	6	6	6.51E+04	Atp5o	5	5	6.39E+04	Dtx3l	4	4	3.03E+04
Hdlbp	6	6	3.54E+04	Stt3b	5	5	2.47E+04	Tubb6	4	4	1.36E+05
Vps11	6	6	5.11E+04	Ddx39a	5	5	4.25E+04	Eef1d	4	4	1.00E+05
Adar	6	6	2.81E+04	Eif5a	5	5	8.01E+04	Sec31a	4	4	3.95E+04
Tcp1	6	6	6.56E+04	BC006779	5	5	2.27E+04	Eif3d	4	4	8.93E+04
Pitpna	6	6	7.45E+04	Lss	5	5	3.63E+04	Capza2	4	4	3.14E+04
Tns3	6	6	4.93E+04	Atp6v1e1	5	5	6.79E+04	Cox5a	4	4	9.40E+04
Psma1	6	6	1.42E+05	Rab11a	5	5	7.32E+04	Atp2a2	4	4	6.43E+04
Stat2	6	6	4.52E+04	Rps2	5	5	7.17E+04	Esyt1	4	4	6.76E+04
Psma3	6	6	7.06E+04	Cct5	5	5	4.59E+04	Hsd17b4	4	4	8.42E+04
Elmo1	6	6	5.21E+04	Rps9	5	5	9.94E+04	Sfxn3	4	4	2.64E+04
Csk	6	6	5.96E+04	Sf3b1	5	5	5.50E+04	Pdxk	4	4	6.53E+04
Iars	6	6	2.77E+04	Glt25d1	5	5	3.79E+04	Csrp1	4	4	6.82E+04
Actb	5	20	4.26E+06	Txnrd1	5	5	1.01E+05	Aaas	4	4	1.01E+05
Tubb3	5	13	2.05E+05	Trappc11	5	5	5.57E+04	Echs1	4	4	9.95E+04
Ctss	5	9	1.21E+05	Naa15	5	5	3.55E+04	Cand1	4	4	1.86E+04
Hnrnpd	5	9	3.17E+05	Syncrip	5	5	5.32E+04	Slc30a5	4	4	1.01E+05
Psmb8	5	8	1.33E+05	Eef1g	5	5	9.42E+04	Mat2a	4	4	4.56E+04
Vdac2	5	8	1.99E+05	Nsf	5	5	3.74E+04	Snx6	4	4	1.02E+05
Lgals3	5	8	4.58E+05	Glo1	5	5	7.32E+04	Asph	4	4	2.02E+04
Fam129b	5	7	7.29E+04	C1qc	5	5	1.51E+05	Slc25a12	4	4	4.15E+04
Pdia6	5	7	9.89E+04	Ndufv1	5	5	5.35E+04	Atp6v1h	4	4	6.68E+04
Lsp1	5	7	4.95E+04	Ap1g1	5	5	3.74E+04	Raly	4	4	5.48E+04
Ywhab	5	7	1.62E+05	Vav1	5	5	9.10E+04	Flnb	4	4	1.47E+04
Dld	5	7	8.88E+04	Ifih1	5	5	3.07E+04	Hgsnat	4	4	8.27E+04
Ube2v1	5	7	7.64E+04	Ndufb9	5	5	6.12E+04	Hnrpll	4	4	4.27E+04
Grb2	5	7	1.09E+05	Tubb1	4	14	2.88E+05	Vps16	4	4	4.69E+04
Txn	5	7	3.81E+05	Tubb4a	4	8	1.67E+05	Psme2	4	4	5.06E+04
Dlat	5	7	1.58E+04	Arpc4	4	8	1.27E+05	Dlst	4	4	5.52E+04
Sqstm1	5	6	7.53E+04	Gnai2	4	7	7.73E+04	Cct7	4	4	3.35E+04
Ctsl1	5	6	2.94E+05	Slc30a7	4	7	2.21E+05	Asns	4	4	4.80E+04
Rpl18	5	6	2.27E+05	Uba52	4	7	1.67E+05	Abi1	4	4	7.33E+04
Eef1b	5	6	9.30E+04	Lyz1	4	7	4.02E+05	Apex1	4	4	3.80E+04
Rab14	5	6	3.49E+04	Fes	4	6	2.91E+04	Ripk1	4	4	3.56E+04
Tbxas1	5	6	4.15E+04	Gnai1	4	6	1.11E+05	Dnm1l	4	4	2.37E+04
Ppp2r4	5	6	3.25E+04	Hnrnpa1	4	6	7.32E+04	Rasa4	4	4	3.98E+04
Slc25a5	5	6	1.49E+05		4	6	4.22E+04	Sfpq	4	4	1.44E+05
Mcm7	5	6	2.94E+04	Psmb6	4	6	1.41E+05	Ptpn1	4	4	4.55E+04
Rpl30	5	6	1.11E+05	Cotl1	4	6	1.03E+05	Psma7	4	4	5.57E+04
Top1	5	6	3.04E+04	Rps29	4	6	1.08E+05	Nampt	4	4	6.22E+04
Wdfy2	5	6	2.56E+04	Rps12	4	6	5.06E+04	Wasf2	4	4	4.65E+04
Rps18	5	6	1.22E+05	Cstb	4	6	1.94E+05	Immt	4	4	3.96E+04
Prdx6	5	6	8.41E+04	Sec13	4	5	8.25E+04	Eif3b	4	4	3.39E+04
Ppp3ca	5	6	4.64E+04	Hnrnpab	4	5	5.01E+04	Hmha1	4	4	2.28E+04
Ddx1	5	6	4.46E+04	Rpl12	4	5	8.82E+04	Jak1	4	4	2.23E+04
Ywhah	5	6	7.31E+04	Uqcrc2	4	5	6.46E+04	Slc9a3r1	4	4	9.67E+04
Elavl1	5	6	8.16E+04	Plin3	4	5	3.83E+04	Amdhd2	4	4	6.67E+04
Apeh	5	6	2.77E+04	Ifi205a	4	5	3.27E+04	Gpx4	4	4	1.14E+05
Cnot1	5	6	1.74E+04	Gnb1	4	5	1.59E+05	Rpl7	4	4	9.57E+04
Sec24c	5	6	4.01E+04	Eif2s3x	4	5	7.34E+04	Tars	4	4	2.04E+04
Ifi47	5	6	2.50E+04	Mpp1	4	5	5.83E+04	Rbbp4	4	4	1.13E+05
H2-D1	5	6	3.79E+04	Rbm39	4	5	2.96E+04		4	4	3.13E+04
Rac1	5	6	1.30E+05	Irg1	4	5	5.44E+04	Kpnb1	4	4	4.72E+04
Rpl13a	5	6	1.28E+05	Mthfd1l	4	5	1.87E+04	Rhoa	4	4	1.29E+05
Pfkl	5	5	1.01E+05	Impdh2	4	5	2.28E+04	Scp2	4	4	5.95E+04
Sqrdl	5	5	8.44E+04	Snrpb	4	5	3.26E+04	Ppp1ca	4	4	6.38E+04
Ogt	5	5	4.55E+04	Rpl7a	4	5	4.44E+04	Myh10	4	4	5.56E+04

Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity
Ppp2r1a	4	4	5.46E+04	Map4	3	4	1.54E+04	Gnai3	3	3	6.46E+04
Oat	4	4	4.80E+04	AI607873	3	3	7.85E+04	Gls	3	3	3.67E+04
Tap1	4	4	5.44E+04	Tardbp	3	3	3.46E+04	Cybb	3	3	4.34E+04
Btk	4	4	4.92E+04	Ankfy1	3	3	2.96E+04	Tle3	3	3	2.95E+04
Dyrk1a	4	4	5.79E+04	Luc7l2	3	3	4.83E+04	Arpc5	3	3	8.01E+04
Atp6v0a1	4	4	7.26E+04	Crmp1	3	3	1.41E+05	Nln	3	3	3.73E+04
Iqsec1	4	4	3.85E+04	Psmb10	3	3	9.53E+04	Memo1	3	3	5.82E+04
Psma5	4	4	7.76E+04	Snrpf	3	3	3.85E+04	Cars	3	3	4.15E+04
Itgam	4	4	5.30E+04	Phf5a	3	3	1.17E+05	Sirt2	3	3	2.86E+04
Wdr26	4	4	3.00E+04	Wdr5	3	3	3.33E+04	Pogz	3	3	2.22E+04
Pstpip1	4	4	2.77E+04	Chchd2	3	3	5.06E+04	Spr	3	3	3.07E+04
Idh3b	4	4	5.49E+04	Pak2	3	3	2.45E+04	Vamp7	3	3	2.42E+04
Rcc2	4	4	5.26E+04	Rae1	3	3	9.67E+04	Rab9a	3	3	4.38E+04
Twf1	4	4	5.00E+04	Tbc1d15	3	3	2.76E+04	Uba7	3	3	5.88E+04
Arpc3	4	4	1.62E+05	Dbnl	3	3	4.11E+04	Pgls	3	3	5.56E+04
Rps15a	4	4	4.85E+04	Prpf4	3	3	5.59E+04	Rps19	3	3	2.11E+05
Acly	4	4	1.89E+04	Bola1	3	3	6.60E+04	Fdx1l	3	3	4.05E+04
Tecr	4	4	6.09E+04	Psmd9	3	3	4.90E+04	Fndc3a	3	3	5.12E+04
Cherp	4	4	7.22E+04	Ndrg1	3	3	7.36E+04	Pi4ka	3	3	3.01E+04
Gusb	4	4	5.89E+04	Abce1	3	3	8.24E+04	Psmb4	3	3	5.77E+04
Agps	4	4	3.24E+04	Igbp1	3	3	3.85E+04	Rab5a	3	3	9.03E+04
Ahsa1	4	4	2.89E+04	Dnmt3a	3	3	2.07E+04	Grpel1	3	3	3.80E+04
Hist1h2ba	3	14	1.20E+06	Rbmx	3	3	7.28E+04	Vasp	3	3	3.32E+04
Gapdhs	3	7	5.58E+05	Pfas	3	3	4.58E+04	Aldh3b1	3	3	7.20E+04
Mogs	3	6	3.81E+04	Dhcr7	3	3	1.12E+05	Ide	3	3	2.28E+04
Eno3	3	5	9.92E+04	Cpeb2	3	3	1.29E+04	Drg2	3	3	1.91E+04
Erp44	3	5	3.64E+04	Ptbp3	3	3	5.64E+04	Heatr7a	3	3	3.70E+04
Rplp2	3	5	6.93E+04	7-Sep	3	3	5.85E+04	Hnrpdl	3	3	1.06E+05
Pgam1	3	5	1.62E+05	Nmt1	3	3	4.43E+04	Nup155	3	3	2.54E+04
Tes	3	5	4.36E+04	Cstf1	3	3	2.96E+04	Tpm3	3	3	6.46E+04
Txndc17	3	5	9.10E+04	Mat2b	3	3	4.72E+04	Gnas	3	3	3.62E+04
Pcbp2	3	5	1.27E+05	Cbx3	3	3	6.74E+04	Shmt2	3	3	5.76E+04
Acbd3	3	5	2.45E+04	Rcc1	3	3	2.65E+04	Psmd4	3	3	4.00E+04
Capza1	3	5	4.95E+04	Sf3b5	3	3	1.31E+05	B2m	3	3	2.34E+05
Mtco2	3	5	4.96E+04	Casp1	3	3	2.21E+04	Tpd52	3	3	1.92E+04
Adssl1	3	4	2.32E+04	Pml	3	3	3.80E+04	Snrnp40	3	3	4.86E+04
Pebp1	3	4	1.29E+05	Impa1	3	3	6.42E+04	Fndc3b	3	3	3.47E+04
Otub1	3	4	3.97E+04	Cyb5r3	3	3	1.17E+05	Pafah1b3	3	3	4.25E+04
Rpn2	3	4	4.26E+04	H6pd	3	3	1.89E+04	Map2k1	3	3	8.39E+04
Slc39a10	3	4	2.87E+04	Psmd12	3	3	7.68E+04	Pygl	3	3	3.58E+04
G3bp1	3	4	2.38E+04	Pik3ap1	3	3	4.26E+04	Rbm47	3	3	4.94E+04
Safb	3	4	2.85E+04	Uba6	3	3	1.24E+04	Snrpd2	3	3	3.72E+04
Ywhaz	3	4	2.55E+05	Nploc4	3	3	5.92E+04	Ppp6c	3	3	1.88E+04
Aldh4a1	3	4	2.95E+04	Wdr91	3	3	4.09E+04	Rap1b	3	3	2.74E+04
Mdh1	3	4	7.55E+04	Ifitm3	3	3	5.87E+04	Rpl10a	3	3	1.69E+05
Ugt1a7c	3	4	3.18E+04	Hsd17b11	3	3	3.47E+04	Npepl1	3	3	2.91E+04
Wars	3	4	2.91E+04	Ddi2	3	3	8.95E+03	Fcgr1	3	3	5.12E+04
Rps14	3	4	9.16E+04	Dhrs1	3	3	2.83E+04	Psmd3	3	3	2.98E+04
Galk2	3	4	6.46E+04	Psat1	3	3	6.84E+04	Srm	3	3	4.82E+04
Vps29	3	4	1.89E+05	Flii	3	3	3.02E+04	Ap2a1	3	3	3.99E+04
Chmp4b	3	4	6.55E+04	Slc3a2	3	3	4.77E+04	Pls3	3	3	2.98E+04
Strap	3	4	4.62E+04	Cmpk1	3	3	4.66E+04	Cpne3	3	3	1.93E+04
Suclg1	3	4	6.88E+04	Fnbp1	3	3	7.41E+04	Rpl3	3	3	8.40E+04
Myadm	3	4	5.69E+04	Kiaa0196	3	3	3.36E+04	Nlrp3	3	3	3.27E+04
Zeb2	3	4	1.95E+04	Dctn4	3	3	6.67E+04	Renbp	3	3	9.95E+04
Rpl5	3	4	5.34E+04	Ubap2l	3	3	1.03E+05	Mov10	3	3	4.18E+04
Blmh	3	4	2.75E+04	Rpl18a	3	3	4.66E+04	Ethe1	3	3	7.64E+04
Cbl	3	4	4.09E+04	Galk1	3	3	3.20E+04	Ncl	3	3	5.38E+04
Pcyox1	3	4	2.43E+04	Akr1c13	3	3	4.34E+04	Ostf1	3	3	1.15E+05
Sf3a2	3	4	2.45E+04	Nop56	3	3	3.52E+04	Nt5c2	3	3	3.88E+04
Gba	3	4	1.60E+04	Myg1	3	3	4.81E+04	Idh3a	3	3	5.72E+04
Tmpo	3	4	8.65E+04	Eml2	3	3	5.30E+04	Zyx	3	3	4.55E+04
Hmgcl	3	4	4.33E+04	Hnrnph2	3	3	6.10E+04	Hdac4	3	3	7.44E+04
Gbp2	3	4	3.23E+04	Hist1h1c	3	3	4.96E+05	Acaa1a	3	3	2.53E+04
Mlec	3	4	3.76E+04	Arf6	3	3	2.61E+04	Cbr2	3	3	2.31E+04
Zfp384	3	4	4.82E+04	Chi3l3	3	3	6.01E+04	Hmgb2	3	3	9.96E+04
Adsl	3	4	6.38E+04	Mtmr6	3	3	4.12E+04	Sh3glb1	3	3	8.28E+04
Sumf1	3	4	4.52E+04	Tmem214	3	3	7.46E+04	Nup35	3	3	2.80E+04
Aak1	3	4	1.55E+04	Wdr77	3	3	3.33E+04	Rpl35a	3	3	1.42E+05
9-Sep	3	4	5.20E+04	Cct4	3	3	8.35E+04	Xpo7	3	3	3.18E+04
Nqo2	3	4	4.88E+04	Tnpo1	3	3	2.56E+04	Ndufs6	3	3	1.23E+05
Cpt2	3	4	3.83E+04	Nit1	3	3	3.89E+04	Cdc5l	3	3	1.41E+04
Hist1h1b	3	4	9.59E+04	Snx1	3	3	2.04E+05	Agl	3	3	5.10E+04
Trim21	3	4	2.81E+04	Eif2ak2	3	3	2.22E+04	Ahcyl1	3	3	1.10E+05
Rps26	3	4	3.99E+04	Lbr	3	3	6.68E+04	Fech	3	3	6.70E+04
Wdr11	3	4	2.06E+04	Plod3	3	3	6.13E+04	Dcxr	3	3	2.10E+04
Nlrx1	3	4	2.33E+04	Picalm	3	3	2.30E+04	Rpl8	3	3	2.38E+05
Ap2b1	3	4	6.40E+04	Pfkp	3	3	2.21E+04	Cblb	3	3	1.83E+04
Cox4i1	3	4	7.94E+04	Rps27l	3	3	1.15E+05	Sec61a1	3	3	1.53E+05
Prdx3	3	4	4.57E+04	Atp2a1	3	3	1.95E+04	Mtch2	3	3	4.79E+04
Hist1h2bb	3	4	1.84E+06	Acadm	3	3	7.24E+04	Pde12	3	3	1.92E+04
Mavs	3	4	3.55E+04	Ftl1	3	3	5.96E+04	Actr1b	3	3	6.23E+04
Rps13	3	4	1.58E+05	Ipo7	3	3	1.72E+04	Psmd8	3	3	6.69E+04
Iars2	3	4	1.79E+04	Tlr2	3	3	3.47E+04	Rbpj	3	3	5.94E+04
Lamp2	3	4	5.29E+04	Rps25	3	3	1.91E+05	Hck	3	3	4.83E+04
Dok3	3	4	1.92E+04	Rpl37a	3	3	8.16E+04	Ranbp10	3	3	4.41E+04
Ascc3	3	4	3.01E+04	Nek9	3	3	2.85E+04	Plin2	3	3	1.80E+04
Copg2	3	4	2.73E+04	Hist1h1a	3	3	2.33E+05	Ttll12	3	3	2.52E+04
Mob1a	3	4	2.80E+04	Pmm2	3	3	4.21E+04	Limd1	3	3	3.08E+04
Rap1a	3	4	1.00E+05	Glul	3	3	4.87E+04	Gsdmdc1	3	3	8.28E+04
Cd68	3	4	4.67E+05	Rpl15	3	3	2.49E+05	Wdfy4	3	3	2.80E+04
Dstn	3	4	1.33E+05	Rab31	3	3	3.43E+04	Eftud2	3	3	3.89E+04
Cyc1	3	4	3.84E+04	Cdc42	3	3	1.09E+05	Rraga	3	3	4.80E+04
Ttc37	3	4	4.08E+03	Nceh1	3	3	5.89E+04	Ilf3	3	3	3.37E+04
Ube2n	3	4	5.77E+04	Rps6	3	3	4.63E+04	Gart	3	3	4.37E+04
Gpnmb	3	4	2.20E+05	Ugp2	3	3	4.28E+04	Prmt1	3	3	2.90E+04

Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity	Gene Symbol	Unique	Total	Avg. Precursor Intensity
Camk2a	3	3	4.64E+04	Tsnax	2	3	6.80E+03	Wdr37	2	2	4.32E+04
Mtss1	3	3	4.64E+04	Arl8b	2	3	1.44E+05	Gla	2	2	1.58E+05
Parp12	3	3	6.96E+04	Safb2	2	3	4.00E+04	Ranbp9	2	2	2.95E+04
Rpl28	3	3	1.32E+05		2	3	2.66E+04	Sp100	2	2	4.88E+04
Hmgcs1	3	3	4.20E+04	S100a4	2	3	3.31E+05	Glod4	2	2	5.07E+04
Rab18	3	3	2.19E+04	Fkbp1a	2	2	1.25E+05	Snf8	2	2	3.27E+04
Gbp5	3	3	4.14E+04	Pter	2	2	4.25E+04	Rps20	2	2	1.52E+05
Eif4b	3	3	3.62E+04	Etv3	2	2	2.44E+04	Ptgr1	2	2	3.30E+04
Rnf114	3	3	4.96E+04	Nit2	2	2	1.49E+04	Akap8	2	2	3.67E+04
Dnase2	3	3	7.23E+04	Ywhaq	2	2	5.59E+04	Polr2b	2	2	1.93E+04
Ube2m	3	3	6.07E+04	Tpm3-rs7	2	2	5.04E+04	Naga	2	2	3.29E+04
Arcn1	3	3	4.37E+04	Aldh7a1	2	2	3.65E+04	Naa50	2	2	5.93E+04
Etf1	3	3	2.92E+04	Rrbp1	2	2	4.89E+04	Sec22b	2	2	5.78E+04
Rps11	3	3	1.07E+05	Trap1	2	2	1.20E+05	Ciao1	2	2	1.32E+04
Hkdc1	3	3	3.95E+04	Hsph1	2	2	3.01E+04	Lap3	2	2	4.55E+04
Cisd2	3	3	4.91E+04	Rpl14	2	2	3.40E+04	Nrp2	2	2	3.41E+04
Hsd17b12	3	3	5.03E+04	Luc7l3	2	2	3.30E+04	Gmppb	2	2	1.66E+04
Etfb	3	3	3.88E+04	Ak3	2	2	2.44E+04	Mapre1	2	2	9.06E+04
Camk1	3	3	4.75E+04	Rps8	2	2	2.68E+04	Zbp1	2	2	2.58E+04
Qars	3	3	3.57E+04	Snrpc	2	2	6.57E+04	H2-Q10	2	2	8.68E+04
Acot10	3	3	5.23E+04	Erlin1	2	2	9.49E+04	Rgs1	2	2	6.03E+04
Tbcb	3	3	6.39E+04	Me2	2	2	7.93E+04	Lancl2	2	2	5.51E+04
Aacs	3	3	3.83E+04	Osbpl8	2	2	5.12E+04	Tmed10	2	2	2.11E+04
Naa25	3	3	1.57E+04	Hebp1	2	2	4.19E+04	Txnl1	2	2	1.93E+04
Tubb5	2	6	2.26E+05	Ythdf3	2	2	8.61E+04	Mcl1	2	2	5.01E+04
Eno2	2	5	7.26E+03	Ssr4	2	2	3.23E+04	Api5	2	2	2.63E+04
Gnb2	2	5	1.30E+05	Aldh16a1	2	2	7.24E+04	Zfp207	2	2	6.50E+04
Mrps7	2	4	1.30E+04	Dpp3	2	2	2.60E+04	Ykt6	2	2	3.03E+04
Des	2	4	3.13E+05	Atp5h	2	2	7.89E+04	Man2b2	2	2	3.79E+04
Capg	2	4	2.75E+05	Napa	2	2	3.48E+04	Psmb5	2	2	8.53E+04
Rpl27a	2	4	1.42E+05	Gnaq	2	2	4.15E+04	Atxn10	2	2	2.96E+04
Hibadh	2	4	1.61E+04	Sfxn1	2	2	6.22E+04	Irf5	2	2	3.94E+04
Scpep1	2	4	1.65E+04	Psmd11	2	2	8.03E+04	Supt16h	2	2	1.41E+04
Hspa1a	2	4	3.46E+05	Lyn	2	2	3.23E+04	Ugdh	2	2	2.85E+04
Ptbp2	2	4	1.23E+05	Parp14	2	2	4.06E+04	Psmd14	2	2	1.41E+05
Yy1	2	4	1.55E+04	Dpf2	2	2	2.54E+04	Pld3	2	2	8.49E+04
Hist1h2af	2	4	3.41E+06	Ppp5c	2	2	7.68E+04	Tpd52l2	2	2	6.33E+04
Snx12	2	4	7.13E+04	Sf3b3	2	2	2.96E+04	Nop58	2	2	2.60E+04
Ncf1	2	3	9.50E+03	Sh3bgrl	2	2	1.23E+05	Fen1	2	2	8.10E+04
Adh5	2	3	4.46E+04	Tbl3	2	2	2.46E+04	Tial1	2	2	1.81E+05
Acsl4	2	3	1.66E+04	Syk	2	2	1.00E+05	Atg3	2	2	7.22E+04
Lgals9	2	3	1.08E+05	Slc37a2	2	2	3.73E+04	Adrbk1	2	2	8.00E+04
Rpl10	2	3	2.69E+04	Lilrb4	2	2	2.65E+04	Ptk2b	2	2	5.92E+04
Ywhag	2	3	1.84E+05	Pyhin1	2	2	5.78E+04	Ewsr1	2	2	6.95E+04
Sh3bgrl3	2	3	6.65E+04	Wbp2	2	2	1.24E+05	Strn4	2	2	5.48E+04
Junb	2	3	3.30E+04	Sec23a	2	2	2.68E+04	Ggh	2	2	3.07E+04
Rbms1	2	3	1.13E+05	Vps26a	2	2	9.16E+04	Tbl2	2	2	3.75E+04
Manf	2	3	4.30E+04	Unc93b1	2	2	4.04E+04	Msra	2	2	8.37E+04
Rasl2-9	2	3	1.38E+05	Eif6	2	2	3.70E+04	Atox1	2	2	7.66E+04
Tsta3	2	3	3.58E+04	Galc	2	2	8.27E+04	Tap2	2	2	1.65E+04
Znf326	2	3	3.86E+04	Nfatc2	2	2	3.54E+04	Ncbp1	2	2	3.47E+04
Fam98a	2	3	3.69E+04	Erh	2	2	5.73E+04	Arf4	2	2	2.72E+04
Cpne2	2	3	1.84E+04	Chordc1	2	2	5.04E+04	Clcc1	2	2	3.24E+04
Caprin1	2	3	1.31E+05	Tbl1xr1	2	2	2.95E+04	Psmb7	2	2	2.27E+05
Rpl11	2	3	8.52E+04	Uso1	2	2	4.12E+04	Atp5i	2	2	4.84E+04
Aars	2	3	4.97E+04	Xpnpep1	2	2	9.56E+04	Emc8	2	2	1.74E+05
Ankle2	2	3	1.93E+04	Cdc37	2	2	3.97E+04	Rps17	2	2	1.16E+05
Sars	2	3	4.54E+04	Eif2c2	2	2	1.49E+04	Frmd4a	2	2	1.10E+05
Ubr4	2	3	3.81E+04	Ola1	2	2	4.43E+04	Eif3k	2	2	2.41E+04
Mtm1	2	3	2.54E+04	Ap3m1	2	2	3.73E+04	Rbm7	2	2	6.20E+04
Rpl22	2	3	9.55E+04	Rpl19	2	2	6.79E+04	Kars	2	2	4.70E+04
Ear6	2	3	5.02E+04	Nhp2l1	2	2	4.97E+04	Eps15	2	2	1.58E+04
Phb	2	3	4.92E+04	Stx7	2	2	9.18E+04	Gltp	2	2	5.86E+04
Slc25a10	2	3	1.53E+04	Rpl31	2	2	1.47E+05	Wdr45l	2	2	2.70E+04
Plod1	2	3	2.08E+04	Gmppa	2	2	3.89E+04	Ergic1	2	2	9.29E+04
Clptm1	2	3	3.52E+04	Lmnb1	2	2	2.25E+04	Arl1	2	2	3.32E+04
Atxn2	2	3	2.51E+04	Prpf6	2	2	3.54E+04	Pmpcb	2	2	6.31E+04
Ctsh	2	3	6.13E+04	Adk	2	2	3.40E+04	Twf2	2	2	6.26E+04
	2	3	1.50E+04	Aifm1	2	2	3.51E+04	Arhgdib	2	2	2.38E+05
Prdx4	2	3	1.11E+06	Ahnak2	2	2	4.32E+04	Glrx3	2	2	3.31E+04
Chchd3	2	3	4.30E+04	Ifit2	2	2	1.28E+05	Cycs	2	2	6.13E+04
Psmb3	2	3	3.88E+04	Strn	2	2	2.50E+04	Acad8	2	2	2.61E+04
Acadvl	2	3	2.54E+04	Mettl7a2-Higd	2	2	4.35E+04	Dpp9	2	2	3.20E+04
Cox6b1	2	3	1.29E+05	Epb41l2	2	2	4.34E+04	Aldh18a1	2	2	9.30E+03
Trmt112	2	3	6.66E+03	Isca2	2	2	1.35E+04	Commd7	2	2	4.35E+04
Celf2	2	3	1.56E+04	Paf1	2	2	2.50E+04	Hspa2	2	2	9.69E+04
Usp14	2	3	1.71E+04	Ypel5	2	2	4.16E+04	Ssb	2	2	5.85E+04
Acsl3	2	3	3.12E+04	Snrnp70	2	2	3.19E+04	Lsm4	2	2	5.55E+04
Rpl17	2	3	3.20E+05	Prkar1a	2	2	4.22E+04	Osbp	2	2	5.35E+04
Parp1	2	3	1.21E+04	Clic4	2	2	7.97E+04	Psma8	2	2	3.95E+04
Tm9sf3	2	3	1.23E+05	Arhgap25	2	2	1.28E+04	Zdhhc13	2	2	2.97E+04
Cfp	2	3	4.20E+04	Fntb	2	2	6.84E+04	Lypla1	2	2	9.70E+04
Rab21	2	3	2.74E+04	Numa1	2	2	3.91E+04	Ikbkg	2	2	6.05E+04
Arrb2	2	3	1.07E+04	Arih2	2	2	4.46E+04	Mrpl37	2	2	2.86E+04
Chd8	2	3	3.74E+04	Lrch1	2	2	3.86E+04	Atxn2l	2	2	3.55E+04
Skap2	2	3	3.26E+04	Tpt1	2	2	1.55E+05	Thyn1	2	2	2.70E+04
Ube1ay	2	3	6.42E+04	Ifi30	2	2	3.92E+04	Ran	2	2	1.67E+05
Arl8a	2	3	9.84E+04	Pcca	2	2	1.10E+04	Gmds	2	2	5.07E+04
Crebbp	2	3	7.92E+03	Txndc5	2	2	3.82E+04	Selrc1	2	2	2.96E+04
Akr1e2	2	3	8.93E+05	Tor1aip1	2	2	5.48E+04	Phb2	2	2	4.38E+04
Smap2	2	3	1.59E+04	Gsr	2	2	6.89E+04	Srrm2	2	2	2.67E+04
Tmem66	2	3	1.81E+04	Psmc6	2	2	6.86E+04	Gaa	2	2	2.31E+04
Ufd1l	2	3	1.09E+04	Slc6a6	2	2	3.03E+04	Eif2s2	2	2	4.34E+04
Vac14	2	3	2.41E+04	Acox3	2	2	5.68E+04	Dhcr24	2	2	3.33E+04
Pdlim4	2	3	3.58E+04	Ndufb7	2	2	2.60E+04	Rab5c	2	2	6.86E+04
Pls1	2	3	1.58E+05	Cpne1	2	2	6.71E+04	Tsg101	2	2	2.78E+04

Gene Symbol	Unique	Total	Avg. Precursor Intensity
Matr3	2	2	4.55E+04
Prkaa1	2	2	3.50E+04
Srsf3	2	2	2.99E+04
H2afy	2	2	3.73E+04
Tle1	2	2	1.00E+05
Sptlc2	2	2	6.79E+04
Prkag1	2	2	4.04E+04
Ptges3	2	2	2.29E+05
Uprt	2	2	2.86E+04
Rasa1	2	2	1.03E+04
Rexo2	2	2	4.32E+04
Atp5c1	2	2	7.01E+04
Lrrc47	2	2	3.68E+04
Samd9l	2	2	2.93E+04
Acaca	2	2	1.18E+04
Smu1	2	2	3.21E+04
Lpcat3	2	2	2.37E+04
Mcm2	2	2	3.42E+04
Snrpd3	2	2	5.20E+04
Prdx2	2	2	3.85E+04
Git1	2	2	4.23E+04
Slc25a1	2	2	4.49E+04
Ptpre	2	2	4.70E+03
Rps28	2	2	4.88E+04
Copg1	2	2	2.28E+04
Arhgef6	2	2	2.52E+04
Hp1bp3	2	2	1.53E+04
Csde1	2	2	2.29E+04
Pla2g15	2	2	4.93E+04
Syngr1	2	2	3.53E+04
Stat6	2	2	2.69E+04
Iah1	2	2	1.38E+04
Rpl6	2	2	1.05E+05
Bcat2	2	2	1.07E+05
Hars	2	2	3.95E+04
Capzb	2	2	1.14E+05
Myo1f	2	2	7.62E+04
Nudt21	2	2	5.52E+04
Vav3	2	2	4.94E+04
Vbp1	2	2	3.69E+04
D10Jhu81e	2	2	5.02E+04
Abr	2	2	4.45E+04
Clta	2	2	8.62E+04
Nup153	2	2	2.08E+04
Rdh11	2	2	5.56E+04
Tpm1	2	2	9.85E+04
Acad9	2	2	2.01E+04
Cmas	2	2	3.09E+04
Tnip1	2	2	4.05E+04
Ctbp1	2	2	6.33E+04
Ppid	2	2	2.73E+04
Mta1	2	2	2.82E+04
Plekhf2	2	2	5.52E+04
Pfkm	2	2	3.54E+04
Acp1	2	2	2.19E+05
Prpf19	2	2	7.45E+04
Psmd6	2	2	2.33E+04
Rpl9	2	2	6.45E+04
Gpd2	2	2	3.76E+04
Vrk1	2	2	2.53E+04
Myo5a	2	2	3.21E+04
Tuba4a	2	2	8.01E+04
Dnm1	2	2	3.18E+04
Ndufs2	2	2	5.86E+04
Parp9	2	2	1.81E+04
Trim34a	2	2	5.15E+04
Aarsd1	2	2	1.62E+04
Plrg1	2	2	4.88E+04
Engase	2	2	1.94E+04
Asna1	2	2	2.02E+04
Reep5	2	2	5.80E+04
Cggbp1	2	2	5.25E+04
Atp12a	2	2	1.69E+05
Zc3hav1	2	2	7.30E+04
Rpl26	2	2	4.45E+04
Hpse	2	2	4.09E+04
Aimp1	2	2	2.37E+04
Mccc1	2	2	9.69E+03
Fubp3	2	2	3.24E+04
Vps13c	2	2	4.61E+04
Isyna1	2	2	4.44E+04
Prkcd	2	2	7.51E+04
Stmn1	2	2	2.84E+04
Srsf10	2	2	3.89E+04
Mtap	2	2	5.97E+04
Cacybp	2	2	3.21E+04
Atp5f1	2	2	8.54E+04
Pggt1b	2	2	1.52E+04
Sucla2	2	2	4.78E+04
Fam129a	2	2	3.76E+04
Cryl1	2	2	3.30E+04
S100a6	2	2	1.95E+05
Prkaca	2	2	7.77E+04
Kpna3	2	2	5.09E+04
Gtf2b	2	2	1.23E+04
Dcaf7	2	2	3.01E+04
Rpl36	2	2	5.93E+04
Farsb	2	2	1.65E+04
Ampd3	2	2	3.49E+04
Idh3g	2	2	3.82E+04
Hgs	2	2	4.10E+04
Usp8	2	2	8.50E+03
Gm2a	2	2	9.86E+04
Apbb1ip	2	2	3.85E+04
Pcyt1a	2	2	5.70E+04
U2af1	2	2	1.09E+05
Rps6ka3	2	2	5.18E+04
Ndufs8	2	2	2.24E+05
Sf3b2	2	2	4.64E+04
Ddb1	2	2	3.15E+04
Osbpl11	2	2	3.35E+04
Tm9sf4	2	2	4.22E+04
Pgm2	2	2	6.12E+04
Lamtor2	2	2	6.89E+04
Csnk2a1	2	2	4.77E+04
Adprh	2	2	2.93E+04
Rbmxl1	2	2	1.57E+04
Gfpt1	2	2	1.37E+04
Eml4	2	2	1.91E+04
Psma2	2	2	1.11E+05
Alox5ap	2	2	3.64E+04
Sec24d	2	2	1.55E+04
Oxct1	2	2	3.77E+04
Smpd4	2	2	5.03E+04
Acaa2	2	2	2.48E+04
Dnajc10	2	2	6.79E+04
Trip12	2	2	2.18E+04
Ptbp1	2	2	7.50E+04
Snx8	2	2	4.64E+04
Lrrc59	2	2	2.32E+04
Rpl10l	2	2	3.86E+04
Rapsn	2	2	1.51E+04
Nutf2	2	2	1.28E+05
Clcn7	2	2	9.55E+03
Actr3b	2	2	8.88E+04
Lyz2	1	3	1.69E+06
Casp6	1	3	3.35E+04
Mvd	1	3	2.96E+03
Ppp2r5d	1	3	1.74E+03
Rpl38	1	3	7.94E+04
Cnbp	1	2	2.82E+04
Brcc3	1	2	1.28E+04
Tpm4	1	2	5.11E+04
Pdia6	1	2	6.02E+04
Edc4	1	2	3.68E+04
Atp6v1g1	1	2	3.77E+05
Ppp2r5c	1	2	2.21E+04
Carm1	1	2	1.97E+04
Xaf1	1	2	1.93E+04
Nup43	1	2	1.60E+04
Pycard	1	2	4.57E+04
Myl6b	1	2	4.12E+05
Lrch3	1	2	7.18E+03
Gls	1	2	5.27E+04
Idua	1	2	3.75E+04
Atp2b1	1	2	5.65E+04
Marcks	1	2	4.17E+04
Mif	1	2	1.85E+05
Rps23	1	2	2.41E+04
H2afv	1	2	1.96E+05
Sf1	1	2	2.22E+04
Mcm4	1	2	2.47E+04
Hist1h1e	1	2	1.99E+05
Sdhc	1	2	1.16E+04
Pdpr	1	2	4.96E+03
Hmox2	1	2	3.24E+04
Ubtf	1	2	2.58E+04
Ostc	1	2	5.03E+03
Cbll1	1	2	2.56E+04
Mrps18a	1	2	3.26E+04
Scfd1	1	2	4.35E+03
Vapa	1	2	2.94E+04
Gpx1	1	2	2.93E+04
Srsf11	1	2	3.38E+03
Rfc3	1	2	2.21E+04
Smad1	1	2	7.67E+03
Sf3a3	1	2	1.12E+04
Aimp2	1	2	4.07E+04
Trim23	1	2	6.82E+03
Rab35	1	2	7.34E+04
	1	2	2.06E+04
Kpna1	1	2	9.24E+03
Serbp1	1	2	1.51E+04
Eif4g1	1	2	3.73E+04
Rpl24	1	2	8.18E+04
Krit1	1	2	3.17E+03
Lgmn	1	2	5.18E+04
Crtc2	1	2	6.55E+03
Rab33b	1	2	3.83E+05
Acin1	1	2	5.26E+03
Gng2	1	2	4.16E+03
Nfxl1	1	2	3.89E+04
Atp5j2	1	2	2.68E+04
Smad2	1	2	7.87E+03
Ncf4	1	2	1.54E+04
Apoa1bp	1	2	2.23E+03
Gm11744	1	2	1.75E+05
Mob4	1	2	5.37E+04
Ppt1	1	2	2.50E+04
Clcn5	1	2	1.80E+04
Prps1	1	2	1.05E+04
Maff	1	2	2.34E+03
Arl11	1	2	5.26E+03
Agpat4	1	2	5.87E+03
Klhl17	1	2	1.03E+05
Ufm1	1	2	4.69E+03

Table 5: Proteins identified in WT macrophages cross-referenced with CRAPome database

User Input	Mapped Gene Symbol	Num of Expt. (found/total)	Ave SC	Max SC
ACACA	ACACA	86 / 411	139.1	840
SRRM2	SRRM2	141 / 411	24.3	284
DHX9	DHX9	205 / 411	15.3	169
PARP1	PARP1	182 / 411	11.8	151
SF3B2	SF3B2	134 / 411	12.7	83
TMPO	TMPO	127 / 411	13.6	78
MCM2	MCM2	95 / 411	7.4	68
POLR2B	POLR2B	58 / 411	6.5	65
HSPA4	HSPA4	133 / 411	11.3	58
SAFB2	SAFB2	75 / 411	5.6	56
AHNAK2	AHNAK2	20 / 411	19.2	56
CNBP	CNBP	16 / 411	11.7	56
RBMXL1	RBMXL1	159 / 411	5.8	53
NOP56	NOP56	96 / 411	5.9	50
PHB2	PHB2	95 / 411	5.9	47
GART	GART	104 / 411	6.6	45
EIF3B	EIF3B	108 / 411	5.4	42
MCM4	MCM4	113 / 411	5.9	42
PLS3	PLS3	41 / 411	6.2	36
PSMD3	PSMD3	111 / 411	6.2	34
MTA1	MTA1	53 / 411	7.5	34
DDX39A	DDX39A	138 / 411	5.2	32
PSMC6	PSMC6	89 / 411	3.8	32
CSDE1	CSDE1	67 / 411	5.9	31
LUC7L3	LUC7L3	122 / 411	6.6	31
PRPF6	PRPF6	92 / 411	5.6	30
MOV10	MOV10	44 / 411	5.3	29
MYO5A	MYO5A	25 / 411	5.6	29
SMU1	SMU1	38 / 411	7.1	29
ZC3HAV1	ZC3HAV1	58 / 411	6.3	28
CLCC1	CLCC1	21 / 411	3.3	26
STMN1	STMN1	78 / 411	3.8	26
TBL3	TBL3	42 / 411	4.3	26
PLRG1	PLRG1	38 / 411	3.8	25
SF3A3	SF3A3	106 / 411	4.2	25
RALY	RALY	44 / 411	4.2	20
KARS	KARS	70 / 411	4.6	20
CAPZA1	CAPZA1	133 / 411	3.7	19
DNM1L	DNM1L	35 / 411	4.1	18
CHD8	CHD8	30 / 411	6.7	17
AIMP1	AIMP1	71 / 411	3.5	17
OLA1	OLA1	41 / 411	3	17
ACSL3	ACSL3	48 / 411	1.7	16
GFPT1	GFPT1	15 / 411	2.8	16
TECR	TECR	54 / 411	2.2	15
PSMD6	PSMD6	82 / 411	3.6	15
SRSF10	SRSF10	71 / 411	3.1	15
IPO7	IPO7	64 / 411	3.5	14
CLTA	CLTA	59 / 411	2.9	14
DCAF7	DCAF7	33 / 411	1.8	14
KPNA3	KPNA3	44 / 411	2.3	14
SLC25A1	SLC25A1	42 / 411	3.4	14
ABI1	ABI1	38 / 411	4.2	13
USP14	USP14	48 / 411	2.3	13
FARSB	FARSB	39 / 411	3.4	13
FUBP3	FUBP3	63 / 411	3.1	13
ISYNA1	ISYNA1	35 / 411	1.9	13
CSTF1	CSTF1	23 / 411	2.8	12
XPO7	XPO7	21 / 411	2.1	12
CACYBP	CACYBP	67 / 411	3.4	12
FLII	FLII	36 / 411	2	11
TBC1D15	TBC1D15	23 / 411	4.5	11
AIMP2	AIMP2	60 / 411	1.9	11
CPD	CPD	4 / 411	3.8	10
ETF1	ETF1	56 / 411	2.9	10
EML4	EML4	19 / 411	2.1	10
AGPS	AGPS	11 / 411	2.3	9
ABR	ABR	10 / 411	3.3	9
VAPA	VAPA	39 / 411	2.5	9
HK2	HK2	9 / 411	2	8
RAB21	RAB21	27 / 411	2.2	8
TBCB	TBCB	30 / 411	3	8
ATP12A	ATP12A	25 / 411	1.8	8
HARS	HARS	34 / 411	2.2	8
LRRC47	LRRC47	30 / 411	2.5	8
ACTR1B	ACTR1B	44 / 411	2.1	7
DNMT3A	DNMT3A	9 / 411	2.2	7
UGP2	UGP2	11 / 411	1.6	7
CMAS	CMAS	28 / 411	2.1	7
SUCLA2	SUCLA2	10 / 411	3.2	7
ATP6V1E1	ATP6V1E1	16 / 411	1.7	6
PPA2	PPA2	25 / 411	2.1	6
CAMK2A	CAMK2A	7 / 411	2.4	6
LIMD1	LIMD1	13 / 411	1.9	6
LAP3	LAP3	20 / 411	1.8	6
LRRC59	LRRC59	80 / 411	2.4	6
RFC3	RFC3	37 / 411	1.6	6
HMGCS1	HMGCS1	14 / 411	1.9	5
NDRG1	NDRG1	19 / 411	2.1	5
ATP2B1	ATP2B1	12 / 411	2.1	5
EIF3K	EIF3K	58 / 411	1.5	5
TMED10	TMED10	19 / 411	1.6	5
TNFAIP3	TNFAIP3	2 / 411	4	4
MLEC	MLEC	4 / 411	2.3	4
ACTR3B	ACTR3B	29 / 411	1.3	4
ATXN10	ATXN10	26 / 411	1.7	4
PPP5C	PPP5C	17 / 411	1.4	4
STRN	STRN	9 / 411	2	4
AAK1	AAK1	6 / 411	1.7	3
SNX12	SNX12	6 / 411	1.3	3
PPP2R5D	PPP2R5D	13 / 411	1.8	3
ADK	ADK	6 / 411	1.5	3
CARM1	CARM1	24 / 411	1.4	3
GTF2B	GTF2B	10 / 411	1.5	3
IDH3G	IDH3G	9 / 411	1.6	3
PCYT1A	PCYT1A	4 / 411	1.8	3
PPID	PPID	3 / 411	1.7	3
PRKAG1	PRKAG1	12 / 411	1.8	3
RDH11	RDH11	9 / 411	1.4	3
REEP5	REEP5	4 / 411	1.5	3
STRN4	STRN4	11 / 411	1.5	3
TOR1AIP1	TOR1AIP1	21 / 411	1.6	3
NLRX1	NLRX1	4 / 411	1.3	2
SCP2	SCP2	7 / 411	1.1	2
ARL8A	ARL8A	9 / 411	1.3	2
TRMT112	TRMT112	25 / 411	1.2	2
TSNAX	TSNAX	9 / 411	1.4	2
AP3M1	AP3M1	14 / 411	1.3	2
ATP5I	ATP5I	6 / 411	1.2	2
CLCN7	CLCN7	2 / 411	1.5	2
DHCR24	DHCR24	3 / 411	1.3	2
NUTF2	NUTF2	6 / 411	1.2	2
OSBP	OSBP	4 / 411	1.5	2
SMPD4	SMPD4	7 / 411	1.1	2
TBL1XR1	TBL1XR1	18 / 411	1.2	2
USP8	USP8	1 / 411	2	2
MALT1	MALT1	1 / 411	1	1
NDUFB9	NDUFB9	2 / 411	1	1
APOE	APOE	6 / 411	1	1
DOK3	DOK3	2 / 411	1	1
MTSS1	MTSS1	1 / 411	1	1
SIRT2	SIRT2	2 / 411	1	1
AARSD1	PTGES3L-AARSD1	1 / 411	1	1
APBB1IP	APBB1IP	2 / 411	1	1
IDUA	IDUA	1 / 411	1	1
KRIT1	KRIT1	4 / 411	1	1
LPCAT3	LPCAT3	1 / 411	1	1
MCL1	MCL1	1 / 411	1	1
OSTC	OSTC	6 / 411	1	1
PARP14	PARP14	2 / 411	1	1
PARP9	PARP9	1 / 411	1	1
PGM2	PGM2	3 / 411	1	1
SEC24D	SEC24D	2 / 411	1	1
SNF8	SNF8	2 / 411	1	1
TM9SF4	TM9SF4	2 / 411	1	1
TNIP1	TNIP1	6 / 411	1	1
YPEL5	YPEL5	2 / 411	1	1
IFI204	gene symbol not found			
ACAA1A	gene symbol not found			
CASP6	gene symbol not found			
EAR6	gene symbol not found			
FCGR1	gene symbol not found			
GBP5	gene symbol not found			
NLRP3	gene symbol not found			
PLIN2	gene symbol not found			
WDFY4	gene symbol not found			
ACOX3	gene symbol not found			
ADRBK1	gene symbol not found			
EIF2C2	gene symbol not found			
ETV3	gene symbol not found			
FRMD4A	gene symbol not found			
IAH1	gene symbol not found			
KLHL17	gene symbol not found			
MAFF	gene symbol not found			
NRP2	gene symbol not found			
PGGT1B	gene symbol not found			
PLEKHF2	gene symbol not found			
RASA1	gene symbol not found			
RGS1	gene symbol not found			
SAMD9L	gene symbol not found			
SDHC	gene symbol not found			
THYN1	gene symbol not found			
TRIM34A	gene symbol not found			
WDR37	gene symbol not found			
WDR45L	gene symbol not found			
ZDHHC13	gene symbol not found			

*SC: Spectral count. Used as a quantitative measure of protein abundance

Chapter 5: DISCUSSION

Some sections in this chapter have been published in Asrat, S., A.S. Dugan, and R.R. Isberg, *The frustrated host response to Legionella pneumophila is bypassed by MyD88-dependent translation of pro-inflammatory cytokines*. PlOS Pathog, 2014. 10(7): p. e1004229.

5.1. Cytokine transcripts bypass translation inhibition

Inhibition of protein translation is a common virulence mechanism used by many viruses and bacteria. In this study, we show that host cells have evolved mechanisms to cope with translation inhibition by selectively translating a subset of genes, including pro-inflammatory cytokines such as IL-1α, IL-1β and TNF-α. The ability to bypass *L. pneumophila* translational inhibition is an important determinant of host protection, as mice defective in the IL-1α/IL-1β response and humans exposed to TNF-α inhibitors are highly susceptible to *L. pneumophila* infection [20, 95].

L. pneumophila challenge of bone-marrow macrophages leads to a dramatic reduction in global protein translation. The bacterium interferes with protein translation both at the initiation step [71] and elongation step [46]. It has been shown previously that this inhibition triggers the transcription of various stress response genes including NF-κB- and MAPK-regulated genes, heat shock proteins and pro-inflammatory cytokines and chemokines [46, 47, 147]. We show here that *L. pneumophila* translocated effectors prevent the translation of these genes, resulting in a "frustrated response," in which there is accumulation of transcripts but no increase in protein levels. A subset of cytokine genes are insensitive to this inhibition and get translated in cells that show the highest level of protein synthesis inhibition.

We observed bypass of translational inhibition as an orderly series of events resulting in the accumulation of IL-1α in cells harboring bacteria as well as in bystanders, followed by release of the cytokine into culture supernatants. Initial transcriptional induction and translation of IL-1α occurred independently of the Icm/Dot system, and was associated with TLR-signaling (as seen in *dotA* infections), consistent with the TLR-

dependent activation of the NF-κB response occurring at early time points after *L. pneumophila* challenge [99]. This was followed by persistent accumulation of pro-IL-1α protein in a process that required both the presence of the Icm/Dot system and MyD88-dependent signaling (Fig 3.4 B), indicating collaborative signaling between the two pathways.

Surprisingly, accumulation of pro-IL-1α was equally robust in both cells harboring bacteria and in bystander cells, in spite of the translocated protein synthesis inhibitors that are deposited by *L. pneumophila*. This observation is particularly striking, in that time points showing the strongest inhibition of protein synthesis also resulted in the fastest rate of pro-IL-1α accumulation, arguing that there is selective ribosomal loading of cytokine transcripts in intoxicated cells (Fig 3.5 B&C). In the absence of MyD88 signaling, no such bypass could be observed in infected cells and we show that this is likely due to transcript abundance (discussed in section 5.2). Accumulation of pro-IL-1α in infected cells was followed by its release, which required both the Icm/Dot system and MyD88.

It is conceivable that the *L. pneumophila* translation elongation inhibitors could be responsible for the induction of IL-1α and IL-1β. It has previously been shown that the 5 *Legionella* translocated effectors that inhibit translation elongation are responsible for activation of MAPKs, pro-inflammatory cytokines and MAPK-dependent host genes (*Dups*, *Fos*) in the absence of pattern-recognition [46, 47]. Inhibition of translation elongation by itself (CHX or ExoA treatment of cells) has also been shown to transcriptionally induce the MAPK pathway [47]. The persistent activation of IL-1 within

infected cells could therefore be the consequence of *L. pneumophila* translocated substrates targeting host elongation and activating MAPK signaling downstream.

Arguing against this model is the fact that a strain that lacks 5 of the known translation inhibitors (Δ5) still induced considerable *Il1α* transcription as well as pro-IL-α accumulation (Fig. 3.13 C, E, F) [20]. The accumulation of cytokine in response to this strain could have resulted from residual translation inhibition that was observed, or trigged by unknown Icm/Dot-signals. It could also be the consequence of translation initiation inhibition [71]. A recent study proposed that infection of macrophages with virulent *L. pneumophila* strains (both Dot+ and Δ5) leads to downregulation of mTOR activity, which is sufficient to suppress cap-dependent protein translation initiation [71]. The second signal that is required for amplifying pattern-recognition could be generated from such translational suppression, and could be the trigger for induction of pro-IL-1α.

5.2 The role of MyD88 in selective translation of pro-inflammatory cytokines

Challenge of MyD88 deficient macrophages with *L. pneumophila* induced cytokine gene transcription, but the transcribed genes failed to be translated. The MyD88-dependent bypass of translation inhibition was independent of transcript stability in the case of *Il1β* transcript, which is a known strategy for post-transcriptional regulation of cytokines [126, 144]. Two different models could explain this surprising result. 1) There may be a previously unrecognized MyD88-dependent signaling pathway that controls post-transcriptional processing of cytokine transcripts, and 2) The role of MyD88 could be totally passive, and merely a consequence of enhancing expression of cytokine gene transcripts.

Evidence supporting the first model comes from studies that show regulation of IL-1β translation by different kinases, mRNA binding proteins and mircoRNAs (miRNAs). For example, it has previously been shown that the translation of IL-1β is controlled by tyrosine kinase 2 (Tyk2) [133]. The mechanism of regulation is not clear but in the absence of Tyk2, there is an increased association of IL-1β mRNA with polysomes, resulting in significantly higher translation of IL-1β [133]. It is possible that MyD88 signaling could be modulating this pathway. Alternatively, MyD88-dependent signals could result in either enhanced ribosome loading, or could regulate translation via action at the 3' untranslated regions (3' UTR). A number of pro-inflammatory cytokines are regulated at a posttranscriptional level by AU-rich elements (AREs) in their 3'UTR, ARE-binding proteins or microRNAs that bind to this region [6, 126]. Similarly, recent studies have highlighted the importance of long non-coding RNAs (lncRNAs) as regulators of cytokine transcription and translation [14, 120]. Interestingly, there are a number of lncRNAs that are induced by TLR-signaling (enriched for NF-κB binding sites), and regulate the innate immune response [14, 120]. For example, *Il1β* is located between an upstream lncRNA (*Il1β-RBT46*) as well as a downstream lncRNA (*Il1β-eRNA*). Both lncRNAs are rapidly induced upon LPS stimulation and regulate IL-1β production [120]. It is possible that the role of MyD88 could be to regulate the production of lncRNAs, ARE-binding proteins or miRNAs, which in turn facilitate selective translation of pro-inflammatory cytokines.

There is equal inhibition of protein synthesis in WT and MyD88$^{-/-}$ macrophages (data not shown), which means that MyD88 does not influence the rate/level of translation inhibition during *Legionella* infection. MyD88-dependent post-transcriptional

processing of cytokines likely occurs under normal conditions but cannot fully explain how cytokine mRNA gets translated when protein synthesis is inhibited by *Legionella* both at the initiation and elongation stages.

5.3. Transcript abundance allows bypass of translation inhibition

Using RNAseq, we showed that pro-inflammatory cytokines (*Tnf*, *Il1α*, *Il1β*) and chemokines (*Cxcl2, Ccl3, Ccl4*) are the most abundantly expressed transcripts in WT macrophages upon *Legionella* infection. This level was significantly higher than house keeping genes, which indicates that an infected macrophage would be full of cytokine and chemokine transcripts. This level of abundance will significantly increase the likelihood of encountering functional ribosomes within an intoxicated cell.

Consistent with this hypothesis, we show that other highly transcribed genes in WT and MyD88$^{-/-}$ macrophages, such as RGS-1, TNFAIP3 and IκB, were selectively translated upon *Legionella* infection. Although *L. pneumophila* infection causes a large induction of cytokine and chemokine transcripts in the absence of MyD88, these levels were still significantly lower than what was seen when pattern recognition was intact (Fig. 4.1). We hypothesize that this added boost in cytokine gene transcription by MyD88 is sufficient to push the concentrations of these transcripts above the minimum threshold necessary to support selective translation.

Based on these findings, we argue that the primary determinant of translation in cells challenged with *L. pneumophila* is the relative abundance of a particular transcript upon infection.

Our attempt to increase transcription of cytokines in MyD88 knockout macrophages by activating NF-κB was not successful. Pre-stimulation of MyD88$^{-/-}$ macrophages with TLR3, TLR4 and NOD agonists resulted in a transient increase of cytokine transcription, followed by a rapid downregulation upon *Legionella* challenge. Macrophages tightly control cytokine transcription and it is possible that in the absence of MyD88, there is a feedback inhibition that prevents overexpression of pro-inflammatory genes. Similarly, it is possible that IκB, which is synthesized in these cells upon infection, results in rapid inhibition of NF-κB.

One way to test if transcript abundance is sufficient to overcome translation inhibition is overexpression of inducible GFP or enzyme readout reporters in macrophages after translation is blocked by *L. pneumophila*. The caveat to this is that it does not mimic physiological conditions and any post-transcriptional regulations that facilitate bypass of translation inhibition would not work under such conditions. It would however allow us to determine if increasing the likelihood of ribosome encounter leads to selective translation.

5.4. Innate immune response to elongation inhibition

Elongation inhibition is a virulence strategy that is beneficial to a number of bacterial pathogens. In the case of *Legionella*, the ability to inhibit translation elongation is crucial for its survival in its natural host amoebae. Δ5 mutant, the strain that lacks the 5 *Legionella* elongation inhibitors, is attenuated for growth in *Dictyostelium discoideum* [46].

Interestingly, pharmacological inhibitors of host protein translation elongation induce significant transcription of various innate immune genes, including pro-inflammatory cytokines such as *Il6*, *Il23*, *Il1α* and *Il1β* [20, 46, 47]. Translation and secretion of these proteins has been reported to take place when the highly conserved host elongation machinery is targeted by toxins such as *P. aeruginosa* Exotoxin A, *Corynebacterium diphtheriae* encoded diphtheria toxins and *E. coli* encoded Shiga toxin [20, 46, 134]. *Diphtheria* toxin and *Pseudomonas* ExoA modify elongation factor 2 (EF2) of eukaryotic cells, which has been shown to trigger a strong host immune response [20, 78]. Similarly, Shiga toxin and Ricin inactivate 60S ribosomal subunit by cleaving an adenine residue from 28S ribosomal RNA, which prevents the EF-1A-aminoacyl-tRNA complex from binding to ribosomes [42]. This mode of elongation inhibition induces the ribotoxic stress response, where there is rapid activation of JNK and p38 and induction of DUSPs, pro-inflammatory cytokines (TNF-α, IL-1β) and chemokines (IL-8, MIP-1) [58, 69, 134]. This suggests the presence of a conserved surveillance mechanism the host uses to detect and respond to inhibition of the translation elongation machinery.

It is possible that transcript abundance facilitates selective translation of stress-response genes upon toxin-induced elongation inhibition. In deed, the vast majority of genes that have been shown to resist elongation inhibition are abundantly transcribed in cells, pointing to a simple yet effective mechanism used by the host immune system to ensure translation of inflammatory mediates regardless of the mode of inhibition.

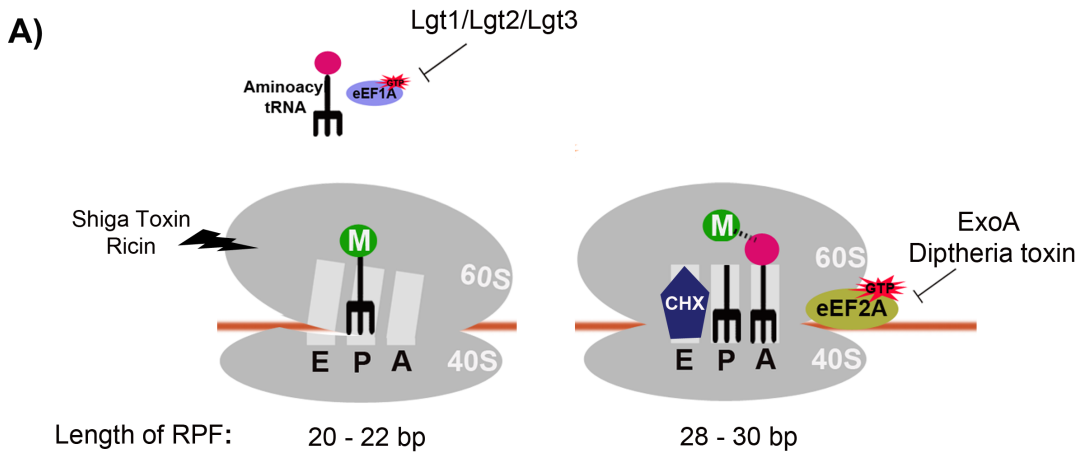
Interestingly, selective translation has been shown to play a crucial role in the response against *Pseudomonas aeruginosa* in the model organism *C. elegans.* When intestinal cells endocytose *P. aeruginosa* Exotoxin A (ExoA), the toxin targets host

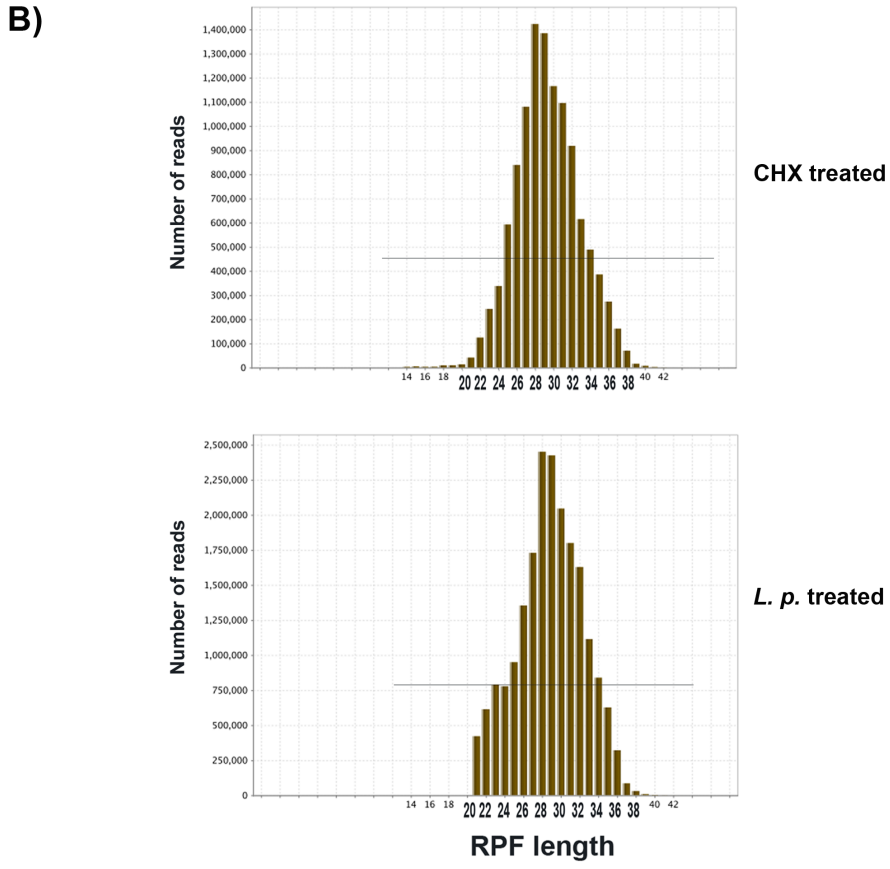
translation elongation factor 2 (EF2) and rapidly blocks protein synthesis [108]. This inhibition leads to the selective translation of ZIP-2, which is required for activation of defense pathways and pathogen clearance [41]. It was proposed that the 5' UTR of zip-2, which contains several upstream open reading frames (uORFs), was required for the selective translation. uORFs facilitate translation reinitiation but it is not clear how they can influence translation elongation. Even in the presence of uORFs, cells will need to acquire new elongation factor 2 (EF2) in order to complete polypeptide chain extension, and it is not clear how and if translation could proceed without EF2.

5.4.1. *Legionella* induced elongation inhibition, how is it bypassed?

A previous study has shown that the cytokine induction seen during *L. pneumophila* can be mimicked by the addition of *P. aeruginosa* Exo A in combination with a TLR2 ligand Pam3CSK4 [20]. We were not able to reproduce this result using a variety of concentrations of the protein synthesis inhibitor cycloheximide (CHX), which instead reduces pro-inflammatory cytokine production in response to PAMP challenge. The mechanism by which cycloheximide inhibits protein synthesis is sufficiently different from these toxins, which could explain why we see differences in the innate immune response against CHX. CHX binds to the E-site of the 60S ribosomal subunit and freezes all translating ribosomes [143] (Fig 5.1 A). The RNA/ribosome complex remains stabilized and does not dissociate, a phenomenon that may not be perceived as danger by eukaryotic cells. In contrast, both *L. pneumophila* and ExoA interfere with elongation factor functions (Fig 5.1 A).

Figure 5.1: Different elongation inhibitors stabilize distinct ribosome conformations. A) Model that illustrates how different toxins/translocated effectors block host translation elongation. The *L. pneumophila* translocated effectors Lgt1/Lgt2/Lgt3 are glycosyltransferases that block host elongation factor eEF1A. Shiga toxin and ricin inactivate 60S ribosomal subunit by cleaving a single adenine residue from 28S rRNA. ExoA and diphtheria toxin modify elongation factor 2 (EF2) of eukaryotic cells by ADP-ribosylation. CHX binds to the E-site of the 60S ribosomal subunit and blocks all translating ribosomes. B) The length of mRNA fragment protected by ribosomes during CHX treatment (top) or *L. pneumophila* infection (botton). RPF: Ribosome protected fragment.


A)
Lgt1/Lgt2/Lgt3
Aminoacyl tRNA
eEF1A
GTP
Shiga Toxin
Ricin
M
60S
E P A
40S
CHX
eEF2A
ExoA
Diptheria toxin
Length of RPF:
20 - 22 bp
28 - 30 bp


B)
Number of reads
CHX treated
L. p. treated
RPF length

A recent study reveled that elongation inhibitors stabilize two different ribosome conformational states depending on the stage at which they block translation elongation [92]. Prior to peptide bond formation, the ribosome is in a non-rotated form, which protects 20-22bp of mRNA. Upon peptide bond formation, there is rotation of the large ribosomal subunit (60S) relative to the small subunit (40S), which leads to protection of a longer mRNA fragment (28-30bp) [92] (Fig 5.1 A). Using ribosome profiling, the study showed that CHX mainly stabilized post-rotated ribosome conformation and protected longer mRNA fragments while another elongation inhibitor, anisomycin, exclusively stabilized a pre-rotation conformation[92].

By looking at the length of ribosome-protected fragments during *Legionella* infection, we observed that a subset (~15%) of *Legionella* treated cells protect 20-24 bp fragments (Fig 5.1 B). (Note: This experiment was performed in unsorted macrophages where there is 15-20% infectivity, the remaining bystander population protects longer mRNA fragments during translation, similar to what is seen for CHX).

Legionella translocated effectors target elongation factor eEF1A, which explains why the pre-rotated ribosome conformation was stabilized during infection. We would expect to see similar shorter fragments protected by ribosomes upon treatment with Shiga toxin and Ricin (Fig 5.1A).

5.4.2. The kinetics of host elongation inhibition by *L. pneumophila* translocated effectors

Transcript abundance and uORF dependent translation reinitiation of innate immune genes can explain how cells overcome host-driven initiation block during *Legionella* infection. However, if there is *Legionella*-induced elongation inhibition throughout the course of infection, we would expect to see a complete block in protein synthesis, similar to what has been observed during CHX treatment. This is however not the case, which can be explained by two models. 1) The pathogen-induced elongation inhibition may only happen transiently during early stages of infection, or 2) There may be previously unrecognized alternative mechanisms of host translation elongation, such as elongation factors that resist modification by *Legionella* effectors/toxins, or there may be a population that are sequestered from modification, allowing them to selectively act on few transcripts.

Previous studies looking at the kinetics of Lgt1, Lgt2 and Lgt3 expression showed that these effectors could only be detected early during infection in *Acanthamoeba castellani* (maximal expression observed at 3hrs POI) [23]. Lgt3 in particular, was undetectable after 3hrs. Consistent with this, we have shown that deleting the 5 elongation inhibitors only rescued protein synthesis at early stages during infection (Fig 3.13 A & B). At later time points (past 6-8hrs), there was an equal level of protein synthesis in WT and Δ5 infected macrophages (*Hempstead, unpublished*), indicating that host-driven initiation inhibition has taken over at these time points.

Based on these observations, we hypothesize that early during infection, *Legionella* actively translocates elongation inhibitors into host cells, which effectively block host protein synthesis. Later during infection, the elongation inhibitors are no longer expressed/translocated and the translation block we see is mainly due to host-driven initiation inhibition. Host-driven initiation inhibition is accompanied by selective translation of pro-inflammatory cytokines and IFN inducible inflammatory mediators (Discussed in section 5.5).

To confirm this hypothesis, the translocation and expression levels of Lgt1/Lgt2/Lgt3 should be determined in macrophages infected with WT *Legionella* at different time points post infection. The exact mechanism of elongation inhibition by these effectors should also be worked out to further understand how it shapes the host innate immune responses.

5.5. The innate immune response to initiation inhibition

Inhibition of translation initiation is a common stress response used by host cells to maintain cellular homeostasis upon viral infection, starvation, ER-stress and UV damage. Under such conditions, host cells phosphorylate initiation factor eIF2α, leading to global translation arrest and selective translation of genes required for stress response.

5.5.1. uORF-mediated translation reinitiation (REI)

~30-40% of mammalian mRNA are predicted to have uORFs but the functional relevance of the vast majority of these uORFs or the mechanism by which they regulate translation initiation has not been experimentally validated. uORF-mediated translation reinitiation in particular, is a process that is not fully understood. The key factor that

determines reinitiation is the ability of the uORF (or the sequence surrounding it) to retain 40S ribosome subunits as well as initiation factors (eIF3 and eIF4F) after termination of translation [19].

The ER-stress response transcription factor ATF4 and the yeast transcription factor GCN4 are good examples of how translation inhibition leads to selective translation of mRNA with uORFs in their 5' UTR [63, 162]. GCN4 contains 4 uORFs its 5'UTR. The first uORF closest to the mRNA cap is translation reinitiation-permissive while the following three uORFs are non-permissive, which allows tight control of translation initiation at the main coding sequence. Under nutrient replete conditions, when there are excess initiation factors available, the first uORF is translated and there is enough ternary complex (eIF2 + Met-tRNA) available to reinitiate at the next 3 uORFs [63]. This will significantly reduce translation of the main coding sequence. However, when there are limited initiation factors available, for example during starvation when eIF2α is phosphorylated, ribosomes will take more time to reacquire a new ternary complex after translation of the first uORF [56, 63, 116]. Due to this, ~50% of ribosomes will scan past the next 3 uORFs and move down to the main coding sequence. This scanning step will give ribosomes enough time to acquire a new ternary complex and start translation at the main coding sequence [56].

The mammalian DNA-binding protein ATF4, which is required for induction of the unfolded protein response (UPR), uses a very similar strategy for translation reinitiation during ER-stress [162]. Other proteins required for the unfolded protein response, such as CHOP, ATF5 and IBTKα, also use uORFs-dependent translation reinitiation when protein synthesis is inhibited during ER-stress [16, 125, 172]. This

points to an evolutionarily conserved translation initiation mechanism used by host cells to maintain translation upon inhibition of eIF2.

We report here that macrophages use a similar strategy to overcome translation inhibition upon bacterial infection. To our surprise, the majority of proteins that show uORF-dependent translation regulation during *Legionella* infection were type I IFN inducible genes. The type I IFN response is crucial for restriction of viral infection, and it does so by activating hundreds of interferon stimulated genes (ISGs) [73]. One of the mechanisms by which ISGs restrict viruses is by phosphorylation of the host initiation factor eIF-2α [73]. Under such conditions, host translation inhibition prevents viruses from synthesizing the proteins they require for replication, as they fully depend on the host translation machinery. It was not clear how host cells maintained the production of ISGs while there was global translational suppression. Our results indicate that a number of ISGs contain *cis*-acting mRNA elements, such as uORFs, which would allow them to get selectively synthesized. Consistent with this, a recent study showed that a number of transcripts involved in antiviral immunity contain alternative translation initiation mechanisms and encode truncation variants which are crucial for protein diversification [30].

Interestingly, some of the ISGs that bypassed translation inhibition during *Legionella* infection, such as Ifih1, Ifit 1 and Nlrp3, had overlapping uORFs. These overlapping uORFs were efficiently translated under normal conditions while the main ORF showed limited ribosome association (Fig 4.7, Ifit1 and Nlrp3 untreated). Upon *Legionella* challenge, this pattern was reversed and we detected less efficient translation of the uORFs, while the main ORF actively associated with ribosomes (Fig 4.7, Ifit1 and

Nlrp3 *L.p*. treated). This pattern of translation is common under ER stress and is seen with ATF4 and ATF5.

The exact mechanism by which the other identified uORFs facilitate translation reinitiation has not been worked out. It is possible that uORF-binding proteins exist that facilitates reinitiation. Such proteins have been shown to modulate mRNA stability and prevent nonsense-mediated mRNA decay (NMD) [140]. The classical yeast transcription factor GCN4 for example, prevent NMD using stabilizer elements (STEs) within its 5'UTR. Pub1, a ribonucleoprotein (hnRNP) particle, directly interacts with STE elements and prevents nonsense mediated decay of the mRNA [140], which in turn enhances translation of GCN4. It is possible that the other genes we identified (Fig 4.8, A) use this strategy to overcome translation inhibition.

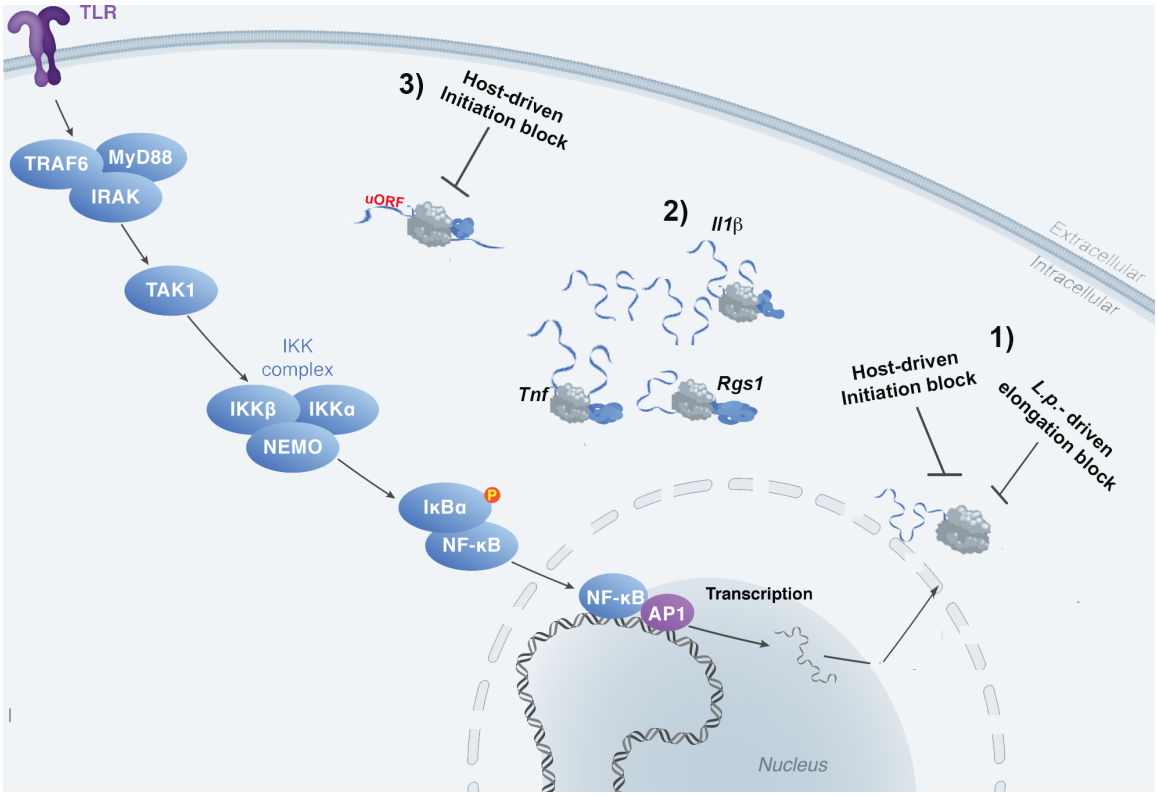
The 5' sequence directly upstream of some uORFs, termed 5' enhancer, has been shown to interact with the N-terminal domain of initiation factor eIF3 [154]. This region contains reinitiation-promoting elements that form secondary structures, such as double-circle hairpins, which are predicted to facilitate translation of the uORF and retention of 40S ribosome [56, 154]. This means that translation can initiate at the main ORF without the need to acquire additional initiation factors. It would be interesting to see if any of the ISGs and stress response regulators identified in this study could directly interact with eIF3 and retain ribosomal subunits.

5.5.2. How do uORFs facilitate bypass of translation inhibition during *Legionella* infection?

We have shown that a number of genes that bypass translation inhibition, independent of transcript abundance, contain upstream open reading frames in their 5'UTR. However, it is not clear how these uORFs regulate translation and if the presence of uORFs alone is sufficient to bypass *Legionella*-induced translation arrest. To conclusively demonstrate that uORFs allow bypass of translation inhibition, 5'UTR sequences can be fused to GFP or enzyme readout reporters that show differential sensitivity to translation inhibitors. Site-specific mutation/deletion analysis can also be performed to check if there will be loss of reinitiation in the absence of uORFs. Similarly, 5'UTR of transcripts that contain uORFs could be used as affinity reagents to identify host complexes that are associated with translation bypass.

Figure 5.2: Summary model: Two distinct mechanisms allow bypass of translation inhibition during *Legionella* infection.

1) Upon *Legionella* challenge, bacterial translocated effectors target host elongation factors and host cells downregulate their own translation initiation. This inhibition leads to a frustrated host response for most genes. 2) Pro-inflammatory cytokines and chemokines overcome translation inhibition due to their transcript abundance. 3) At later time points during infection, host-driven translation initiation inhibition takes full effect. Type I IFN inducible genes and other stress response regulators use uORFs to selectively get translated when initiation is blocked.


TLR
TRAF6
MyD88
IRAK
TAK1
IKK
complex
IKKβ
IKKα
NEMO
IκBα
P
NF-κB
NF-κB
AP1
Transcription
Nucleus
Extracellular
Intracellular
3)
Host-driven
Initiation block
uORF
2)
Il1β
Tnf
Rgs1
1)
Host-driven
Initiation block
L.p.- driven
elongation block

5.6. Conclusion

In this study, we elucidated how the innate immune system is regulated in response to perturbed host cell processes, such as protein synthesis inhibition. In particular, we address an interesting paradox of how translation inhibition leads to selective synthesis of genes associated with inflammation. Translation inhibition is a common virulence mechanism that is used by a number of bacteria and viruses, but the immune response to such inhibition has not been characterized. We dissect this common response seen in a number of pathogens, using *L. pneumophila* and provide a universal model for how pathogens uniquely regulate pro-inflammatory gene production in a fashion not observed with harmless pathogens.

We find that upon *Legionella* infection, protein synthesis inhibition leads to a frustrated host response, where there is constant transcription of various innate immune genes that fail to get translated due to the bacterium-induced translation inhibition. Host cells have devised unique mechanisms to evade this blockade and initiate a successful inflammatory response. One mechanism is transcript abundance, where highly transcribed innate immune genes, such as Il-1β and TNF, selectively get translated under conditions of intoxication. The other mechanism for bypass is upstream open reading frames (uORFs) within 5' untraslated regions (5'UTR), which facilitate translation reinitiation.

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