Type I Interferon is Not Just for Viruses: Cytosolic Sensing of Bacterial Nucleic Acids

by

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Abstract

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Initial detection of invading microorganisms is one of the primary tasks of the innate immune system. However, the molecular mechanisms by which pathogens are recognized remain incompletely understood. I used the intracellular gram-negative *Legionella pneumophila* to study mechanisms by which the innate immune system distinguishes virulent bacteria from avirulent bacteria. I have made the surprising observation that a cytosolic RNA immunosurveillance pathway (called the RIG-I/MDA5 pathway), thought primarily to detect viruses, is also involved in the innate immune response to the intracellular vacuolar bacterial pathogen, *Legionella pneumophila*. In the response to viruses, the RIG-I/MDA5 immunosurveillance pathway has been shown to respond to viral RNA or DNA. We found that the RIG-I pathway was required for the response to *L. pneumophila* RNA, but was not required for the response to *L. pneumophila* DNA. Thus one explanation of my results is that *L. pneumophila* RNA accesses the host cell cytosol via its type IV secretion system, where it triggers the RIG-I/MDA5 pathway. This is unexpected since bacteria have not previously been thought to translocate RNA into host cells. I was able to isolate IFN-stimulatory activity by immunoprecipitating RIG-I from cells infected with T4SS-competent *Legionella*. In the future, I will utilize deep sequencing technology to pinpoint the origin and identity of RIG-I bound ligands during $T4SS^+$ *L. pneumophila* infection.

I also found that *L. pneumophila* suppresses the RIG-I/MDA5 pathway by a translocated effector protein, SdhA. Several viral repressors of the RIG-I/MDA5 pathway have been described, but bacterial repressors of RIG-I/MDA5 are not known. Thus, this study provides novel insights into the molecular mechanisms by which the immune system detects bacterial infection, and conversely, by which bacteria suppress innate immune responses.

While all bacteria are capable of inducing type I interferon, many species do so independently of the cytosolic RNA sensing pathway that responds to *Legionella*. Therefore, I, along with many Vance lab members, investigated the mechanism by which cyclic dinucleotides (c-di-GMP and c-di-AMP), bacterial second-messenger molecules, activate a robust and specific host response in macrophages. c-di-GMP has been shown to activate TBK-1, IRF3, NF-κB, and MAP kinases to induce type I

interferon, in manner independently of known TLR and cytosolic nucleic acid sensing pathways. In parallel studies in the lab, ENU mutagenesis of mice identified a mouse mutant that completely abrogates the host response to cyclic dinucleotides. Sequencing identified a missense mutation in the open reading frame of *Sting*, which converts an isoleucine to aspargine in the C-terminal globular domain, rendering STING protein undetectable in mutant macrophages. Previous reports of *Sting* demonstrated a role for this multiple transmembrane domain containing protein that localizes to the endoplasmic reticulum and/or mitochondrial associated-membrane (MAM) in cytosolic DNA and RNA sensing pathways. I found that overexpression of the ENU-induced mutant *Sting* allele failed to induce type I interferon, despite robust expression of the protein. Surprisingly, studies in the Vance lab have shown that wild type STING is capable of binding c-di-GMP, in contrast to the ENU mutant allele, which does not bind. I found that a soluble C-terminal truncation of STING (amino acids 138-379), which removes most predicted transmembrane domains, is sufficient to bind c-di-GMP. Taken together, genetic studies have demonstrated an essential role for *Sting* in the innate immune response to cyclic dinucleotides and biochemical data shows that the C-terminal region of the protein functions as the direct sensor of cyclic dinucleotides.

The experimental line of investigation presented in my thesis dissects the pathways by which the innate immune system recognizes infection of virulent bacteria. Both stories discussed herein demonstrate the importance of innate immune detection of nucleic acids; molecules microbes cannot live without. Interestingly, it is the compartment in which nucleic acids are present that ultimately triggers innate immune receptors. The demonstration that cytosolic RNA sensors detect secretion system competent *Legionella* illustrates the consequence of breaching the phagosomal barrier. While bacterial replication may be restricted to membrane bound compartments, accessing the host cell cytosol via the T4SS alerts host sensors to the pathogen's presence. Previously thought only to recognize viral infection, I have demonstrated the breadth of cytosolic RNA sensors in recognizing not only viruses, but also bacteria. STING's ability to detect cyclic dinucleotides delivered to the host cell cytosol exemplifies how the innate immune system has honed in on a uniquely bacterial nucleic acid molecule. Cyclic dinucleotides are not only structurally distinct, but their role in regulating virulence factor expression makes them an excellent target for innate immune detection of pathogens.

Dedication

To Donald Morisato, an inspiring and motivating mentor.

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List of Abbreviations

IFN: Type I interferon
TLR: Toll-like receptor
NLR: Nod-like receptor
RIG-I: Retinoic acid inducible gene I
MDA5: Melanoma differentiation-associated gene 5
IPS-1: Interferon-beta promoter stimulator 1
STING: Stimulator of interferon genes
***Sting*^{gt}**: ENU-induced *Goldenticket* (*gt*) allele of *Sting*
IRF3*:* Interferon regulatory factor 3
c-di-GMP: 3',5'-Cyclic diguanylic acid
c-di-AMP: 3',5'-Cyclic adenylic acid
DGC: Diguanylate cyclase
PDE: Phosphodiesterase
T4SS: Type IV secretion system
LPS: Lipopolysaccharide
Δdot: *L. pneumophila* lacking a functional T4SS
Δfla: *L. pneumophila* lacking flagellin
ΔsdhA: *L. pneumophila* lacking the gene *sdhA*

Acknowledgements

I am indebted to Donald Morisato who guided me down the path of investigating the mysterious ways of nature at the cellular and molecular level.

I thank my advisor Russell Vance, for taking a chance on a student who didn't know what endotoxin was at the start of my rotation. I thank my committee members, Chair Russell Vance, Mark Schlissel, Britt Glausinger, and Ellen Robey for advice and constructive criticism along the way. I am particularly thankful to Mark Schlissel, who acted as my chair during the Qualifiying Exam, and proved to be a thoughtful, immensely helpful, and insightful mentor during that process, and throughout graduate school.

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I am indebted to the members of team ENU: Katia Sotelo-Troha, Sky Brubaker, Jakob von Moltke, Norver Trinidad, John-Demian Sauer, and Chris Rae, for their amazing hard work and dedication to the ENU mutagenesis screen.

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I am so grateful to Anthea Letsou, who pushed me to go to graduate school and served as an important role model as a successful woman in science.

Chapter 1.

Introduction

This doctoral dissertation focuses on experiments designed to probe host-pathogen interactions. In this work, I investigated the molecular mechanisms by which the host innate immune system senses bacterial infection and distinguishes virulent bacteria from avirulent bacteria. My work focused on the innate immune system's response to bacterial infection by activation of the cytokine IFNβ. Chapter 1 presents the known host pathways that induce IFNβ, and the general mechanisms by which various species of bacteria have been shown in the literature to activate an IFNβ host response. Much of this text was published in a review in *Cellular Microbiology (Monroe et al., 2010).* Chapter 2 describes experiments investigating ways *Legionella pneumophila* activates and suppresses IFNβ. Chapter 3 describes my quest to identify the *Legionella* IFN-stimulatory ligand. Chapter 4 describes my contributions to understanding how bacterial second messenger molecules, cyclic dinucleotides, are sensed by the innate immune system. Finally, Chapter 5 presents my concluding perspectives and future directions for the field.

1.1 Induction of Type I Interferons by Bacteria

Type I interferons (IFNs) are secreted cytokines that orchestrate diverse immune responses to infection. Although typically considered to be most important in the response to viruses, type I IFNs are also induced by most, if not all, bacterial pathogens. Although diverse mechanisms have been described, bacterial induction of type I IFNs occurs upon stimulation of two main pathways: (1) Toll-like receptor (TLR) recognition of bacterial molecules such as lipopolysaccharide (LPS); (2) TLR-independent recognition of molecules delivered to the host cell cytosol. Cytosolic responses can be activated by two general mechanisms. First, viable bacteria can secrete stimulatory ligands into the cytosol via specialized bacterial secretion systems. Second, ligands can be released from bacteria that lyse or are degraded. The bacterial ligands that induce the cytosolic pathways remains uncertain in many cases, but appear to include various nucleic acids. This introduction discusses recent advances in our understanding of how bacteria induce type I interferons and the roles type I IFNs play in host immunity during bacterial infections.

Type I interferons (IFNs) are secreted cytokines that include a single IFNβ protein, as well as numerous IFNα and other IFN family members (Decker et al., 2005). All type I IFNs signal via a heterodimeric receptor (IFNAR) and act locally and systemically to coordinate diverse responses to infection. An important local effect of type I IFN is the induction of the "anti-viral state", which involves expression of host genes that interfere with viral replication (Zuniga et al., 2007). Some genes induced by type I IFN also exhibit anti-bacterial activity, such as the p47 GTPases (Taylor et al., 2004). Type I IFN can also sensitize host cells to apoptosis, which is thought to

counteract the ability of viruses or bacteria to utilize the host's intracellular niche for replication. In addition to local responses, type I IFN functions systemically, for example to activate Natural Killer and $CD8^+$ T cell cytotoxicity, or to induce the upregulation of genes required for antigen presentation and activation of adaptive immunity.

The ability to produce type I IFN appears to be a universal property of all cells in the body, but the proximal pathogen-sensing receptors and signaling mechanisms leading to type I IFN induction differ significantly depending on the stimulatory ligand and responding cell type. Despite their diversity, the signaling pathways leading to induction of type I IFN do converge upon some common downstream elements, including the ubiquitin ligase TRAF3 and transcription factors such as IRF3 and IRF7. Once activated by phosphorylation in the cytosol, the IRFs enter the nucleus and assemble with NF-kB and other transcription factors on the *Ifnb* promoter in a complex (Panne et al., 2007) that activates extremely robust (e.g., 1000-fold) transcriptional induction of the *Ifnb* gene. Here, we discuss the current understanding of the type I IFN host response to bacteria, which was the focus of my thesis research.

1.1.1 TLR-dependent pathways

The Toll-like receptors (TLRs) are a family of cell surface or endosome localized receptors that recognize a variety of conserved microbial molecules (Kumar *et al.*, 2009). TLR2, 3, 4, 5, 7, 8 and 9 are the primary TLRs that are potentially able to recognize bacterial products, and with the exception of TLR5, all have been linked to the induction of type I IFN. Interestingly, the mechanism of IFN induction by these TLRs varies considerably, and in many cases, TLR signaling only results in IFN induction in dedicated cell types (Figure 1).

Type I IFN induction by TLR4. The best-characterized mechanism by which bacteria induce type I IFN is via TLR4, a cell-surface localized receptor that recognizes the lipid A moiety of lipopolysaccharide (LPS) from the outer membrane of gram-negative bacteria. TLR4 may recognize other bacterial ligands (Ashkar et al., 2008; Mossman et al., 2008; Thanawastien et al., 2009). TLR4 signals via two cytosolic adaptor proteins, MyD88 and TRIF, which are recruited sequentially to the cytoplasmic tail of TLR4 (Kagan et al., 2008). TRIF, but not MyD88, is required for induction of type I IFN by TLR4. TLR4 signaling induces type I IFN in many cell types and this broad capacity to induce type I IFN is shared by TLR3, which recognizes double-stranded RNA and is the only other TLR that utilizes TRIF for its downstream signaling (Kumar *et al.*, 2009). However, there are few examples of TLR3-dependent recognition of bacteria. The other TLRs that induce type I IFN do so only in specialized cell types such plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) in a MyD88-dependent pathway (Figure 1). It is not clear why TLR4 would have evolved the unique capacity to stimulate type I IFN in many cell types in response to LPS. As discussed below, it does not appear that type I IFN is particularly critical for defense against gram-negative bacteria.

The microbes that are best recognized by TLR4 tend to be gram-negative commensals that reside on mucosal surfaces, such as *E. coli* in the gut, or closely

related pathogenic genera, such as *Salmonella*. These microbes tend to produce hexaacylated lipid A that is the optimal ligand for TLR4. There is speculation that some mucosal bacteria may benefit by producing LPS that is recognized by TLR4 (Munford and Varley, 2006), but how they might benefit is not yet clear. Despite a widespread portrayal of TLR ligands as highly conserved across diverse bacterial species, gram-negative bacteria produce a tremendous variety of LPS molecules, many of which are poor ligands for TLR4. For example, the LPS of many human pathogens, including *Legionella pneumophila, Helicobacter pylori, Francisella tularensis, Coxiella burnetii,* and *Brucella abortus*, is poorly detected by TLR4 (Munford and Varley, 2006). In the case of *Yersinia pestis*, production of a specific LPS that evades TLR4 recognition is essential for virulence (Montminy et al., 2006). In other cases, it remains unclear if evasion of TLR4 is critical for virulence. Indeed, as discussed below, most TLR4-evasive gram-negative bacteria still induce type I IFNs via TLR-independent pathways.

Type I IFN induction by other TLRs. In contrast to TLR4, which is localized to the cell surface, the other TLRs that stimulate type I IFN (i.e., TLR3, 7, 8 and 9) localize to intracellular compartments (Figure 1). TLR2 was recently reported to induce type I IFN, but only in inflammatory monocytes and selectively in response to viral, not bacterial, ligands (Barbalat et al., 2009). TLR7, and its paralog TLR8, recognize single-stranded RNA ligands, whereas TLR9 recognizes DNA containing unmethylated CpG motifs. These TLRs signal exclusively via MyD88 and can stimulate the production of inflammatory cytokines such as TNFα in many cell types, but exhibit the ability to induce type I IFNs only in specialized cell types, most notably pDCs. Although pDCs are not numerous, they are capable of producing vast amounts of type I IFN on a per cell basis, and are important for inducing systemic levels of type I IFN in the response to viruses. However, the role of pDCs in bacterial infections has not been extensively investigated. In fact, although DNA from many bacterial species contains the unmethylated CpG motifs that can be recognized by TLR9, there is remarkably little evidence that TLR9 participates in the response to bacterial infections. One report shows that TLR9 can recognize *Salmonella typhimurium* and subsequent acidification of the phagosome is required to induce SPI-2 (a key intracellular *Salmonella* virulence factor) (Arpaia et al., 2011). A recent study provided surprising evidence for cell-type specific TLR-dependent IFN responses to bacteria. In this study, cDCs, but not macrophages or pDCs, were shown to produce type I IFN in a TLR7-MyD88-IRF1-dependent manner via phagolysosomal degradation of group A and B *Streptococcus* (GAS, GBS) (Mancuso et al., 2009). Previously, in response to viruses, TLR7 was thought to induce type I IFN primarily in pDCs, not cDCs. Thus, the results of Mancuso *et al.* may describe a bacterial-specific TLR-dependent pathway for induction of type I IFN.

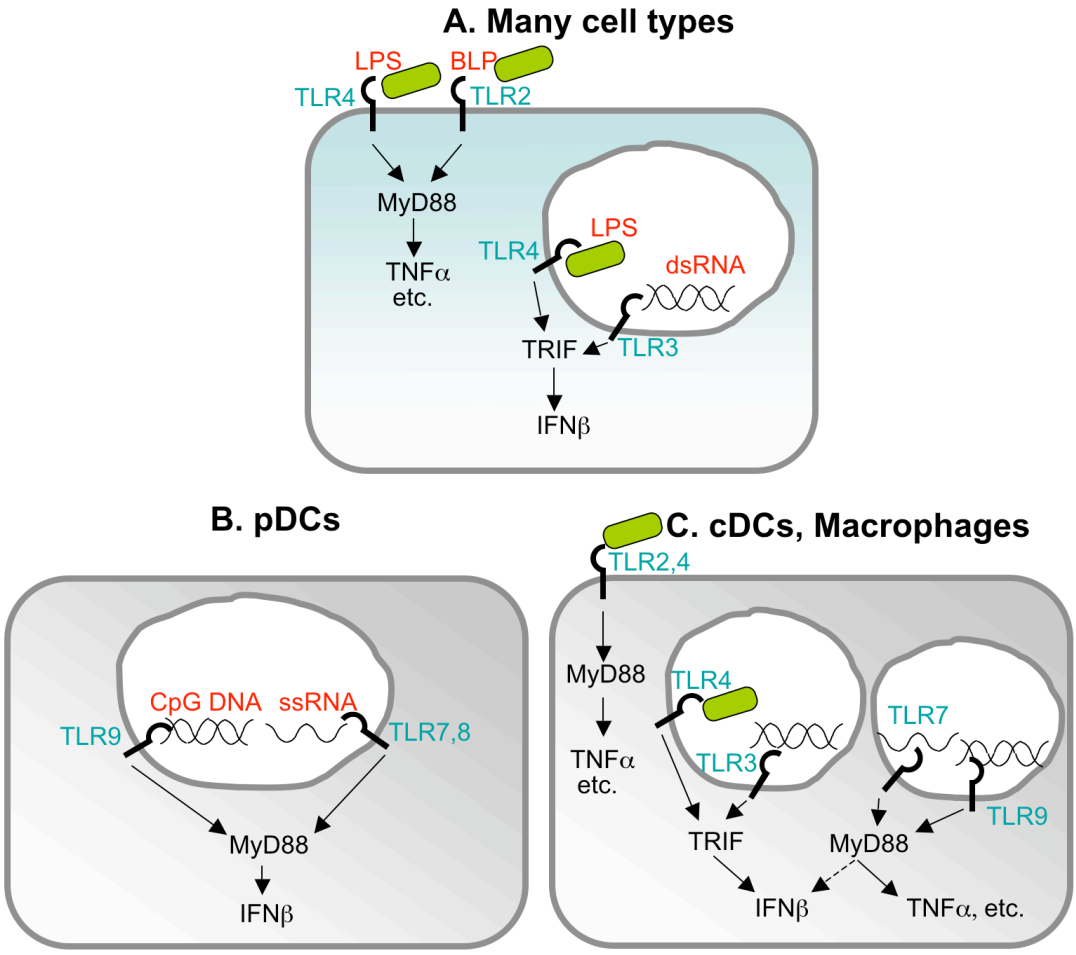

Figure 1.1 TLR Receptors and Ligands. (A) TLR2 and 4 recognize bacterial lipoprotein (BLP) and lipopolysaccharide (LPS), respectively, and signal from the cell surface via the adaptor MyD88 to activate proinflammatory cytokines including TNFα and IL-6. TLR3 recognizes double stranded RNA (dsRNA) and signals via the adaptor TRIF from an intracellular compartment to induce IFNβ. Upon endocytosis, TLR4 can also signal via TRIF to induce type I IFN. (B) Plasmacytoid dendritic cells primarily express nucleic acid sensing TLRs, which are localized to intracellular compartments. pDCs produce vast amounts of IFNβ upon stimulation via a MyD88-dependent pathway. (C) Conventional dendritic cells and macrophages express many TLRs. However, only cDCs have been reported to induce IFNβ via TLR7 and 9. In macrophages, TLR7 and 9 signaling induces proinflammatory cytokines such as TNFα.

1.1.2 Cytosolic pathways that induce type I IFN

In addition to surface- or endosome-localized TLRs, host cells also express several cytosolic sensors that induce type I IFN in response to nucleic acid ligands, such as RNA, DNA, and cyclic-di-nucleotides (cyclic-di-GMP and cyclic-di AMP) (Figure 2A). The mechanisms by which bacteria stimulate cytosolic sensors is one of the

subjects of this thesis. As discussed below, one current model is that nucleic acids can be released from lysed bacteria. Additionally, bacterial secretion systems may leak or secrete nucleic acid ligands during infection (Figure 2B).

Cytosolic RNA Sensing. RIG-I, MDA5, and LPG2 (collectively called RIG-I-like receptors or RLRs) are cytosolic receptors that bind directly to RNA and induce a type I IFN response to many RNA viruses (Wilkins and Gale, 2010) (Figure 2A). All three RLRs bind RNA via a DExD/H box-containing RNA helicase domain. RIG-I and MDA5 contain caspase-recruitment domains (CARDs), which are required for signaling through a downstream signaling adaptor, IPS-1 (also called MAVS, Cardif, VISA). IPS-1 appears to localize to the mitochondria where it serves as an essential adaptor for coordinating activation of IRF3/7, NF-kB, and MAP kinases (Yoneyama and Fujita, 2009).

The precise role of LGP2 remains to be fully clarified, but it is clear that RIG-I and MDA5 function non-redundantly in viral recognition due to distinct specificities for different RNA structures. RIG-I preferentially recognizes 5'-triphosphate RNA, a motif modified in self RNAs by capping (mRNA) or removal (tRNA, rRNA) (Hornung et al., 2006; Pichlmair et al., 2006). RIG-I can also recognize short double-stranded synthetic RNAs, which may or may not contain a 5' triphosphate (Kato et al., 2008; Schlee et al., 2009; Schmidt et al., 2009; Takahasi et al., 2008). In contrast, MDA5 ligands are less well characterized, but appear to include double-stranded RNAs greater than one kilobase in length that lack a 5' triphosphate (Kato et al., 2008). While studies with synthetic or purified ligands have been informative as to what ligands *can* stimulate RLRs, not much is known about the physiological ligands that *do* activate RLRs during infection. A few exceptions are two recent studies that investigated viral RIG-I bound ligands (Baum et al., 2010; Rehwinkel et al., 2010). One study showed that during influenza or Sendai virus infection, full length ssRNA viral genomes with 5'-triphosphates serve as the dominant RIG-I ligand, whereas RNAs from viral transcripts, replication-derived dsRNA intermediates, or processed self RNAs do not contribute (Rehwinkel et al., 2010). However, Baum et al. revealed a distinct species of RIG-I bound 5' triphosphate RNAs via deep sequencing. This RNA species consists of the genomes of defective interfering (DI) particles during Sendai virus infection, and short genomic, as well as subgenomic DI particles during influenza infection (Baum et al., 2010). Since bacterial mRNAs are not capped and can contain 5' triphosphates (Bieger and Nierlich, 1989), bacteria are potentially able to generate RNA ligands that can be recognized by RLRs, though there are few specific examples (see below).

Two reports recently suggested an unusual mechanism by which DNA could stimulate a cytosolic RNA sensor. In this mechanism, RNA polymerase III transcribes cytosolic DNA thereby generating RNA ligands for RIG-I (Ablasser et al., 2009; Chiu et al., 2009). Only highly AT-rich DNA appears to be a suitable template for Pol III. Epstein-Barr Virus, which produces Pol III-transcribed EBER RNAs, is an example of a pathogen sensed via the Pol III pathway (Ablasser et al., 2009; Chiu et al., 2009).

Cytosolic DNA Sensing. In addition to the Pol III-dependent pathway, both mouse and human cells express at least one additional cytosolic DNA-sensing pathway (Ablasser et al., 2009; Stetson and Medzhitov, 2006a). A few recent reports claim to

identify a DNA sensor, and taken together these data suggest that the innate immune system exhibits cell-type specificity, and in some cases redundancy, in the recognition of cytosolic DNA. One characteristic of cytosolic DNA sensor(s) are clear: it appears to be able to sense dsDNA from many sources without a requirement for specific sequence motifs. One candidate sensor, ZBP-1 (DLM-1/DAI), was reported to bind DNA and activate IRF3 to induce type I IFN (Takaoka et al., 2007; Wang et al., 2008). ZBP-1 has been reported to be important for the type I IFN response to human cytomegalovirus (HCMV) (DeFilippis et al., 2010). However, *Zpb1* deficiency shows no discernable defect in IFN induction in response to other viral or bacterial infections (Ishii et al., 2008; Lippmann et al., 2008; Monroe et al., 2009) possibly because of redundancy with other DNA sensors. Another candidate DNA sensor, IFI16, which contains a pyrin and 2 DNA-binding HIN domains, was recently reported to function as a DNA sensor in human THP-1 cells (Unterholzner et al., 2010). HSV-1 was shown to require *Ifi16* and p204 (the proposed mouse ortholog of *Ifi16*) for a robust IFNβ response. At this time, there are no other reports of viral or bacterial pathogens that are sensed by *Ifi16.*

STING (stimulator of interferon genes, also known as MITA, ERIS, TMEM173) was recently identified as a downstream signaling adaptor required for IFN induction in response to cytosolic DNA (Ishikawa and Barber, 2008; Sun et al., 2009; Zhong et al., 2008). In addition, STING has been reported in some instances to function as a signaling adaptor downstream of RIG-I, but not MDA5 (Ishikawa and Barber, 2008; Zhong et al., 2008) (Figure 2A). *Listeria monocytogenes, Chlamydia muridarum,* and cyclic-di-nucleotides (discussed below) all require STING for type I IFN induction *in vitro* (Ishikawa and Barber, 2008; Prantner et al., 2010; Sauer et al., 2011). Many other bacteria probably require STING for type I IFN induction since it appears to function in multiple cytosolic nucleic acid sensing pathways.

Cytosolic cyclic-di-nucleotoide sensing. Cyclic-di-GMP is a bacterial second messenger signaling molecule produced by nearly all bacterial species, but not by mammalian cells, and therefore, could be a more specific target for innate immune recognition of bacteria than other nucleic acids. In fact, cyclic-di-GMP has been shown to exhibit immunostimulatory properties (Karaolis et al., 2007a), including robust induction of a cytosolic pathway leading to type I IFN production (McWhirter et al., 2009). Furthermore, identification of diadenylate cyclase activity in *Bacillus subtilius* (Witte et al., 2008) led to speculation that another cyclic-di-nucleotide, c-di-AMP, may also elicit a type I IFN response (McWhirter et al., 2009). In fact, elegant work by Woodward et al. demonstrated that *Listeria monocytogenes* transports c-di-AMP into the host cell cytosol via multidrug efflux pumps to activate a host IFN response (Woodward et al., 2010). Induction of type I IFN by c-di-GMP and c-di-AMP requires *Sting* and *Irf3*, and is independent of other cytosolic sensors or TLRs (McWhirter et al., 2009; Sauer et al., 2011). Moreover, STING plays a critical role in the *in vivo* IFN response to *Listeria monocytogenes* and cyclic-di-GMP (Sauer et al., 2011). At present, the cytosolic DNA-sensing and c-di-nucleotide-sensing pathways are genetically indistinguishable, yet in spite of this, biochemical evidence from our lab suggests that STING functions as the c-di-nucleotide sensor, but not as a direct DNA sensor (Burdette

D., in preparation). There is no evidence that induction of type I IFN by any bacterial species requires c-di-GMP, unlike the evidence that innate sensing of c-di-AMP accounts for recognition of *Listeria* infection (Sauer et al., 2011; Woodward et al., 2010). It is possible that c-di-AMP is the biologically relevant molecule targeted by the innate immune system, and that the immunostimulatory nature of c-di-GMP, a very similar molecule, is the result of this selective pressure. However, there are many bacterial species that remain untested and further investigation is required.

Other cytosolic pathways that affect IFN induction. There are few examples of non-nucleic acid molecules contributing to induction of type I IFN via cytosolic pathways. One example is the recognition of bacterial cell wall fragments, such as muramyl dipeptide (MDP), by the cytosolic sensors NOD1 and NOD2. NOD1 and NOD2 signal through the kinase RIP2, which leads to NF-κB activation (Park et al., 2007). Reports vary as to whether NOD signaling is sufficient for IFN-β induction. Although stimulation of NOD2 by MDP appears insufficient to induce type I IFN, stimulation with an N-glycolyl-modified form of MDP made by *Mycobacterium tuberculosis* was sufficient to induce significant type I IFN via NOD2 and the transcription factor IRF5 (Pandey et al., 2009). In response to viruses, ssRNA has been reported to induce *Ifnb* via NOD2 (Sabbah *et al.*, 2009). However, in response to bacteria, NOD signaling most often appears to contribute to induction of type I IFNs primarily via NF-kB, which synergizes with other transcription factors, but alone is insufficient to induce *Ifnb* (Leber et al., 2008).

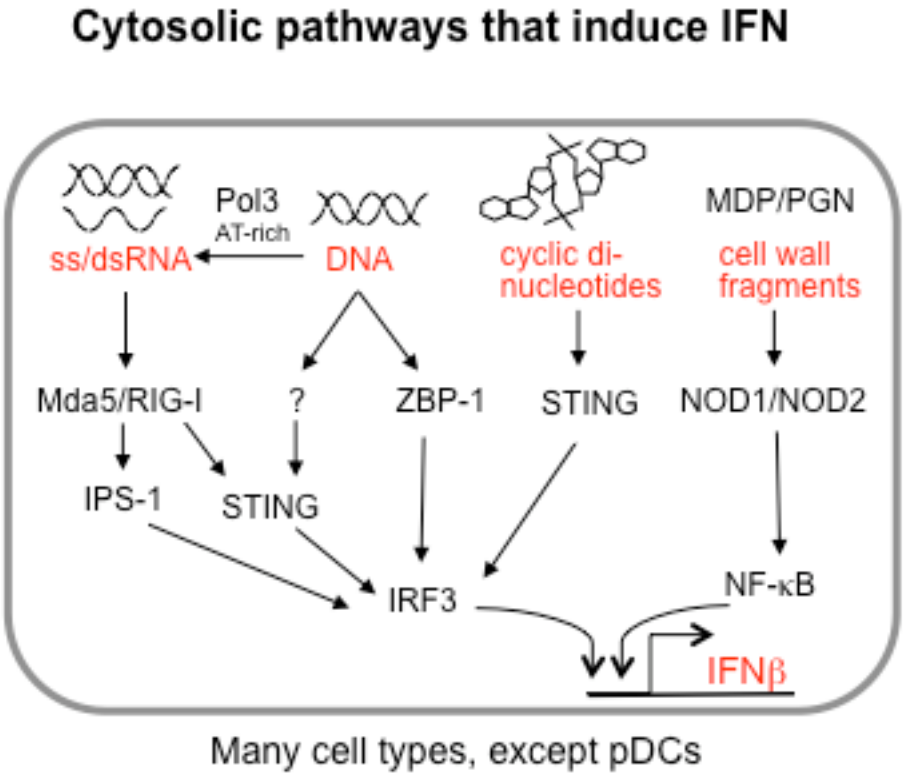

Figure 1.2 Induction of Type I IFN via Cytosolic Receptors and Ligands. In many cell types, except pDCs, cytosolic IFN-inducing receptors are expressed that sense nucleic acids, including RNA, DNA, and cyclic-di-nucleotides. AT-rich DNA can be transcribed by RNA polymerase III to generate ligands for the RNA-sensing pathway. Other sensors for DNA and c-di-nucleotides appear to exist, but remain to be identified. Cytosolic pathways that recognize bacterial cell wall fragments can synergize with nucleic acid sensing pathways to induce IFNβ.

1.2 Induction of type I IFN via Bacterial Secretion Systems

Bacteria can stimulate cytosolic signaling pathways by various mechanisms. As outlined in the following examples, one common mechanism appears to involve delivery of bacterial ligands to the host cell cytosol via a variety of specialized secretion systems. Secretion systems are commonly employed by bacterial pathogens to deliver effector proteins to the cell cytosol from a phagosome or extracellular location. Although translocated effectors allow pathogens to manipulate their hosts, molecules delivered to the host cell cytosol can become targets of innate immune recognition.

1.2.1 Legionella.

Much of my thesis work focused on the mechanism by which *L. pneumophila* induced type I IFN and my findings on this topic can be found in Chapter 2. Here I will briefly introduce my work as well as others' in the field.

Few bacteria have been shown to induce type I IFN via the RNA sensing pathway involving MDA5, RIGI or IPS-1. One exception is *Legionella pneumophila*, a gram-negative pathogen that replicates in macrophages by employing a type IV secretion system (T4SS) to translocate effectors into the macrophage cytosol and orchestrate the creation of its replicative vacuole (Isberg et al., 2009). Interestingly, induction of type I IFN by *L. pneumophila* requires its type IV secretion system but not TLRs (Stetson and Medzhitov, 2006a), suggesting that IFN is induced upon cytosolic recognition of a translocated *L. pneumophila* molecule. In human epithelial-like A549 cells, knockdown of *IPS-1*, the signaling adaptor for RIG-I and MDA5, reduced the induction of type I IFN by *L. pneumophila* (Opitz et al., 2006). Two other studies demonstrated that mouse bone marrow macrophages carrying a targeted deletion or an shRNA to knockdown *Ips-1*, *Rig-i* or *Mda5* were partially defective in IFN induction in response to *L. pneumophila* (Chiu et al., 2009; Monroe et al., 2009). Despite agreement that an RNA-sensing pathway can respond to *L. pneumophila*, there is uncertainty over the underlying molecular mechanism. Chiu et al. favor a model in which *L. pneumophila* translocates DNA into host cells, leading to production of RNA ligands via Pol III transcription. This model is consistent with the ability of the *L. pneumophila* T4SS to conjugate DNA plasmids to recipient bacteria (Vogel et al., 1998), but no there is no direct evidence DNA translocation occurs during infection. Monroe et al. demonstrated that transfection of macrophages with *L. pneumophila* RNA, but not DNA, induced *Rig-i*-dependent type I IFN in macrophages. Identification of the physiological ligand translocated through the T4SS is the focus of Chapter 3. *Helicobacter pylori* is another gram-negative pathogen with a T4SS that may stimulate an IFN response in host cells via a mechanism similar to that of *L. pneumophila* (Rad et al., 2009).

1.2.2 Listeria.

The gram-positive bacterium *Listeria monocytogenes* employs a pore-forming toxin, listeriolysin O (LLO), to disrupt the phagosomal membrane and escape into the cell cytosol where it replicates (Portnoy et al., 1988). LLO-deficient *Listeria* are trapped in a vacuole and induce a MyD88-dependent response, but do not induce type I IFN, whereas wildtype *Listeria* that reach the cytosol induce a distinct, non-overlapping IRF3-dependent type I IFN response (Leber et al., 2008; O'Riordan et al., 2002). Induction of type I IFN by *Listeria* requires *Sting* and *Irf3,* but is independent of TLRs and the cytosolic RNA-sensing pathway. An unbiased genetic screen identified a role for multidrug resistant transporters in the induction of the cytosolic IFN response to *Listeria* (Crimmins et al., 2008). Mutation of MDRs and their repressors has shown that they are capable of transporting the small molecule c-di-AMP, which induces a robust Sting-dependent type I IFN response (Sauer et al., 2011; Woodward et al., 2010).

1.2.3 Francisella.

Francisella tularensis is a gram-negative bacterium that is the causative agent of tularemia. *F. tularensis* utilizes a type VI secretion system, encoded within the *Francisella* pathogencitiy island (FPI), to escape into the macrophage cytosol where it replicates. The FPI is also required for induction of type I IFN, via a pathway that requires IRF3, but is independent of TLRs or the cytosolic RNA sensors (RIG-I, MDA5) (Henry et al., 2007). It is possible that *Francisella* either secretes an IFN-inducing ligand, or leaks immunostimulatory DNA after lysis in the cytosol. It has also been suggested that nucleic acids from phagosomally degraded *Francisella* are released into the cytosol upon disruption of the phagosomal membrane via the T6SS (Fernandes-Alnemri *et al.*, 2010). Francisella DNA that reaches the cytosol activates IRF3-dependent type I IFN signaling, which is critical for activation of the DNA-sensing AIM2 inflammasome (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010).

1.2.4 Yersinia.

LPS from the gram-negative genus *Yersinia* can serve as a potent ligand for TLR4, but in addition, a recent report identified a TLR-independent type I IFN response to extracellular *Yersinia* expressing a functional T3SS (Auerbuch et al., 2009). The TLR-independent response to *Yersinia* occurs in the absence of known translocated effectors, yet requires the pore-forming proteins YopB or YopD (Auerbuch et al., 2009). These data are consistent with a model in which a bacterial molecule reaches the host cytosol in a T3SS-dependent manner and stimulates a cytosolic pathway leading to IFN induction. Neither the stimulatory bacterial molecule nor the cytosolic sensor or host factors mediating this response have been identified.

1.2.5 Mycobacterium.

Mycobacterium tuberculosis resides in a membrane bound compartment within infected host cells and gains access to the cytosol via a type VII secretion system (T7SS, formerly known as ESX-1). Like other pathogens discussed above, *M. tuberculosis* relies on its secretion system for virulence, and in addition, the secretion system is required for type I IFN induction *in vitro* and *in vivo* (Stanley et al., 2007). Despite conflicting reports as to which host pathways are required, there is agreement that TLR signaling is not required (Pandey et al., 2009; Stanley et al., 2007). Leber et al. and Pandey et al. found a partial requirement for NOD2, whereas Stanley et al. found no requirement for RIP2 (a kinase downstream of NOD2) in IFNb induction. As previously mentioned, NOD2 has been proposed to induce type I IFN in response to N-glycolyl-MDP from *M. tubercuolosi*s (Pandey et al., 2009). Taken together, it appears that *M. tuberculosis* induces type I IFN by delivery of nucleic acids and/or cell wall fragments to the cytosol, but it remains unclear whether the T7SS translocates these molecules, or simply permeabilizes the phagosomal membrane, allowing for leakage of bacterial molecules to the cytosol.

1.3 Induction of type I IFN by Ligands Released by Bacteria Degraded in the Phagosome

As discussed above, it is thought that viable bacteria induce type I IFNs by secretion of molecules into host cells. However, there are several reports of bacteria that induce type I IFN upon degradation by innate immune cells. Degraded or lysed bacteria that remain confined in a phagosome can activate TLRs, as exemplified by TLR7 recognition of *Streptococcus* in cDCs (see above; (Mancuso et al., 2009)). Different cell types appear to vary in their degradative capacity. For example, it was found that cDCs and macrophages, but not pDCs, generate nucleic acid ligands for TLR7 and 9 upon bacterial infection, alternatively, pDCs may have a reduced ability to phagocytose bacteria (Mancuso *et al.*, 2009). In addition, there are several examples of bacteria that activate cytosolic IFN-inducing pathways once degraded in a phagosome. In macrophages, Group B *Streptococcus* was shown to induce TLR-independent type I IFN in manner requiring degradation of phagolysosomal bacteria and disruption of the phagosomal membrane by pore-forming toxins (Charrel-Dennis et al., 2008). Cytosolic recognition of GBS required IRF3, but not IPS-1, RIP2 or ZBP-1. The data presented were consistent with a model in which liberated bacterial genomic DNA activates an unknown cytosolic DNA sensor (Charrel-Dennis et al., 2008). Studies have also suggested that live *Borrelia burgdorferi* induces type I IFN by a TLR-independent mechanism likely involving degradation of bacteria in the phagosome (Miller et al., 2008; Salazar et al., 2009).

A similar mechanism was previously found to be relevant in type II IFN (IFNγ)-activated macrophages infected with *Listeria* (Herskovits et al., 2007). IFNγ pretreatment of macrophages mimics conditions expected to exist *in vivo* after the innate immune response has already been initiated. In contrast to naïve macrophages, IFN-γ-activated macrophages are able to produce type I IFN during infection with *Listeria* deficient in hemolysin (LLO), a pore-forming toxin required for bacterial entry into the cytosol. Induction of type I IFN seemed to result from rapid phagosomal degradation of LLO-deficient *Listeria* and subsequent release of ligands into the cytosol that signal, in part, through NOD2 and IRF3 (Herskovits et al., 2007). The involvement of NOD2 suggested PGN fragments were being delivered to the cytosol, potentiating IFN induction by activating NF-kB (Leber et al., 2008). In this case, the primary IFN-inducing signal could be nucleic acid or another ligand, released from degraded bacteria. Lysozyme-sensitive mutants of *Listeria* that were rapidly degraded in naïve macrophages were found to induce type I IFNs, but this induction was unexpectedly found to be entirely TLR2-dependent and only partially MyD88-dependent (Boneca et al., 2007), which is difficult to reconcile with the existing literature. An additional unresolved issue is how ligands generated by phagosomally degraded *Listeria* reach the host cell cytosol. Nevertheless, it has become clear that many extracellular and intracellular vacuolar bacteria induce a cytosolic type I IFN response. Type I IFN induction by phagosomal degradation of bacteria may or may not be independent of bacterial secretion systems, and leads to the release of ligands capable of activating cytosolic IFN-inducing pathways.

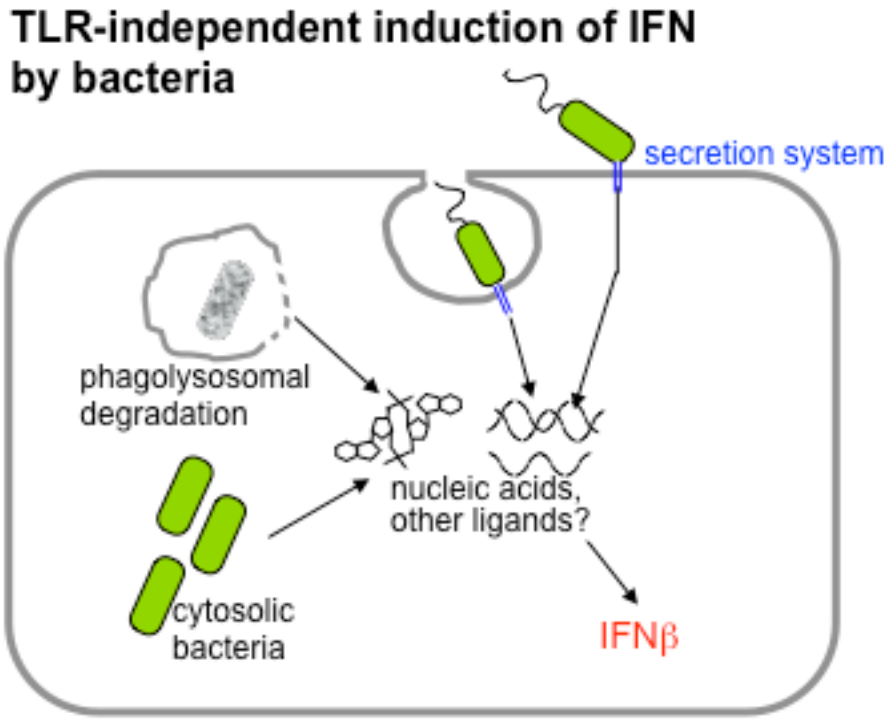

Figure 1.3 Mechanisms of Bacterial Induction of Type I IFN. Extracellular or phagosomal bacteria utilizing secretion systems can leak or secrete nucleic acids that are sensed via cytosolic pathways outlined in A. Bacteria replicating in the cytosol activate type I IFN either by transport or lysis that releases IFN-inducing ligands. Degradation of phagocytosed bacteria can lead to IFN induction in many ways, and in some cases ligands generated in the phagolysosome access the cytosol via a pathway that remains to be elucidated.

1.4 The Function of Type I Interferons in the Host Response to Bacteria

Although type I IFNs are well known to induce a robust antiviral host response, the role of type I IFNs in response to bacterial infection is variable, and is even sometimes detrimental to the host. For example, type I IFN plays an important role in mediating the pathology of LPS-induced toxic shock (Karaghiosoff et al., 2003). A bigger surprise has been several studies demonstrating that type I IFN can actually impair bacterial clearance. For example, *Ifnar*-deficient mice exhibit lower *Listeria monocytogenes* burdens in the liver and spleen, as compared to wild type mice (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). Type I IFN signaling is also detrimental to the clearance of *Mycobacterium tuberculosis* from the spleen (Stanley et al., 2007) and the lung during infection with various *Mtb* strains (Ordway *et al.*, 2007). Furthermore, type I IFN impairs clearance of *Chlamydia* from the genital tract and lungs (Nagarajan et al., 2008; Qiu et al., 2008), and is detrimental to host survival during infection with *Francisella tularensis* (Henry et al., 2010a). The *in*

vivo mechanisms by which type I IFN signaling increases host susceptibility to bacterial infection remain uncertain. One suggestion is that abundant type I IFN predisposes lymphocytes to apoptosis, resulting in suppression of innate responses via increased IL-10 (Carrero and Unanue, 2006). The observation that type I IFN stimulates production of IL-27, a cytokine that strongly suppresses IL-17A production (Guo et al., 2008), hints at another mechanism (Henry et al., 2010a). IL-17A is a cytokine produced by γδ T cells that appears to play an important role in restricting *Listeria* replication by orchestrating neutrophil responses (Hamada et al., 2008; Meeks et al., 2009). In fact, *Ifnar*-deficient mice induce more IL-17A in response to *Francisella* and *Listeria* (Henry et al., 2010a). Therefore, one way type I IFN signaling could result in increased host susceptibility is by suppressing IL-17 responses, which are necessary for neutrophil-mediated bacterial clearance. Another report shows that crosstalk between cytokine signaling pathways can reduce the host's ability to mount an appropriate innate immune response. Induction of type I IFN by *Listeria* was shown to suppress macrophage activation by reducing the ability to respond to IFNγ, a critical cytokine for resistance to *Listeria* (Rayamajhi *et al.*, 2010).

Given that type I IFN appears to be a universal host response to bacterial infection, it would be surprising if type I IFN never played a role in host protection. In fact, type I IFN is crucial for host resistance to some bacterial infections. For example, *Ifnar*-deficient mice exhibit decreased survival and increased bacterial burdens upon infection with Group B *Streptococcus*, *Streptococcus pneumoniae*, and *E. coli* (Mancuso et al., 2007). The susceptibility of *Ifnar*-deficient mice to these infections correlated with reduced cytokine production such as TNFa and IFNg. Type I IFN also plays a role in restricting *L. pneumophila* replication in macrophages (Coers et al., 2007), but *Ifnar*$^{-/-}$ mice do not appear to exhibit increased susceptibility *in vivo* (Monroe et al., 2009), potentially due to redundancy. In response to *F. tularensis*, type I IFN signaling has also been observed to induce the expression of inflammasome components, a molecular signaling complex involved in interleukin-1b and IL-18 production (Henry *et al.*, 2007). Thus, in the context of bacterial infection, type I IFN appears to modulate a broad range of pro- and anti-inflammatory effects. The mechanisms by which the immunomodulatory effects of type I IFNs are regulated are only beginning to be understood (Rothlin et al., 2007).

The large number of recent studies on the induction and function of type I IFNs in response to bacterial infections has led to an increasing appreciation for the complexity of this family of cytokines. Given that most, if not all, bacteria as well as parasitic protozoa, induce type I IFNs, via multiple pathways, it is clearly too simplistic to fall back on the old notion that type I IFNs are primarily 'antiviral' cytokines. On the other hand, in the context of bacterial infections, it is difficult to provide a simple statement of the function of type I IFNs. It appears, instead, that in response to bacteria, types I IFNs serve a variety of beneficial and detrimental immune functions, many of which remain to be fully understood.

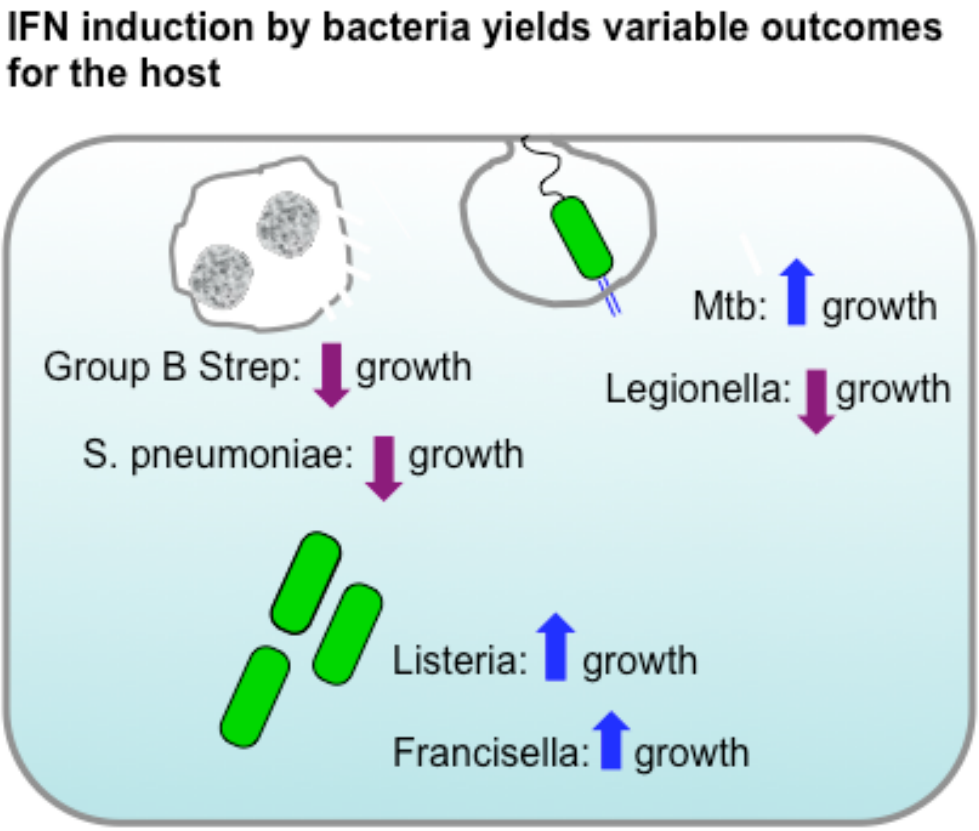

Figure 1.4 Bacterial Induction of Type I IFN yields Variable Outcomes for the Host. Species of bacteria respond to host induction of type I IFN differently. Some species are restricted by type I IFN, some species of bacteria actually replicate better upon induction of type IFN. Notably, this is unlike effect on viruses in which viral replication is robustly restricted.

Chapter 2.

Type I Interferon Response to *Legionella pneumophila*

This chapter describes experiments addressing the host and bacterial factors that play a role in the type I IFN response to infection with *L. pneumophila*. These studies illustrate a dynamic host-pathogen interaction, which is remarkable considering *Legionella* has not evolved in a mammalian host to our knowledge. This work, in part, was published in the journal *PLoS Pathogens* (Monroe et al., 2009).

2.1 Introduction

The intracellular bacterium *Legionella pneumophila* has become a valuable model for the study of immunosurveillance pathways. *L. pneumophila* is a motile gram-negative bacterium that is the cause of a severe pneumonia called Legionnaires' Disease (Fields et al., 2002). In the environment, *L. pneumophila* is believed to replicate in various species of freshwater amoebae. In humans, *L. pneumophila* causes disease by replicating within alveolar macrophages in the lung (Horwitz and Silverstein, 1980). Replication in macrophages and amoebae requires a type IV secretion system that the bacterium uses to inject effector proteins into the host cell cytosol (Isberg et al., 2009). These effectors are believed to orchestrate the creation of an intracellular vacuole in which *L. pneumophila* can replicate. Interestingly, there appears to be considerable redundancy among the effectors, and there are few examples of single effector mutations that have a large effect on intracellular replication of *L. pneumophila*. One *L. pneumophila* effector required for intracellular replication is SdhA (Laguna et al., 2006), but the mechanism by which SdhA acts on host cells remains uncertain (Laguna et al., 2006).

A variety of immunosurveillance pathways that detect *L. pneumophila* infection have been described (Archer and Roy, 2006; Hawn et al., 2007; Hawn et al., 2006; Shin et al., 2008). The best characterized cytosolic immunosurveillance pathway requires the host proteins Naip5 and Ipaf to detect the cytosolic presence of *L. pneumophila* flagellin, leading to activation of caspase-1, rapid pyroptotic macrophage death, and efficient restriction of bacterial replication (Amer et al., 2006; Lightfield et al., 2008; Molofsky et al., 2006; Ren et al., 2006; Zamboni, 2004). *L. pneumophila* has also been observed to induce transcriptional activation of type I interferon (IFN) genes in macrophages and epithelial-like cell lines by a mechanism that remains incompletely characterized (Opitz et al., 2006; Stetson and Medzhitov, 2006a). Induction of type I IFNs by *L. pneumophila* is independent of the flagellin-sensing pathway (Coers et al., 2007), but also appears to contribute to restriction of bacterial replication in macrophages (Coers et al., 2007; Schiavoni et al., 2004) and epithelial-like cell lines (Opitz et al., 2006).

Type I IFNs are an important class of cytokines that orchestrate diverse immune responses to pathogens (Stetson and Medzhitov, 2006b). Encoded by a single IFNβ gene as well as multiple IFNα and other (e.g., IFNε, κ, δ, ζ) genes, type I IFNs are

transcriptionally induced by a number of immunosurveillance pathways, including Toll-like receptors (TLRs) and a variety of cytosolic sensors (Pichlmair and Reis e Sousa, 2007). For example, cytosolic RNA is recognized by two distinct helicase and CARD-containing sensors, RIG-I and MDA5 (Yoneyama et al., 2004), that signal through the adaptor IPS-1 (also called MAVS, CARDIF, VISA) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Sun et al., 2006; Xu et al., 2005). The cytosolic presence of DNA also induces type I IFNs, but this phenomenon is less well understood and currently under intense investigation (Ishii et al., 2006; Ishikawa et al., 2009; Stetson and Medzhitov, 2006a; Unterholzner et al., 2010). Studies with *Ips-1*-deficient mice have indicated that cytosolic DNA can signal independently of IPS-1 in many cell types, including macrophages (Sun et al., 2006). However, cytosolic responses to DNA appear to require IPS-1 in certain cell types, including 293T cells (Cheng et al., 2007; Ishii et al., 2006). Indeed, two recent reports have described a pathway by which AT-rich DNA can signal via IPS-1 (Ablasser et al., 2009; Chiu et al., 2009). In this pathway, DNA is transcribed by RNA polymerase III to form an RNA intermediate that can be sensed by RIG-I. The RNA Pol III pathway appears to be operational in macrophages, but is redundant with other DNA-sensing pathways in these cells. A couple of reports have proposed that DAI (also called ZBP-1) is a cytosolic DNA-sensor (Takaoka et al., 2007; Wang et al., 2008), but *Zbp1*-deficient mice appear to respond normally to cytosolic DNA (Ishii et al., 2008), consistent with the existence of multiple cytosolic sensors for DNA. Other small molecule compounds, such as cyclic-di-GMP, cyclic-di-AMP, and DMXAA, can also trigger cytosolic immunosurveillance pathways leading to induction of type I IFNs (Karaolis et al., 2007a; McWhirter et al., 2009; Roberts et al., 2007; Woodward et al., 2010).

Type I IFNs are typically considered antiviral cytokines that act locally to induce an antiviral state and systemically to induce cellular innate and adaptive immune responses (Pichlmair and Reis e Sousa, 2007). Mice deficient in the type I IFN receptor (*Ifnar*) are unable to respond to type I IFNs, and are highly susceptible to viral infections. Interestingly, most bacterial infections also trigger production of type I IFNs, but the physiological significance of type I IFNs in immune defense against bacteria is complex. Type I IFN appears to protect against infection with group B *Streptococcus* (Mancuso et al., 2007), but this is not the case for many other bacterial infections. For example, the intracellular gram-positive bacterium *Listeria monocytogenes* induces a potent type I IFN response (Leber et al., 2008; O'Riordan et al., 2002), but *Ifnar*-deficient mice are actually more resistant to *L. monocytogenes* infection than are wildtype mice (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). Many bacterial pathogens, including *Francisella tularensis*, *Mycobacterium tuberculosis*, *Brucella abortus*, and group B *Streptococcus*, induce type I IFN production by macrophages via a cytosolic TLR-independent pathway (Charrel-Dennis et al., 2008; Henry et al., 2007; Roux et al., 2007; Stanley et al., 2007), but the bacterial ligands and host sensors required for the interferon response of macrophages to these bacteria remain unknown.

It was demonstrated that induction of type I IFN by *L. pneumophila* in macrophages did not require bacterial replication or signaling through the TLR-adaptors MyD88 or Trif, but did require the bacterial Dot/Icm type IV secretion system (Stetson

and Medzhitov, 2006a). Because the IFN response could be recapitulated with transfected DNA (Ishii et al., 2006; Stetson and Medzhitov, 2006a) and because Dot/Icm system has been shown to conjugate DNA plasmids to recipient bacteria (Vogel et al., 1998), it was proposed that perhaps *L. pneumophila* induced type I IFN via a cytosolic DNA-sensing pathway (Stetson and Medzhitov, 2006a). Another report used RNA interference to implicate the signaling adaptor IPS-1 (MAVS) in the IFN response to *L. pneumophila* in human A549 epithelial-like cells (Opitz et al., 2006). However, the significance of this latter finding is unclear since RNAi-mediated knockdown of RIG-I and MDA5, the two sensor proteins directly upstream of IPS-1, did not have an effect on induction of type I IFN by *L. pneumophila* (Opitz et al., 2006). Moreover, the A549 response to *L. pneumophila* may be distinct from the macrophage or *in vivo* response.

Recently, one report proposed that *L. pneumophila* DNA was recognized in the cytosol by RNA polymerase III (Chiu et al., 2009), resulting in the production of an RNA intermediate that triggered IFN production via the IPS-1 pathway. Apparently consistent with this proposal, *Ips-1*-deficient mouse macrophages did not produce type I IFN in response to *L. pneumophila* (Chiu et al., 2009). Moreover, since Pol III acts preferentially on AT-rich substrates, it is plausible that Pol III would recognize the *L. pneumophila* genome, which has a high proportion (62%) of A:T basepairs. However, the response to *L. pneumophila* DNA was not investigated (Chiu et al., 2009). In addition, the same report, as well as others (Ablasser et al., 2009; McWhirter et al., 2009), observed that the type I IFN response to AT-rich (or any other) DNA is not *Ips-1*-dependent in mouse cells. Thus, if *L. pneumophila* DNA was reaching the cytosol, the simplest prediction would be that the resulting type I IFN response would be independent of *Ips-1*, instead of *Ips-1*-dependent, as was shown (Chiu et al., 2009). Thus, the mechanism of IFN induction by *L. pneumophila* remains unclear.

In this Chapter, I describe my efforts to define bacterial and host factors controlling the macrophage type I IFN response to *L. pneumophila*. In agreement with previous studies (Chiu et al., 2009; Opitz et al., 2006), I found that *Ips-1* is required for optimal induction of type I IFN in response to *L. pneumophila* infection *in vitro*. I extended this observation by demonstrating that *Ips-1* also contributes to the type I IFN response in an *in vivo* model of Legionnaires' Disease. Furthermore, I provided the first evidence that two RNA sensors upstream of *Ips-1, Rig-i* and *Mda5*, are involved in the macrophage interferon response to *L. pneumophila*. Importantly, however, I did not observe a role for the Pol III pathway in the type I IFN response to *L. pneumophila*. Instead, I found that *L. pneumophila* genomic DNA stimulates an *Ips-1*/*Mda5*/*Rig-i*-independent IFN response in macrophages, which contrasts with the *Ips-1*-dependent response to *L. pneumophila* infection. On the other hand, I found that *L. pneumophila* RNA stimulated a *Rig-i*-dependent IFN response. Thus, my data are consistent with a model in which *L. pneumophila* RNA, or host RNA, rather than *L. pneumophila* DNA, is the primary ligand that stimulates the host IFN response. In this Chapter, I also describe my work investigating bacterial factors that modulate the host type I IFN response. Although numerous viral proteins that interfere with IFN signaling have been described, similar bacterial proteins have not been documented. It is therefore interesting that we were able to identify a secreted bacterial effector, SdhA, as an inhibitor of the *Ips-1*-dependent IFN response to *L. pneumophila*. Taken together, our

findings provide surprising evidence that cytosolic RNA-sensing pathways are not specific for viral infections but can also respond to bacterial infections, and moreover, our data provide a specific example of a bacterial factor that suppresses the host IFN response.

2.2 Experimental Procedures

Ethics statement. Animal experiments were approved by the University of California, Berkeley, Institutional Animal Care and Use Committee.

Mice, cell lines and plasmids. Bone marrow derived macrophages were derived from the following mouse strains: C57BL/6J (B6), *Ips-1*$^{-/-}$ (Sun et al., 2006), *Mda5*$^{-/-}$ (Gitlin et al., 2006), *Ifnar*$^{-/-}$ (Muller et al., 1994), *Zbp1*$^{-/-}$ (Ishii et al., 2008), *MyD88/Trif*$^{-/-}$, and *Casp1*$^{-/-}$ (Li et al., 1995). C57BL/6J mice were purchased from the Jackson Laboratory. *Ips-1*$^{-/-}$ mice were from Z. Chen (University of Texas Southwestern Medical Center). *Ips-1*$^{-/-}$ mice were obtained on a mixed B6/129 background and *Ips-1*$^{-/-}$ and *Ips-1*$^{+/-}$ littermate controls were generated by breeding (*Ips-1*$^{-/-}$ x B6) F1 mice to *Ips-1*$^{-/-}$. *Mda5*$^{-/-}$ mice were from M. Colonna and S. Gilfillan (Washington University). L929-ISRE IFN reporter cells were from B. Beutler (The Scripps Research Institute). Viruses to immortalize *MyD88*$^{-/-}$*Trif*$^{-/-}$ immortalized bone marrow derived macrophages were the generous gift of Kate Fitzgerald, Doug Golenbock (U. Mass, Worcester) and Dhan Kalvakolanu (U. Maryland). The complementation plasmid (pJB908-SdhA) was generously provided by Ralph Isberg (Tufts). Expression constructs pEF-BOS-RIG-I and pEF-BOS-MDA5 were generously provided by Jae Jung (Harvard University).

Bacterial strains. LP02 is a streptomycin-resistant thymidine auxotroph derivative of *Legionella pneumophila* strain LP01. LP02Δ*sdhA* and LP02Δ*sdhA*Δ*sdhB*Δ*sidH* were a generous gift from R. Isberg (Tufts University). The Δ*flaA*Δ*sdhA* strain was generated by an unmarked deletion of *flaA* in LP02Δ*sdhA* using the allelic exchange vector pSR47S-Δ*flaA* (Ren et al., 2006).

Cell culture. L929-ISRE and HEK293T cells were cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, and 100U/mL penicillin. Macrophages were derived from bone marrow cells cultured for eight days in RPMI supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, 100U/mL penicillin, and 10% supernatant from 3T3-CSF cells, with feeding on the fifth day of growth. *MyD88*$^{-/-}$*Trif*$^{-/-}$ immortalized macrophages were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, and 100U/mL penicillin.

Reagents. Poly I:C was from GE Biosciences, pA:T (poly(dA-dT):poly(dA-dT)) was from Sigma, and Sendai Virus was from Charles River Laboratories. Wildtype Theiler's Virus GDVII was from M. Brahic and Eric Freundt (Stanford University). Pol III inhibitor (ML-60218) was from Calbiochem.

Isolation of nucleic acids from *L. pneumophila.* Total bacterial RNA was isolated

using RNAprotect Bacterial Reagent (Qiagen) and RNeasy kit (Qiagen). Genomic DNA was isolated by guanidinium thiocyanate followed by phenol:chloroform extraction. Nucleic acids were treated with RQ1 RNase-Free DNase (Promega) and/or RNaseA (Sigma).

Quantitative RT-PCR. Bone marrow derived macrophages were plated at a density of 2 x10^6 per well in 6 well plates and infected with an MOI of 1. Macrophage RNA was harvested 4 hours post infection and isolated with the RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA was DNase treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed with Superscript III (Invitrogen). Quantitative PCR assays were performed on the Step One Plus RT PCR System (Applied Biosystems) with Platinum Taq DNA polymerase (Invitrogen) and EvaGreen dye (Biotium). Gene expression values were normalized to *Rps17* (mouse) or S9 (human) levels for each sample. The following primer sequences were used: mouse *Ifnb*, F, 5'-ATAAGCAGCTCCAGCTCCAA-3'and R, 5'-CTGTCTGCTGGTGGAGTTCA-3'; mouse *Rps17*, F, 5'-CGCCATTATCCCCAGCAAG-3' and R, 5'-TGTCGGGATCCACCTCAATG-3'; mouse *Rig-i,* F, 5'-ATTGTCGGCGTCCACAAAG-3' and R, 5'-GTGCATCGTTGTATTTCCGCA-3', human *Ifnb,* F, 5'-AAACTCATGAGCAGTCTGCA-3' and R, 5'- AGGAGATCTTCAGTTTCGGAG G-3'; human S9, F, 5'-ATCCGCCAGCGCCATA-3' and R, 5'-TCAATGTGCTTCTGGGAATCC-3'.

Cell stimulation and transfection. Cell stimulants were transfected with Lipofectamine 2000 (LF2000, Invitrogen) according to the manufacturer's protocol. Nucleic acids were mixed with LF 2000 in Optimem (Invitrogen) at a ratio of 1.0 µl LF2000/µg nucleic acid and incubated for 20 minutes at room temperature. The ligand-lipid complexes were added to cells at a final concentration of 3.3 µg/ml (96-well plates) and 1.0 µg/ml (6 well plates). For poly I:C, the stock solution (2.5 mg/ml) was heated at 55°C for 10 minutes and cooled to room temperature immediately before mixing with LF2000. Transfection experiments were incubated for 8 hours, unless otherwise stated. RIG-I, MDA5, TRIF and SdhA expression plasmids, along with an IFNβ-firefly luciferase reporter and TK-*Renilla* luciferase plasmids, were transfected with FuGENE 6 (Roche) according to the manufacturer's protocol. Nucleic acids were mixed with FuGENE 6 in Optimem at 0.5 µl/96 well and incubated for 15 minutes. Total transfected DNA was normalized to 200ng per well using an empty pcDNA3 plasmid. Cells were stimulated 20 hours after transfection of expression plasmids.

Type I IFN bioassay and luciferase reporter assay. Cell culture supernatants or bronchoalveolar lavage fluid (BALF) was overlayed on L929-ISRE IFN reporter cells in a 96-well plate format and incubated for 4 hours at 37°C and 5%CO_2. L929-ISRE IFN reporter cells and HEK293T cells expressing an IFNβ-firefly luciferase reporter and TK-*Renilla* luciferase were lysed in Passive Lysis Buffer (Promega) for 5 minutes at room temperature and relative light units were measured upon injection of firefly luciferin substrate (Biosynth) or *Renilla* substrate with the LmaxII^{384} luminometer (Molecular

Devices). For transient transfection reporter assays, luciferase values were normalized to an internal *Renilla* control.

Cytotoxicity Assays. Cytotoxicity of bacterial strains was determined by measuring lactate dehydrogenase release essentially as previously described (Decker and Lohmann-Matthes, 1988). Macrophages were plated at a density of $1x10^5$ in a 96-well plate and infected with stationary phase *L. pneumophila* at a multiplicity of infection (MOI) of 1. Plates were spun at 400x*g* for 10 minutes to allow equivalent infectivity of non-motile and motile strains (Ren et al., 2006). Plates were re-spun 4 hours post infection and cell culture supernatants were assayed for LDH activity. Specific lysis was calculated as a percentage of detergent lysed cells.

Growth curves. Bacterial growth was determined as previously described (Coers et al., 2007). Bone marrow derived macrophages were plated at a density of $1x10^5$ per well in white 96-well plates (Nunc) and allowed to adhere overnight. Macrophages were infected with stationary-phase *L. pneumophila* at a multiplicity of infection (MOI) of 0.01. Growth of luminescent *L. pneumophila* strains was assessed by RLU with the LmaxII^{384} luminometer (Molecular Devices). Nonluminescent bacterial strains were analyzed for colony-forming units on buffered charcoal yeast extract plates.

Transposon Mutagenesis. Transposon mutagenesis of LP02 was previously described (Ren et al., 2006). Briefly, the pSC123 mariner transposon was mated from *E.coli* SM10 λpir into the *L. pneumophila* strain LP02. Matings were plated on buffered yeast extract charcoal plates with streptomycin (100µg/ml) and kanamycin (25µg/ml). Single colonies were isolated and grown in overnight cultures and used to infect bone marrow derived *MyD88*$^{-/-}$ *Trif*$^{-/-}$ macrophages. After overnight incubation, levels of type I interferon in the supernatant was determined by bioassay. The site of transposon insertion was determined by Y-linker PCR (Kwon and Ricke, 2000).

***In vivo* studies.** Age and sex-matched *lps-1*$^{-/-}$ and littermate *lps-1*$^{+/-}$ mice were infected intranasally with $2.5 x10^6$ LP01 Δ*flaA* in 20µl PBS. Bronchoalveolar lavage was performed 20 hours post infection via the trachea using a catheter (BD Angiocath 18g, 1.3 x 48mm) and 800µl PBS. Type I interferon induction was determined by bioassay. Type I interferon amounts were calculated using a 4-parameter standard curve determined by dilution of recombinant IFNβ (R&D Systems). CFUs were determined by hypotonic lysis of cells from the brochoalveolar lavage fluid (BALF). In parallel experiments, it was determined that CFU in the BALF was representative of total CFU in the lung.

shRNA knockdown. Knockdown constructs were generated with the MSCV/LTRmiR30-PIG (LMP) vector from Open Biosystems. shRNA PCR products were cloned into the LMP vector using XhoI and EcoRI sites. *Rig-i* sequence: 5'-GCCCATTGAAACCAAGAAATT-3', control shRNA sequence: 5'-TGACAGTGTCTTCGCTAATGAA-3'. *MyD88*$^{-/-}$ *Trif*$^{-/-}$ immortalized bone marrow derived macrophages were transduced with retrovirus as previously described (Lightfield et al.,

2008). GFP^+ macrophages were sorted with the DAKO-Cytomation MoFlo High Speed Sorter.

2.3 Results

2.3.1 The cytosolic RNA-sensing pathway is involved in the macrophage response to L. pneumophila.

We hypothesized that a cytosolic innate immune sensing pathway controls the type I IFN response to *L. pneumophila*. To test this hypothesis, we determined whether macrophages deficient in known cytosolic RNA and DNA sensing pathway components can induce type I IFNs in response to *L. pneumophila*. Macrophages were infected with *L. pneumophila* at a multiplicity of infection (MOI) of 1 and induction of interferon beta (*Ifnb*) transcription was analyzed by quantitative RT-PCR after 4 hours (Figure 2.1A-D). As previously reported (Chiu et al., 2009), *Ips-1*$^{-/-}$ macrophages showed a significantly reduced induction of *Ifnb* in response to infection with wild type *L. pneumophila* compared to *Ips-1*$^{+/+}$ macrophages ($p<0.05$; Figure 2.1A). Induction of *Ifnb* was not completely eliminated in *Ips-1*$^{-/-}$ macrophages, however, as *Irf3*$^{-/-}$ macrophages exhibited an even lower induction of *Ifnb* compared to *Ips-1*$^{-/-}$ ($p<0.05$; Figure 2.1A). Consistent with previous reports (Stetson and Medzhitov, 2006a), we found that the Dot/Icm type IV secretion system was required to elicit the macrophage type I interferon response since Δ*dot L. pneumophila* did not induce a robust type I interferon response (Figure 2.1A). These results suggest that *L. pneumophila* induces type I IFN *via* a cytosolic RNA immunosurveillance pathway that involves the adaptor *Ips-1*.

We hypothesized that a cytosolic RNA sensor that functions upstream of *Ips-1* could be involved in the type I interferon host response to *L. pneumophila*. However, knockdown experiments previously failed to reveal a role for the known sensors (MDA5 and RIG-I) upstream of IPS-1 (Opitz et al., 2006). Therefore, we tested *Mda5*$^{-/-}$ knockout macrophages (Figure 2.1B) and found reduced induction of *Ifnb* transcription as compared to control *Mda5*$^{+/+}$ macrophages. Importantly, however, *Dot*-dependent induction of type I IFN was not completely abolished in *Mda5*$^{-/-}$ macrophages, implying that other redundant pathways are also involved.

Rig-i knockout mice die as embryos, so we were unable to obtain *Rig-i*$^{-/-}$ knockout macrophages. To circumvent this problem, we stably transduced immortalized macrophages with a retrovirus expressing an shRNA to knock down *Rig-i* expression. Quantitative RT-PCR demonstrated that the knockdown was effective, even in infected macrophages (Figure 2.1C), and that *Rig-i* knockdown had a significant effect on the induction of type I interferon by *L. pneumophila* (Figure 2.1D). In the experiments in Figures 2.1C and 2.1D we used the Δ*flaA* strain of *L. pneumophila*, but similar results were obtained with wildtype, and it was previously shown that flagellin is not required for the IFN response to *L. pneumophila* (Coers et al., 2007; Opitz et al., 2006). It is unusual, but not unprecedented, that a pathogen would stimulate both the RIG-I and MDA5 RNA-sensing pathways (Fredericksen et al., 2008).

To determine whether a candidate DNA sensor, Dai (or Zpb1), is involved in the type I interferon response to *L. pneumophila*, we tested whether *Zbp1*$^{-/-}$ macrophages respond to *L. pneumophila.* We observed similar levels of *Ifnb* induction in *Zbp1*$^{+/+}$ and *Zbp1*$^{-/-}$ macrophages (data not shown). Differences in *Ifnb* transcript levels between *Zbp-1*$^{+/+}$ and *Zbp-1*$^{-/-}$ macrophages infected with *L. pneumophila* were not statistically

significant (ns, $p>0.1$, Student's t-test) (data not shown). Taken together, these results imply that the RNA sensors Rig-i and Mda5, but not the DNA sensor Zbp1, are involved in sensing *L. pneumophila* infection.

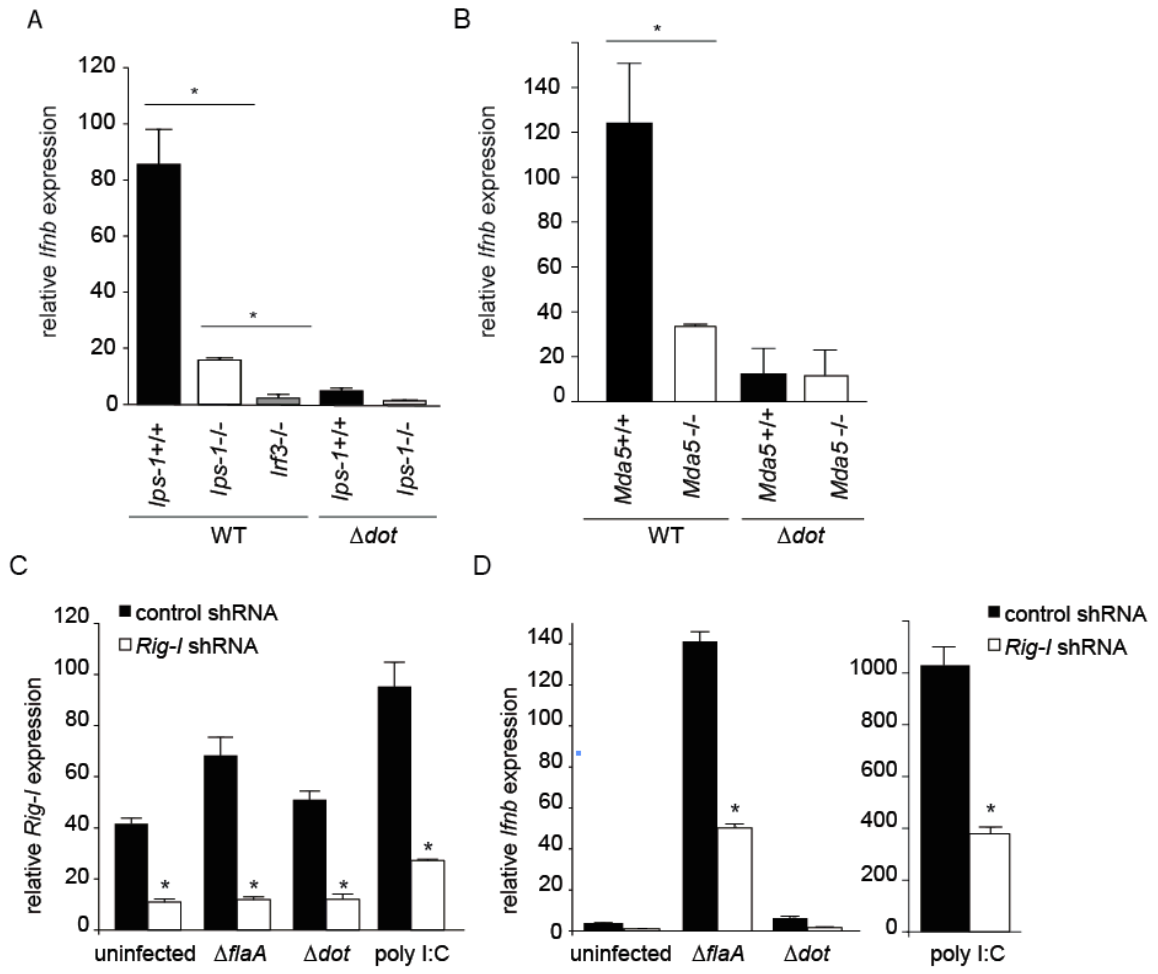

Figure 2.1 The cytosolic RNA-sensing pathway is involved in the host type I interferon response to *L. pneumophila*. (A) Induction of interferon beta (*Ifnb*) by *L. pneumophila* is largely dependent on *Ips-1.* Bone marrow derived *Ips-1*$^{+/+}$, *Ips-1*$^{-/-}$, and *Irf3*$^{-/-}$ macrophages were infected with wild type and Δ*dot L. pneumophila* at a multiplicity of infection (MOI) of 1. *Ifnb* induction was analyzed by quantitative RT-PCR 4 hours post infection. *Ifnb* message was normalized to ribosomal protein *rps17* levels. Differences in *Ifnb* transcript induction were statistically significant in *Ips-1*$^{+/+}$ versus *Ips-1*$^{-/-}$ macrophages (*, p<0.05) and *Ips-1*$^{-/-}$ versus *Irf3*$^{-/-}$ (*, p<0.05, Student's t-test) when infected with wild type *L. pneumophila.* (B) Induction of *Ifnb* by *L. pneumophila* is partially dependent on *Mda5*. Bone marrow derived *Mda5*$^{+/+}$ and *Mda5*$^{-/-}$ macrophages were infected with wild type and Δ*dot L. pneumophila* at a multiplicity of infection (MOI) of 1. *Ifnb* induction was assessed by quantitative RT-PCR as in (A). Differences in *Ifnb* induction were statistically significant (*, p<0.05, Student's t-test) between *Mda5*$^{+/+}$ and *Mda5*$^{-/-}$ infected with wild type *L. pneumophila.* (C) Retroviral transduction of a *Rig-i* shRNA, but not the control shRNA, knocks down expression of *Rig-i* in *MyD88*$^{-/-}$*Trif*$^{-/-}$ immortalized macrophages. Stable transduction of *MyD88*$^{-/-}$*Trif*$^{-/-}$ immortalized macrophages was performed with a retroviral vector containing a control and *Rig-i*

shRNA. Level of *Rig-i* knockdown was determined by quantitative RT-PCR under uninfected, infected, and poly I:C stimulation conditions. Differences in *Rig-i* transcript levels were statistically significant (*, $p<0.05$, Students t-test) under resting, infected, and ligand-stimulated conditions. (D) *Rig-i* is involved in the host type I interferon response to infection with *L. pneumophila***.** *Rig-i* knockdown leads to reduced *Ifnb* expression in response to infection with Δ*flaA L. pneumophila,* as well as stimulation with poly I:C. Quantitative RT-PCR was carried out 4 hours post infection. Control knockdown macrophages induced a statistically significant (*, $p<0.05$) higher level of *Ifnb* transcript in response to Δ*flaA L. pneumophila* and poly I:C. No significant difference was found in uninfected or Δ*dot L. pneumophila* infected macrophages.

We tested whether loss of signaling through the RNA sensing components *Ips-1* or *Mda5* could mimic the previously observed permissiveness of *Ifnar*$^{-/-}$ macrophages (Coers et al., 2007). However, neither *Ips-1*$^{-/-}$ nor *Mda5*$^{-/-}$ macrophages were permissive to *L. pneumophila*, suggesting that the low levels of IFNβ produced in the absence of *Ips-1* or *Mda5* are sufficient to restrict *L. pneumophila* growth (Figure 2.2).

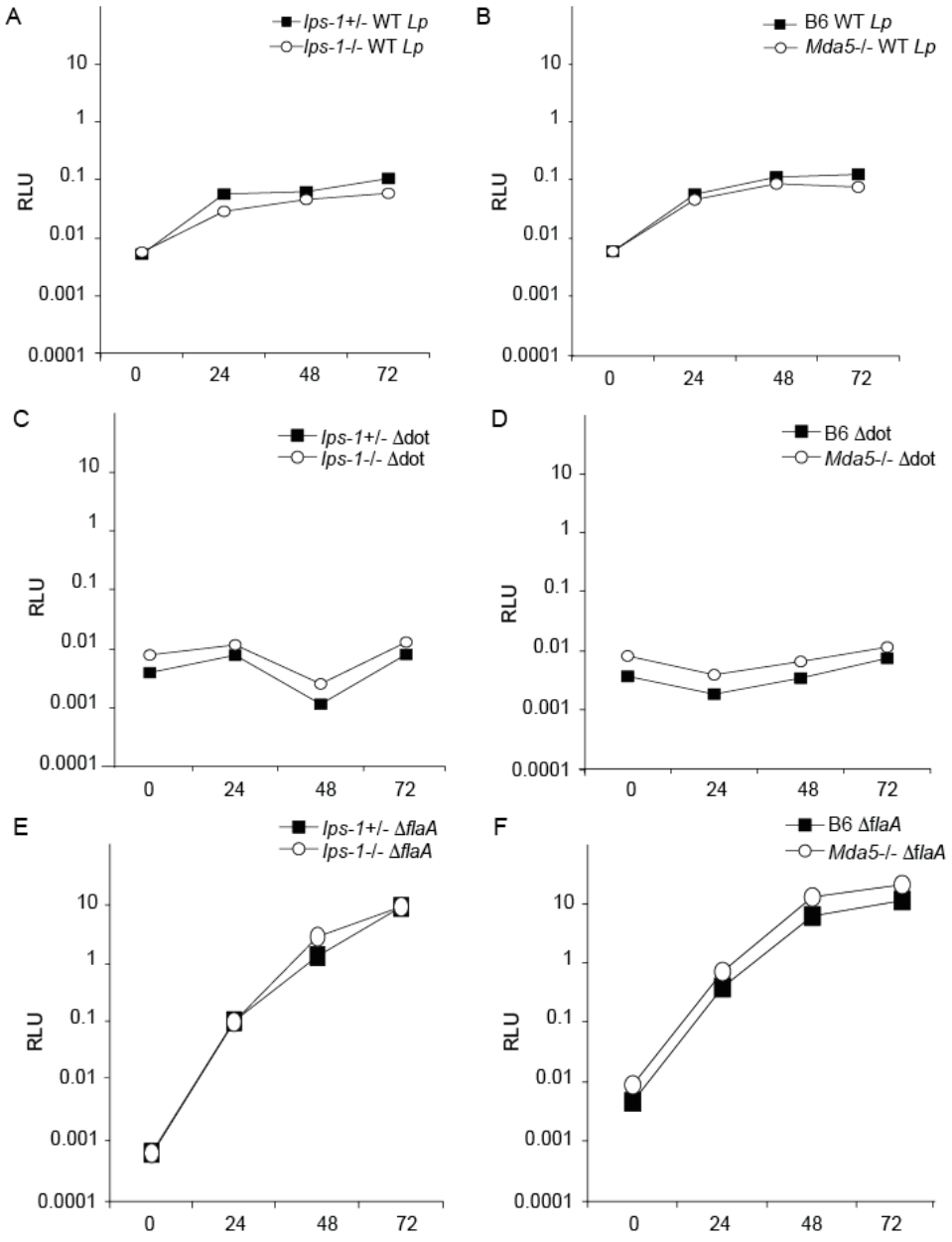

Figure 2.2 *L. pneumophila* replication is restricted in *lps-1*$^{-/-}$ and *Mda-5*$^{-/-}$ macrophages. *lps-1*$^{+/-}$, *lps-1*$^{-/-}$, C57BL/6 (B6) and *Mda5*$^{-/-}$ macrophages were infected at an MOI of 0.01 and growth of luminescent *L. pneumophila* strains was determined by RLU at 0, 24, 48, and 72 hours post infection. (A) *lps-1*$^{+/-}$and *lps-1*$^{-/-}$ macrophages were infected WT (LP02) *L. pneumophila* (B) C57BL/6 (B6) and *Mda5*$^{-/-}$ macrophages were infected as in A (C) *lps-1*$^{+/-}$and *lps-1*$^{-/-}$ macrophages were infected with Δ*dot L. pneumophila* (D) C57BL/6 (B6) and *Mda5*$^{-/-}$ macrophages were infected as in C (E) *lps-1*$^{+/-}$and *lps-1*$^{-/-}$ infected with Δ*flaA L. pneumophila* (F) C57BL/6 (B6) and *Mda5*$^{-/-}$ macrophages were infected as in E.

2.3.2 The type IV secreted effector SdhA suppresses induction of interferon by L. pneumophila.

To identify bacterial components that modulate the type I interferon response to *L. pneumophila*, Sarah McWhirter conducted a transposon mutagenesis screen. The LP02 strain of *L. pneumophila* was mutagenized with a *mariner* transposon as described previously (Ren et al., 2006). Individual transposon mutants were used to infect *MyD88*$^{-/-}$ *Trif*$^{-/-}$ bone marrow-derived macrophages at an MOI of 1, and after approximately 16 hours, supernatants were collected and overlayed on type I IFN reporter cells (Jiang et al., 2005). Induction of type I IFN was compared to wild type (LP02) and Δ*dot L. pneumophila* controls. Approximately 2000 independent mutants were tested and eight mutants were isolated that were confirmed to be defective in induction of type I IFN (S. McWhirter). All these mutants harbored insertions in genes required for the function of the *Dot/Icm* apparatus (e.g., *icmB, icmC, icmD, icmX, icmJ*), thereby validating the screen.

Interestingly, a single transposon mutant, 11C11, was found that consistently hyperinduced the type I interferon response. The transposon insertion mapped to the 3' end (nucleotide position 3421 of the open reading frame) of a gene, *sdhA*, that was previously shown (Laguna et al., 2006) to encode a type IV secreted effector protein of 1429 amino acids (166kDa) (Figure 2.3A) (S. McWhirter). SdhA has previously been shown to be essential for bacterial replication in macrophages (Laguna et al., 2006), but a connection to type I IFNs was not previously noted. To confirm that the hyperinduction of type I interferon was due to mutation of *sdhA,* I compared the 11C11 transposon mutant to an unmarked clean deletion of *sdhA* (Figure 2.3B). Both the 11C11 mutant and Δ*sdhA L. pneumophila* showed similar levels of hyperinduction of type I interferon. The *L. pneumophila* genome contains 2 paralogs of *sdhA*, called *sidH* and *sdhB*. A triple knockout strain, Δ*sdhA*Δ*sdhB*Δ*sidH,* was compared to single deletion of *sdhA* to determine if either paralog regulated the induction of type I IFNs. Similar levels of IFNβ were induced Δ*sdhA*Δ*sdhB*Δ*sidH* and Δ*sdhA* (Figure 2.3B). Similar results were obtained when induction of *Ifnb* was assessed by quantitative RT-PCR (Figure 2.3C). A role for *sdhA* in regulating the interferon response was further confirmed by complementing the Δ*sdhA* mutation with an *sdhA* expression plasmid. As expected, the complemented strain induced significantly less type I IFN than the control Δ*sdhA* strain harboring an empty plasmid (Figure 2.3D). These results indicate that SdhA functions, directly or indirectly, to repress the induction of type I IFN by *L. pneumophila*.

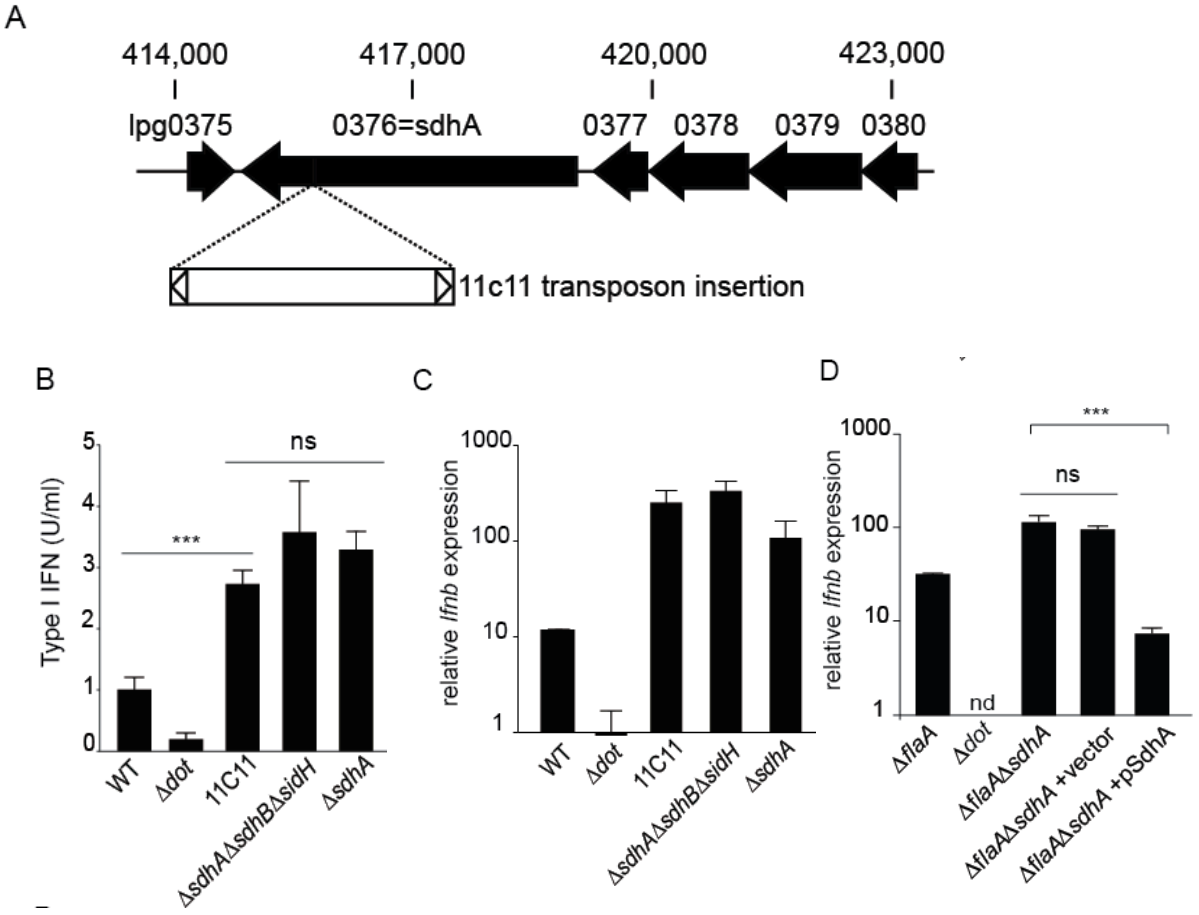

Figure 2.3 The type IV secreted effector SdhA suppresses induction of interferon by *L. pneumophila*. (A) The 11C11 mutant harbors a transposon insertion in *sdhA*. The transposon insertion site is in the 3' end of the open reading frame of the *sdhA* locus at nucleotide position 3421. (B) A clean deletion mutant of *sdhA* recapitulates the 11C11 transposon mutant and hyperinduces type I interferon. Bone marrow derived *Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages were infected with stationary phase *L. pneumophila* strains at a MOI of 1. Cell supernatants were harvested 8 hours post infection and assayed for type I interferon induction by an L929-ISRE luciferase bioassay. Type I interferon levels were determined by generating a standard curve with recombinant IFNβ. An unmarked clean deletion of *sdhA* was compared to wild type, Δ*dot*, the transposon mutant 11C11, and a triple deletion of *sdhA* and the two *L. pneumophila* paralogs, *sidH* and *sdhB*. Differences in IFNβ induction were statistically significant between WT *L. pneumophila* and the transposon mutant 11C11 (***, $p<0.0005$, Student's t-test). Differences between 11C11, Δ*sdhA* and Δ*sdhA*Δ*sdhB*Δ*sidH* were not statistically significant (ns, $p>0.05$, Student's t-test). (C) A clean deletion mutant of *sdhA* recapitulates the 11C11 transposon mutant and hyperinduces transcriptional activation of *Ifnb*. Bone marrow derived *Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages were infected with wild type, Δ*dot*, Δ*sdhA,* 11C11*,*

Δ*sdhA*Δ*sdhB*Δ*sidH* stationary phase *L. pneumophila* and transcriptional induction of *Ifnb* was analyzed by quantitative RT-PCR. (D) Complementation of the *sdhA* mutant results in loss of the *Ifnb* hyperinduction phenotype. *MyD88*$^{-/-}$*Trif*$^{-/-}$ BMDM were infected at an MOI of 1 with Δ*flaA*, Δ*dot*, Δ*flaA*Δ*sdhA* and Δ*flaA*Δ*sdhA L. pneumophila* carrying vector or a plasmid expressing full length SdhA. Expression of *Ifnb* message was assessed by quantitative RT-PCR 4 hours post infection.

2.3.3 Hyperinduction of type I IFN by the sdhA mutant involves the cytosolic RNA-sensing pathway.

It was possible that Δ*sdhA* mutants hyperinduced type I IFN via a pathway distinct from the normal cytosolic RNA-sensing pathway that responds to wildtype *L. pneumophila*. Therefore, to determine whether hyperinduction of type I interferon by Δ*sdhA* occurs through the same pathway that responds to wild type *L. pneumophila*, we infected *Ips-1*$^{-/-}$ and *Mda5*$^{-/-}$ macrophages with Δ*sdhA L. pneumophila.* Transcriptional activation of *Ifnb* was determined by quantitative RT-PCR. The hyperinduction of *Ifnb* seen in *Ips-1*$^{+/+}$ macrophages was almost abolished in *Ips-1*$^{-/-}$ macrophages ($p<0.001$; Figure 2.4A). As a control, induction of *Ifnb* by poly I:C, a double-stranded synthetic RNA analog, was also *Ips-1*-dependent as expected. Similarly, the hyperinduction of *Ifnb* was also reduced in *Mda5*$^{-/-}$ macrophages ($p<0.01$; Figure 2.4B). However, the *Mda5*$^{-/-}$ macrophages still induced significant amounts of *Ifnb,* suggesting that the requirement for *Mda5* is not complete. We also tested the Δ*sdhA* mutant in *Rig-i* knockdown macrophages. Rig-i knockdown appeared to be effective (Figure 2.4) and specifically diminished *Ifnb* expression (Figure 2.4D). Thus, the residual *Ifnb* induction in *Mda5*$^{-/-}$ may be due to *Rig-i,* or to another uncharacterized pathway. As a control, Theiler's virus (TMEV) induced *Ifnb* in a completely *Mda5*-dependent manner, as expected (Figure 2.4B).

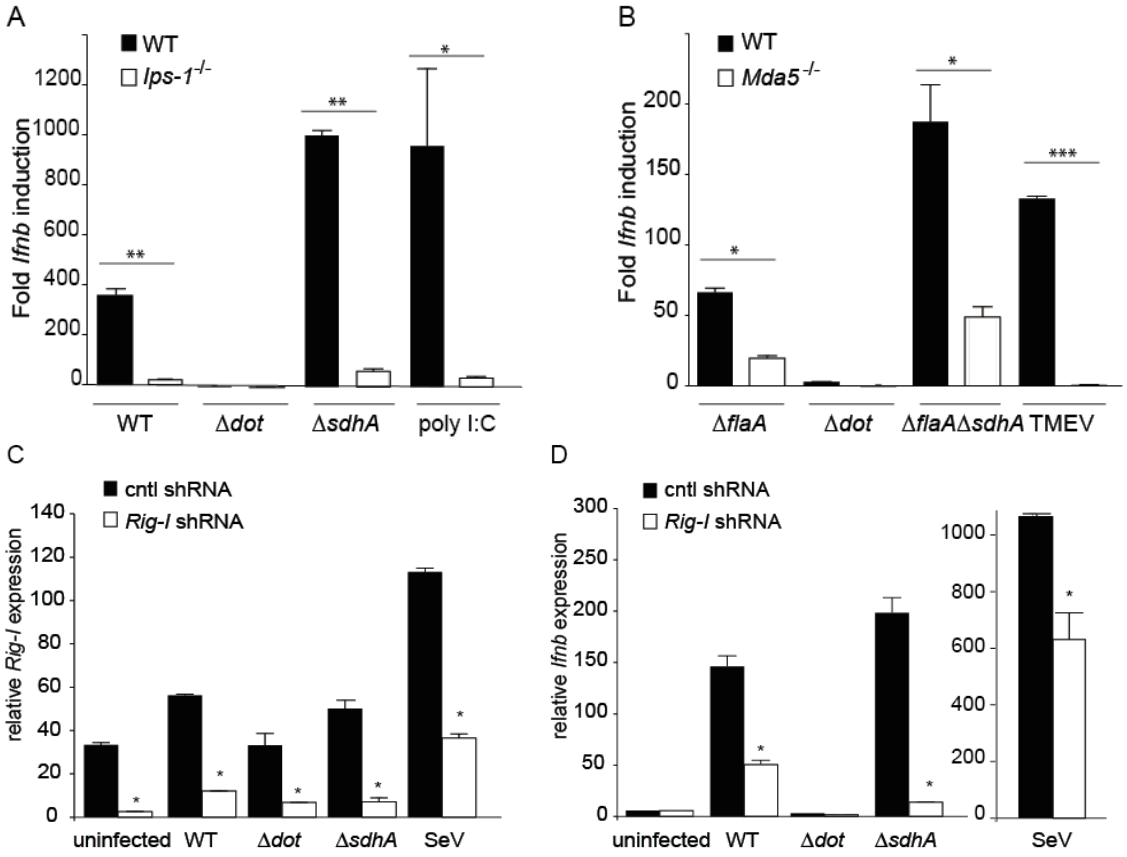

Figure 2.4 Hyperinduction of type I IFN by *sdhA* mutants involves cytosolic RNA sensing pathway components *Ips-1*, *Rig-i*, and *Mda5*.
(A) Hyperinduction of *Ifnb* by Δ*sdhA L. pneumophila* is largely dependent on *Ips-1.* Bone marrow derived *Ips-1*$^{+/+}$ and *Ips-1*$^{-/-}$ macrophages were infected with wild type, Δ*dot*, and Δ*sdhA L. pneumophila* at an MOI of 1. *Ips-1*$^{+/+}$ and *Ips-1*$^{-/-}$ macrophages were transfected with 1.0 µg/ml poly I:C. 4 hours post infection and stimulation, macrophages were harvested and assessed for *Ifnb* induction as in Figure 1. *Ips-1*$^{+/+}$ infected with WT *L. pneumophila* induced statistically significant higher levels of *Ifnb* transcript than *Ips-1*$^{-/-}$ (**, p<0.005, Student's t-test). The same phenotype was seen in *Ips-1*$^{+/+}$ infected with Δ*sdhA L. pneumophila* (**, p<0.005) and transfected with poly I:C (*, p<0.05) when compared to *Ips-1*$^{-/-}$. (B) Hyperinduction of *Ifnb* by Δ*sdhA L. pneumophila* is partially dependent on *Mda5.* Bone marrow derived *Mda5*$^{+/+}$ and *Mda5*$^{-/-}$ macrophages were infected with Δ*flaA,* Δ*dot*, and Δ*flaA*Δ*sdhA L. pneumophila* at an MOI of 1. Theiler's virus (TMEV) was overlaid onto *Mda5*$^{+/+}$ and *Mda5*$^{-/-}$ macrophages. 4 hours post bacterial and viral infection, macrophages were harvested and assessed for *Ifnb* induction by qRT-PCR as in Figure 1. *Ifnb* message was induced statistically significantly in *Mda5*$^{+/+}$ macrophages infected with Δ*flaA L. pneumophila* versus *Mda5*$^{-/-}$ (*, p<0.05, Student's t-test). *Mda5*$^{+/+}$ also responded statistically significantly to Δ*flaA*Δ*sdhA L. pneumophila* over *Mda5*$^{-/-}$ (*, p<0.05, Student's t-test), while Theiler's virus elicited a robust *Ifnb* response from *Mda5*$^{+/+}$ not seen in *Mda5*$^{-/-}$

(***, p<0.005, Student's t-test). (C) Retroviral transduction of a *Rig-i* shRNA, but not the control shRNA, knocks down expression of *Rig-i.* *MyD88*$^{-/-}$*Trif*$^{-/-}$ immortalized macrophages were stably transduced with retroviral vector containing a control and *Rig-i* shRNA. Level of *Rig-i* knockdown was determined by quantitative RT-PCR under uninfected and infected conditions. Differences in *Rig-i* transcript levels were statistically significant (*, p<0.05, Students t-test) under resting and infected conditions. (D) *Rig-i* is involved in the hyperinduction of type I interferon by Δ*sdhA L. pneumophila***.** *Rig-i* knockdown leads to reduced *Ifnb* expression in response to infection with WT and Δ*sdhA L. pneumophila,* as well as Sendai virus*.* Quantitative RT-PCR was carried out 4 hours post infection. Control knockdown macrophages induced a statistically significant (*, p<0.05) higher level of *Ifnb* transcript in response to WT and Δ*sdhA L. pneumophila* and Sendai virus. No significant difference was found in uninfected or Δ*dot L. pneumophila* infected macrophages.

2.3.4 The effects of the sdhA mutant are independent of caspase-1 activation and the type I interferon receptor.

It was previously shown that Δ*sdhA* mutants induce a rapid death of infected macrophages that is dependent upon activation of multiple cell death pathways (Laguna et al., 2006). Consequently, we hypothesized that the hyperinduction of type I IFN by the Δ*sdhA* mutant might be due to the release of molecules from dying cells, such as DNA, that could induce *Ifnb* expression. To rule out this explanation, we infected *Casp1*$^{-/-}$ macrophages, which are resistant to cell death at the early timepoints examined (e.g., 4h post infection), and asked whether type I interferon was still hyperinduced in response to Δ*sdhA L. pneumophila*. In fact, we found that *Casp1*$^{-/-}$ macrophages infected with the Δ*sdhA* mutant hyperinduced *Ifnb* to levels above that observed in B6 macrophages (Figure 2.5A). We suspect that the increased *Ifnb* induction seen in *Casp1*$^{-/-}$ cells was an indirect consequence of the lower levels of cell death in these cells, and was not due to a specific suppression of type I interferon transcription by *Casp1* activation. In any case, our results indicated that the hyperinduction of type I IFN by the Δ*sdhA* mutant was not due to increased cell death induced by the mutant. As a control, we confirmed that *Casp1*$^{-/-}$ macrophages were resistant to cell death at the 4h timepoint tested (Figure 2.5B).

Induction of *Ifnb* is often regulated by a positive feedback loop in which initial production of IFNβ results in signaling through the type I IFN receptor (*Ifnar*) and synergistically stimulates the production of additional type I IFN. We therefore examined whether the hyperinduction of *Ifnb* by the Δ*sdhA* mutant might be due to positive feedback through the type I IFN receptor. To test this possibility we examined induction of *Ifnb* by the Δ*sdhA* mutant in *Ifnar*$^{-/-}$ macrophages. We found that hyperinduction of *Ifnb* by Δ*sdhA L. pneumophila* occurs even in the absence of signaling from the type I interferon receptor, since *Ifnar*$^{-/-}$ macrophages hyperinduce *Ifnb* in response to infection with Δ*sdhA L. pneumophila* (Figure 2.5A).

The mechanism by which the Δ*sdhA* mutant induces cell death remains unclear (Laguna et al., 2006). Studies with the intracellular bacterial pathogen *Francisella tularensis* have demonstrated the existence of a type I IFN-inducible caspase-1-dependent cell death pathway (Henry et al., 2007). Therefore, we sought to establish if

caspase-1-dependent cell death occurred in the absence of Ifnar signaling in response to wild type and Δ*sdhA L. pneumophila*. *Ifnar*$^{-/-}$ macrophages were infected at an MOI of 1 and assayed for release of the intracellular enzyme lactate dehydrogenase (LDH) 4 hours post infection. *Ifnar*$^{-/-}$ macrophages exhibited similar LDH release as B6 macrophages, whether infected with WT or Δ*sdhA L. pneumophila*, and this LDH release was dependent upon caspase-1 activation (Figure 2.5B). These data demonstrate that caspase1-dependent pyroptotic death occurs independently of the type I interferon receptor during infection with wild type and Δ*sdhA L. pneumophila.*

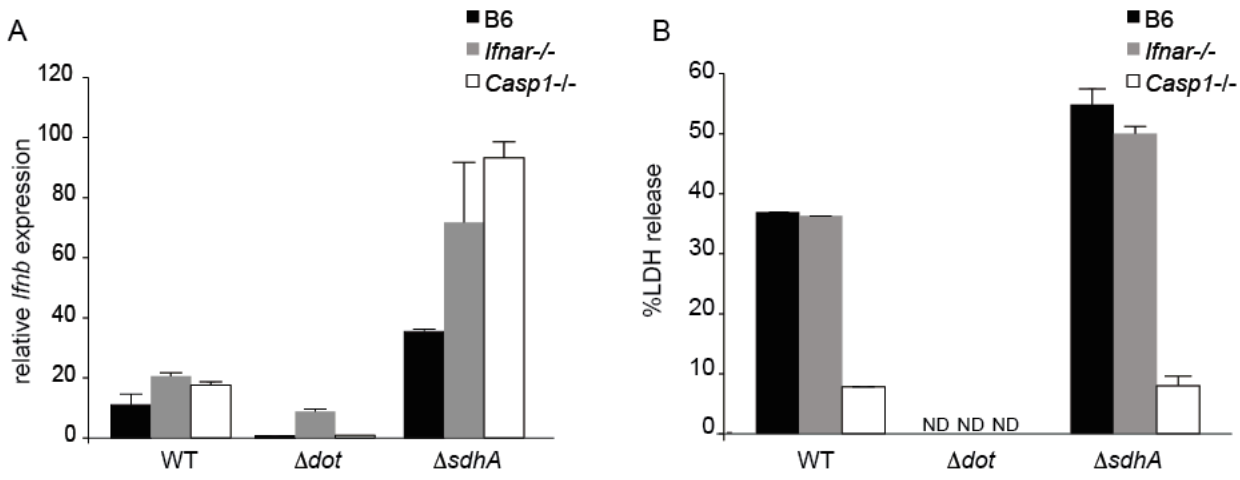

Figure 2.5 The effects of the *sdhA* mutation on type I interferon induction are independent of caspase-1 activation and the type I interferon receptor.
(A) Type I interferon receptor signaling and caspase1-dependent pyroptotic cell death are not required for superinduction of *Ifnb* by the *sdhA* mutant.
Bone marrow derived C57BL/6, *Ifnar*$^{-/-}$ and *Casp1*$^{-/-}$ macrophages were infected with wild type, Δ*dot*, and Δ*sdhA L. pneumophila* at an MOI of 1. *Ifnb* message was analyzed by qPCR from macrophage RNA harvested 4 hours post infection.
(B) Caspase1-dependent pyroptotic cell death occurs independently of the type I interferon receptor. Bone marrow derived C57BL/6, *Ifnar*$^{-/-}$ and *Casp1*$^{-/-}$ macrophages were infected with wild type, Δ*dot*, and Δ*sdhA L. pneumophila* at an MOI of 1 and release of lactate dehydrogenase (LDH) in cell supernatants was measured 4 hours post infection. Specific cell lysis was calculated as a percentage of detergent lysed cells with spontaneous LDH release subtracted. No statistically significant difference was found between B6 and *Ifnar*$^{-/-}$ macrophages infected with Δ*sdhA L. pneumophila* ($p>0.1$, Student's t-test). ND, not detected.

2.3.5 Loss of type I interferon receptor signaling alone does not permit growth of Δ*sdhA mutant.*

Since growth of the Δ*sdhA* mutant is severely attenuated in macrophages (Laguna et al., 2006), we hypothesized that hyperinduction of type I interferon might contribute to the restriction of replication of the Δ*sdhA* mutant. To test this hypothesis, we infected *Ifnar*$^{-/-}$ macrophages with luminescent strains of *L. pneumophila* at an MOI of 0.01 and monitored bacterial replication over a 72 hour time period. As previously reported (Coers et al., 2007), *Ifnar*$^{-/-}$ macrophages were more permissive to WT and Δ*flaA L. pneumophila* as compared to C57BL/6 macrophages (Figure 2.6A, C). However, the Δ*sdhA* or Δ*flaA*Δ*sdhA L. pneumophila* strains were still significantly restricted in *Ifnar*$^{-/-}$ macrophages (Figure 2.6B, D). Thus, SdhA is required for bacterial replication in macrophages primarily via a mechanism independent of its role in suppressing type I IFN. As expected, Δ*dot L. pneumophila* did not replicate in WT or *Ifnar*$^{-/-}$ macrophages (Figure 2.6E).

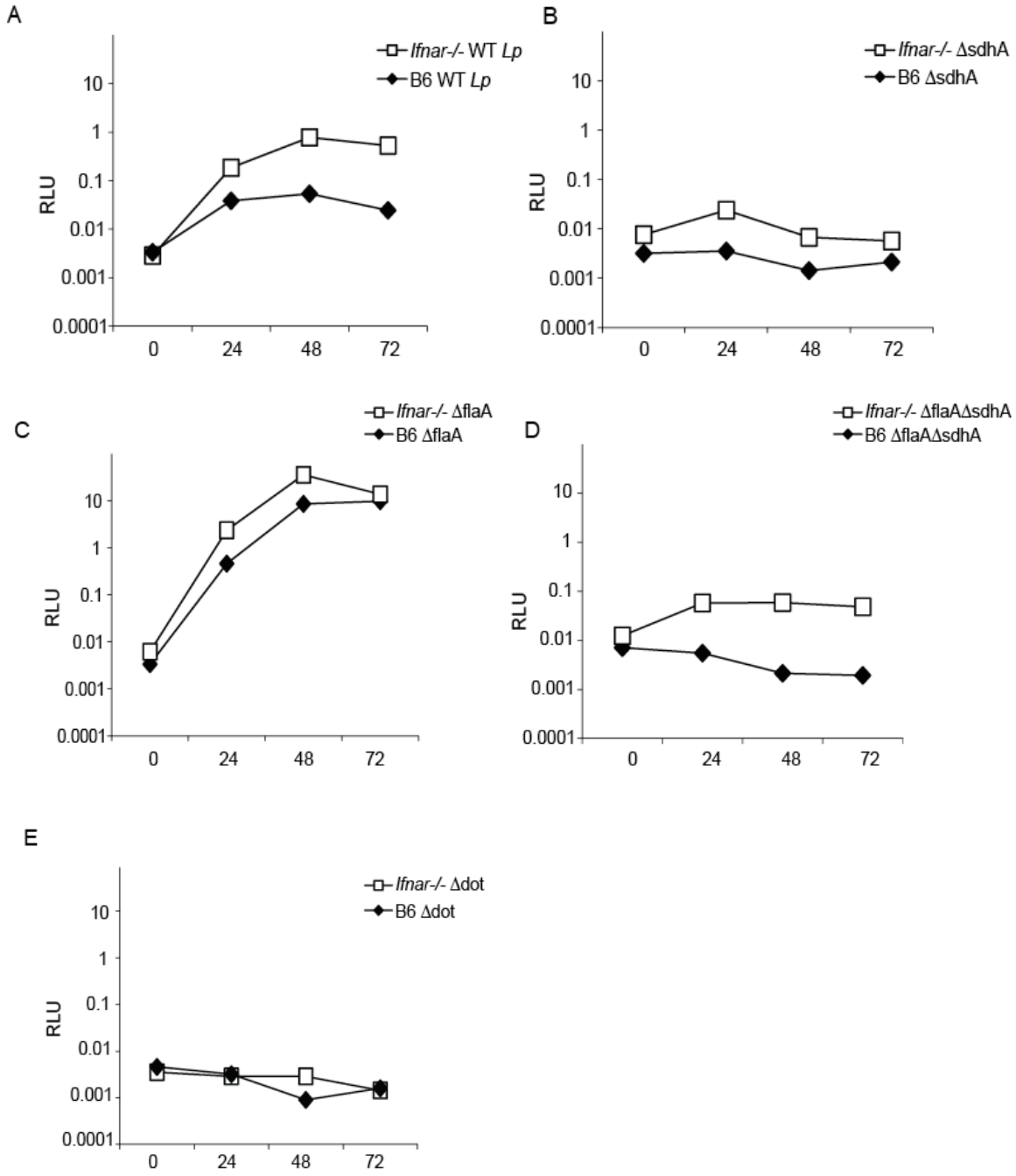

Figure 2.6 Abrogation of type I interferon receptor signaling alone does not permit growth of Δ*sdhA* mutant. C57BL/6 (B6) and *Ifnar*$^{-/-}$ macrophages were infected at an MOI of 0.01 and growth of luminescent *L. pneumophila* strains was determined by RLU at 0, 24, 48, and 72 hours post infection. (A) C57BL/6 (B6) and *Ifnar*$^{-/-}$ macrophages were infected WT (LP02) *L. pneumophila* (B) macrophages were infected as in A but with Δ*sdhA L. pneumophila* (C) Δ*flaA L. pneumophila* (D) Δ*flaA*Δ*sdhA L. pneumophila* (E) Δ*dot L. pneumophila.*

2.3.6 Δ*sdhA L. pneumophila* hyperinduces IFNβ *in vivo.*

To test whether the Δ*sdhA* mutant hyperinduces type I IFN *in vivo* as we observed *in vitro*, we intranasally infected C57B/6 mice with either Δ*flaA* or Δ*flaA*Δ*sdhA L. pneumophila* and harvested broncheoaveolar lavage fluid 16 hours post infection and assessed IFNß levels, as well as CFU (Figure 2.7). Despite being better restricted (*, p<0.5), Δ*flaA*Δ*sdhA L. pneumophila* hyperinduces IFN *in vivo* (**, p<0.01).

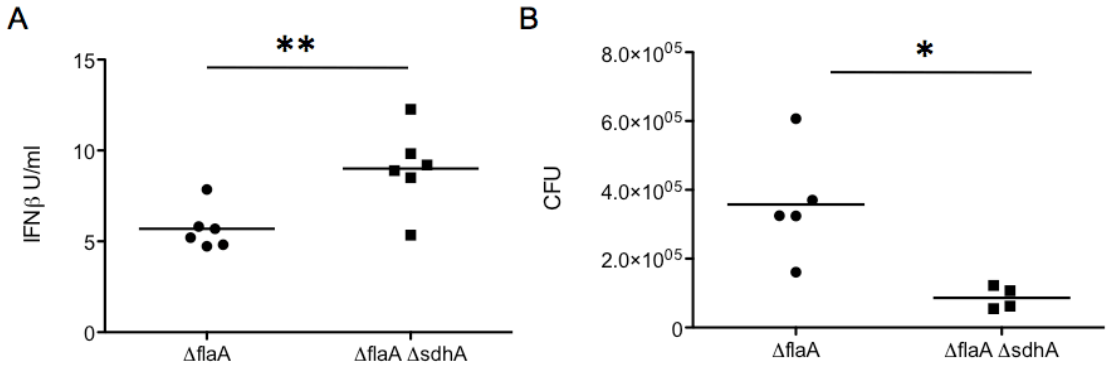

Figure 2.7 Δ*sdhA L. pneumophila* hyperinduces IFNß *in vivo.* (A) Age and sex-matched C57B/6 mice were infected intranasally with 2.5 x 10^6 Δ*flaA or* Δ*flaA* Δ*sdhA L. pneumophila* expressing thymidine on a plasmid to complement thy auxotrophy. Bronchoalveolar lavage with PBS was performed 16 hours post infection. Type I interferon levels in the bronchoalveolar lavage fluid (BALF) were analyzed by bioassay and recombinant IFNβ was used to determine a standard curve. A two-tailed t-test determined the differences in IFNβ levels were statistically significant (**, p<0.01, Student's t-test) upon comparison of Δ*flaA or* Δ*flaA*Δ*sdhA L. pneumophila* infected mice. (B) *L. pneumophila* colony forming units are significantly different in Δ*flaA or* Δ*flaA*Δ*sdhA L. pneumophila* infected mice. Bronchoalveolar lavage fluid from infected mice was centrifuged to isolate cells. Hypotonic lysis of cells was performed and CFU were plated on buffered yeast extract charcoal plates with antibiotic selection for *L. pneumophila.* A two-tailed t-test determined that CFU in mice 16 hours post infection were statistically significantly different (*, p<0.5, Student's t-test).

2.3.7 Overexpression of SdhA suppresses Mda5 and RIG-I induced IFN.

Since SdhA is a secreted effector, we hypothesized that SdhA may act in the host cell cytosol, rather than in the bacterium, to repress *Ifnb* induction. To test this hypothesis, I co-expressed SdhA with MDA5 or RIG-I, by transient transfection of HEK293T cells, and assessed interferon expression with an IFNβ-luciferase reporter. Expression of either MDA5 or RIG-I robustly induced the IFNβ-luc reporter upon stimulation with poly I:C (Figure 2.8). When SdhA was co-expressed with MDA5, a dose-dependent repression of the IFNβ-luc reporter was observed (Figure 2.8A). Co-expression of SdhA also resulted in a dose-dependent repression of RIG-I-dependent

induction of the IFNβ-luc reporter (Figure 2.8B). However, SdhA co-expression did not affect TRIF-dependent induction of the IFNβ-luc reporter (Figure 2.8C), arguing against the possibility that SdhA expression has non-specific effects on IFNβ-luc induction. These results must be interpreted with caution since the 293T IFNβ-luc reporter system is highly artificial; moreover, I have not demonstrated a direct interaction of SdhA with signaling components in the RNA-sensing pathway. In fact, the reported effects of SdhA on mitochondria (Laguna et al., 2006) suggest the effect may be somewhat indirect (see Discussion). Nevertheless, the 293T transfection results suggest that SdhA can act in the host cytosol to specifically repress induction of the RIG-I/MDA5 pathway.

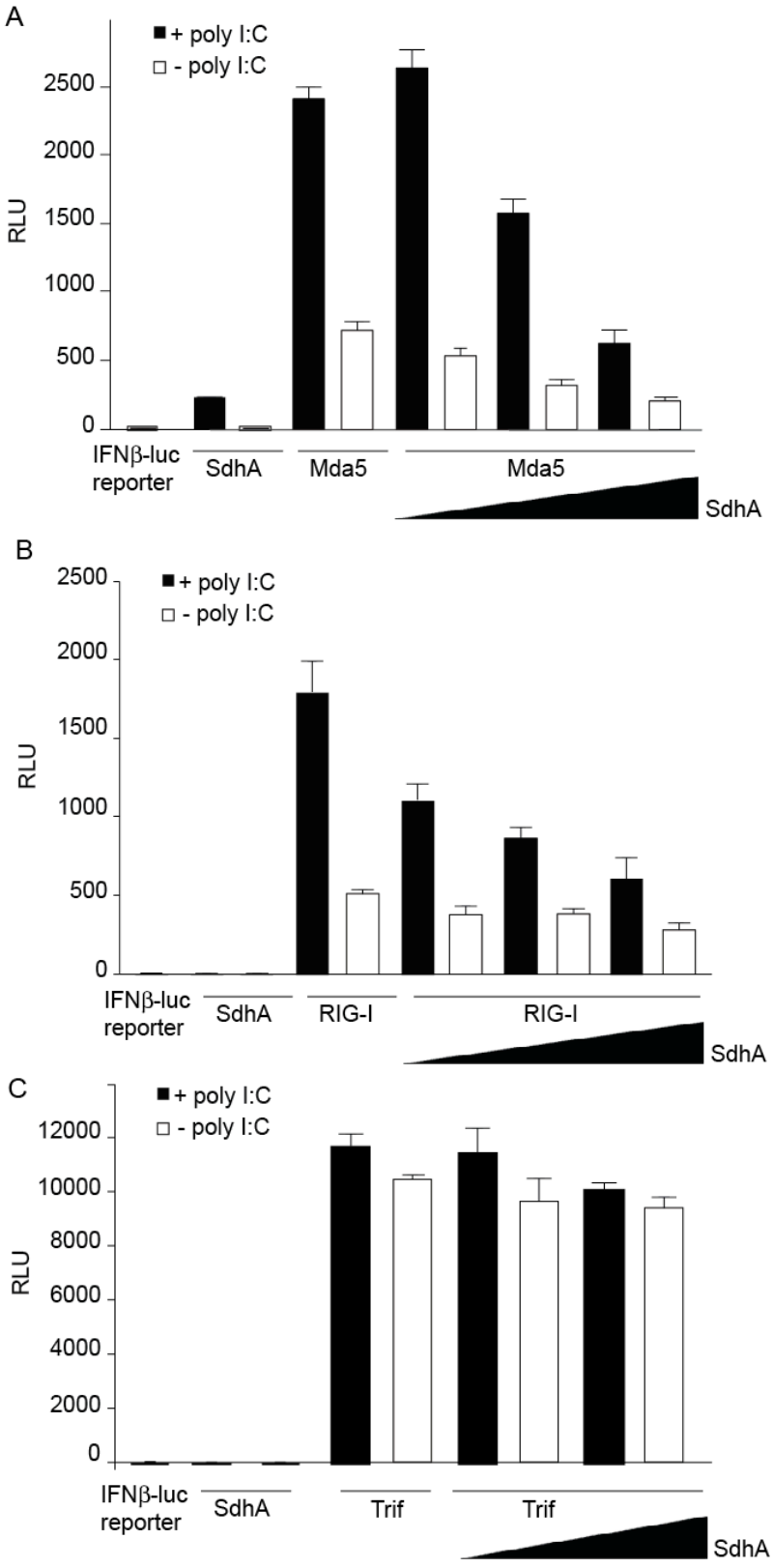

Figure 2.8 SdhA represses MDA5 and RIG-I induction of interferon. Overexpression of SdhA in HEK293T cells results in repression of interferon induction mediated by MDA5 or RIG-I but not TRIF. (A) HEK293T cells were transfected with plasmids encoding the IFNβ-firefly luciferase reporter, TK-*Renilla* luciferase reporter (for normalization), full length MDA5 and/or increasing amounts of full length SdhA. At 20 hours post transfection, cells were transfected with poly I:C and then firefly luciferase and *Renilla* luciferase levels were determined 8 hours later. (B) Transfection and stimulation were performed as in A, except with a RIG-I expression plasmid and/or increasing amounts of full length SdhA expression plasmid. (C) Transfection and stimulation were performed as in A, except with a Trif expression plasmid and/or SdhA.

2.3.8 L. pneumophila genomic DNA does not appear to stimulate an Ips-1-dependent IFN response.

Based on the observation that the host type I IFN response requires the *L. pneumophila* Dot/Icm type IV secretion system and was at least partly *Ips-1*, *Rig-i*, and *Mda5*-dependent, we hypothesized that *L. pneumophila* nucleic acids (RNA, DNA or both) might gain access to the macrophage cytosol *via* the type IV secretion system and induce a host type I interferon response. To test if *L. pneumophila* nucleic acids are sufficient to induce type I interferon, I transfected *MyD88*$^{-/-}$ *Trif*$^{-/-}$ macrophages with purified *L. pneumophila* genomic DNA or total RNA and determined the induction of type I interferons by bioassay. Poly(dA-dT):poly(dA-dT) (abbreviated as pA:T) was used as a non-CpG containing DNA control and poly I:C was used as an RNA control. Nucleic acid preparations were treated with DNase and/or RNase to eliminate contaminating nucleic acids. Both purified *L. pneumophila* DNA and the crude RNA preparation induced IFNβ (Figure 5A). *L. pneumophila* RNA treated with RNase also induced IFNβ, presumably due to (contaminating) DNA in the preparation (Figure 2.9A). However, *L. pneumophila* RNA treated with DNase induced type I interferon to a level above that induced by *L. pneumophila* RNA treated with both RNase and DNase, suggesting that *L. pneumophila* RNA alone can induce type I interferon production (Figure 2.9A). The induction of type I IFN by *L. pneumophila* RNA was modest, possibly because bacterial RNA is less stable than DNA. Nevertheless, these results suggest that both *L. pneumophila* RNA and DNA can induce a type I interferon host response.

Next, I determined if *L. pneumophila* nucleic acids could induce type I interferon in an *Ips-1*-dependent manner in macrophages. In certain cell types, though not mouse macrophages, AT-rich DNA has been shown to induce type I IFN via IPS-1 (Ablasser et al., 2009; Cheng et al., 2007; Chiu et al., 2009; Ishii et al., 2006). It was important to assess whether *L. pneumophila* DNA, in particular, might signal in an *Ips-1*-dependent manner since the *L. pneumophila* type IV secretion system has previously been shown to translocate DNA (Vogel et al., 1998). *Ips-1*$^{+/-}$ and *Ips-1*$^{-/-}$ macrophages were transfected with pA:T and *L. pneumophila* DNA, as well as infected with Sendai virus, a virus previously determined to induce an *Ips-1*-dependent IFN response. Stimulation with pA:T or *L. pneumophila* DNA failed to induce *Ifnb* in an *Ips-1*-dependent manner, whereas Sendai virus induced significantly more *Ifnb* in *Ips-1*$^{+/-}$ versus *Ips-1*$^{-/-}$ macrophages (Figure 2.9B). Similar results were obtained in *Mda5*$^{-/-}$ macrophages: induction of type I IFN with pA:T or *L. pneumophila* genomic DNA showed no requirement for *Mda5,* whereas a control simulation, Theiler's Virus, showed *Mda5*-dependent induction of IFNβ, as expected (Figure 2.9C). It was possible that at high concentrations of DNA, an *Ips-1*-independent DNA-sensing pathway overwhelmed any putative *Ips-1*-dependent recognition of DNA. However, induction of *Ifnb* was independent of *Ips-1* even when titrated amounts of pA:T or *L. pneumophila* genomic DNA were transfected into macrophages (Figure 2.9D, 2.9E). Thus, these results suggest that while transfected *L. pneumophila* DNA robustly induces type I interferon, *L. pneumophila* genomic DNA does not appear to induce the *Ips-1*-dependent IFN response that is characteristic of *L. pneumophila* infection.

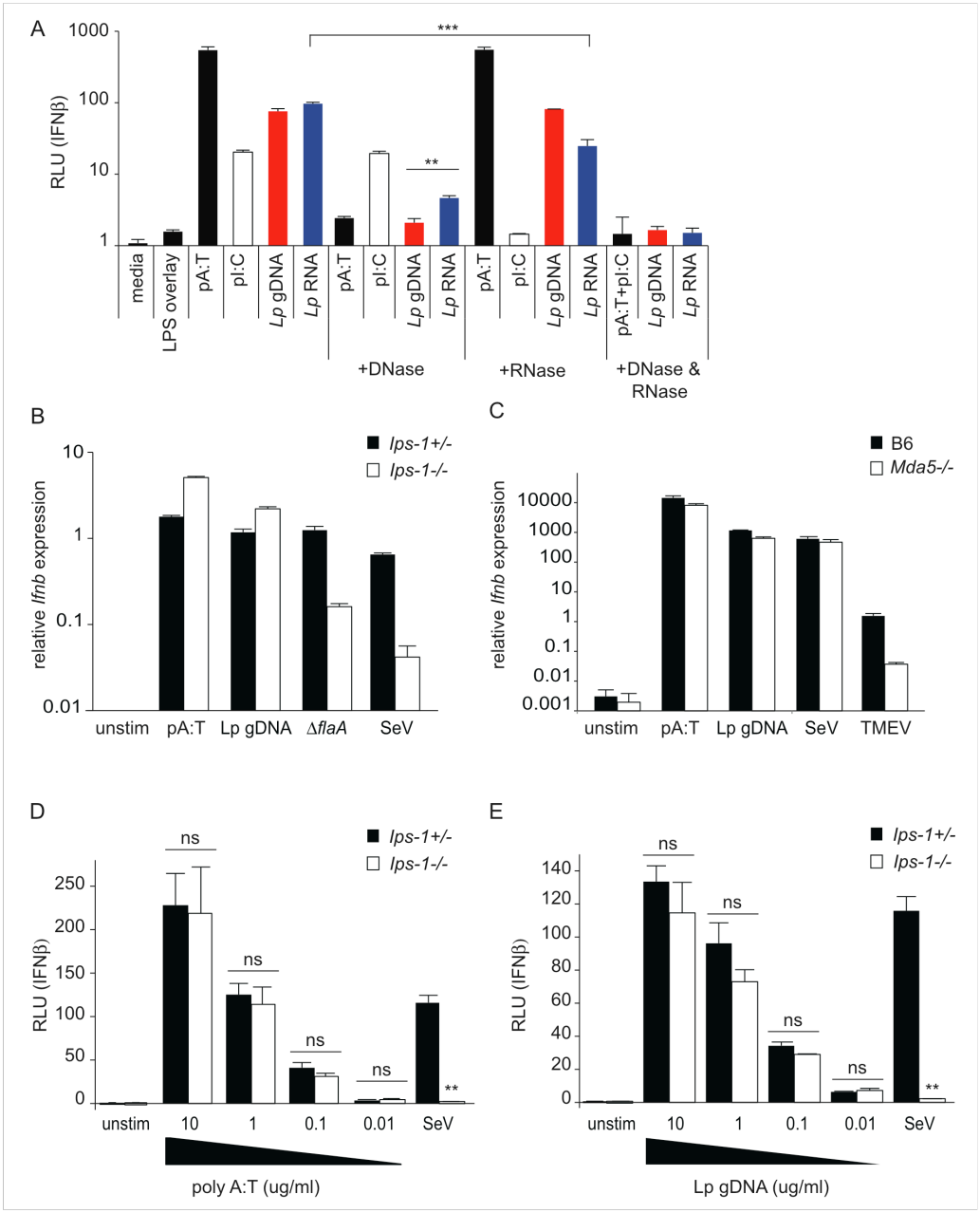

Figure 2.9 *L. pneumophila* DNA and RNA stimulate type I IFN production in macrophages. (A) Purified genomic DNA and RNA from *L. pneumophila* induces type I interferon independently of *MyD88* and *Trif*. Bone marrow derived *Myd88*$^{-/-}$ *Trif*$^{-/-}$ macrophages were stimulated by transfection of 3.3 µg/ml purified *L. pneumophila* DNA, *L. pneumophila* RNA, pA:T (DNA), and pI:C (RNA). Nucleic acids were treated with DNase and/or RNase A before transfection. Macrophage supernatants were harvested 8 hours post stimulation and analyzed for IFNβ levels by L929-ISRE luciferase bioassay. IFNβ production by DNase-treated *L. pneumophila* RNA was statistically

significantly higher compared to DNase-treated *L. pneumophila* DNA (**, p<0.005). In addition, RNase A-treated *L. pneumophila* RNA produced statistically significant lower levels of IFNβ (***, p<0.0001, Student's t-test) than *L. pneumophila* RNA and RNase-treated *L. pneumophila* DNA. (B) Genomic *L. pneumophila* DNA does not induce type I interferon in a *Ips-1*-dependent manner. *Ips-1*$^{-/-}$ and heterozygous littermate bone marrow derived macrophages were stimulated by transfection of 1.0 µg/ml pA:T and purified genomic *L. pneumophila* DNA. Macrophages were infected with Δ*flaA L. pneumophila* at an MOI of 1. Sendai virus (SeV) was overlaid onto *Ips-1*$^{-/-}$ and heterozygous littermate macrophages. Transcriptional activation of *Ifnb* was determined by quantitative RT-PCR as described in Figure 1. (C) The viral RNA sensor *Mda5* is not required for induction of type I interferon by *L. pneumophila* DNA. WT (C57BL/6) and *Mda5*$^{-/-}$ bone marrow derived macrophages were stimulated by transfection of 1.0 µg/ml pA:T and purified genomic *L. pneumophila* DNA. Sendai virus (SeV) and Theiler's virus (TMEV) were overlaid onto WT and *Mda5*$^{-/-}$ macrophages. Quantitative RT-PCR was used to determine *Ifnb* gene expression. (D) Non-CpG containing DNA (pA:T) does not induce *Ips-1*-dependent *Ifnb* at all concentrations tested. *Ips-1*$^{-/-}$ and heterozygous littermate bone marrow derived macrophages were stimulated with a titration of pA:T by transfection of 10, 1.0, 0.1, 0.01 µg/ml pA:T. The difference between *Ips-1*$^{+/-}$ and *Ips-1*$^{-/-}$ macrophages transfected with pA:T was not statistically significant (ns, p>0.1, Student's t-test). Sendai virus (SeV) was overlaid onto *Ips-1*$^{-/-}$ and heterozygous littermate macrophages (**, p<0.005). Cell supernatants were collected 8 hours post stimulation/infection. Induction of type I interferon was determined by L929-ISRE luc bioassay. Units are relative light units (RLU). (E) Genomic *L. pneumophila* DNA induces type I interferon independently of *Ips-1* at all concentrations tested. *Ips-1*$^{-/-}$ and heterozygous littermate bone marrow derived macrophages were stimulated with a titration of purified genomic *L. pneumophila* DNA by transfection of 10, 1.0, 0.1, 0.01 µg/ml *L. pneumophila* DNA. No statistically significant difference was found between *Ips-1*$^{+/-}$ and *Ips-1*$^{-/-}$ macrophages transfected with genomic *L. pneumophila* DNA (ns, p>0.1, Student's t-test). Sendai virus (SeV) was overlaid onto *Ips-1*$^{-/-}$ and heterozygous littermate controls (**, p<0.005). Macrophage supernatants were collected 8 hours post stimulation/infection. Type I interferon levels were determined by L929-ISRE luc bioassay, units are relative light units (RLU).

2.3.9 L. pneumophila RNA stimulates type I interferon via Rig-i.

To determine whether *L. pneumophila* RNA could be recognized by Rig-i, we transfected *L. pneumophila* RNA into macrophages in which *Rig-i* expression had been stably knocked down. Importantly, the *Rig-i* knockdown was performed in immortalized bone-marrow-derived macrophages that lack MyD88 and Trif, in order to avoid potential activation of known RNA-sensing TLRs. Knockdown of *Rig-i* was effective under our transfection conditions, as *Rig-i* message was significantly lower in macrophages transduced with a *Rig-i* shRNA compared to a control shRNA (p<0.05; Figure 2.10A). Crude *L. pneumophila* RNA (which also contains genomic DNA contaminants) induced *Ifnb* robustly in both control shRNA and *Rig-i* shRNA macrophages, even upon treatment with RNase A (Figure 2.10B). However, transfection of DNase-treated *L.*

*pneumophil*a nucleic acids induced significantly less *Ifnb* in *Rig-i* knockdown macrophages as compared to control knockdown macrophages ($p<0.05$; Figure 2.10B). This result suggests that *L. pneumophila* RNA can induce *Rig-i*-dependent type I interferon. It was not possible to perform a similar experiment in the *Ips-1*$^{-/-}$ macrophages because these macrophages were MyD88/Trif^{+} and exhibited background interferon, presumably due to TLR3-signaling.

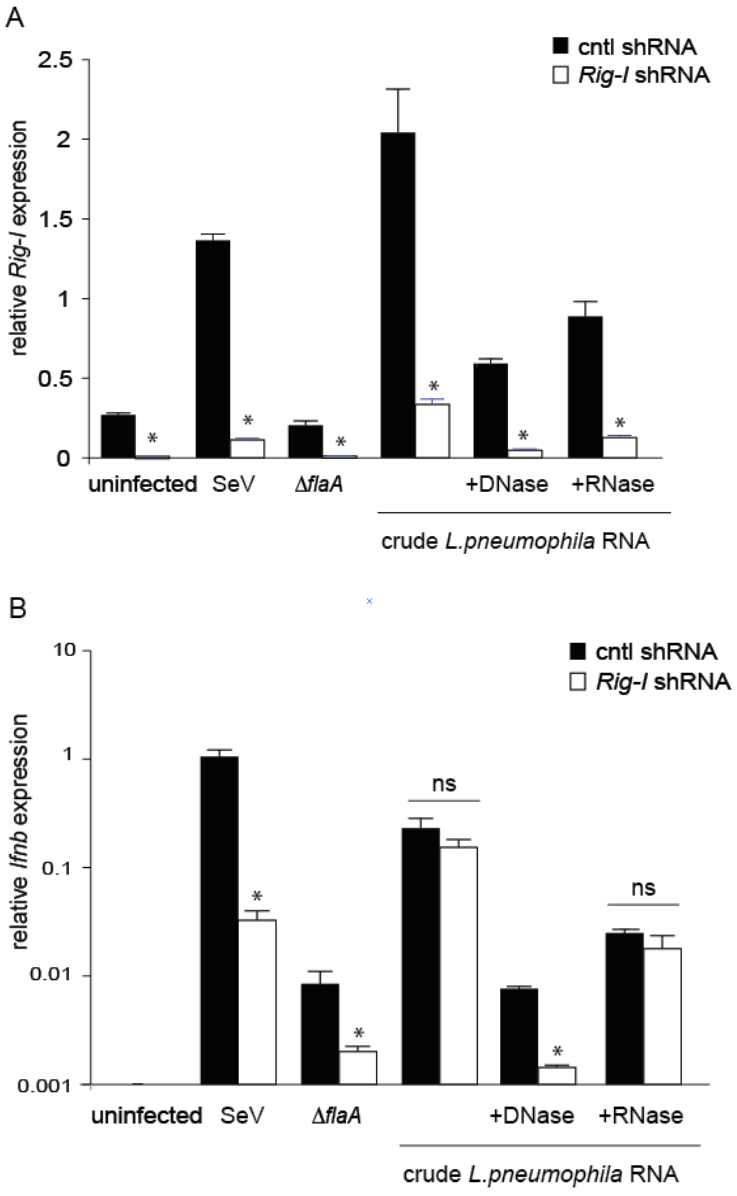

Figure 2.10 *L. pneumophila* RNA induces type I interferon *via Rig-i*. (A). The efficiency of *Rig-i* knockdown was determined by quantitative RT-PCR under uninfected, viral and bacterial infected, and transfected conditions. Differences in *Rig-i* transcript levels were statistically significant (*, $p<0.05$, Students t-test) under resting, infected, and transfected conditions. (B) *Rig-i* is involved in the host type I interferon response to *L. pneumophila* RNA. *Rig-i* knockdown leads to reduced *Ifnb* expression upon transfection with DNase-treated *L. pneumophila* RNA. Quantitative RT-PCR was carried out 4 hours post stimulation. Control knockdown macrophages induced a statistically significant (*, $p<0.05$) higher level of *Ifnb* transcript in Δ*flaA L. pneumophila* and Sendai virus infected macrophages. No significant difference was found in response to untreated and RNase-treated *L. pneumophila* nucleic acids.

2.3.10 RNA Polymerase III does not appear to be required for the IFN response to L. pneumophila.

A recent report found that an inhibitor of RNA polymerase III, ML-60218 (Wu et al., 2003), blocked the type I IFN response to *L. pneumophila* (Chiu et al., 2009). It was proposed that *L. pneumophila* DNA is translocated into macrophages and transcribed by Pol III into a ligand that could be recognized by RIG-I (Chiu et al., 2009). In contrast, I did not see an effect of ML-60218 on induction of type I IFN by *L. pneumophila* in bone marrow-derived macrophages (Figure 2.11A). The lack of an effect does not appear to be due to redundant recognition by another DNA sensor in macrophages because the interferon induction was still largely *Ips-1*-dependent (Figure 2.11A). Nevertheless, we also tested 293T cells, which express only the Pol III pathway for cytosolic recognition of DNA (Ablasser et al., 2009; Chiu et al., 2009). As expected, 293T cells responded to pA:T in an ML-60218-inhibitable manner, but did not respond well to *L. pneumophila* genomic DNA (Figure 2.11B), again suggesting that *L. pneumophila* genomic DNA is not an efficient substrate for the Pol III pathway. The Pol III inhibitor also appeared to have little effect on *L. pneumophila* replication in bone-marrow macrophages (Figure 2.11C-E).

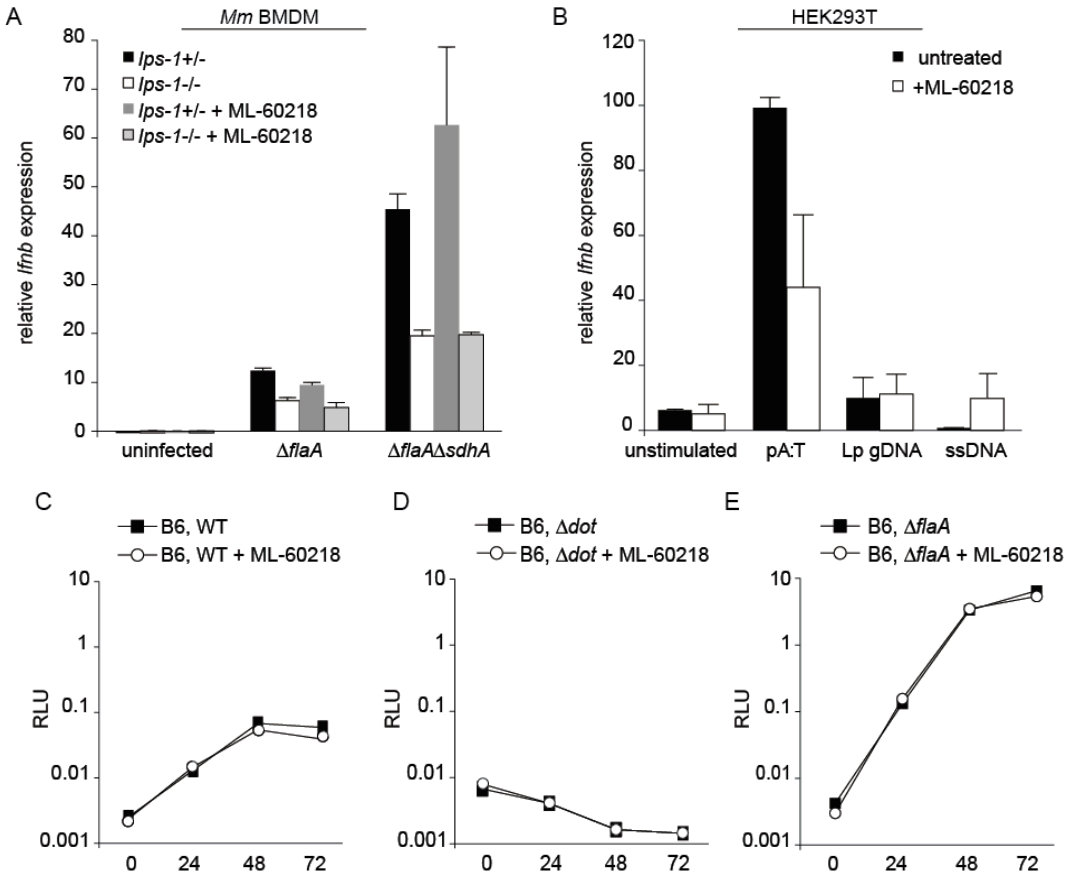

Figure 2.11 The Pol III pathway does not appear to recognize *L. pneumophila* DNA or affect *L. pneumophila* replication. (A) Inhibition of Pol III had no effect on *lps-1*-dependent *Ifnb* induction by *L. pneumophila*. *lps-1*$^{+/-}$ and *lps-1*$^{-/-}$ macrophages were pretreated (controls were untreated) with 20μM ML-60218 10 hours before infection with Δ*flaA* and Δ*flaA*Δ*sdhA L. pneumophila* at an MOI of 1. *Ifnb* induction was analyzed by quantitative RT-PCR 4 hours post infection. *Ifnb* message was normalized to ribosomal protein *rps17* levels. (B) *L. pneumophila* genomic DNA does not induce *IFNB* in HEK293T cells. HEK293T cells were pretreated, or left untreated, with 20μM ML-60218 10 hours before transfection with 1.0μg/ml pA:T, *L. pneumophila* genomic DNA, or salmon sperm DNA. *Ifnb* induction was analyzed by quantitative RT-PCR 4 hours post infection. *Ifnb* message was normalized to S9 levels. (C) WT (C57BL/6) macrophages were infected at an MOI of 0.01 in the presence or absence of 20μM ML-60218 and growth of luminescent *L. pneumophila* strains was determined by RLU at 0, 24, 48, and 72 hours post infection. For inhibitor conditions, macrophages were pretreated with 20μM ML-60218 10 hours before infection. Macrophages were infected with WT (LP02) *L. pneumophila* or with isogenic Δ*dot L. pneumophila* (D) Δ*flaA L. pneumophila* (E).

2.3.11 In vivo role of *Ips-1* in the host type I interferon response to *L. pneumophila*.

In order to validate our findings *in vivo*, we infected *Ips-1*$^{-/-}$ and littermate *Ips-1*$^{+/-}$ mice with *L. pneumophila* (2.5 x 10^6 LP01 Δ*flaA* per mouse, infected intranasally) and assayed type I interferon production in bronchoalveolar lavage fluid 20 hours post infection by bioassay. *Ips-1*$^{+/-}$ mice induced an IFN response that was statistically significantly greater than the response of *Ips-1*$^{-/-}$ mice (Student's t-test, p=0.01; Figure 2.12A). The difference in IFN production was not explained by a difference in bacterial burden in the *Ips-1*$^{+/-}$ and *Ips-1*$^{-/-}$ mice, since both genotypes exhibited similar levels of bacterial colonization (p=0.76, Student's t-test; Figure 2.12B). The lack of an effect of *Ips-1*-deficiency on bacterial replication *in vivo* was not surprising given that I also failed to observe an effect of *Ifnar*-deficiency on bacterial replication *in vivo* (data not shown). We suspect that type II IFN (IFNγ), which is not made by macrophages *in vitro,* or another *in vivo* pathway, may compensate for loss of type I IFN *in vivo*.

Nevertheless, our results provided an important validation of our *in vitro* studies and affirm a role for *Ips-1* in the *in vivo* type I interferon response to *L. pneumophila*. Since *Ips-1*-deficient mice still mounted a measurable IFN response *in vivo*, it appears that additional *Ips-1*-independent pathways (e.g., TLR-dependent pathways, possibly involving other cell types (Mancuso et al., 2009)) also play a role *in vivo*.

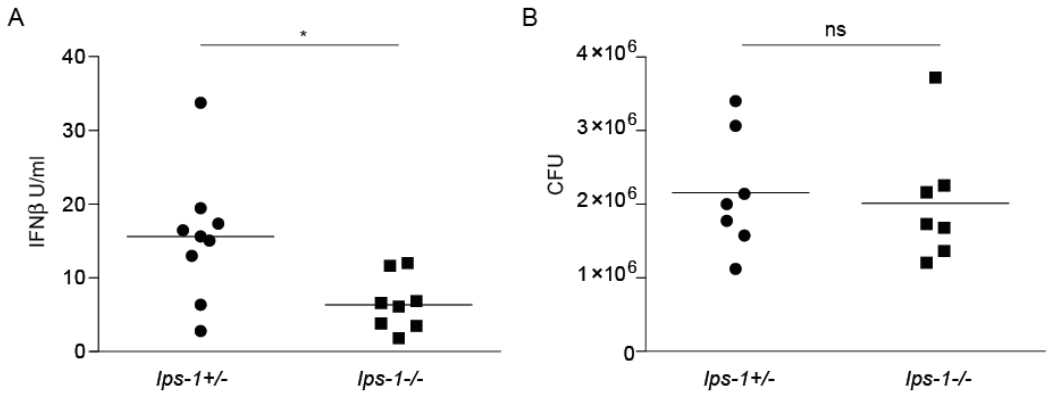

Figure 2.12 Role of *lps-1* in the *in vivo* response to *L. pneumophila*. (A) The type I interferon response to *L. pneumophila* involves *lps-1 in vivo.* *lps-1*$^{-/-}$ and heterozygous littermate mice were infected intranasally with 2.5 x 10^6 LP01Δ*flaA.* Bronchoalveolar lavage with PBS was performed 20 hours post infection. Type I interferon levels in the bronchoalveolar lavage fluid (BALF) were analyzed by bioassay and recombinant IFNβ was used to determine a standard curve. A two-tailed t-test determined the differences in IFNβ levels were statistically significant (*, $p<0.01$, Student's t-test) upon comparison of *lps-1*$^{+/-}$ and *lps-1*$^{-/-}$ mice. (B) *L. pneumophila* colony forming units are not significantly different in *lps-1*$^{+/-}$ and *lps-1*$^{-/-}$. Bronchoalveolar lavage fluid from infected *lps-1*$^{+/-}$ and *lps-1*$^{-/-}$ mice was centrifuged to isolate cells. Hypotonic lysis of cells was performed and CFU were plated on buffered yeast extract charcoal plates with antibiotic selection for *L. pneumophila.* A two-tailed t-test determined that CFU in *lps-1*$^{+/-}$ and *lps-1*$^{-/-}$ mice 20 hours post infection were not statistically significantly different (ns, $p>0.5$, Student's t-test).

2.4 Discussion

Type I interferons (IFNs) have long been appreciated as critical players in antiviral immune defense, and recent work has identified several molecular immunosurveillance pathways that induce type I IFN expression in response to viruses (Pichlmair and Reis e Sousa, 2007; Stetson and Medzhitov, 2006b). In contrast, the roles of type I IFNs in response to bacteria, and the pathways by which bacteria induce type I IFNs, are considerably less well understood. In the work described in this Chapter, I sought to characterize the type I IFN response to the gram-negative bacterial pathogen *Legionella pneumophila*. My work focused on the type I IFN response mounted by macrophages, since this is the cell type that is believed to be the primary replicative niche in the pathogenesis of Legionnaires' Disease.

In agreement with previous work (Stetson and Medzhitov, 2006a), I found that *L. pneumophila* induces type I IFNs in macrophages *via* a TLR-independent pathway that requires expression of the bacterial type IV secretion system. These results suggested that a cytosolic immunosurveillance pathway controls the IFN response in macrophages. In this report I identify the cytosolic RNA-sensing pathway as a key responder to *L. pneumophila* infection (Figure 2.1) and, in agreement with previous results using human A549 cells (Lippmann et al., 2008), I did not observe a role for *Dai* (*Zbp1*), a gene implicated in the response to cytosolic DNA (Takaoka et al., 2007; Wang et al., 2008). A previous study using RNA interference in the human A549 epithelial-like cell line also found a role for IPS-1 in the type I IFN response to *L. pneumophila* (Opitz et al., 2006). However, knockdown of RIG-I or MDA5 did not appear to affect the IFN response, so the role of the IPS-1 pathway was unclear. In my study, I used mice harboring targeted gene deletions to establish a role for *Mda5* and *Ips-1* in the type I IFN response to *L. pneumophila* in macrophages, and uncovered a role for *Rig-i* using an shRNA knockdown strategy. I also found that the cytosolic RNA-surveillance pathway regulated the IFN response *in vivo* in a mouse model of Legionnaires' Disease.

After our manuscript was submitted, a report published by Chiu and colleagues also concluded that *Ips-1* is required for the macrophage type I IFN response to *L. pneumophila* (Chiu et al., 2009). However, the report of Chiu et al differs considerably from our current work by proposing that the type I IFN response to *L. pneumophila* occurs via a novel and unexpected pathway in which *L. pneumophila* DNA is transcribed by RNA polymerase III to generate an RNA intermediate that is sensed by RIG-I. Others have found that the Pol III pathway can be activated by viral and AT-rich DNA in certain cell types (Ablasser et al., 2009). Our data, however, are not easily reconciled with a role for the Pol III pathway in recognition of *L. pneumophila*. First, and perhaps most important, is the observation that the response to DNA (in contrast to the response to *L. pneumophila* infection) has never been seen to be *Ips-1*-dependent in macrophages [(McWhirter et al., 2009); Figure 2.9]. This suggests that the response to *L. pneumophila* is not simply a response to DNA, regardless of the mechanisms by which potentially translocated DNA might be recognized.

We considered the possibility that *L. pneumophila* DNA exhibits unique properties that cause it to be a particularly efficient substrate for the Pol III pathway. Indeed, the *L. pneumophila* genome does contain stretches of highly AT-rich DNA, and it has been reported that only highly AT-rich DNA is an efficient substrate for the Pol III

pathway (Ablasser et al., 2009; Chiu et al., 2009). Therefore I tested whether *L. pneumophila* genomic DNA, unlike other DNA, could induce an *Ips-1*-dependent response in macrophages. Although *L. pneumophila* DNA induced a robust IFN response, the response was not *Ips-1*-dependent (Figure 2.9C, E). Indeed, even the optimal Pol III substrate poly(dA–dT):poly(dA–dT) (abbreviated as pA:T) does not appear to induce an *Ips-1*-dependent IFN response in macrophages [(Figure 2.9B, D) and (McWhirter et al., 2009)]. The lack of *Ips-1*-dependence in the response to pA:T appears to be due to *Sting*-dependent, *Ips-1*-independent DNA-sensing pathway which dominates over the Pol III pathway in bone marrow macrophages (Ablasser et al., 2009; Ishikawa et al., 2009). Thus, if translocated DNA is the relevant bacterial ligand that stimulates the *Ips-1*-dependent host type I IFN response, an explanation is required for how the dominant *Sting*-dependent DNA-sensing pathway is not activated. While *L. pneumophila* could selectively inhibit or evade Sting mediated DNA-sensing, there is at present no evidence to support this mechanism. Moreover, in our hands, the Pol III inhibitor used by Chiu et al (ML-60218) failed to affect IFN induction or bacterial replication in macrophages (Figure 2.11), as would be predicted if the Pol III pathway was selectively activated in response to *L. pneumophila* infection. Therefore, my data lead us to consider alternative models.

Although Chiu et al primarily used the RAW macrophage-like cell line in their experiments with *L. pneumophila*, we do not believe that cell-type-specific effects can account for the discrepancy in results. Although it is possible that RAW cells express only the Pol III pathway, this would not change the fact that the proposed model of Chiu et al invokes DNA as the primary IFN-inducing ligand produced by *L. pneumophila*. The simplest prediction of such a model would be that the response of bone marrow macrophages to *L. pneumophila* would be *Ips-1-independent*, as is the response of macrophages to all forms of DNA that have been tested. In contrast, as documented here (Figure 2.1) and by Chiu et al. (Chiu et al., 2009), the response to *L. pneumophila* is *Ips-1-dependent*. Moreover, 293T cells, which express only the Pol III DNA-sensing pathway (Ablasser et al., 2009; Chiu et al., 2009), failed to respond significantly to *L. pneumophila* genomic DNA, despite a robust response to pA:T (Figure 2.11B). Therefore, our data suggest that recognition of *L. pneumophila* genomic DNA by Pol III is not responsible for the *Ips-1*-dependent IFN response to *L. pneumophila*.

We considered two other models to explain how *L. pneumophila* induces a type I interferon response. The first is that *L. pneumophila* translocates RNA into host cells. In support of this model, I demonstrate that *L. pneumophila* RNA, unlike any form of DNA tested, induced a *Rig-i*-dependent type I IFN response in macrophages (Figure 2.10B). However, I did not demonstrate that *L. pneumophila* RNA species are translocated into host cells, and this will be important to examine in future studies (see Chapter 3). Interestingly, it was recently reported that purified *Helicobacter pylori* RNA stimulates RIG-I in transfected 293T cells (Rad et al., 2009). A second model to explain type I IFN induction by *L. pneumophila* is that infection induces a host response that indirectly results in signaling via the MDA5/RIG-I/IPS-1 pathways. *L. pneumophila* secretes a large number of effectors into the host cytosol and these effectors disrupt or alter a large number of host cell processes (Ensminger and Isberg, 2009). Such disruption may either lead to the generation of host-derived RNA ligands for the RIG-I and MDA5

sensors, or may result in signaling through these sensors in the absence of specific ligands. It was previously proposed that a host nuclease, RNaseL, can generate self-RNA ligands for the RIG-I and MDA5 pathways in response to viral infection (Malathi et al., 2007). Although I did not observe a role for RNaseL in the response to *L. pneumophila* (K.M. Monroe, unpublished data), it is conceivable that a different host enzyme can fulfill a similar function.

Identification by S. McWhirter and my characterization of a secreted bacterial effector, SdhA, previously shown to suppress host cell death, that also suppresses the IFN response to *L. pneumophila*, is consistent with a model in which a host cell stress response leads to direct or indirect activation of the cytosolic RNA-sensing pathway. However, the mechanism by which SdhA acts on host cells remains mysterious. Laguna and colleagues provided evidence that SdhA is critical for prevention of mitochondrial disruption that occurs when host cells are infected with the Δ*sdhA* mutant (Laguna et al., 2006). Given that Ips-1 localizes to mitochondria and requires mitochondrial localization for its function (Seth et al., 2005), it is tempting to speculate that SdhA acts on mitochondria in a way that both prevents their disruption and interferes with the function of Ips-1. To provide evidence that SdhA acts specifically on the RIG-I/MDA5 pathway, I used transient transfections of 293T cells. SdhA repressed induction of *Ifnb* when co-expressed with Mda5 or Rig-I but not Trif (Figure 2.8). Given these results and the evidence that SdhA is translocated into host cells (Laguna et al., 2006), I favor the idea that SdhA acts within host cells. Mutation of *sdhA* was reported not to affect translocation of other effectors into host cells (Laguna et al., 2006); thus, we tend not to support the alternative possibility that SdhA blocks translocation of the putative IFN-stimulatory ligand through the type IV secretion system. SdhA is a large protein of 1429 amino acids, but does not contain domains of known function, except for a putative coiled coil (a.a. 1037-1068). In future studies it will be important to address whether subdomains of SdhA can be identified that are required for suppression of the IFN response. It will also be important to determine whether these subdomains are distinguishable from any putative subdomains required for suppression of host cell death. In fact, our data have suggested that suppression of cell death and the IFN response may be separable functions of SdhA. I found that cell death was not required for hyperinduction of IFN by the Δ*sdhA* mutant, and conversely, I also found that hyperinduction of type I IFN does not lead to increased cell death (Figure 2.5).

This study demonstrates a partial role for both *Mda5* and *Rig-i* RNA sensors in response to *L. pneumophila.* Although these sensors are typically thought to respond to distinct classes of viruses, there are indications that they can also function cooperatively in response to certain stimuli, e.g., West Nile Virus (Fredericksen et al., 2008). My results suggest that *L. pneumophila* produces ligands that can stimulate both *Mda5* and *Rig-i* and that these two sensors cooperatively signal via *Ips-1*. Fitting with this model, I found that *Ips-1*-deficiency generally had a more severe impact on type I IFN induction than did *Mda5* or *Rig-i* deficiency.

Cytosolic RNA-sensing pathways are believed to respond exclusively to viral infection, and it is therefore surprising that *L. pneumophila* appears to trigger these pathways. Other bacterial species, such as *Listeria monocytogenes* and *Francisella tularensis,* have been shown to induce an *Ips-1*-independent cytosolic pathway leading

to type I IFN induction (Crimmins et al., 2008; Henry et al., 2007; Sun et al., 2006). *Ips-1* or *Mda5*-deficiency, as well as *Rig-i* knockdown, did not result in a complete elimination of the type I IFN response (Figure 2.1, Figure 2.4). Thus, a cytosolic DNA-sensing pathway may also be stimulated in response to *L. pneumophila* infection. A minor role for a cytosolic DNA-sensing pathway would be consistent with the observation that the *L. pneumophila* Dot/Icm type IV secretion system can translocate DNA into recipient cells (Vogel et al., 1998). However, as discussed above, my results with purified *L. pneumophila* DNA suggest that cytosolic sensing of *L. pneumophila* DNA does not account for the *Ips-1*-dependent induction of IFN that I observe (Figure 2.9). Another more likely possibility is that *Sting* plays a role, in parallel with *Ips-1,* to transduce signals from activated RIG-I during *Legionella* infection. Consistent with this hypothesis, I have observed a requirement for *Sting* in bone marrow derived macrophages (K.M. Monroe, unpublished data). One difficulty is that I cannot distinguish *Sting's* role in RNA versus DNA sensing. We are currently generating a useful tool for the future by breeding *Ips-1*$^{-/-}$ with *gt*$^{*/*}$ (a nonfunctional ENU-induced allele of *Sting*) mice to be able to test the IFN response in the absence of both pathways. One last possibility that we cannot eliminate is that a non-DNA, non-RNA ligand is translocated into host cells and stimulates the *Ips-1* pathway. In fact, in separate work, we have found that a small bacterial cyclic dinucleotide, c-di-GMP, can trigger a type I IFN response in macrophages, but importantly, this response is entirely independent of the *Ips-1* pathway (McWhirter et al., 2009). Nevertheless, there may be other small nucleic acid molecules that can be translocated by the Dot/Icm secretion system and signal in host cells via *Ips-1*.

Taken together, these results lead to new insights into the host immunosurveillance pathways that provide innate defense against bacterial pathogens. I demonstrate an unexpected role for a viral RNA-sensing pathway in the response to *L. pneumophila*, and together with S. McWhirter, identify a secreted bacterial effector, SdhA, that can suppress this response. These results therefore open new possibilities for immunosurveillance of bacterial pathogens.

2.5 Acknowledgements

I would like to thank Sarah McWhirter for her amazing efforts on the transposon mutagenesis screen in *Legionella* and initial identification of the *sdhA* insertion. I would like to thank R. Vance, G. Barton, J. Coers and members of the Vance and Barton labs for helpful discussions, G. Lam, K. Sotelo-Troha, and L. Tieu for technical assistance, R. Isberg for *sdhA* mutants and advice, D. Rookhuizen for help with the mutagenesis screen, K. Fitzgerald, D. Golenbock and D. Kalvakolanu for virus to immortalize macrophages, K. Ishii and S. Akira for *Zpb1*$^{-/-}$ bone marrow, M. Colonna and S. Gilfillan for *Mda5*$^{-/-}$ mice, D. Portnoy for *Ifnar*$^{-/-}$ mice, and Z. Chen for *Ips-1*$^{-/-}$ mice.

Chapter 3.

Searching for the endogenous *L. pneumophila* T4SS-dependent IFN-inducing ligand.

The work in Chapter 3 follows up on the observations and resulting hypotheses suggested by my work in Chapter 2. The experiments presented here focus on identifying the IFN-inducing ligand, which I hypothesize is bacterial RNA translocated via the T4SS during *L. pneumophila* infection. The experiments in this chapter are an unpublished work in progress and include many failed approaches and one experimental approach that proved promising.

3.1 Introduction

The results from Chapter 2 elicit the question: what is the endogenous IFN-inducing ligand during infection with T4SS-competent *Legionella?* There have been many hypotheses that have been put forth to try to answer this question and many experimental approaches to address it. In this chapter, I will discuss the hypotheses, from simple to complex, as well as the approaches that proved successful and unsuccessful.

In the field of innate immunity there is a desire to move away from use of synthetic purified ligands and towards identifying the endogenous infection-induced or microbe-derived ligands that directly activate innate immune receptors. Few reports in the literature have made the leap from purified ligands to dissecting the receptor-bound ligand in the presence of the microbe (Baum et al., 2010; Rehwinkel et al., 2010). In this work, I seek to identify the endogenous ligand that activates the host IFN response during T4SS^{+} *Legionella*.

The simplest hypothesis suggested by my data in Chapter 2 is that *Legionella* leaks or translocates RNA via the T4SS to stimulate cytosolic host RNA sensors. I showed that purified *Legionella* RNA, when delivered to the cytosol, activates a *Rig-i/Mavs*-dependent response. Purified genomic *Legionella* DNA induces a cytosolic IFN response that doesn't require *Rig-i,* suggesting that *Legionella* DNA does not activate the Pol III pathway. Taken together, this data strongly suggested to us that Occam's razor might apply in this case, in that the simple model of *Legionella* RNA accessing the host cell cytosol via the T4SS functions as the source of IFN-stimulatory activity during infection.

The proposal that RNA translocates or leaks through the T4SS is not popular among those in the field that believe that the T4SS is a tightly regulated machine with specific cargo. However, bacterial effector proteins are not the sole molecules permitted across the Rubicon of the T4SS. It can undergo conjugative transfer of plasmid DNA (Vogel et al., 1998) and flagellin, a potent inflammasome-activating ligand, can pass through the *Salmonella typhimurium* T3SS (Sun et al., 2007). It is likely that secretion system dependent delivery of flagellin to the host cell cytosol holds true during

Legionella infection, thought the amount of flagellin required to activate the inflammasome is probably very small and difficult to detect (Lightfield et al., 2008). Certainly allowing flagellin to access the host cell cytosol is not "intentional" as it has dire consequences for restricting the growth of bacteria (Ren et al., 2006). Therefore, the T4SS has come to be thought of as *Legionella's* Achilles' heel (Vance, 2010). While it is required to translocate effector molecules which set up a replicative niche, it also enables activation of multiple innate immune sensing pathways that eventually restrict bacterial growth (Coers et al., 2007). RNA translocation across a bacterial secretion system remains to be demonstrated, and I propose that it is a potential mechanism that would explain my results.

Another hypothesis I have entertained is that a specific host RNA may be generated in response to *Legionella* infection and act as a ligand to activate cytosolic RNA sensors. This hypothesis proposes that infection with T4SS-competent *Legionella* induces a change in host RNA biology, perhaps cleavage or degradation of host RNA. It has been proposed that a host endoribonuclease, RNAse L, can act in this manner in response to infection with Sendai virus or EMCV (Malathi et al., 2007). In this case, the authors suggest that small (<200 nucleotides) self-RNAs increase the IFN response initiated by viral RNA ligands. The authors did not do a comprehensive study of the actual ligands bound to RIG-I or MDA5. It is possible that other mechanisms of generating host-derived RNA ligands function in the cell. For instance, could by-products of miRNA-directed Dicer cleavage of mRNAs generate ligands that could be sensed by cytosolic RNA sensors? Or, does sequestration of the RISC in P bodies, for example, reduce potential interactions with cytosolic RNA sensing pathways? Consistent with these speculations, there are a number of host miRNAs that are slightly upregulated during T4SS-competent, but not T4SS-deficient *Legionella* infection (K.M Monroe, unpublished data).

A derivative of the host RNA hypothesis questions whether mitochondrial RNA can serve as an IFN-inducing ligand during infection. This idea originates from the observation that mitochondria serve as a platform for coordinating cytosolic RNA sensing pathways. MAVS, a key adapter for the RNA sensors RIG-I and MDA5, can signal only when its mitochondrial transmembrane domain is functional (Seth et al., 2005). Moreover, STING, which functions in RNA, DNA, and cyclic di nucleotide sensing (see Chapter 4) has been reported to localize exclusively to the outer mitochondrial membrane (Zhong et al., 2008), though further study is needed since other reports show ER localization (Ishikawa and Barber, 2008; Sun et al., 2009). The relationship between innate immunity and mitochondria is a provocative mystery (McWhirter et al., 2005). I hypothesized that this organelle, with its prokaryotic origins, is best poised to act as a stimulator of the RIG-I/MDA5/MAVS pathway since it contains endogenous RNA that could be released upon MAVS signaling. Importantly, mitochondrial mRNA is not capped (Clayton, 1984). Host-derived mRNAs have a 5' 7-methylguanosine cap over the 5'-triphosphate of the initial nucleotide. The 5' cap is key to prevent recognition by RIG-I, which exploits the 5'-triphosphate on foreign RNAs (Hornung et al., 2006). Moreover, *Legionella* harbors a translocated effector, SdhA, that appears to target the mitochondria by a mechanism that is incompletely understood (Laguna et al., 2006). Infection of cells with *ΔsdhA L. pneumophila* results in abnormal

mitochondrial morphologies and increased host cell death (Laguna et al., 2006). As shown in Chapter 2, *ΔsdhA L. pneumophila* hyperinduces IFN and leads to an increase in pyroptotic host cell death (Monroe et al., 2009). Therefore, I reasoned there were compelling reasons to investigate the link between IFN activation during *Legionella* infection and mitochondria.

3.2 Experimental Procedures

Isolation of Mitochondria from Mouse Livers. (Protocol communicated by Alyssa Rosenbloom of the Bustamante lab.) Livers were harvested from 3 C57BL/6 mice. The livers were placed in a 15ml conical on ice and mitochondria were isolated in M1 buffer (0.32M sucrose, 1mM EDTA, 1mM KCl, 10mM Tris pH 7.5) with 3-4 strokes of a Dounce homogenizer. The homogenized livers were centrifuged at 1000xg for 5 minutes, supernantant transferred to a new conical and centrifuged at 13,000xg for 20 minutes. The pellet was resuspended in M3 buffer (210mM Mannito, 70mM sucrose, 5mM Tris pH 7.5, 10mM $MgCl_2$, +protease inhibitors) and centrifuged at 1000xg for 5 minutes. The supernatant was saved and centrifuged at 13,000xg for 5 minutes. The pellet was then lysed in RLT buffer and RNA harvested with the Qiagen RNeasy kit. IFN stimulatory activity of mitochondrial RNA was measured by transfection into RIG-I or MDA5 overexpressing HEK293Ts with an IFN-luciferase reporter. Relative light units of luciferase were normalized to a constitutive TK Renilla reporter, or mitochondrial RNA was transfected into *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages, after 8 hours supernatants were overlayed onto L929-ISRE reporter cells as previously described (see Chapter 2 or (Monroe et al., 2009).)

Cell Culture. THP-1 cells were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, and 100U/mL penicillin. Upon plating, THP-1 cells were treated for 24 hours with 1μg/ml phorbol 12-myristate 13-acetate (PMA), media was replaced, and cells were incubated for 24 hours before infection. Macrophages were derived from bone marrow cells cultured for eight days in RPMI supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, 100U/mL penicillin, and 10% supernatant from 3T3-CSF cells, with feeding on the fifth day of growth.

Radiolabeling *Legionella* RNA. *Legionella pneumophila* (Δ*flaA and ΔdotAΔflaA* strains) stationary phase cultures were diluted to O.D. 2.0, fresh supplements were added to the cultures along with 10μCi α-P^{32} ATP or UTP (Perkin Elmer). Bacterial cultures undergoing RNA radiolabeling were grown shaking at 37°C overnight in a beta shield box. Radiolabled *Legionella* were pelleted, washed 3 times with PBS and used to spinfect bone marrow derived macrophages at an MOI of 10. Macrophage lysates were collected 2 hours post infection with the specified detergent, lysates were centrifuged at 10,000xg to pellet cell debris, and the remaining cytosolic fraction was filtered through a 0.2μM low retention filter to remove any remaining whole bacteria. Remaining cytoplasmic fractions were analyzed for radioactivity (Beckman LS 6000IC).

***L. pneumophila* Infection of THP-1 cells.** THP-1 cells were plated at a density of 10^7 per 10cm dish, activated with 10μg/ml PMA for 24 hours, media was replaced, and cells were spinfected (1400rpm for 10 minutes at RT in the Centra GP8R benchtop centrifuge, plastic adapters were removed to place the 10cm dishes stacked in the metal containers with only the skeleton adapter) with Δ*flaA* or *ΔdotAΔflaA L.* pneumophila at and MOI of 20, or Sendai virus at an MOI of 5,000 the following day.

Preparation of THP-1 lysates. 4-5 hours post infection, the cells were washed 4 times with cold PBS, and incubated with 3mls of citrate saline buffer (135mM KCl, 15mM NaCitrate) for 5 minutes at 37C. Infected THP-1 cells were gently collected by scraping, pipetted to break up cell clumps, an equal volume of PBS was added and cells were pelleted. Wash one last time with only PBS and resuspend cells in 100μl of Ready to Use 1X Polysome Lysis Buffer (10X: 100mM HEPES ph7.0, 1M KCl, 50mM $MgCl_2$, 0.25M EDTA, 5% NP-40, Ready to Use includes added fresh: 1 tablet complete proteinase inhibitor, RNAseOUT, SUPER RNAse IN (Ambion), 0.002M DTT) per 10cm dish, incubated on ice for at least 5 minutes and stored -80C for **only** 1-2 days. Preliminary experiments indicated that cell lysates **must** be frozen at -80°C and for only a few days.

Antibody Coating Beads. Antibody coat Protein G Sepharose beads (GE Healthcare) by washing in NT2 buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1mM $MgCl_2$, 0.05% NP-40) 3 times at 4°C, use 50μl of beads per 10cm dish in 500μl of NT2 buffer with 4.0μl 1mg/ml anti-human RIG-I antibody (Enzo Life Sciences). Incubate beads plus antibody rotating overnight at 4°C. Immediately before use wash antibody coated beads with 1ml of room temp NT2 buffer 4 times. Spin down beads after each wash and aspirate of any liquid. After final wash resuspend beads in 900μl of ice-cold NET2 buffer (850μl NT2, 10μl 0.1M DTT, 30μl 0.5M EDTA, 5μl RNAse OUT, 5μl SUPER RNAse IN).

RIG-I Immunoprecipitation. (Modified from Keene et al., Nature Protocols vol.1 no.1, 2006, and communicated by Alina Baum from the Garcia-Sastre lab. Originally performed in A549 cells with Sendai virus, which serves as an important positive control in these experiments.) Lysates were thawed on ice and centrifuged at 15,000xg for 15 minutes at 4C to clear lysate of cell debris. Lysates were transferred to new tube and store on ice. No preclear was performed. Lysates were added to antibody-coated beads. Samples were mixed, and spun briefly at 8,000-10,000xg to pellet beads. 50μl of supernatant was removed and frozen at -80C to represent total cellular RNA (TCR). IP samples were incubated, rotating overnight at 4C. The next morning beads were washed 4-5 times with 1 ml of ice-cold NT2 buffer. Tubes were kept on ice the entire time. (Beads can be resuspended in 1X SDS +DTT running buffer and analyzed by western blot analysis for RIG-I pulldown.) Beads were resuspended in 100μl NT2 buffer with 100ul Prot K buffer and 2μl Proteinase K (**must** be RNA grade). Samples were incubated at 55C for 30 minutes with occasional mixing. Supernantant was transferred to a new tube after bead settling. Follow manufacturer's protocol for TRIzol (Invitrogen) extraction of RNA. RNA pellets were resuspended in 20μl nuclease free water.

Macrophage Stimulation. RIG-I immunoprecipitated, TRIzol extracted RNA was transfected **immediately** after resuspension in nuclease free water into *MyD88*$^{-/-}$*Trif*$^{-/-}$ bone marrow derived macrophages. *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages were plated in 24 well plates at 200,000 cells per well in CSF conditioned media without antibiotics. Resuspended RNA samples and appropriate control stimulants (titrated from 1.0μg to 5ng of 5'triphosphate RNA and dsRNA control from Invivogen) were transfected with Lipofectamine 2000 as per the manufacturer's instructions.

Quantitative RT-PCR. Cellular RNA was harvested after overnight stimulation and isolated with the RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA was DNase treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed with Superscript III (Invitrogen). Quantitative PCR assays were performed on the Step One Plus RT PCR System (Applied Biosystems) with Platinum Taq DNA polymerase (Invitrogen) and EvaGreen dye (Biotium). Gene expression values were normalized to *S9* levels for each sample. The following primer sequences were used: human *Ifnb*, F, 5'-AAACTCATGAGCAGTCTGCA-3'and R, 5'-AGGAGATCTTCAGTTTCGGAGG-3'; human *S9*, F, 5'-ATCCGCCAGCGCCATA-3' and R, 5'-TCAATGTGCTTCTGGGAATCC -3'.

3.3 Results and Discussion

3.3.1 Host RNA does not stimulate IFN.

To test the hypothesis that degradation of host RNA could serve as a source of IFN stimulatory activity during infection with *Legionella*, I isolated RNA from uninfected and infected (with *ΔflaAΔsdhA L. pneumophila,* a potent IFN-inducing strain of *Legionella*) macrophages. When purified host RNA was transfected into HEK293T cells overexpressing RIG-I (100ng per 96 well) it did not activate an IFN reporter (data not shown). This suggested that an infection generated host RNA species likely does not serve as a RIG-I ligand during *Legionella* infection. However, it is difficult to be completely confident with this negative result, as I did not have a good positive control for this experiment. To address this hypothesis in a different way, I tested a role for a host RNase, RNase L, in the IFN response to *Legionella*. As discussed in the introduction, RNase L was reported to play positive feedback role in the IFN response to viruses by generating small (<200 nucleotide) self RNAs (Malathi et al., 2007). These self-RNAs were proposed to activate both cytosolic sensors, RIG-I and MDA5 (Malathi et al., 2007). Therefore, we reasoned that *Legionella* infection might activate cleavage of host RNA in an Rnase L-dependent manner. I found no change in IFN induction in RNase L-deficient macrophages infected with wild type, *ΔflaA,* or *ΔdotA L. pnuemophila*, compared to wild type macrophages (data not shown). This data suggests that Rnase L does not play a role in the host IFN response to *Legionella.* However, the positive control (poly I:C) did not demonstrate RNase L-dependence in my hands (data not shown). Malathi et al. assessed the role of RNase L in mouse embryonic fibroblasts (MEFs) and *in vivo* during infection with EMCV or Sendai virus. Here I am testing bone marrow derived macrophages, which have been shown to respond to viral infection and

poly I:C via the same cytosolic pathways that function in MEFs. Certainly there are other ways to generate self-RNA degradation and I have not yet completely ruled out this mechanism as a possibility. However, these results suggest that cleaved host RNA is not a predominant source of IFN stimulation during *Legionella* infection.

3.3.2 Testing the hypothesis that mitochondrial RNA may serve as an IFN-stimulatory ligand during infection with T4SS-competent Legionella.

Next, I set out to test a derivative of the host-RNA hypothesis, namely, that mitochondrial-derived RNA could have the ability to stimulate IFN when "activated" by infection. I reasoned that my previous experiment collected total cellular host RNA and that if mitochondrial RNA was the stimulatory fraction, it may be of minimal proportion in those samples. To test this hypothesis, I isolated RNA from mitochondria purified from mouse livers, transfected it into *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages, and determined IFNβ levels in the supernatant by L929-ISRE luciferase bioassay (Figure 3.1). I found that mitochondrial RNA induces IFNβ and that this response is abrogated upon RNase A treatment, but not DNase treatment (Figure 3.1). Poly I:C was used as a control stimulus. This result shows that mitochondrial RNA can induce an IFN host response. While provocative, I still need to address the question of whether mitochondrial RNA serves as an IFN-inducing ligand during infection.

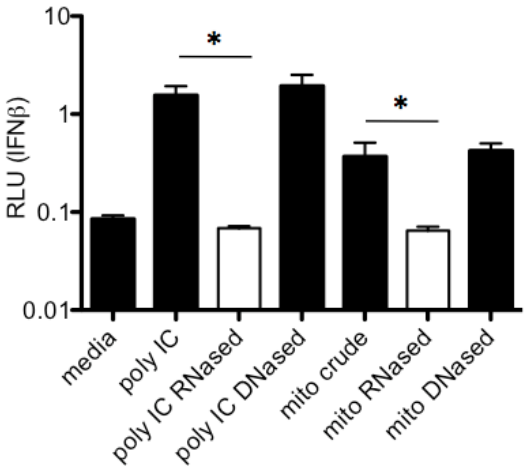

Figure 3.1 Purified mitochondrial RNA induces IFNβ. RNA was isolated from mitochondria that were harvested from mouse livers. Mitochondrial RNA preparations (mito crude) were lipofected into *MyD88*$^{-/-}$*Trif*$^{-/-}$ bone marrow macrophages. 8 hours post stimulation supernatant was harvested and overlaid onto L292-ISRE luciferase reporter cells. Units are relative light units (RLU). White bars= RNase treated samples. The difference between crude RNA preparations and RNased samples were statistically significant (*, $p<0.05$, Student's t test). The differences between crude RNA preparations and DNase treated samples were not statistically significant (not indicated).

To test whether mitochondrial RNA could serve as a RIG-I or MDA5 ligand in a reconstituted system, I expressed RIG-I or MDA5 in HEK293T cells and transfected in crude mitochondrial RNA preparation, RNased or DNased mitochondrial RNA. Contrary to the IFN response in *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages, I did not see activation of the IFN-luc reporter (data not shown). This result led me to question whether either RNA sensor can respond to mitochondrial RNA directly. It is possible that the reconstituted system is an inappropriate method for assessing responsiveness to particular ligands. Notably, purified *Legionella* RNA behaves in a similar manner, in that it induces IFNβ when transfected into *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages, but there is no measurable response in HEK293Ts expressing either RIG-I or MDA5 (data not shown). The explanation for this discrepancy is not known. Poly I:C, another RIG-I ligand, is able to stimulate IFN in HEK293T cells. In addition, I did not test mitochondrial RNA that has been somehow "activated" by infection, which is a caveat to these experiments. Therefore at this time, I cannot rule out a role for mitochondrial RNA functioning/contributing as an endogenous IFN-inducer during *Legionella* infection. Fortunately, the approach I am currently working towards, which is discussed further on, will be able to address this remaining question.

3.3.3 Radiolabeling Legionella RNA to assess T4SS translocation of RNA into the host cell cytosol.

The next approach I took to determine if *Legionella* translocates RNA via the T4SS was to radiolabel *Legionella* RNA, infect macrophages, and look for T4SS-dependent radioactivity in the macrophage cytosol. I grew log phase *Legionella* (*ΔflaA* and *ΔdotAΔflaA)* cultures with α-P^{32}ATP or UTP, which selectively incorporates into RNA and not DNA, until the bacteria reached stationary phase. I then infected macrophages with the radiolabeled bacteria, and assessed the radioactive signal in filtered cytosolic fractions of infected macrophages. Unfortunately, I found that *Legionella* lysed at low levels during lysate preparation, and this low level bacteriolysis contaminated the filtered preparations with measurable P^{32} (Figure 3.2A). Therefore with this experimental approach, I observed no greater than a 2-fold increase in P^{32} signal in T4SS-competent *Legionella* compared to T4SS-deficient *Legionella* (Figure 3.2B). I tested a multitude of macrophage lysate conditions and saw this modest, however strikingly consistent, 2-fold increase in P^{32} signal in T4SS^{+} *Legionella* in all lysis buffers (data not shown). While this data hints that RNA can leak or translocate through the T4SS, it was not a robust enough signal to feel confident that I could biochemically track down the RNA species present in the macrophage cytosol.

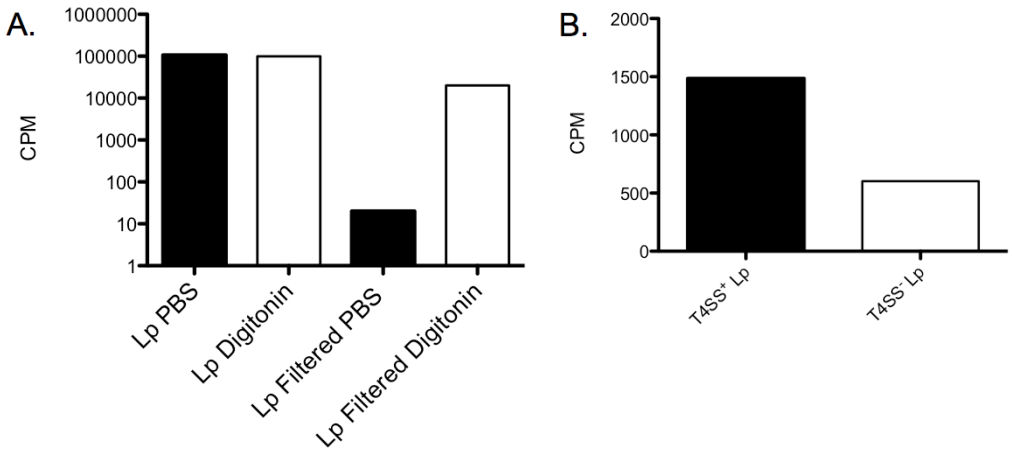

Figure 3.2 Infection of macrophages with P^{32} radiolabeled *Legionella* RNA leads to modest T4SS-dependent signal in the macrophage cytosol despite bacteriolysis by lysate buffer. (A) *Legionella* was grown with α-P^{32}ATP until stationary phase, pelleted and resuspended in PBS or 0.05% Digitonin in PBS, bacteria were filtered with a 0.2μM low retention filter and P^{32} signal was measured by CPM. (B) Macrophages were infected with $T4SS^{+}$ or $T4SS^{-}$ *Legionella* radiolabeled with α-P^{32} ATP incorporated into RNA, lysates were harvested with 0.05% Digitonin in PBS 2 hours post infection, centrifuged, filtered, and CPM measured. Similar results were obtained with lysis buffers in PBS containing 0.01% NP40, 0.05% Saponin, or 0.05% Triton-X-100.

3.3.4 Immunoprecipitation of RIG-I to identify IFN-stimulatory RNA.

Finally, I decided to take a more direct approach to identifying the IFN-inducing ligand during *Legionella* infection. To this end, I infected cells with T4SS-competent or deficient *Legionella*, immunoprecipitated RIG-I with an endogenous antibody (which recognizes human RIG-I), eluted bound RNA species, TRIzol purified the RNA, and transfected it into *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages and assessed *Ifnb* induction by quantitative RT-PCR. In previous experiments, I have worked with mouse bone marrow macrophages, so for the RIG-I immunoprecipitations with the human-specific antibody, I needed to find a human cell line that responded to *Legionella.* Opitz et al. showed that A549 lung epithelial cells induce IFN in a *dot*-dependent and *Mavs*-dependent manner (Opitz et al., 2006). I found that A549 cells induce IFN upon infection with T4SS+ *Legionella*, however, this response is not very robust (<10-fold induction) (Figure 3. 3). I hypothesized that *Legionella* may not efficiently infect lung epithelial cells since they are not phagocytic and *Legionella* is not invasive. To overcome this barrier, I coated the bugs in antibody and expressed Fc receptor on the A549 cells; however, this did not improve the IFN response (data not shown). In contrast, a human monocytic cell line, THP-1, is phagocytic, and so I infected these cells and assessed *Ifnb* levels. THP-1 cells infected with *Legionella* induce *Ifnb* approximately 100-fold over uninfected cells, and importantly this IFN response requires the T4SS (Figure 3.3). Presently, I need to

determine if THP-1 cells require *Rig-i/Mavs* to mount an IFN response to *Legionella*. I will need to perform siRNA oligo-mediated knockdown or transduce a targeted knockdown construct directed against the *Rig-i* or *Mavs* transcript.

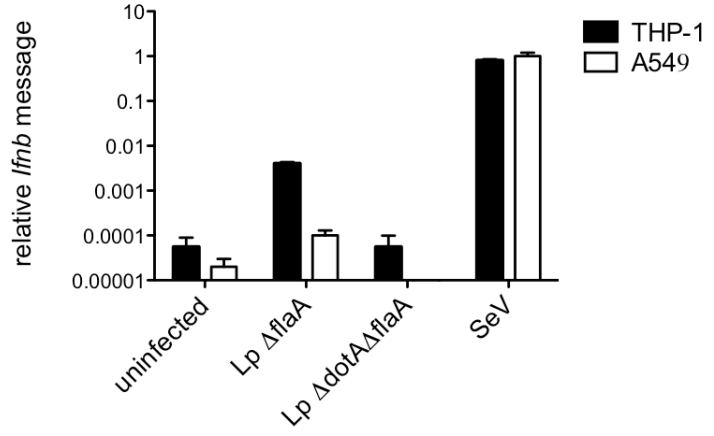

Figure 3.3 THP-1 human monocytic cells mount a more robust IFN response than A549 cells to *L. pneumophila* infection. Indicated human cell lines were infected at an MOI of 5 with *ΔflaA* or *ΔdotAΔflaA L. pneumophila* or Sendai virus. RNA was harvested 4 hours post infection and human *Ifnb* levels, normalized to S9, were determined by quantitative RT-PCR.

I then immunoprecipitated RIG-I from a cell line known to respond to *Legionella* via the *Rig-i/Mavs* pathway (A549) and one that responds robustly (THP-1) and tested whether I could isolate IFN-stimulatory activity. With this approach, I was able to immunoprecipitate IFN-stimulating activity in both cell types, though the IFN response was predictably higher in THP-1 infected cells (Figures 3.4 and 3.5). The next step, which I plan to accomplish in the next few months, is to deep sequence a library of these samples using Illumina technology. This approach will identify the origin, whether bacterial, host, or mitochondrial or a mixture of the aforementioned sources, and any distinct characteristics of RIG-I bound molecules. The IFN response is not completely absent during infection with T4SS-deficient *Legionella.* However, I predict that deep sequencing will show that the pool of RIG-I bound RNA will differ depending upon the T4SS. There are a few controls that need to be performed in the immediate future. For example, is the RIG-I immunoprecipitated IFN-stimulatory activity RNase sensitive and *Mavs*-dependent, as would be expected?

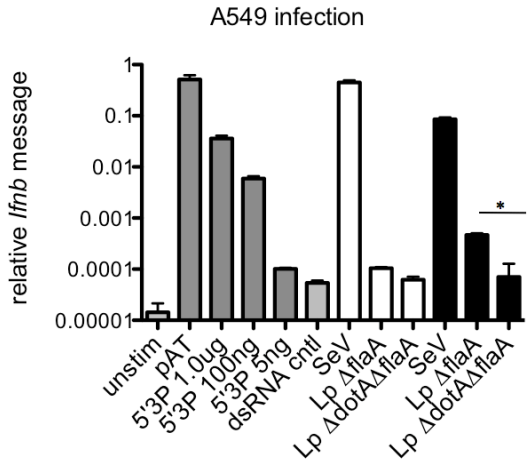

Figure 3.4 RIG-I immunoprecipitation from A549 infected cells results in isolation of a T4SS dependent IFN-stimulating activity. A549 cells were infected with Sendai virus or T4SS-competent (*ΔflaA*) or T4SS-deficient *(ΔdotAΔflaA*) *Legionella* at an MOI of 20 for 6h. Lysates were immunoprecipitated with anti-RIG-I antibody, and TRIzol extracted RNA was transfected into *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages. Quantitative RT-PCR was used to determine relative *Ifnb* induction, normalized to S9 transcript. Student's t test was used to calculate statistical significance (*, p=0.001). Dark grey bars= positive stimulation controls, Light grey bars= negative controls, White bars= total cellular RNA samples, Black bars= anti-RIG-I IP samples.

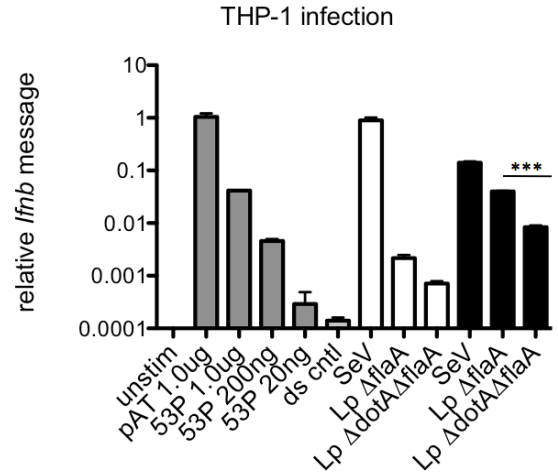

Figure 3.5 RIG-I immunoprecipitation from THP-1 infected cells results in isolation of a T4SS-dependent IFN-stimulating activity. THP-1 cells were infected with Sendai virus or T4SS-competent (*ΔflaA*) or T4SS-deficient *(ΔdotAΔflaA*) *Legionella* at an MOI of 20 for 5h. Lysates were immunoprecipitated with anti-RIG-I antibody, and TRIzol extracted RNA was transfected into *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages. Quantitative RT-PCR was used to determine relative *Ifnb* induction, normalized to S9 transcript. Student's t test was used to calculate statistical significance (***, p<0.00001). Dark grey bars=

positive stimulation controls, Light grey bars= negative controls, White bars= total cellular RNA samples, Black bars= anti-RIG-I IP samples.

3.4 Acknowledgements

I would like to thank Alina Baum and Adolfo Garcia Sastre for the generous gift of the human RIG-I antibody, as well as communicating the RIG-I immunoprecipitation protocol. I would like to thank Greg Lam for his time and effort on many failed experiments that were relevant to this line of research. I also thank Alyssa Rosenblum for sharing knowledge regarding mitochondria preparations.

Chapter 4.

A critical function for Sting in the type I interferon response to cyclic dinucleotides.

This chapter describes experiments that address the observation that cyclic diguanylate (c-di-GMP), a common bacterial second messenger molecule, stimulates a potent *Sting*-dependent innate immune response upon delivery to the cytosol of macrophages. Experiments presented in this chapter were published in *Journal of Experimental Medicine (McWhirter et al., 2009)* and *Infection and Immunity (Sauer et al., 2011)* in which I was a contributing author. Some of the data presented here is part of a manuscript in process (Burdette et al., submitted). In this chapter I highlight my specific experimental contributions to these manuscripts, and summarize the contributions of others in order to provide context for my work.

4.1 Introduction

This story has been influenced by a few coincidental events along the way and the original reason we embarked on studying cyclic di-nucleotides did not escape serendipity. A transposon mutagenesis screen was initiated in the lab to look for *Legionella pneumophila* mutants that either no longer activate a type I IFN host response or hyperinduce IFN. A mutant was found that induced low levels of IFNβ. This strain harbored a transposon insertion in the gene *pleD*, which encodes for a protein involved in both synthesis and hydrolysis of c-di-GMP (S. McWhirter). This was the first arrow that pointed us towards investigating the role of cyclic dinucleotides in innate immune responses. However, it turned out that this mutant was uninformative, as a clean deletion of *pleD* did not recapitulate the transposon mutant (data not shown), and a second insertion was found in a type IV secretion system gene, thereby explaining the mutant phenotype without needing to invoke a role for the PleD insertion or cyclic dinucleotides. However, by the time the mutant had been properly characterized, we had already initiated a fruitful series of experiments, described below, in which we described and characterized a mechanism for innate immune sensing of c-di-GMP. Together with studies on c-di-AMP in the Portnoy lab (Woodward et al., 2010), this work has propelled the field into investigating new areas of cyclic di-nucleotides as they relate to innate immunity and bacterial physiology.

One goal of studying the innate immune system is to understand what molecular features of microbes the germ-line encoded receptors have evolved to recognize, thereby alerting the presence of a potential pathogen. It is clear from the work of numerous labs that dedicated cytosolic sensors detect nucleic acids from bacteria and viruses. Chapter 1 and Chapter 2 describe cytosolic RNA and DNA sensors in detail. In our investigation of innate immune sensing of cyclic di-nucleotides, we hypothesized that even though they are nucleic acids in their chemical nature, the unique structure of

cyclic di-nucleotides may require a devoted sensor. Studies of DNA sensing have shown that DNA oligomers of at least 30-40 basepairs are necessary for triggering an IFN response (Ablasser et al., 2009; Unterholzner et al., 2010). The most common ligand utilized for DNA sensing studies, poly(dA:dT) (pAT) is kilobases in length. In light of our hypothesis, we ruled out all known nucleic acid sensors as cyclic di-nucleotide sensors (McWhirter et al., 2009). This data further supports the idea that a unique cyclic di-nucleotide sensor exists in the toolbox of the mammalian innate immune system.

All cells utilize small nucleic acid molecules as important intracellular information transducers. Cyclic AMP (cAMP) is a signal transduction molecule utilized in both eukaryotic and prokaryotic cells for a wide variety of biological processes. Cyclic GMP (cGMP) is a second messenger used solely in eukaryotes (Tamayo et al., 2007). On the other hand, bacteria exclusively utilize guanosine tetra-phosphate (ppGpp) as a global gene regulator during amino acid starvation to conserve energy (Magnusson et al., 2005). Another bacterial specific second messenger molecule is cyclic diguanylate (c-di-GMP), which is synthesized from two GTP molecules that are hydrolyzed and circularized to form monophosphate linkages. c-di-GMP is actually a cyclic ribonucleotide, and is not deoxygenated at the 2' position. Synthesis of c-di-GMP occurs by diguanylate cyclases (DGCs), of which the catalytic GGDEF domain is restricted to bacteria (Galperin et al., 2001). Phosphodiesterases (PDEs) hydrolyze c-di-GMP to pGpG via an EAL domain. Often both of these domains can be found encoded in the same protein. The roles c-di-GMP plays in bacterial physiology is becoming better understood, and it is clear that c-di-GMP regulates key processes necessary for pathogenesis such as, biofilm formation, motility, and virulence gene expression (Tamayo et al., 2007).

Recent discovery of diadenylate cyclase activity in the *Bacillus subtilis* protein DisA suggested a role for c-di-AMP prokaryotic regulation (Witte et al., 2008). Remarkably, structural analysis of DisA showed c-di-AMP bound to the nucleotide binding domains, which is notably unrelated in both sequence and structure to the GGDEF domains of DGCs (Witte et al., 2008). Importantly, the DAC domain is predicted in other bacteria, including pathogenic bacteria, and archeae genomes (Romling, 2008). *Listeria monocytogenes* induces a type I IFN response dependent upon multidrug efflux pumps (MDRs), leading to the hypothesis that a small molecule is responsible for activating IFN (Crimmins et al., 2008). Mass spectrometry analysis identified c-di-AMP in supernatants of wild type *Listeria* and increasing amounts in *Listeria* overexpressing MDRs (Woodward et al., 2010). Purified c-di-AMP induces a robust, dose-dependent type I IFN response in macrophages (Woodward et al., 2010). These results suggest that c-di-AMP plays a key role in innate immune recognition of the bacterial pathogen *Listeria monocytogenes.* Notably, the type I IFN response contributes to *Listeria* pathogenesis, rather than resolving infection for the host (Auerbuch et al., 2004).

Previous studies have shown that c-di-GMP is immunostimulatory, can function as an adjuvant, and is protective against bacterial challenge (Ebensen et al., 2007; Karaolis et al., 2007a; Karaolis et al., 2007b). Characterization of the immune response to c-di-GMP showed that intraperitoneal injection in mice activated monocyte and

granulocyte recruitment (Karaolis et al., 2007a). Immature human DCs upregulate costimulatory molecules, MHC class II, and activate cytokines when cultured with c-di-GMP (Karaolis et al., 2007a). Vaccination of mice with antigen +c-di-GMP, but not antigen alone, elicited Ag-specific IgG antibodies in two studies (Ebensen et al., 2007; Karaolis et al., 2007a). Most strikingly, pretreatment of mice with c-di-GMP lead to restriction of *Staphylococcus aureus* and *Klebsiella pneumoniae* (Karaolis et al., 2007a; Karaolis et al., 2007b). Here, we demonstrate the recognition of cytosolic cyclic di-nucleotides by the innate immune system, and dissect the host pathways that are required to sense cyclic di-nucleotides to mount an immune response.

4.2 Experimental Procedures

Mice. Wild type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Tmem173*$^{-/-}$ femurs were a kind gift from Dr. Glen Barber (University of Miami, FL). *Tmem173*$^{gt/gt}$ mice were generated by N-ethyl-N-nitrosourea (ENU) induced mutation of C56BL/6 mice as previously described (Sauer et al., 2011).

Cell culture. HEK293T cells were cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, and 100U/mL penicillin. Macrophages were derived from bone marrow cells cultured for eight days in RPMI supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, 100U/mL penicillin, and 10% supernatant from 3T3-CSF cells, with feeding on the fifth day of growth. Immortalized macrophages were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, and 100U/mL penicillin. THP-1 cells were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 100μM streptomycin, and 100U/mL penicillin. Upon plating, THP-1 cells were treated for 24 hours with 1μg/ml phorbol 12-myristate 13-acetate (PMA), media was replaced and cells were incubated for 24 hours before stimulation.

Cell stimulation and overexpression. Bone marrow macrophages were harvested as described above and plated at 10^6 cells/well in six well dishes. 500 μg/ml polydAdT or polyI:C were transfected into macrophages using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturers protocol. Macrophages were infected with Sendai virus (Charles River Laboratory, Wilmington, MA) at 150 HAU/ml. Macrophages were treated with 100ng/ml LPS. 4μg/ml of c-di-GMP was transfected into macrophages using Lipofectamine2000 according to manufacturers protocol. 4μg/ml of c-di-AMP was delivered to the cytosol using digitonin permeabilization as previously described (Woodward et al., 2010). Four hours following infection/treatment RNA was harvested and qRT-PCR was performed.
The wild type *Tmem173* pcDNA vector was a kind gift from Dr. Glen Barber. *Tmem173*$^{gt/gt}$ pcDNA constructs were made using Stratagene's Quick Change Site-directed Mutagenesis (Agilent Technologies, Santa Clara, CA) (Sauer et al., 2011). *Tmem173*$^{+/+}$, and *Tmem173*$^{gt/gt}$ pcDNA constructs, along with an IFNβ-firefly luciferase reporter and TK-*Renilla* luciferase plasmids, were transfected with FuGENE 6 (Roche) according to the manufacturer's protocol. Nucleic acids were mixed with FuGENE 6 in

Optimem at 0.5 µl/96 well and incubated for 15 minutes. Total transfected DNA was normalized to 200ng per well using an empty pcDNA3 plasmid. Luciferase production was measured 24 hours post transfection.

Quantitative RT-PCR. Immortalized macrophages were plated at a density of 2 x10^6 per well in 6 well plates and stimulated with 4 ug/ml cyclic di nucleotide. Macrophage RNA was harvested 4 hours post infection and isolated with the RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA was DNase treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed with Superscript III (Invitrogen). Quantitative PCR assays were performed on the Step One Plus RT PCR System (Applied Biosystems) with Platinum Taq DNA polymerase (Invitrogen) and EvaGreen dye (Biotium). Gene expression values were normalized to *Rps17* levels for each sample. The following primer sequences were used: mouse *IFNb*, F, 5'-ATAAGCAGCTCCAGCTCCAA-3'and R, 5'-CTGTCTGCTGGTGGAGTTCA-3'; mouse *Rps17*, F, 5'-CGCCATTATCCCCAGCAAG-3' and R, 5'-TGTCGGGATCCACCTCAATG-3'.

Western Blotting. Whole cell lysates were collected in Laemmli Buffer 24 hours following transfection as indicated. Lysates were run on SDS-PAGE, transferred to PVDF membranes. Overexpression of wild type and *Gt* Sting was detected with anti-HA antibody (Roche**,** Indianapolis, IN) and β-actin was detected with anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL).

Macrophage Immortalization and Complementation. B6 and *Tmem173*$^{gt/gt}$ bone marrow was infected with a v-myc/v-raf expressing retrovirus (Blasi et al, Nature1985, 318(6047):667-70), followed by differentiation in 10% L929-MCSF supernatant. After one month in culture, L929-MCSF supernatant was removed from the media and the surviving macrophages established an immortalized cell line. MCSV complementation constructs were made by subcloning wild type or gt *Tmem173* from pcDNA into the BglII and NotI sites of MSCV2.2. Immortalized macrophages were transduced by infecting Vsv-g pseudotyped MSCV IRES-GFP retrovirus packaged with wild type *Tmem173* or *Tmem173*$^{gt/gt}$ in GP2 cells. After one week in culture, GFP$^+$ macrophages were sorted with the DAKO-Cytomation MoFlo High Speed Sorter**.**

4.3 Results and Discussion

4.3.1 Cyclic di-GMP induces a robust type I interferon response in macrophages.

McWhirter et al. demonstrated that delivery of cyclic di-GMP via liposomes to the cytosol of bone marrow macrophages, but not overlay, activated a robust, dose-dependent type I IFN response (McWhirter et al., 2009). We hypothesized that the IFN response was specific to the di-cyclic form of guanosine, and not other permutations of mono- or di-guanosine. c-di-GMP may alarm the host that virulent bacteria are present

since it plays a role in bacterial physiology that includes activating virulence gene expression, biofilm formation, and motility (Tamayo et al., 2007; Vance et al., 2009). GTP and GMP are molecules made by both bacteria and eukaryotic cells, therefore not good targets for identifying foreign microbes by the innate immune system. pGpG is a hydrolyzed form of c-di-GMP and we wanted to determine whether metabolites of c-di-GMP are capable of being sensed or perhaps represent the physiological form of the molecule being sensed by the host. ppGpp is a bacterial specific molecule, which functions as a regulator of global gene transcription in bacteria (Magnusson et al., 2005). Since ppGpp regulates gene expression in both virulent and avirulent bacteria, we predict the host does not sense it, as it does not represent a pathogenic state. To test the specificity of the host response to c-di-GMP, we transfected c-di-GMP, GTP, GMP, pGpG, ppGpp into bone marrow macrophages and assessed levels of *IFNb* induction. Only c-di-GMP induces *IFNb* and the response is comparable to the multiple log-fold induction observed with other known stimulants, pAT and pIC (Figure 4.1). GTP, GMP, pGpG, and ppGpp transfected macrophages express levels of *IFNb* equivalent to unstimulated macrophages (Figure 4.1). Additionally, c-di-GMP hydrolyzed with snake venom phosophdiesterase does not stimulate IFNβ (McWhirter et al., 2009), further supporting the data that suggest that c-di-GMP is exclusively recognized by a cytosolic immunosurveillance pathway.

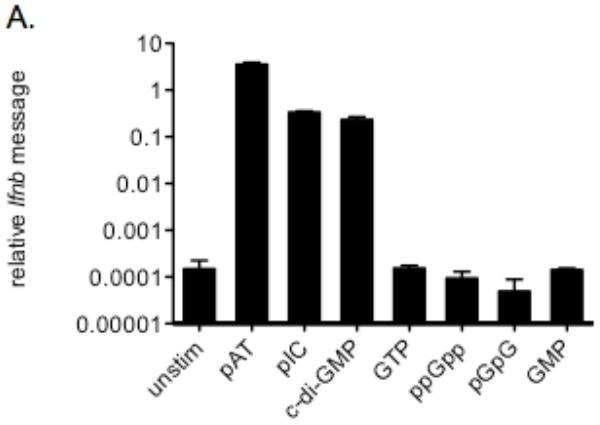

Figure 4.1 Cyclic-di-GMP induces a robust type I interferon response in macrophages. (A) Bone marrow derived macrophages were transfected with 3.3μg/ml of indicated molecules and transcriptional induction of the interferon-β gene (*IFNb*) was determined by quantitative RT-PCR. All samples were normalized to *rps17,* a ribosomal protein mRNA.

4.3.2 The ENU-induced goldenticket mutation reveals a critical function for Sting in innate immune sensing of cyclic di-nucleotides.

McWhirter et al. showed that the IFNβ host response to c-di-GMP is independent of TLR signaling, cytosolic RNA sensing pathways, and Zbp-1 (putative DNA sensor). Sensing of c-di-GMP requires the type I IFN receptor, TBK1 and IRF3; key distal signaling components in many cytosolic sensing pathways (McWhirter et al., 2009).

These results led us to question what more proximal host signaling components were necessary to sense c-di-GMP.

In parallel studies, a screen for ENU-induced mouse mutants that either no longer responds or hyperinduces IFNβ upon infection with *Listeria monocytogenes* or *Legionella pneumophila* was ongoing. A mutant arose from this screen that did not induce IFNβ during wild type *Listeria* infection (Sauer et al., 2011). Importantly, it has been shown that *Listeria* induces type I IFN in a manner that correlates with transport of c-di-AMP from multidrug efflux pumps (Woodward et al., 2010).

Sequencing of the *Sting* locus in *gt* mice identified a T to A basepair conversion at position 596, which is located in exon 4 (K. Sotelo-Troha). All other candidate genes had wild type sequences. This and other supporting data discussed below suggested that we had generated a new allele of *Sting*, which will be referred to as *Goldenticket* (*gt*). This missense mutation alters protein coding at amino acid 199 from isoleucine to asparagine (Sauer et al., 2011). In macrophages harboring homozygous *gt* mutations STING protein is undetectable (JD Sauer, unpublished data). Perhaps Gt STING is misfolded and rapidly degraded. We *de novo* generated the $Sting^{gt}$ allele by site directed mutagenesis of the wild type *Sting* HA-tagged construct (K. Sotelo-Troha). Overexpression in 293T cells showed that wild type *Sting* signals increasing *Ifnb* levels correlating to increasing *Sting* levels. However, *gt Sting* is incapable of inducing *Ifnb* at any expression level (Figure 4.2A). In this system, we confirmed that both WT STING and Gt STING are detected at similar levels suggesting the lack of signaling by Gt STING is not due to lack of expression (Figure 4.2B).

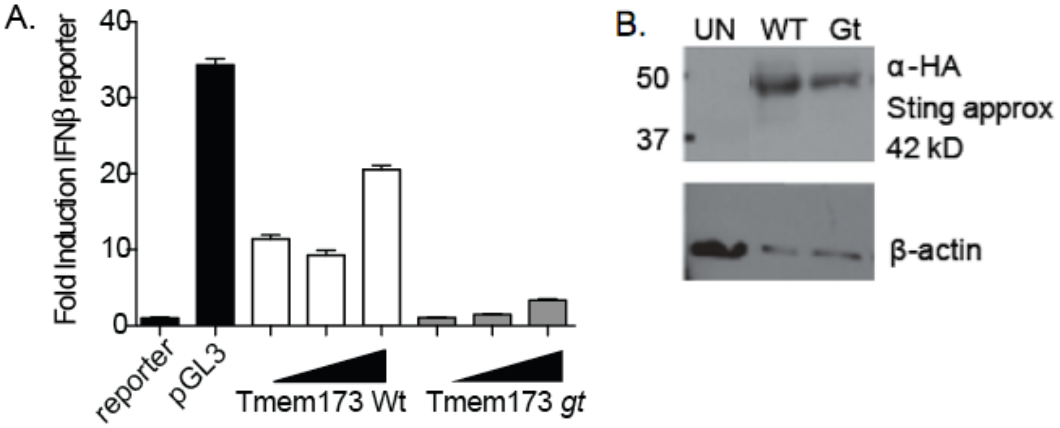

Figure 4.2 T596A mutation abrogates the function of Sting. (A) Luciferase production was measured 24 hours after co-transfection of 293T cells with an IFNβ luciferase reporter and increasing concentrations (50ng, 100ng, 200ng per well) of wild type or *gt Sting*. pGL3 is a positive control luciferase expression vector. (B) Whole cell lysates were collected from 293T cells 24 hours after transfection with 200ng/ml of wild type (WT) or *gt Sting*. Sting was detected using anti-HA antibody and β-actin was used as a loading control.

We tested whether bone marrow macrophages from *Sting*$^{gt/gt}$ mice responded to transfected c-di-GMP and found a complete abrogation of *IFNb* (Figure 4.3A). Interestingly, we found *Sting*$^{-/-}$ macrophages phenocopied the *gt* mutants (Figure 4.3B). *In vitro* results with purified c-di-AMP also demonstrated complete abrogation of *Ifnb* in both *gt* mutants and *Sting*$^{-/-}$ macrophages (Sauer et al., 2011). Therefore, these results suggest that *Sting* is an essential host component required for recognition of cyclic di-nucleotides.

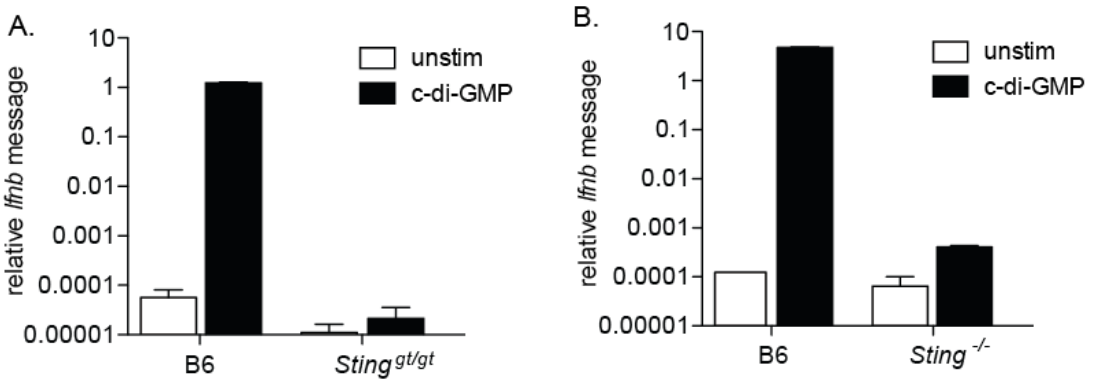

Figure 4.3 *Sting* is required for *Ifnb* induction upon stimulation with cyclic di-GMP. *Sting*$^{+/+}$, (A) *Sting*$^{gt/gt}$ or (B) *Sting*$^{-/-}$ bone marrow-derived macrophages were treated with 4μg/ml (A) c-di-GMP for 4 hours. RNA was harvested and *Ifnb* transcripts were measured relative to *rps17*, respectively.

To ensure that the *Sting*^{gt} mutation was the causative mutation leading to loss of cyclic di-nucleotide responsiveness, we transduced immortalized *Sting*$^{+/+}$ or *Sting*$^{gt/gt}$ macrophages with either WT *Sting* or *gt Sting* and assessed *IFNb* levels upon stimulation with c-di-GMP and c-di-AMP. WT *Sting* complemented *Sting*$^{gt/gt}$ macrophages restoring the *IFNb* response to stimulation with both c-di-GMP (Figure 4.4A) and c-di-AMP (Figure 4.4B). As expected, transduction with *gt Sting* did not restore responsiveness in *Sting*$^{gt/gt}$ macrophages to stimulants. These results suggest that the *gt* lesion is the causative mutation responsible for the loss of signaling in response to cyclic di-nucleotides, and that no other linked mutations can account for the phenotype.

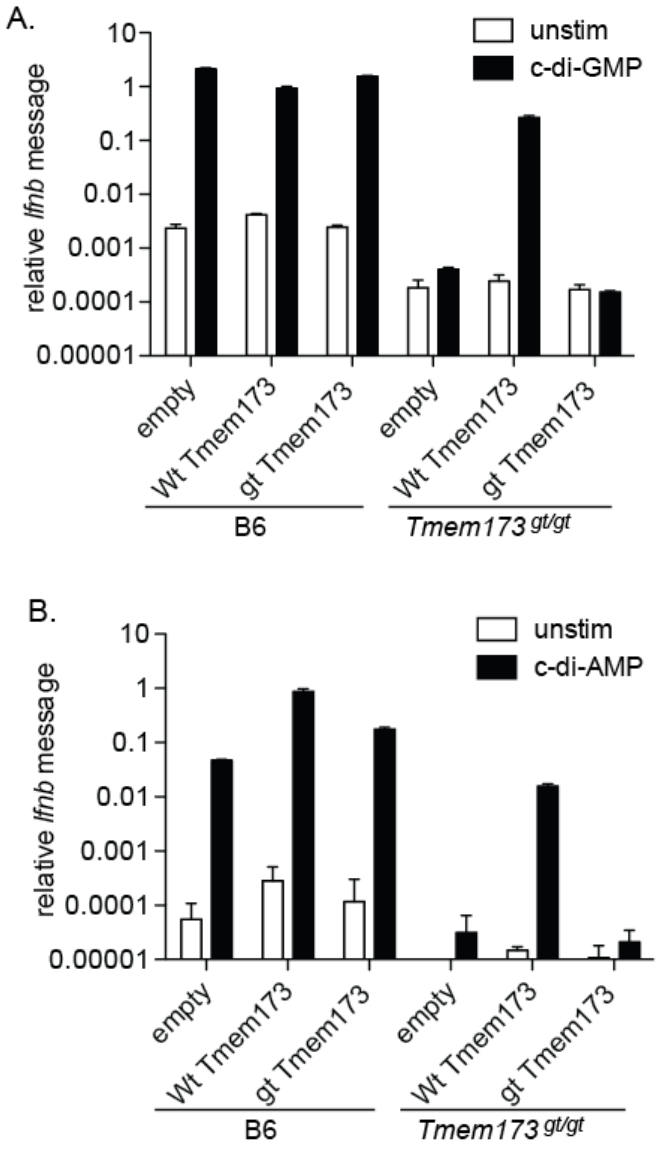

Figure 4.4 Wild type *Sting* restores *IFNb* response to cyclic di-nucleotides in *Sting*$^{gt/gt}$ mutant macrophages. *Sting*$^{+/+}$ or *Sting*$^{gt/gt}$ immortalized bone marrow-derived macrophages were transduced with empty MSCV2.2 vector, or wild type (WT) *Sting,* or goldenticket (*gt*) *Sting*. Transduced cells were stimulated with 4μg/ml of (A) c-di-GMP or (B) c-di-AMP for 4 hours. RNA was harvested and *IFNb* transcripts were measured relative to *rps17*. *IFNb* message induction is statistically significantly higher (*, $p<0.05$) in wild type *Sting* transduced macrophages compared to *gt Sting*.

Sting is reported to be required for the sensing of cytosolic DNA (Ishikawa et al., 2009; Sun et al., 2009). We tested whether the complemented macrophages were rescued for their inability to respond to cytosolic DNA. Macrophages transduced with WT *Sting*, but not macrophages transduced with *gt Sting*, exhibited a normal *Ifnb* response to pAT and bacterial DNA (Figure 4.5A). Both $Sting^{+/+}$ and $Sting^{gt/gt}$ macrophages responded robustly to stimulation with pIC, demonstrating that other cytosolic sensing pathways are intact and capable of proper signaling (Figure 4.5B). Reduced responsiveness to Sendai virus was not rescued by WT *Sting* transduction suggesting that the observed defect is not due to the *gt* mutation and is probably due to an unlinked defect in the immortalized cell line (Figure 4.5B). Similarly, LPS responses in $Sting^{gt/gt}$ macrophages are reduced, but not complemented by WT *Sting* (Figure 4.5C).

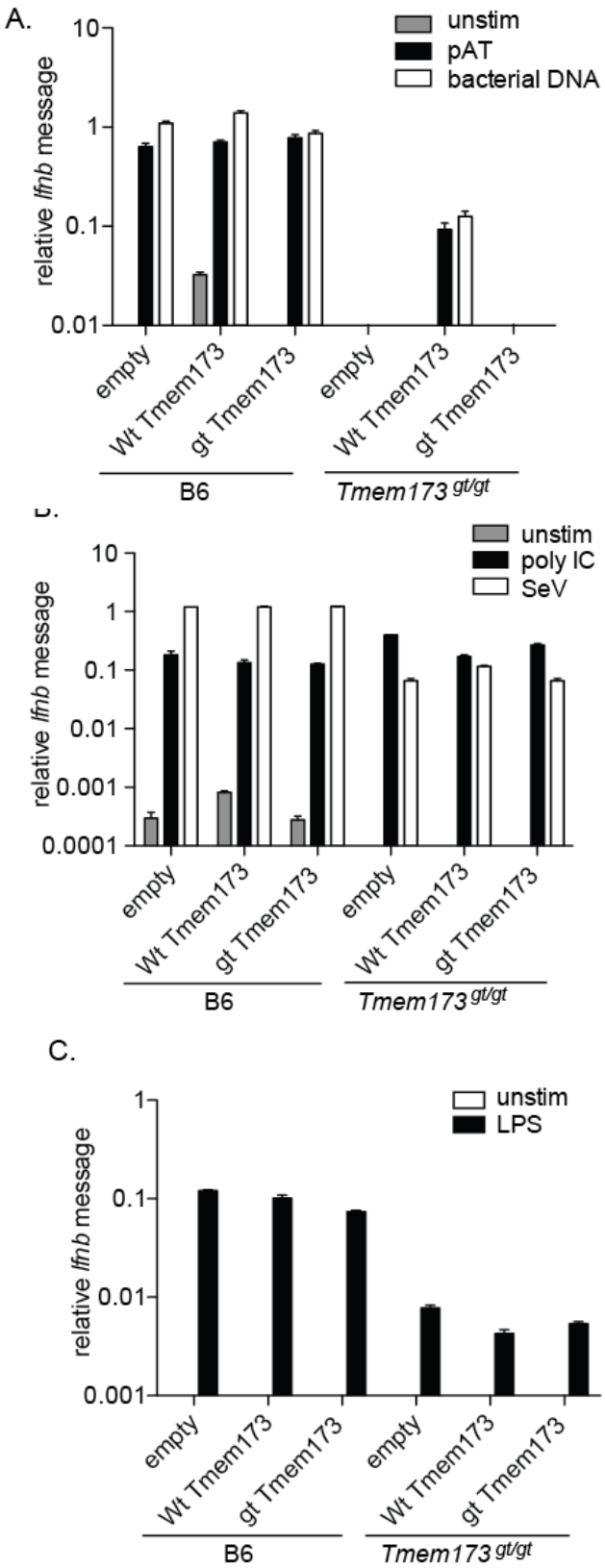

Figure 4.5 Wild type *Sting* restores *IFNb* response to transfected DNA. *Sting*$^{+/+}$ or *Sting*$^{gt/gt}$ immortalized bone marrow-derived macrophages were transduced with empty MSCV2.2 vector, or wild type (WT) *Sting,* or goldenticket (*gt*) *Sting*. (A) Transduced cells were transfected with 500μg/ml poly-dAT:dTA or 500μg/ml purified bacterial (*L. pneumophila*) genomic DNA for 4 hours, then RNA was harvested and *IFNb* transcripts were measured relative to *rps17*. (B) Transduced cells were transfected with 500μg/ml poly(I:C), infected with Sendai Virus at 150 HAU/ml or overlaid with 100ng/ml of LPS for 4 hours, RNA was harvested and *IFNb* transcripts were measured relative to rps17. Wild type *Sting* transduced macrophages induced a statistically significantly higher level of *IFNb* transcript (*, $p<0.05$, Student's t test) when stimulated with DNA, but not poly I:C, Sendai virus or LPS, when compared to *gt Sting* transduced macrophages.

4.3.3 Human THP-1 cells respond to cyclic di-nucleotide stimulation.

All of our characterization of innate immune sensing of cyclic di-nucleotides has been focused on the response in mice or murine cells. We were curious as to whether cyclic di-nucleotides elicit an IFN response in humans to determine whether this response has been maintained during human evolution and to provide relevance beyond the *mus musculus* model organism. HEK 293T cells do not induce an IFN response when stimulated with c-di-GMP (McWhirter et al., 2009). This is due to a notable characteristic of 293T cells in that they lack STING expression in certain lineages (D. Burdette, personal communication). Human cell lines tested that do not respond to c-di-GMP include A549 lung epithelial cells, U373 glioma cells, and Hela cells (K.M. Monroe, unpublished data). Despite these results, the THP-1 human peripheral blood monocyte line induces a potent *Ifnb* response to both c-di-GMP and c-di-AMP when delivered to the cytosol (Figure 4.6). Correlating with these observations, THP-1 cells robustly express STING, whereas Hela cells do not (Sun et al., 2009). In the future, it will be important to determine whether STING is required for the IFN response to cyclic dinucleotides in THP-1 cells, as we would predict.

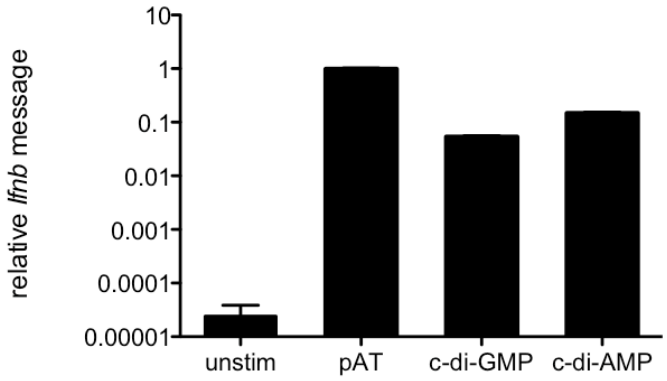

Figure 4.6 The human peripheral blood monocytic cell line, THP-1, induces *Ifnb* upon stimulation with cyclic di-nucleotides. THP-1 cells were treated with PMA (1μg/ml) for 24 hours, media was replaced and cells incubated for 24 hours before stimulation with nucleic acids. pAT (1.0 μg/ml) and c-di-GMP (4.0 μg/ml) were transfected with Lipofectamine 2000. c-di-AMP (6.7 μM) was delivered with digitonin permeabilization solution. RNA was harvested 4 hours post stimulation and assessed for *Ifnb* levels by qRT-PCR.

4.3.4 Sting functions as the innate immune sensor of c-di-GMP.

A question of great interest to us was: what is the direct host sensor of cyclic dinucleotides? To this end, we sought to reconstitute the IFN response to cyclic dinucleotides in HEK293T cells. As previously discussed, HEK293T cells do not respond to c-di-GMP and do not express STING. Since our previous results demonstrated that *Sting* is required for the IFN response to cyclic dinucleotides, we

transfected HEK293T cells with WT and *gt Sting* and measured their IFN response to c-di-GMP. To our surprise, low levels of WT *Sting*, but not *gt Sting,* expression was sufficient to restore responsiveness to c-di-GMP, but not to DNA (Burdette et al., submitted). This suggested to us that STING alone or STING acting with an endogenous protein(s) could function as a c-di-GMP sensor. Importantly, this experiment separated the c-di-nucleotide sensing pathway from the (non-pAT) DNA sensing pathway, since in either case (STING functioning alone or acting with an endogenous protein), it was not enough to restore responsiveness to DNA. We next tested whether STING could bind radiolabeled c-di-GMP (c-di-GMP^{32}) and found that WT STING, but not empty vector nor *gt* STING expressing cell lysates identified a 42kD band, the mass of a STING monomer (Burdette et al. submitted). Further evidence supporting that STING specifically binds c-di-GMP, we showed that immunoprecipitated STING-HA, but not empty vector nor *gt* STING, binds radiolabeled c-di-GMP, furthermore, binding can be competed with cold c-di-GMP and c-di-AMP, but not with cold DNA (Burdette et al., submitted).

STING is a multi-transmembrane domain containing protein, with a large globular C-terminal domain. It has been reported to reside on the ER membrane (Ishikawa and Barber, 2008; Sun et al., 2009) and in a conflicting report, on the outer mitochondrial membrane (Zhong et al., 2008). The topology prediction programs SOSUI, TMHMM, HMMTOP, and TMPRED suggest that there are 5 transmembrane (TM) domains (Figure 4.7A), although there is disagreement in the literature about whether there are 4 or 5 TM domains (Ishikawa and Barber, 2008; Sun et al., 2009; Zhong et al., 2008). The orientation of STING in the membrane is currently unknown. To determine the minimum domain required for STING's responsiveness to cyclic dinucleotides, we generated a series of truncations from both the N- and C-terminus (Figure 4.7B,C).

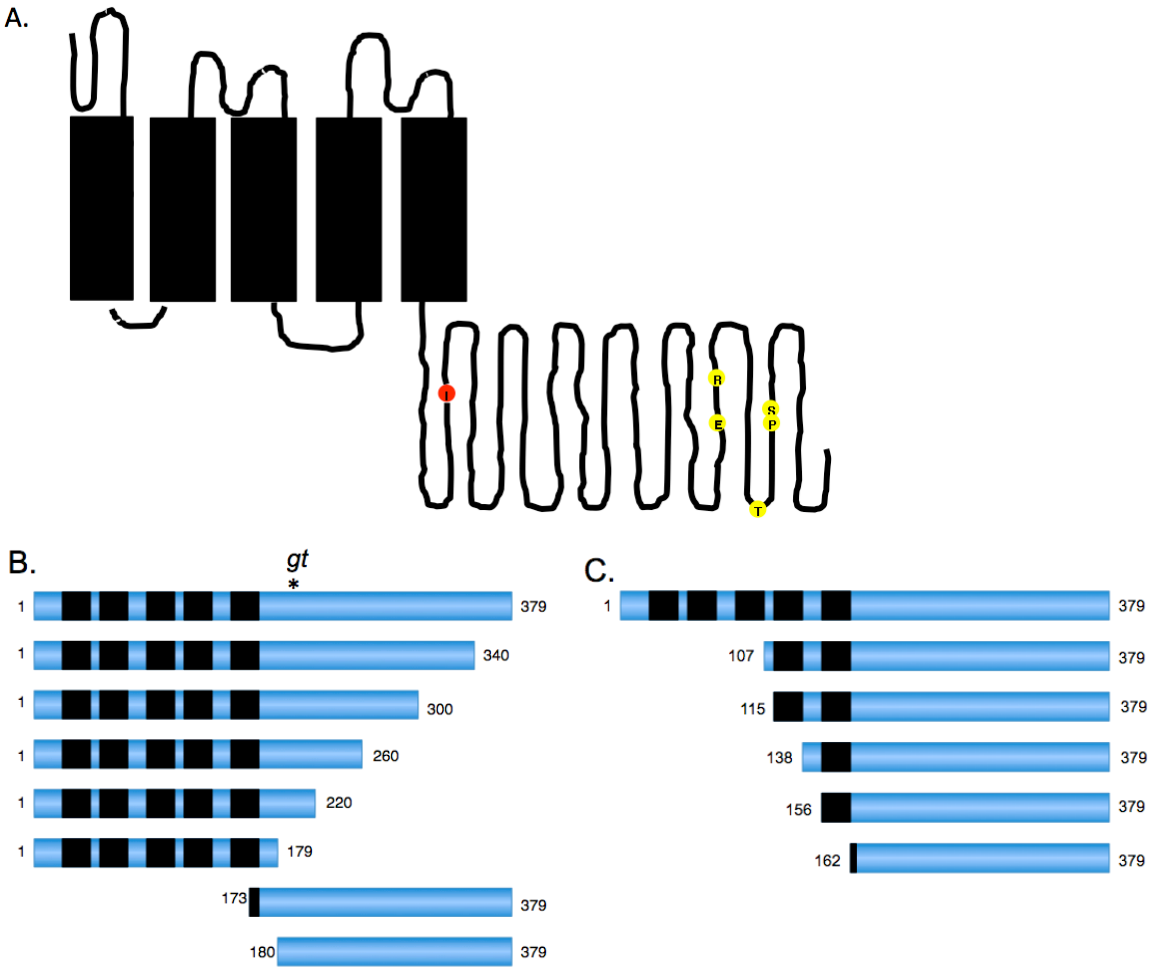

Figure 4.7 Predicted STING topology and diagram of STING N- and C-terminal truncation constructs. (A) Protein topology predication programs SOSUI, TMHMM, HMMTOP, and TMPRED suggest that STING is a 5 pass transmembrane domain protein with a large globular C-terminal domain. Black rectangles indicate TMs. The *gt* mutation occurs in amino acid 199 (red circle) and is predicted to be in the C-terminal globular domain. The regions of STING homology (which are not statistically significant) to the *Listeria* diadenylate cyclase (DAC) are indicated in yellow. (B) Diagram of C-terminal STING truncations and C-terminal only constructs, which were cloned into the mammalian expression vector pcDNA3.1. Full length (FL) STING is 379 amino acids. TM domains are noted in black. (C) N-terminal truncations of STING transmembrane domains, which were cloned into the mammalian expression vector pcDNA3.1.

First, we assessed the C-terminal truncations ability to induce IFN when overexpressed in HEK293T cells along with a luciferase IFN reporter. Full length (FL) Sting induces more than 100-fold induction of the luciferase IFN reporter when overexpressed in HEK293T cells (Figure 4.8A,B). However, only the most conservative C-terminal truncation (1-340aa) induces the IFN reporter and at low levels (6-fold induction) (Figure 4.8A). The reduced signaling is likely due to lower expression of the 1-340 truncation compared to WT STING (Figure 4.8B). When subjected to a radiolabeled c-di-GMP binding assay, only the 1-340 truncation binds c-di-GMP with similar robustness to WT STING (Figure 4.8C) (D. Burdette). The 1-340 truncation suggests that both binding and signaling can occur in the absence of the last 39 amino acids, which seem to impart stability to the protein. All other C-terminal truncations are not expressed at any detectable level, therefore, do not activate the IFN reporter (Figure 4.8A,B). This data suggests that the C-terminus is required for protein stability and thus expression. The C-terminal only constructs (173-379 and 180-379) are expressed at comparable levels to WT STING, however, do not activate the IFN reporter because they do not bind c-di-GMP (Figure 4.8A,B,C). We hypothesize that even though the C-terminal only constructs are well expressed, they may not be able to properly fold to generate a c-di-GMP binding pocket. Overexpression of the *gt* allele of *Sting* does not activate the IFN reporter, despite being expressed at detectable levels (Figure 4.2A,B). $STING^{GT}$ does not bind c-di-GMP (Figure 4.8C), explaining the lack of responsiveness (D. Burdette).

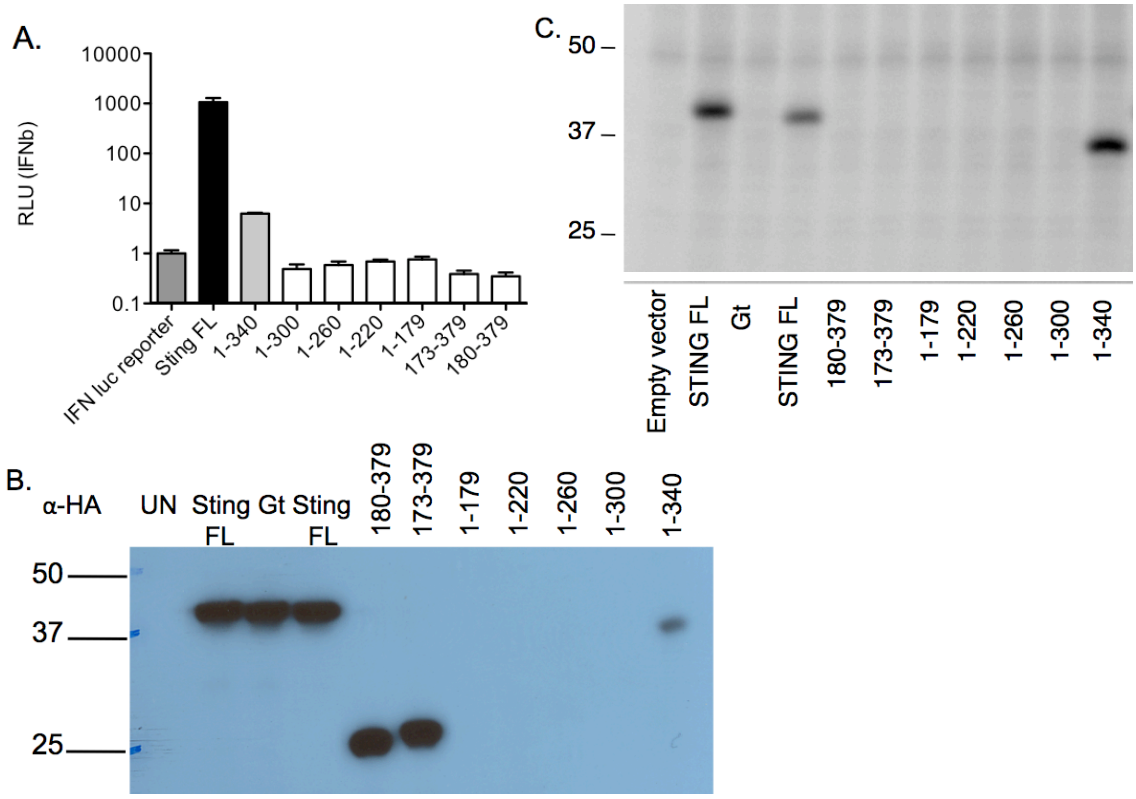

Figure 4.8 C-terminal STING truncation results in loss of IFN induction, protein expression, and c-di-GMP binding. (A) HEK293T cells were transfected with deletion constructs of the C-terminus of STING (50ng per 96 well) with an IFN-luciferase reporter. Activation of IFN was measured by relative light units (RLU) of luciferase and normalized to constitutive expression of TK Renilla. Fold induction over the reporter is shown. (B) Transfected HEK293T cell lysates were subjected to western blot analysis of the C-terminal deletions of STING (α-HA) after separation on SDS-PAGE. (C) HEK293T lysates transfected as in (A) were used in an *In vitro* UV crosslinking binding assay with radiolabeled c-di-GMP. Crosslinked lysates were separated on SDS-PAGE and detected on a phosphor screen (autoradiograph).

Since truncating the C-terminus of STING did not provide much insight into the minimum domain or fragment required to respond to cyclic dinucleotides, we took a hint from the binding assay with STING^{gt}. We reasoned that the C-terminal domain likely contains the residues necessary to bind cyclic di nucleotides. Additional data incriminating the C-terminal domain is that regions of homology to the *Listeria* DAC reside there, however, the homology is not statistically significant and the relevance of this homology is unclear at present. More interestingly, mass spectrometry identified a number of residues in the C-terminal domain that were crosslinked to c-di-GMP (D. Burdette, personal communication). Therefore, we set out to generate a soluble C-terminal fragment capable of binding c-di-GMP. To this end, we designed and

produced N-terminal truncations of STING (Figure 4.7C). Characterization of the N-terminal deletion constructs found that none are able to induce IFN (Figure 4.9A), despite being robustly expressed (Figure 4.9B). However, we identified a soluble N-terminal deletion (138-379aa), which binds c-di-GMP (Figure 4.9C) (D. Burdette). Expression and purification of this C-terminal domain in *E.coli* showed that it is sufficient to bind c-di-GMP in the absence of other host proteins (Burdette et al., submitted).

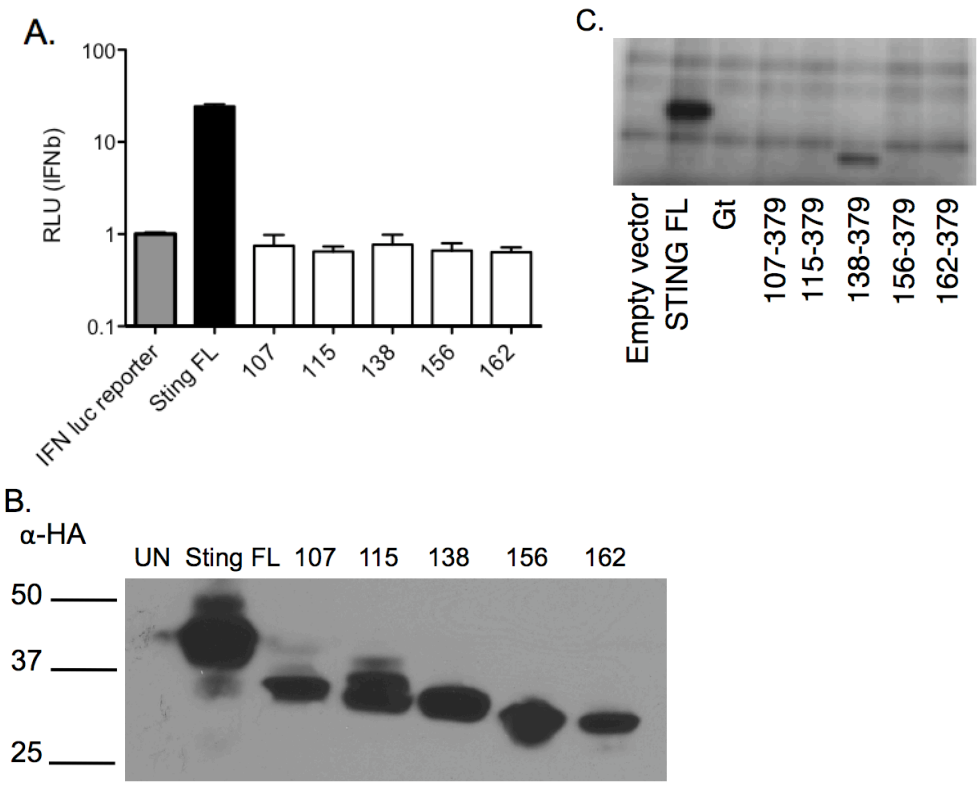

Figure 4.9 Identification of a soluble C-terminal fragment of STING (138-379) that binds c-di-GMP. (A) HEK293T cells were transfected with various deletions of the N-terminus of STING (50ng per 96 well) with an IFN-luciferase reporter. Activation of IFN was measured by relative light units (RLU) of luciferase and normalized to constitutive expression of TK Renilla. Fold induction over the reporter is shown. (B) Transfected HEK293T cell lysates were subjected to western blot analysis of the N-terminal deletions of STING (α-HA) after separation on SDS-PAGE. (C) HEK293T lysates transfected as in (A) were used in an *In vitro* UV crosslinking-binding assay with radiolabeled c-di-GMP. Crosslinked lysates were separated on SDS-PAGE and detected on a phosphor screen (autoradiograph).

Future work will need to focus on identifying the residues of the C-terminal domain of STING that are required to bind c-di-GMP. It is important to note that human STING also binds c-di-GMP (D. Burdette, data not shown). Therefore, we hypothesize that it will be fruitful to investigate conserved residues between mouse and human STING. More extensive studies need to be carried out to understand the complexities

of the protein dynamics of STING. What is the function of the transmembrane domains? Immunosensors responding to cytosolic stimulants are as a rule, until now, cytosolic proteins that are recruited to localized adapters upon ligand binding. While the 138-379 C-terminal domain of STING is sufficient to bind c-di-GMP, it is incapable of signaling (Figure 4.9A). It has been reported that the N-terminal transmembrane domains are required for dimerization and signaling (Sun et al., 2009). It is also possible that the transmembrane domains have another function, such as formation of a channel. Structural studies will be difficult due to the TM domains, but will likely prove informative. The soluble C-terminal domain shows that it alone is required for binding c-di-GMP, while the truncations of the C-terminal domains suggest that parts of this region are important for protein stability/expression. Further mutational analysis will hopefully help dissect the functions of STING's domains and roles in innate immune sensing pathways. Additionally, the dual function of Sting as a cyclic dinucleotide sensor, and DNA sensor adapter is novel. How does STING function in these dual roles? One ongoing goal of mutational analysis of STING is to dissect the cyclic dinucleotide sensing function from the DNA sensing pathway. This mutant would ideally no longer respond to c-di-GMP, but respond to cytosolic DNA or vice versa. Taken together, these data demonstrate that STING directly binds cyclic di nucleotides to function as a direct mammalian innate immune sensor.

4.3.5 Attempt at generating a model c-di-GMP sensed bacteria.

Efforts to identify a bacterial species that is sensed via c-di-GMP have yielded negative results. Therefore, in the absence of a known bacterial species that induces IFN via c-di-GMP, I made an effort to demonstrate that c-di-GMP can be sensed from bacteria in the context of infection. The idea was to show that c-di-GMP could be sensed in the host cell cytosol in manner that requires transport via a key virulence factor, in this case, the type 4 secretion system (T4SS) of *Legionella pneumophila*. To this end, I electroporated wild type *Legionella* and *Legionella* lacking the T4SS, also known as the *dot* apparatus, with either an empty vector or a vector containing *vdcA,* a diguanylate cyclase from *Vibro cholera*, which is known to robustly synthesize c-di-GMP (Tamayo et al., 2008). All *Legionella* strains used in this study were in a Δ*flaA* background to reduce pyroptotic cell death of macrophages. After antibiotic selection for transformed *Legionella* strains, I infected *MyD88/Trif*$^{-/-}$ macropages and assessed *Ifnb* induction (Figure 4.7). I found that *Legionella* overexpressing VdcA hyperinduced IFN in a manner that requires the T4SS (Figure 4.10). 2D-TLC demonstrated that strains containing the VdcA construct robustly produced c-di-GMP compared to *Legionella* with empty vector (H. Carlsson, data not shown). These results suggest that c-di-GMP can be transported through the T4SS and sensed in the host cell cytosol. However, there are a few caveats to this approach that complicate the interpretation of these results. The *Legionella* strains that overexpress VdcA display altered filamentous morphologies some of the time. It is known that c-di-GMP plays a role in biofilm formation, which may be occurring. Despite this, VdcA expression leads to hyperinduction of IFN in a *dot*-dependent manner regardless of normal or altered bacterial morphologies. In these strains, numerous components of the T4SS appear normally expressed at the protein level (K.M. Monroe and D. Burdette, data not shown).

It remains a possibility that expression of VdcA has physiological effects on the bacteria that we have not measured. For instance, bacteria could be lysing and releasing genomic DNA, which could be cause of IFN hyperinduction.

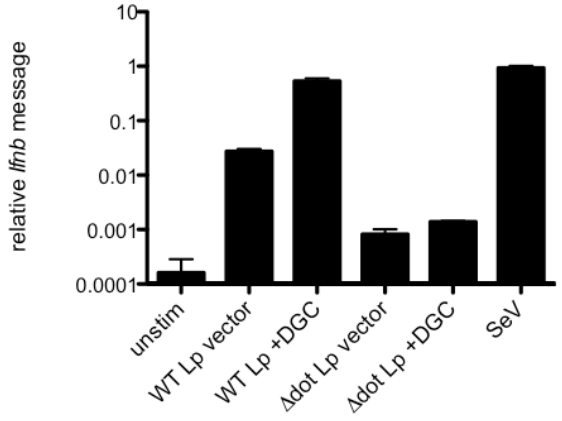

Figure 4.10 *Legionella pneumophila* expressing a *Vibrio cholera* diguanylate cyclase (DGC) hyperinduces IFN in a *dot*-dependent manner. *Legionella* strains were grown in 1mM IPTG overnight and used to infect *MyD88/Trif*$^{-/-}$ macrophages at an MOI of 1, RNA was harvested 4 hours post infection, and qRT-PCR was performed to assess *Ifnb* expression. *Ifnb* transcripts were normalized to *rps17*. Sendai virus was infected at 150 HAU/ml.

We hypothesized that *Sting* would be required to sense wild type *Legionella* +VdcA. To test this idea, we infected *Sting*$^{gt/gt}$ macrophages with the strains described in Figure 4.10 and assessed *Ifnb* expression (Figure 4.11). The *Ips-1* pathway that recognizes wild type *Legionella* without VdcA (see Chapter 2) is not required to sense VdcA expressing *Legionella* (K.M. Monroe, data not shown). We found that *Sting* is required to sense *Legionella* +VdcA (Figure 4.11). However, both DNA (pAT) and c-di-GMP signal in a manner that requires *Sting,* and we are currently unable to genetically distinguish the two pathways. Therefore, while the *Sting*-dependent, *dot*-dependent IFN induced by *Legionella* +VdcA is probably due to c-di-GMP sensing, we can not rule out that DNA or other bacterial ligands may play a role in IFN induction in this experiment.

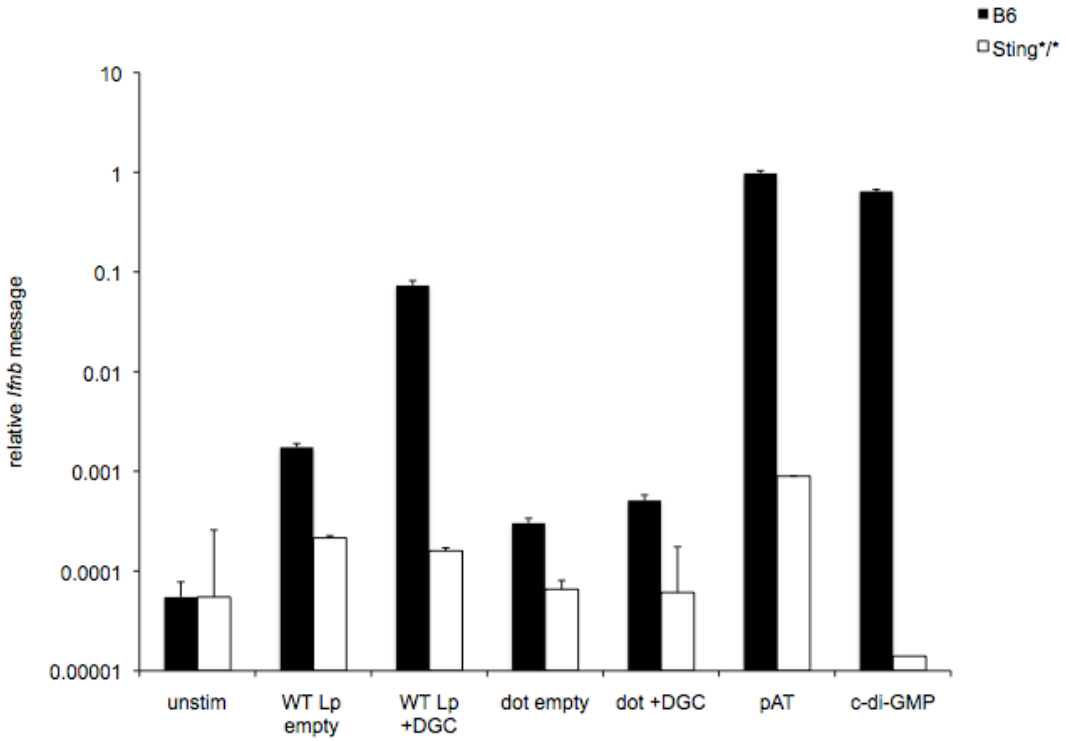

Figure 4.11 *Sting* is required for IFN induction during infection with *Legionella* expressing a *Vibrio cholera* diguanylate cyclase. *Legionella* strains were grown overnight with 1mM IPTG to induce DGC expression. C57BL/6 and *Sting*$^{gt/gt}$ macrophages were infected at an MOI of 1, RNA was harvested 4 hours post infection, and assessed for *Ifnb* expression. *Ifnb* message was normalized to *rps17*.

4.4 Acknowledgements

This project has been a collaboration of many people's efforts all along the way. This work would not have been possible without the persistence of Katia Sotelo-Troha, John Demian Sauer, Sky Brubaker, Jakob von Motlke, and Chris Rae who all performed the daily hard work on the ENU mutagenesis screen in mice. I would also like to thank Russell Vance for the guts to embark on such an undertaking and chance/luck for targeting the mouse genome in such a serendipitous manner. I would like to thank Sarah McWhirter and Roman Barbalat for their initial characterization of the host response to cyclic di-GMP. I would like to acknowledge Dara Burdette for her heroic efforts in identifying the host sensor of cyclic-di-GMP/AMP. I also thank Josh Woodward and Dan Portnoy for the insight into the identification of cyclic-di-AMP as the relevant *Listeria monocytogenes* IFN-inducing molecule.

Chapter 5.

Conclusions, Perspectives, and Future Directions

The work presented in this thesis focuses on understanding innate immune detection of bacterial nucleic acids. In Chapter 2, I demonstrate that a cytosolic RNA detection pathway, widely characterized to respond to viral infection, senses bacterial infection with *L. pneumophila*. Only virulent *L. pneumophila* with a functional type IV secretion system triggers a type I IFN host response, thereby enabling the innate immune system to distinguish virulent bacteria from avirulent bacteria. Surprisingly, I found that *L. pneumophila* encodes a suppressor of the IFN host response, a translocated effector called SdhA. In Chapter 3, I investigated the hypothesis that *Legionella's* T4SS can translocate bacterial RNA into the host cell cytosol where it triggers host RNA sensors. Using purified ligands, I showed that host mitochondrial RNA, as well as *Legionella* RNA activates type I IFN. Total host RNA did not activate a type I IFN response. To begin to determine the identity of the IFN-activating ligand during infection with T4SS^{+} *Legionella*, I immunoprecipitated endogenous human RIG-I, and found IFN-stimulation was greater during infection with T4SS^{+} *Legionella* compared to T4SS^{-} *Legionella*. In future work, I plan to identify these RIG-I bound RNAs by deep sequencing. In Chapter 4, I present my contributions to investigating the host type I IFN response robustly induced by bacterial signaling molecules cyclic dinucleotides. In collaboration with many Vance lab members, I contributed to the finding that a multi-pass transmembrane host protein, STING, is the direct sensor of cyclic dinucleotides.

As highlighted in Chapter 1, the role of type I IFNs in response to bacterial pathogens is still unclear and is an area of much needed study. In some instances, type IFN signaling restricts bacterial growth, however, there are instances where IFN induction is detrimental to the host and leads to increased pathogen replication (Auerbuch et al., 2004; Henry et al., 2010b). Notably, the latter outcome is typically to bacteria that have had an opportunity to evolve the ability to evade or manipulate innate immune responses. In the case of *Legionella*, the bacteria's life cycle does not allow for selection of evasive mechanisms in its mammalian host. Thus *Legionella* activates multiple innate immune sensing pathways, and type I IFN signaling is required in conjunction with these other pathways for bacterial restriction (Coers et al., 2007). I consider the innate immune system to be a generalist, in that a few conserved receptors recognize general classes of conserved molecules called pathogen associated molecular patterns (PAMPs) (Janeway, 1989). More recently these molecules are being referred to as microbial associated molecular patterns (MAMPs), since they are found on commensal as well as pathogenic microbes (Didierlaurent et al., 2005). In contrast, the adaptive immune system utilizes the specificity and diversity of one unique receptor per cell to generate B and T cell receptors that are highly specific to a wide array of antigenic peptides to target foreign invaders. While the innate immune system strives to deal with a battery of pathogens that it encounters with a limited set of receptors, it has been documented in the literature that some host responses are inappropriate for curbing pathogens and can even break self-tolerance to drive

autoimmunity. For instance, type I IFN response to *Listeria* is detrimental to the host (Auerbuch et al., 2004), and TLR7, 8, and 9 have been implicated in driving systemic lupus erythematosus (Baccala et al., 2007). Therefore, future studies should focus on elucidating downstream effects of the type I IFN response during pathogenic bacterial infections and determining how to direct responses towards benefitting the host.

Many questions come to light by the data presented in this thesis. In Chapter 2, I characterized the translocated effector protein, SdhA, as a suppressor of the host type I IFN response. I found that SdhA suppresses the cytosolic RNA sensing pathways activated by wild type *Legionella*. Many questions still remain regarding the mechanism of action of SdhA as an IFN suppressor, in addition to its uncharacterized role in suppressing host cell death (Laguna et al., 2006; Monroe et al., 2009). Does SdhA interact with RIG-I/MDA5 and/or MAVS at the mitochondria? Infection of cells with *Legionella* lacking *sdhA* clearly leads to disruption of the mitochondria (Laguna et al., 2006). My overexpression studies hinted at a role for SdhA interacting with both RIG-I and MDA5 since suppression of the IFN-luc reporter was seen. However, further attempts to address this question ran into difficulties with levels of SdhA expression. In addition, protein domain prediction programs are not currently able to define any domains, other than a small coiled-coil, in SdhA, which is a large protein with 1429 amino acids. Therefore, truncations of the protein are difficult to begin to tackle. SdhA has a key role in the pathogenesis of *Legionella* in that deletion of the gene renders *Legionella* incapable of growth in macrophages (see Chapter 2). For a bacteria that utilizes >200 effector proteins, rarely is a phenotype this striking seen upon loss of one effector (Laguna et al., 2006). There is certainly much work to be done to decipher the mechanism of action of SdhA, however, understanding this effector will likely prove very interesting and rewarding.

In Chapter 3, I demonstrate the ability to isolate IFN-stimulatory RIG-I-bound RNA, which only furthers the interest in the identity of these RNA molecules. I found that both *Legionella* RNA and mitochondrial RNA *can* induce a host IFN response when purified and transfected into macrophages. Now the question remains: what species is actually bound to RIG-I during infection? It may be that only one RNA species interacts with RIG-I or that both bacterial and mitochondrial RNA species bind and activate RIG-I during T4SS^{+} *Legionella* infection. I speculate that during infection with wild type *Legionella*, the majority of the RNA bound to RIG-I is bacterially derived, but that perhaps during infection with *ΔsdhA L. pneumophila* mitochondrial RNA is readily released from the mitochondria enabling it access to RIG-I. By deep sequencing the RIG-I-bound RNAs, I hope to be able to identify the origin of the endogenous RNA ligands, and if found to be of bacterial origin, identify whether a particular RNA species is translocated across the T4SS.

In Chapter 4, I presented my contributions to dissecting the host type I IFN response to cyclic dinucleotides. In this work, I collaborated with members of the Vance lab to identify STING as the direct host sensor of cyclic dinucleotides. While this work is a significant advancement for the field, there are many interesting questions left to address. One question of great interest is what is the biologically relevant cyclic dinucleotide? The current thinking is that *Listeria* is sensed via MDR-dependent transport of c-di-AMP into the host cell cytosol (Woodward et al., 2010) where it binds

STING to activate type I IFN (see Chapter 4, Burdette et al, submitted). Studies from the Vance lab initially identified c-di-*GMP* as a robust inducer of type I IFN (McWhirter et al., 2009). The predicted levels of c-di-GMP transported by *Listeria* MDRs are below the limit of detection by mass spectrometry, however other data point to c-di-AMP as the relevant cyclic dinucleotide during *Listeria* infection (J. Woodward, personal communication). Since c-di-GMP is widely used amongst bacterial species as a signaling molecule, I hypothesize that it does contribute to innate immune detection of a pathogen. However, this bacterial species remains to be identified. Understanding whether either c-di-AMP alone or both cyclic dinucleotides play biologically relevant roles in stimulating the innate immune system during infection with pathogenic bacteria is important to determine.

The relevance of cyclic dinucleotides during viral infections has not yet been explored to my knowledge. A publication from 1991 demonstrated that HIV integration generates cyclic dinucleotides that are hetero-dinucleotides (G-T) (Engelman et al., 1991), unlike the bacterial signaling molecules. A few interesting questions are brought forth by this observation: 1. Do cyclic hetero-dinucleotides elicit a host type I IFN response? 2. Can cyclic hetero-dinucleotides bind and activate STING? 3. Do cyclic hetero-dinucleotides, which are generated during integration of nuclear genomic DNA, ever gain access to STING? Positive answers to these questions would open up a whole new level of importance for the cyclic dinucleotide-STING interaction.

I identified THP-1s as a cyclic dinucleotide responsive human cell line. It will be important to characterize this response. The most pressing question to address is do human cells respond to cyclic dinucleotides via the same pathway and receptor we have identified using mouse STING? I predict that human *Sting* is required for the type I IFN response to cyclic dinucleotides since it can bind STING (D. Burdette, personal communication). Hopefully utilizing the conserved residues in human and mouse STING will provide insight into identifying the residues that function as the cyclic dinucleotide binding pocket.

Finally, there is much to learn about how STING functions as a direct sensor of cyclic dinucleotides, and moreover, how it parses its dual roles in cyclic dinucleotide and DNA sensing. Additionally, the DNA sensor(s), which are predicted to function upstream of STING, are not defined, nor well understood. The domain structure of STING, which contains 3-5 transmembrane domains depending on the program used, is particularly peculiar for a sensor of cytosolic stimulants. It is not yet clear the orientation of STING in the membrane. I predict that the globular C-terminal domain, which is sufficient to bind c-di-GMP, faces the cytosol in whichever membrane it resides. The N-terminal transmembrane domains have been suggested to function in dimerization of STING (Sun et al., 2009), however, how this works is not understood. It has been suggested that STING is phosphorylated by TBK-1 (Zhong et al., 2008), but how the downstream components of the signaling pathway interact with STING to transduce a signal are not yet clear. Importantly, the intracellular compartment that STING signals from or the dynamics of the protein when responding to infection are also not understood. Better understanding STING's mechanism of action will bring clarity to many innate immune sensing pathways since it plays a role in sensing cyclic

dinucleotides, RNA and DNA (Ishikawa et al., 2009; Sauer et al., 2011; Zhong et al., 2008).

These studies dissect the pathways by which the innate immune system activates type I IFN during infection with non-viral pathogens. The work presented here sheds light on functions of the innate immune system and mechanisms of bacterial pathogenesis. By studying the innate immune system, we obtain a better understanding of both organisms engaged in the initial host-pathogen interactions that can either lead to disease or resolution of an infection. While cytosolic RNA sensors have been well characterized in response to viral infections, the breadth of their role in responding to bacterial pathogens is illustrated by my work. Our group's elucidation of the role of STING as the direct host sensor of cyclic dinucleotides defines a key interaction in the fields of innate immunity and bacterial pathogenesis.

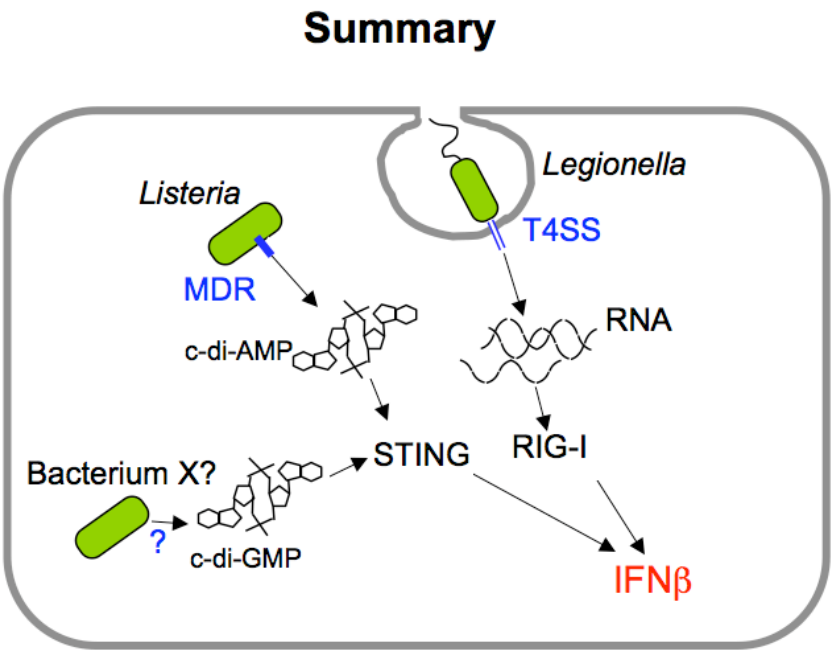

Figure 5.1 Summary model. This diagram summarizes the current model of *Legionella* and cyclic dinucleotide sensing. Insights from my thesis work, which focused on elucidating mechanisms by which bacteria activate a type I IFN innate immune response, contributed to this model.

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