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Hot or Not? Novel Mechanisms of Innate Immune Discrimination Between Pathogens and Non-Pathogens

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Novel Mechanisms of Innate Immune Discrimination Between Pathogens and Non-Pathogens

By

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Abstract

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The innate immune system responds to infectious threats by detecting specific molecular structures conserved among microbes, such as bacterial lipopolysaccharide or flagellin. However, these conserved molecules are found on harmless and pathogenic microbes alike. In order to discriminate between harmful and harmless microbes, it has been proposed that the innate immune system may also sense 'patterns of pathogenesis', the disruptions to host physiology orchestrated specifically by pathogens to infect, replicate within, and spread among their hosts. Immune recognition in plants is known to be based in part upon recognition of specific pathogen-associated activities, but few analogous examples have been described in mammals.

The intracellular bacterial pathogen *Legionella pneumophila* can infect macrophages in the mammalian lung, causing a severe inflammatory pneumonia called Legionnaires' Disease. For virulence, *L. pneumophila* requires a Dot/Icm Type IV secretion system that translocates bacterial effectors to the host cytosol. In this dissertation, *L. pneumophila* was used as a tool to reveal two novel immunosurveillance mechanisms that can discriminate between virulent and avirulent bacteria. The two distinct pathways integrate detection of both microbial molecules and pathogen-associated activities to generate specific responses to Dot/Icm$^+$ *L. pneumophila*.

The first of these novel mechanisms leads to a potent transcriptional response, termed the 'Effector-Triggered Response' (ETR), in macrophages infected with virulent *L. pneumophila*, but not an avirulent Dot/Icm$^-$ mutant. I demonstrate that this unique transcriptional response is due to secretion of five bacterial effector molecules that inhibit host protein synthesis. Upon infection of macrophages with Dot/Icm$^+$ *L. pneumophila*, these five effectors caused a global decrease in host translation, thereby preventing synthesis of IκB, an inhibitor of the NF-κB transcription factor. Thus, macrophages infected with wildtype *L. pneumophila* exhibited prolonged activation of NF-κB, which was associated with transcription of ETR target genes such as *Il23a* and *Csf2*. *L. pneumophila* mutants lacking the five effectors still activated TLRs and NF-κB, but because the mutants permitted normal IκB synthesis, NF-κB activation was more transient and was not sufficient to fully induce the ETR. Translation inhibition also activated other host pathways, including MAP kinase signaling. *L. pneumophila* mutants expressing enzymatically inactive effectors were also unable to fully induce the ETR, whereas multiple compounds or bacterial toxins that inhibit host protein synthesis via distinct mechanisms recapitulated the ETR when administered with TLR ligands. Thus, a a pathogen-encoded activity, namely translation inhibition, can elicit a specific immune response, both in cultured macrophages and *in vivo*.

The second novel mechanism consists of two different TNF-inducible inflammasomes that initiate an inflammatory host cell death, called pyroptosis, in macrophages infected with virulent *L. pneumophila* but not with an avirulent Dot/Icm$^-$ mutant. One of these inflammasomes begins with the previously reported detection of bacterial flagellin by the host proteins Naip5 and Nlrc4, but then leads to the activation of a novel downstream 'death effector'. The other

inflammasome involves activation of the protease Caspase-11 by cIAP1, a host protein that has not previously been implicated in inflammatory death. This latter form of cell death is antagonized by a bacterial effector, SdhA, which is required for growth of *L. pneumophila* within macrophages. The data presented here are consistent with a model in which increased host cell death upon infection with *L. pneumophila* aids in restriction of bacterial growth within these macrophages. The activity of these novel inflammasomes may explain the long-standing observation that TNF is crucial for complete restriction of *L. pneumophila* growth in macrophages.

Previous studies have demonstrated that the host response to bacterial infection is induced primarily by specific microbial molecules that activate TLRs or cytosolic pattern recognition receptors. Our results add to this model by providing several striking illustration of how the host immune response to a virulent pathogen can also be shaped by pathogen-encoded activities, such as (1) inhibition of host protein synthesis and (2) delivery of ligands to the cytosol via specialized secretion systems. Elucidation of these immunosurveillance pathways increases our understanding of how the innate immune system may integrate multiple signals to sense a microbe, determine whether that microbe is a pathogen, and finally generate an appropriately scaled response.

For
Robert and Lori Fontana, who taught me to be curious
and for
Jakob von Moltke, a scientist after my own heart.

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Chapter One:
An Introduction to Pathogen Recognition in Metazoans

1.1. Strategies of the innate immune system.

The innate immune system serves as the first line of host defense against pathogens. More ancient in evolutionary history than the adaptive immune system, and thus more widely distributed among metazoans, innate immune pathways are activated quickly upon exposure to infectious microbes. The two primary functions of the innate immune system are (i) to induce inflammation and other acute responses that may restrict and contain an infection until adaptive immunity develops; and (ii) to aid in the induction and the direction of an effective adaptive response.

In mammals, the innate immune system senses infection primarily through the use of germline-encoded pattern recognition receptors (PRRs) that recognize conserved microbial molecules, such as bacterial peptidoglycan or 5' triphosphate-containing viral RNA. PRRs can be grouped into several classes based on structure and localization. Membrane-bound Toll like receptors (TLRs) monitor the extracellular and endosomal spaces (1), while the structurally distinct Nucleotide Binding Domain/Leucine Rich Repeat Containing Receptors (NLRs) (2, 3), Rig-I like receptors (RLRs) (4), and Aim2 like receptors (ALRs) (5) detect microbial components in the cytosol. A unifying property of each of these families is their recognition of pathogen associated molecular patterns, or PAMPs: molecules that are broadly distributed among specific classes of microbes, but absent in the host (6). By targeting microbial molecules, the host is able not only to distinguish microbe from self, but also to glean information about the class of microbe present, and thereby respond in an effective manner. For example, PRRs that recognize viral nucleic acid motifs tend to elicit production of cytokines called Type I Interferons, which promote an anti-viral state.

A large body of research has demonstrated the central role of microbial pattern recognition in the induction of the innate immune response to pathogens. However, most of the microbes that we encounter from day to day are not normally pathogenic; for example, we are constantly exposed to trillions of commensal bacteria in the gut. An important task of the immune system, therefore, may be to distinguish between pathogens and non-pathogens, mounting effective responses to the former while avoiding unnecessary and potentially damaging responses to the latter. Yet the PRR-PAMP model of innate immune recognition, while providing the basis for distinction between microbe and self, does not offer a straightforward mechanism to distinguish pathogens from commensals. PAMPs are found on non-pathogenic and pathogenic microbes alike, and consequently, even commensal microbes are capable of activating PRRs (6). Thus, it has been proposed that additional innate immune mechanisms may exist to discriminate between pathogens and non-pathogens (7-9).

In plants, selective recognition of pathogens is accomplished by detection of the enzymatic activities of "effector" molecules that are delivered specifically by pathogens into host cells. Typically, the effector is an enzyme that disrupts host cell signaling pathways to the benefit of the pathogen. Host sensors monitoring or "guarding" the integrity of the signaling pathway are able to detect the pathogen-induced disruption and initiate a protective response. This mode of innate recognition is termed "effector-triggered immunity" (10) and represents a significant component of the plant innate immune response. In principle, mammalian innate recognition of pathogen-encoded activities—termed "patterns of pathogenesis" in metazoans (7)—could act in concert with PRRs to distinguish pathogens from non-pathogens, and to mount qualitatively distinct responses that are commensurate with the potential threat. However, few if any examples of effector-triggered immunity have been described in metazoans.

In this work, I will demonstrate that the mammalian innate immune system does indeed mount a specific response to a pathogen-encoded activity. I will also present evidence for novel interactions between innate immune pathways that recognize molecules, and those that respond to pathogen-encoded activities; and I will show that these interactions have important

consequences for the quality and magnitude of the immune response. Before turning to these novel findings, however, it will be helpful to review previously published work that may hint at other examples of recognition of pathogen-encoded activities in metazoans.

1.2. Effector-triggered immunity in metazoans.

Several groups have postulated the existence of metazoan surveillance mechanisms that respond to pathogen-associated activities (7-9), but until recently, there was little evidence to support these speculations. In the last few years, however, multiple papers have documented specific innate immune responses that depend on the activity of pathogen-derived toxins or effector proteins, which themselves target vital host processes such as cytoskeletal remodeling and ion flux across membranes. In some cases, these 'effector-triggered' responses act in concert with classical PRR signaling to induce robust, qualitatively distinct responses to virulent bacteria and viruses. While few, if any, of these responses have been worked out in mechanistic detail, they do provide evidence for effector-triggered immunity as a general mode of innate immune surveillance in animals. Importantly, the "patterns of pathogenesis" discussed below could potentially aid in the detection of multiple classes of pathogens, including intracellular and extracellular bacteria and viruses.

1.2.1. Detection of cytoskeletal remodeling.

Multiple bacterial and viral pathogens manipulate the host actin cytoskeleton in order to avoid phagocytosis by immune cells or, alternatively, to invade and move within host cells. Thus, cytoskeletal dynamics make a logical candidate for monitoring by the innate immune system. In particular, a common host target for pathogens is the Rho family of GTPases, whose members direct cytoskeletal dynamics. Several groups have now demonstrated innate immune responses to bacterial effectors or toxins that constitutively activate members of the Rho GTPase family, including the *Salmonella typhimurium* effectors SopE, SopE2 and SopB (11) and the uropathogenic *Escherichia coli* toxin Cytotoxic Necrotizing Factor-1 (CNF-1) (12). These innate immune responses, which include activation of NF-κB and MAP kinases and secretion of cytokines, are independent of TLR and NLR signaling and, in the case of *S. typhimurium*, require a functional bacterial secretion system (11). It is perhaps significant that these studies were done in epithelial cells, which are not specialized immune cells and do not express a wide complement of PRRs. While many epithelial cells do possess some TLRs, their expression is often spatially restricted to the basal membrane, which may minimize inappropriate recognition of gut commensals (13). Thus, 'effector-triggered' responses might be particularly important (and non-redundant) in epithelial cells, since other modes of pathogen recognition are somewhat restricted. Interestingly, it has been proposed that *S. typhimurium* may actually induce intestinal inflammation as a survival strategy, resulting in decreased competition from commensals (14). Thus, as for classical PRR-based immune recognition, effector-triggered immune responses may be exploited by pathogens in order to gain a survival advantage (11, 14).

A further role for the cytoskeleton in innate immune detection has been suggested, though not conclusively demonstrated, by studies on the cytosolic NLR proteins Nod1 and Nod2. These closely related proteins recognize cytosolic fragments of bacterial peptidoglycan (PGN) (di-amino-pimelic acid and muramyl dipeptide, respectively) and activate host transcription via their common adaptor, Rip2 (15-19). Several papers have reported physical and functional interactions between the Nods, the actin cytoskeleton, and the plasma membrane, that may have consequences for pathogen recognition (reviewed in (20)). Both Nod1 and Nod2 appear to associate with the plasma membrane in resting epithelial cells. This association requires an intact actin cytoskeleton, since pharmacological perturbation of actin polymerization abrogates membrane association (21, 22). Furthermore, physical interactions have been observed between between Nod2 and the small GTPase Rac1 (22), which regulates multiple cell processes including cytoskeletal remodeling. Interestingly, pharmacological inhibitors of actin polymerization actually enhance the responses of both Nod1 and Nod2 to purified ligands (21, 22), while point mutants of Nod1 that cannot localize to the plasma membrane also fail to activate NF-κB (21) and other downstream pathways (23-25). Additionally, in epithelial cells

infected with the invasive pathogen *Shigella flexneri*, Nod1 relocalizes to membrane sites of bacterial internalization, where it associates with proteins involved in NF-κB activation (21) and autophagy (23-25). Taken together, these observations suggest that dynamic associations with both the plasma membrane and the cytoskeleton are important for Nod signaling, although the precise roles of each interaction remain to be dissected (20).

It is not yet clear whether these interactions between Nods and the cytoskeleton are actually important for pathogen recognition. However, given the number of bacterial pathogens (*e.g. Listeria monocytogenes, Shigella flexneri, Rickettsia sp.*) that manipulate host actin, it is tempting to speculate that perturbation of the cytoskeleton may in fact be a "pattern of pathogenesis" that acts in concert with Nod recognition of peptidoglycan to trigger a strong immune response.

1.2.2. Detection of a viral ion channel.

Viruses are also experts in manipulating host cell biology, raising the possibility that they too are sensed indirectly by host pathways that monitor the integrity of the cell. Multiple groups have observed that influenza virus activates the NLR protein Nlrp3 in dendritic cells and macrophages, leading to release of the cytokine IL-1β (26-29). Ichinohe *et al*. (30) recently reported that the activation of Nlrp3 by influenza appears to require the activity of the viral protein M2, a proton channel that plays multiple roles in the influenza replication cycle. It is not yet understood how M2 activity results in Nlrp3 activation, however.

1.2.3. Summary.

While a body of evidence is beginning to accumulate demonstrating that the mammalian innate immune system can respond to pathogen-encoded activities, none of the responses here reported have been worked out in molecular detail. In most or all cases, the precise nature of the "signal" sensed by the innate immune system is not clear; nor are the signal transduction pathways that result in transcriptional and other responses downstream. A more complete picture of an "effector-triggered response" is necessary to understand both the mechanics and the importance of this novel mode of innate immune recognition.

1.3. *Legionella pneumophila*: a model intracellular pathogen.

Historically, the experimental use of model antigens and purified PAMPs has yielded a broad understanding of PRR-based immune surveillance pathways. However, pathogens are defined in large part by their ability to evade and manipulate these same pathways (9). Thus, in order to understand how the immune system detects and combats a virulent microbe—and in particular, to examine the role of pathogenic activities in immune recognition—it is vital to study the interactions between virulent microbes and the immune system in the context of an infection. For this purpose, we employ the gram-negative bacterial pathogen *Legionella pneumophila*.

Common in water sources throughout the world, *L. pneumophila* evolved as a parasite of freshwater amoebae. However, this opportunistic pathogen can also infect alveolar macrophages in the mammalian lung, causing a severe inflammatory pneumonia called Legionnaires' Disease (31). The ability to infect distinct cell types that have been separated by at least a billion years of evolution is perhaps surprising, but may be due to the fact that *L. pneumophila* targets highly conserved host cell pathways in order to survive and replicate.

Upon phagocytosis by a host amoeba or macrophage, *L. pneumophila* employs a Type IV secretion system, called the Dot/Icm system, to translocate over two hundred bacterial effector proteins into the host cytosol (32-34). These effectors manipulate various host cell processes, resulting in delayed fusion with lysosomes, recruitment of ribosomes and ER-derived vesicles to the bacterial phagosome, and formation of a replicative vacuole. While the Dot/Icm system is absolutely required for bacterial replication and virulence, it also may inadvertently deliver bacterial ligands to immunosurveillance pathways in the host cytosol (discussed in detail in **1.4**). Thus, the Dot/Icm system provides both a key determinant of pathogenicity, and a potential way for the immune system to discriminate between virulent and avirulent *L. pneumophila*.

The evolutionary history of *L. pneumophila* makes it an excellent model pathogen for revealing innate immune surveillance strategies in mammals (35). It is believed that *L.*

pneumophila is not transmissible from one mammal to another (36, 37). Because its evolution has occurred primarily or exclusively in amoebae, *L. pneumophila* appears not to have evolved significant immune-evasive mechanisms. Indeed, most healthy individuals mount a robust protective inflammatory response to *L. pneumophila*, resulting from engagement of multiple redundant innate immune pathways (38). We hypothesize, therefore, that *L. pneumophila* may reveal novel innate immune responses that better adapted pathogens may evade or disable (35). Once such a response is unmasked in *L. pneumophila* infection, we can study its role during infection with other, immune-evasive pathogens, where its involvement may have initially been less clear. This approach has led us to a broader understanding of immune recognition not only of *L. pneumophila*, but also of intracellular bacterial pathogens in general.

1.4. Innate immune recognition of *L. pneumophila*.

Multiple PRR-dependent innate immune surveillance pathways detect *L. pneumophila* infection in mammalian macrophages. These pathways can be divided into two classes: those that recognize both virulent and avirulent *L. pneumophila*, and those that respond specifically to pathogenic *L. pneumophila*, but not to an avirulent mutant deficient in Type IV secretion. These two classes of PRR differ primarily in their localization: the former class monitors the endosomal or extracellular space, while the latter is restricted to the cytosol, access to which a secretion-deficient bacterium lacks.

1.4.1. Extracellular and endosomal Toll like receptors.

Among the first category of PRRs that recognize *L. pneumophila* are several Toll like receptors, likely including TLRs 2, 5, and 9, all of which signal through the common adaptor Myd88. While TLR4 is an important sensor for many gram-negative bacteria, the atypical LPS of *L. pneumophila* contains long hydrophobic carbon chains that prevent efficient signaling through TLR4 (39). Instead, TLR2 appears to play the most important role in TLR-dependent recognition of *L. pneumophila*; it is strongly activated by lipoproteins associated with *L. pneumophila* peptidoglycan, resulting in production of TNF and other cytokines that aid in restriction of bacterial growth (39-44). Additionally, mouse alveolar macrophages can recognize *L. pneumophila* flagellin *in vivo* through ligation of TLR5 (45); however, TLR5 is not expressed on bone marrow derived macrophages and therefore does not sense *L. pneumophila* infection *in vitro*. In principle, TLR9 could signal in response to bacterial DNA, but its role in recognition of *L. pneumophila* appears redundant at most (46). On the whole, though, Myd88-dependent signaling plays a crucial role in host restriction of *L. pneumophila* growth, as both macrophages (40) and mice (38, 41, 46) that lack this adaptor protein are permissive to *L. pneumophila* replication. However, it is likely that much of the *Myd88*$^{-/-}$ phenotype, at least *in vivo*, is due to impairment in IL-1 signaling, which also requires Myd88 (42).

*1.4.2. Cytosolic Pattern Recognition Receptors***.**

A separate group of cytosolic PRRs responds specifically to virulent bacteria that are competent to perform Dot/Icm secretion. These pathways provide an interesting case study in attempting to understand how the innate immune system distinguishes pathogens from non-pathogens: while each of these PRRs recognizes a PAMP that is common to both virulent and avirulent strains of *L. pneumophila* (and other bacteria), immune recognition hinges upon delivery of the PAMP to its receptor in the host cytosol. Thus, *L. pneumophila*'s employment of the Dot/Icm system becomes a double-edged sword: in addition to its essential roles in bacterial replication and virulence, the secretion system also translocates bacterial PAMPs into the cytosol (38, 47-52). Meanwhile, secretion-deficient (and avirulent) bacteria do not trigger these pathways. Thus, the cytosolic localization of certain PRRs appears to comprise one mechanism for discrimination of pathogens and non-pathogens.

The Naip5/Nlrc4 inflammasome.

The first of these cytosolic immunosurveillance pathways was discovered by genetic analysis of mouse bone marrow derived macrophages that either supported or restricted the intracellular growth of *L. pneumophila* (53, 54). These experiments have led to the identification of an entire class of multiprotein complexes, termed inflammasomes, that are important for

pathogen recognition and restriction. Inflammasomes assemble in the macrophage cytosol when an NLR directly or indirectly recognizes its cognate ligand, which may be a bacterial or viral PAMP or a host-derived indicator of cell stress (55). Once assembled, inflammasomes serve as scaffolds for the recruitment and activation of the protease Caspase-1. In *L. pneumophila* infection, monomeric flagellin, translocated to the host cytosol via the Dot/Icm secretion system, activates an inflammasome containing the closely related NLRs Naip5 and Nlrc4, resulting in rapid Caspase-1-dependent macrophage death and release of the inflammatory cytokines IL-1β and IL-18 (48, 50, 52, 56-58). Macrophages that lack a functional Naip5 inflammasome are permissive for growth of *L. pneumophila*. Though the mechanism of bacterial restriction by the inflammasome has not been formally elucidated, it is thought that rapid inflammasome-dependent host cell death may serve to deprive the bacteria of their replicative niche, resulting in growth restriction.

Nucleic acid sensors and Type I Interferons.

The use of *L. pneumophila* as a model pathogen yielded early insight into the existence of inflammasomes. Similarly, early evidence for another novel class of cytosolic innate immune receptors came from infection studies with *L. pneumophila*: Stetson *et al*. observed that macrophages infected with *L. pneumophila* produced the cytokine Interferon-β (IFN) in a manner dependent on a functional Dot/Icm system, but independent of TLR signaling (51). Since the Dot/Icm system is evolutionarily related to bacterial conjugation systems, it was initially suggested that the Dot/Icm system might inadvertently translocate bacterial DNA into the host cytosol, where it might trigger an as yet unidentified DNA sensor. Subsequent research has shown that the cytosolic RNA sensors Mda5 and Rig-I, as well as their common adaptor Mavs, are involved in IFN induction by *L. pneumophila* (47, 49, 59). The precise nature of the bacterial ligand is still uncertain; although Mda5 and Rig-I sense RNA, two reports have described an unusual mechanism in which RNA Polymerase III can transcribe double-stranded DNA into an RNA ligand for Rig-I (47, 60). In any case, it appears likely that *L. pneumophila* induces IFN from the cytosol via multiple mechanisms.

Detection of peptidoglycan by Nod1 and Nod2.

In a third cytosolic pathway, the canonical NLRs Nod1 and Nod2 induce a transcriptional response to *L. pneumophila* peptidoglycan fragments via their common adaptor protein, Rip2 (16, 17, 61-64). In a wildtype host, the contribution of these sensors is probably modest; Nod1 and Nod2 perform somewhat redundant roles with TLRs during *L. pneumophila* infection both in macrophages and *in vivo* (46, 65). Yet while TLRs can recognize both wildtype *L. pneumophila* and the avirulent Dot/Icm$^-$ mutant, the cytosolic Nod1 and Nod2 specifically respond to virulent *L. pneumophila* that can access the cytosol via a functional Dot/Icm system (65-67). Several studies have noted a role for Nod1 and/or Nod2 in *L. pneumophila* infection in the mouse lung, although Myd88-dependent pathways (likely to include both TLR and IL-1 signaling) are also crucial (38, 41-43, 45, 46). Frutuoso *et al*. (68) observed that neutrophil recruitment to *L. pneumophila*-infected lungs was almost entirely dependent on Nod signaling, while several other groups have noted a more modest requirement for the Nods in both neutrophil recruitment and clearance of bacteria ((38, 66); M. Fontana and R. Vance, unpublished observations). The effects of Nod1 and Nod2 deficiency become much more pronounced in the absence of compensatory Myd88-dependent signaling; for example, *Myd88*$^{-/-}$*/Rip2*$^{-/-}$ mice, which lack both TLR and Nod signaling, are strikingly susceptible to low doses of *L. pneumophila*, while *Myd88*$^{-/-}$ mice are moderately resistant (38).

Addressing functional redundancy.

The activation of multiple redundant innate signaling pathways by *L. pneumophila* is perhaps to be expected, given the pathogen's lack of significant immune evasion mechanisms. The problem of redundancy sometimes makes it difficult to measure the contributions of any one innate immune pathway *in vivo*. Indeed, the activity of sensing pathways such as the Naip5 inflammasome, which efficiently (up to 1000-fold) restrict the growth of *L. pneumophila* in cultured macrophages, often yield much more modest (~10-fold) effects in the mouse lung.

Several approaches, including the use of gene targeting simultaneously in both mice and bacteria (an approach called "genetics squared" (69)) and the use of purified bacterial ligands, have in some instances allowed the characterization of host pathways in the absence of redundant signals. However, as will be seen throughout this work, host redundancy during *L. pneumophila* infection *in vivo* remains a challenge.

1.5. Dissertation overview.

In this chapter, I have broadly framed the questions that drive this dissertation, and described the system I used to address them. I will turn now to the data. In Chapter Two, I present a comprehensive characterization of the host transcriptional response to *L. pneumophila*. Using both macrophages and bacteria deficient in various factors (host TLR signaling, bacterial flagellin, Type IV secretion, and specific effectors) I determine the separate contributions of these factors to the host transcriptional response. This analysis leads to the identification of a previously unappreciated pathway that participates in *L. pneumophila* recognition.

Chapter Three uncovers the mechanism of this novel pathway, which we term the Effector-Triggered Response (ETR). Unlike previously described pathways, which recognize *L. pneuomophila* molecules, the ETR responds to a pathogen-encoded *activity*: namely, inhibition of host translation by *L. pneumophila*. This novel mode of innate immune detection leads to activation of host signaling pathways (*e.g.* NF-κB and MAP kinases), enhanced transcription of specific gene targets, and secretion of pro-inflammatory cytokines, and appears to be active both in cultured macrophages and *in vivo*.

Chapter Four turns to the question of how multiple innate sensing pathways can be integrated to elicit a specific response to a pathogen. Therein, I examine novel interactions between several innate immune sensing pathways that are known to restrict growth of *L. pneumophila* in macrophages and *in vivo*. Specifically, I present evidence that two novel TNF-inducible inflammasomes recognize *L. pneumophila* in infected macrophages, resulting in cell death and restriction of bacterial growth. These inflammasomes, each of which incorporates some known inflammasome constituents and at least one currently uncharacterized protein, add complexity to our understanding of inflammasomes as guardians of the cytosol. Furthermore, they provide an intriguing example of how "crosstalk" between distinct innate immune pathways can enhance the scale, specificity, and efficacy of the outcome.

In Chapter Five, I will discuss the results of my dissertation research, with a particular emphasis on integrating the models drawn from Chapters Three and Four. I will consider some questions that remain unanswered, and will close with a philosophical look at the innate immune system: what we have learned about its wiring, why it might have evolved this way, and what lines of questioning might bear fruit in the future.

Chapter Two:
The Host Transcriptional Response to *L. pneumophila*

2.1. Introduction.

Which pathways does the mammalian innate immune system use to recognize the model intracellular pathogen *L. pneumophila*? And can these pathways distinguish between virulent and avirulent *L. pneumophila* to tailor an appropriate immune response? These were the questions I set out to answer in my dissertation. While much research has focused on the Naip5/Nlrc4 sensing pathway, which does indeed respond specifically to virulent Dot/Icm$^+$ *L. pneumophila*, I wanted to widen the scope and look for evidence of other immune responses that were specific to pathogenic bacteria. I needed a method that was broad and unbiased, but also sensitive and quantifiable, in order to identify both qualitative and quantitative differences between the immune responses to wildtype and Dot/Icm$^-$ *L. pneumophila*. I therefore began with transcriptome-wide microarray analysis of the macrophage response to both virulent and avirulent *L. pneumophila*.

Two previous publications have also characterized the host transcriptional response to *L. pneumophila* using microarrays. The first of these studies was performed in a human macrophage cell line (70), while the second utilized bone marrow derived macrophages from mice deficient in TLR and Nod signaling (*Myd88*$^{-/-}$*/Rip2*$^{-/-}$; (67)). In contrast, I examined the host response in primary mouse macrophages with intact TLR and Nod signaling. As described below, I also used additional mouse and bacterial mutants to dissect host and microbial factors that modulate the transcriptional response. Thus, my microarray data provide an important complement to the previous studies.

Multiple immunosurveillance pathways are already known to recognize *L. pneumophila*. Some of these, such as Nod1/Nod2 (67) and the Rig-I/Mda5/Mavs/Irf3 axis (47, 49), respond specifically to secretion-competent *L. pneumophila* that can access the cytosol and (inadvertently) deliver ligands to cytosolic host receptors. Thus, at least some of the differences between the transcriptional responses to virulent and avirulent bacteria are likely to be explained by selective triggering of these known cytosolic pathways. After obtaining microarray data from macrophages infected with Dot/Icm$^+$ and Dot/Icm$^-$ *L. pneumophila,* I compared my target lists to existing microarray datasets that report both the transcriptional response to *L. pneumophila* (67, 70) and the canonical gene targets of individual cytosolic pathways (51, 67, 71). Through cross-referencing, I was able to assign many *L. pneumophila*-induced genes to one or more known innate immune pathways. This effort was aided by the use of both mice and bacteria deficient in specific factors: for example, infection of *Myd88*$^{-/-}$ macrophages revealed the contribution of Toll like receptors to the host transcriptional response, while infection with Dot/Icm$^-$ bacteria allowed identification of host genes whose induction depended on a functional bacterial secretion system.

Ultimately, the transcriptional analyses presented here led to identification of a subset of genes whose induction could not be explained by known innate sensing pathways. These "orphan" genes, robustly induced by pathogenic *L. pneumophila* but not by the avirulent Dot/Icm$^-$ strain, provided a readout for a novel host sensing pathway that incorporates recognition not only of bacterial molecules (which are largely shared between the Dot/Icm$^+$ and Dot/Icm$^-$ strains), but also of a pathogen-encoded activity that is unique to the virulent strain.

2.2. *L. pneumophila* induces genes involved in inflammation, vesicle trafficking, and more.

For the initial experiment, *Caspase1*$^{-/-}$ macrophages were infected with three strains of *L. pneumophila*: the wildtype strain LP02, a thymidine auxotrophic derivative of the clinical isolate strain LP01; the isogenic flagellin-deficient mutant *ΔflaA*, which is fully virulent and evades recognition of the Nlrc4/Naip5 inflammasome and subsequent macrophage death; and the avirulent, Dot/Icm$^-$ strain *ΔdotA,* also in the LP02 background. *Caspase1*$^{-/-}$ macrophages were used here to minimize flagellin-dependent macrophage death; however, results were later validated by quantitative RT-PCR in wildtype B6 macrophages.

Table 2.1. Host gene families induced by *L. pneumophila*.

Gene Function	Number of Genes	Examples
Actin Dynamics	4	RasGEF, RhoGAP
Antimicrobial Activity	7	antimicrobial peptides (defensin, cathelicidin); complement components
Modulation of Host Cell Death	5	*Bclaf1*, Fas, cIAP1
Chemokines and Chemokine Receptors	9	*Cxcl5, Ccl20, Cxcl2, Ccr1*
Cytokines	16	*Csf2, Il23a, Il12b, Il10, Il1a, Il6a*
Interferon-Related	12	*Ifnb, Irf1, Irf4, Ifit1, Gbp5, Mx1, Socs3*
Inflammation and Stress	12	*Ier2, Ier3, Egr1, Hif1, Hsp1a, Gadd45a, Gadd45b*
MAP Kinase Signaling	5	*Dusp1, Dusp2, Jdp2*
NF-κB Signaling	8	*Nfkbiz, Ikkb, Malt1, Tnfaip3*
Signal Transduction	15	*Traf1, Trim13, Irak3, Jak2, Rip2*
Cell-Cell Interactions	6	CD83, CD69, E-selectin
Tissue Remodeling	11	Plasminogen activator, *Mmp13*, Factor X
Vesicle Trafficking	3	RabGEF2, *Stx11*

After six hours of infection, microarray analysis revealed two-fold or greater induction of approximately 350 host genes in response to wildtype *L. pneumophila* (Appendix 1). Upregulated genes included cytokines and chemokines involved in inflammation and immune cell recruitment; macrophage surface markers; and stress-associated genes (Table 2.1). Overall, and unsurprisingly, *L. pneumophila* induces a gene signature similar to that induced by several other intracellular bacterial pathogens (71-73), characterized by upregulation of IFN-co-regulated genes (e.g. *Ifit1, Ifit2, Irf1, Gbp5, Mx1, Mx2*) and NF-κB -dependent genes (*e.g. Tnfa*, *Il6*, *Il1a/b*, *Sele*).

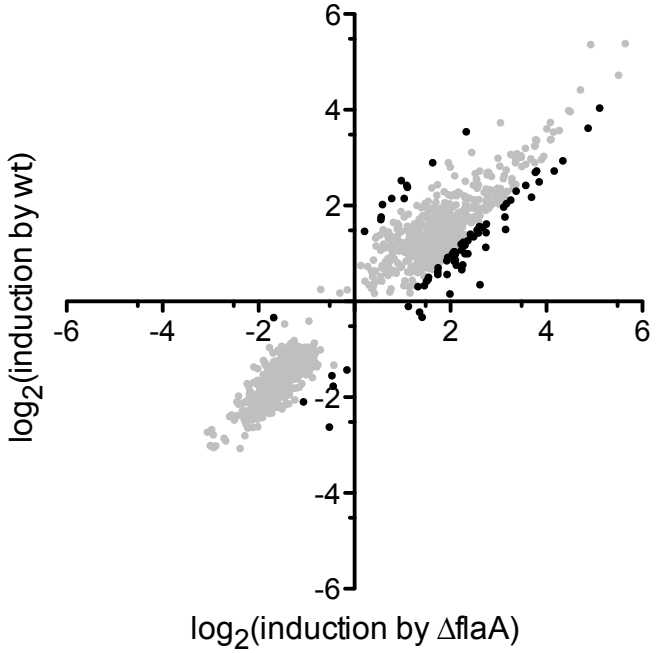

Figure 2.1. The transcriptional response of *Caspase1*$^{-/-}$ macrophages infected with wildtype or flagellin-deficient (*ΔflaA) L. pneumophila*. Black circles indicate genes with a twofold or greater difference in induction by wildtype and *ΔflaA*. Results from one microarray experiment, of two performed, are shown.

2.3. Flagellin does not induce a distinct transcriptional response.

We were curious to test whether flagellin, which triggers Caspase-1-dependent host cell death via the Nlrc4/Naip5 inflammasome, might also induce transcriptional responses in the context of *L. pneumophila* infection. However, comparison of the macrophage response to wildtype and flagellin-deficient bacteria revealed no gene targets that were specifically elevated in wildtype infection (Appendix 2 and Figure 2.1). Indeed, some genes were more highly expressed in *ΔflaA*-infected macrophages, presumably due to increased survival of the infected host cells. Since these macrophages did lack Caspase-1, it is formally possible that there is a transcriptional response to flagellin that is also dependent on Caspase-1 and that was therefore undetectable in this experiment. However, transcriptional responses downstream of Caspase-1 have never been clearly demonstrated, and we favor the interpretation that flagellin does not affect the transcriptional program of the infected cell. (The bone marrow derived macrophages used in these experiments do not express TLR5, a PRR that does induce transcription in other cell types in response to flagellin.)

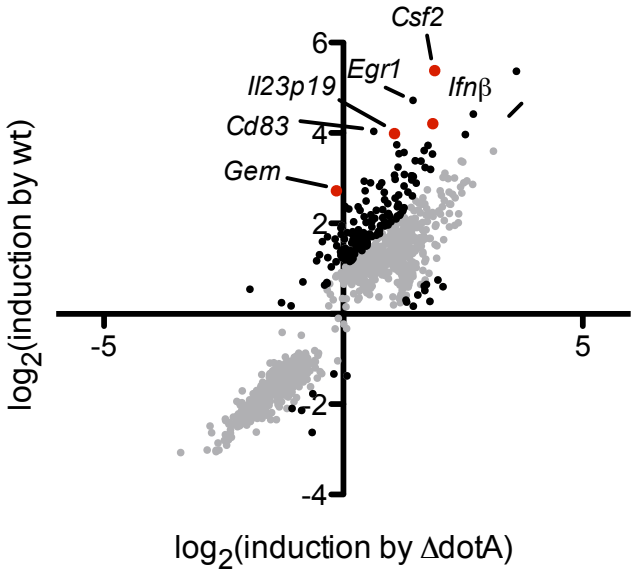

Figure 2.2.
The transcriptional response of *Caspase1*$^{-/-}$ macrophages infected with wildtype or Dot/Icm-deficient (*ΔdotA) L. pneumophila*. Black and red circles indicate Dot/Icm-dependent genes, with a twofold or greater difference in induction by wildtype and *ΔdotA*. Red circles indicate labeled genes. Two replicates were performed; results shown are from one set of arrays.

2.4. Induction of a subset of host genes is dependent on bacterial secretion.

By comparing the host transcriptional responses during infection with wildtype and avirulent bacteria, we identified approximately 150 genes whose induction required an intact Dot/Icm secretion system (Figure 2.2, and Appendix 3). In agreement with previously published work (51, 67, 70), this Dot/Icm-dependent cluster included the cytokine IFN-β and other IFN-related genes, which *L. pneumophila* is known to induce via the Mavs/Irf3 signaling axis (47, 49). Other Dot/Icm-dependent genes result from Nod1/Nod2-dependent recognition of *L. pneumophila* peptidoglycan (67). However, in addition to targets of these known PRRs, I identified a subset of Dot/Icm-dependent genes whose induction could not be explained simply by invoking known pathways. These genes included cytokines (e.g. *Il23a*, *Csf2*), surface markers of macrophage activation (e.g. *Sele, Cd86*), and stress response proteins (e.g. *Egr1*, *Egr3*, *Gadd45a*) as well as proteins of unknown function (e.g. *Gem*, *Rgs1*). Since we were ultimately interested in uncovering novel host pathways that recognize *L. pneumophila*, we were particularly interested in this cluster of genes. We reasoned that these "orphan" targets might represent the output of a novel host pathway that can distinguish between virulent and avirulent *L. pneumophila*.

2.5. Contributions of Toll like receptors to the transcriptional response.

In order to determine which transcriptional targets required TLR signaling for their induction, we infected Myd88-deficient macrophages with Dot/Icm^{+} and Dot/Icm^{-} bacteria. These macrophages lack most signaling downstream of the TLRs that recognize *L. pneumophila* (most significantly, TLR2 (39, 43, 74)). Since we previously determined that flagellin does not alter the host transcriptional response, we used flagellin-deficient bacteria in this experiment to minimize host cell death.

We predicted that most responses to *ΔdotA* bacteria (deficient in Type IV secretion) would be Myd88-dependent, since these bacteria can presumably only access PRRs at the extracellular and endosomal membranes. Indeed, we found that nearly all genes induced by *ΔdotA* in B6 macrophages were greatly diminished in *Myd88*$^{-/-}$ macrophages (Figure 2.3, and Appendix 4). In contrast, we hypothesized that most Dot/Icm-dependent responses would be Myd88-independent, stemming from cytosolic sensing pathways rather than TLR signaling. While this was largely the case (Appendix 4), we were surprised to find that some genes (*e.g. Il23a*, *Csf2*) were both Myd88- and Dot/Icm-dependent (Appendix 4, and see Chapter Three). These same genes had previously been noted as "orphan" transcriptional targets, induced by *L. pneumophila* via an unidentified host pathway. The dual requirement for TLR signaling and bacterial secretion further highlighted these genes as interesting targets for study; the mechanism of their induction, including the puzzling dependence on both extracellular PRRs and cytosolic access, is explored extensively in Chapter Three of this work.

A final observation to be made from these data is that some genes that are induced independently of Type IV secretion in a wildtype macrophage become Dot/Icm-dependent in a macrophage deficient in TLR signaling (Figure 2.3, and Appendix 5). We hypothesize that the Dot/Icm-dependent genes are the products of cytosolic sensing pathways; thus, it follows that genes that become secretion-dependent in the absence of TLR signaling are targets that can be induced *either* by TLR signaling *or* by a cytosolic pathway. Such redundancy between distinct immunosurveillance pathways is a common theme in studies of *L. pneumophila*. However, in this case, use of bacterial and host mutants has allowed us to dissect the contributions of each host pathway.

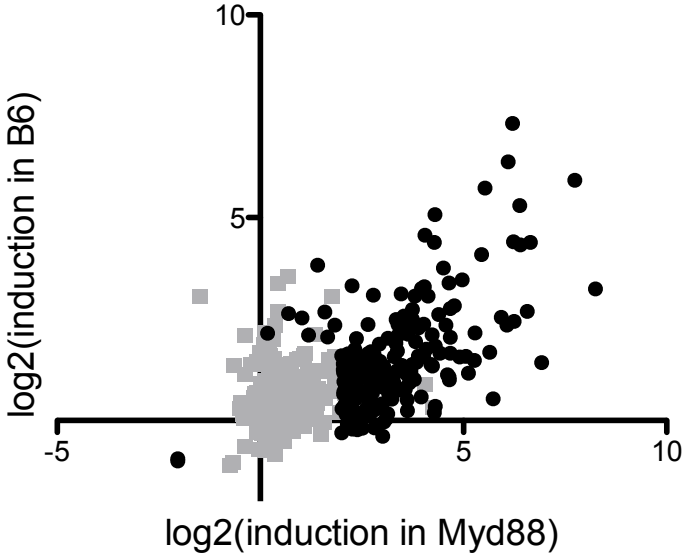

Figure 2.3. The transcriptional responses of wildtype (B6) or TLR-deficient (*Myd88*$^{-/-}$) macrophages to wildtype *L. pneumophila* (black circles) or a Dot/Icm-deficient mutant (grey squares). Results shown are from one experiment.

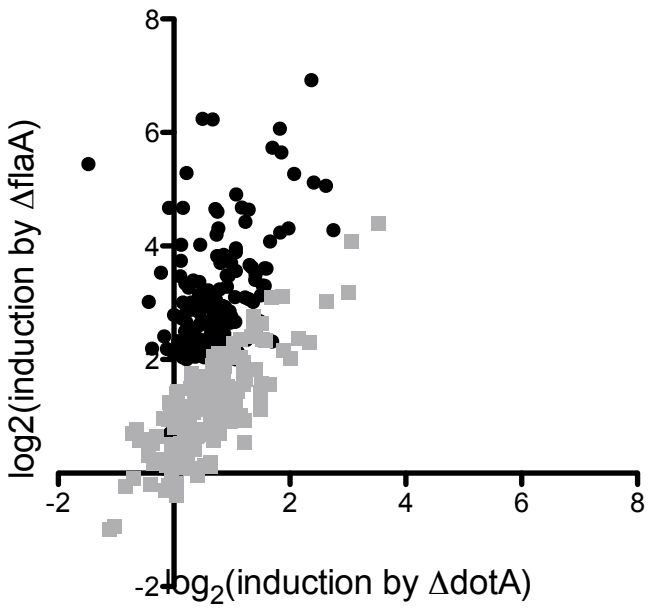

Figure 2.4. The host transcriptional responses of B6 (grey squares) or *Myd88*$^{-/-}$ (black circles) macrophages infected with *ΔflaA L. pneumophila* or the isogenic secretion-deficient mutant. Genes depicted are those with less than a 3-fold difference in induction by the two strains in B6 macrophages. Results are from one experiment.

2.6. Secretion of bacterial effectors shapes the host response.

L. pneumophila secretes upwards of 200 effector proteins into the host cytosol (32-34). Many of these effectors have known activities against host proteins, and some are reported to affect host transcription, for example through activation of the transcription factor NF-κB (75, 76). To assess the role of *L. pneumophila* effectors in induction of the host transcriptional response, we made use of a mutant bacterial strain that lacks the chaperone proteins IcmS and IcmW. These chaperones aid in secretion of most bacterial effectors, including some that are required for replication and growth in macrophages (77-79). Hence, the *ΔicmSΔicmW* mutant strain fails to secrete a large number of effectors, cannot replicate in macrophages, and is avirulent. Importantly, unlike the *ΔdotA* mutant, it does possess a functional secretion system, enabling it to translocate immunostimulatory ligands to the cytosol (51).

We infected B6 and Myd88-deficient macrophages with *ΔicmSΔicmW* mutant bacteria or their isogenic wildtype strain, LP01, and analyzed the host transcriptional response at six hours post infection—a time when bacterial burdens have not yet diverged between the two strains. Quantitative RT-PCR was used to confirm that the *ΔicmSΔicmW* mutants induced IFN-β, an indicator of functional type IV secretion (data not shown). By microarray analysis we identified approximately 20 genes that were induced at least fourfold more strongly by wildtype *L. pneumophila* than by the *ΔicmSΔicmW* mutant in B6 macrophages (Appendix 6). Interestingly, among these 20 genes were many of the same targets (e.g. *Sele, Gadd45a, Egr1*) that were not accounted for in previous characterization of innate pathways recognizing *L. pneumophila*. In *Myd88*$^{-/-}$ macrophages, the number of IcmS/IcmW-dependent genes increased to about 74, indicating that some genes can be induced either by TLR signaling or by a bacterial signal that requires the chaperone proteins IcmS and IcmW (Appendix 7).

The observation that macrophages make altered transcriptional responses to *ΔicmSΔicmW L. pneumophila* could be interpreted in several ways. IcmS and IcmW are required for secretion of many individual effectors, but more broadly, they are also required for formation of the *Legionella*-containing vacuole and for bacterial replication (79). Therefore, altered

recognition of *ΔicmSΔicmW* could be due to the lack of specific effectors, or the activities of those effectors; or it could be due to differential trafficking of these bacteria, or failure to replicate. The latter possibility is perhaps unlikely, since *L. pneumophila* undergoes little replication at the timepoint examined (six hours post infection). Even so, all of these alternatives allow the possibility that the host immune system responds to *L. pneumophila* by recognition of a pathogen-encoded activity, whether it is a specific activity encoded by one or a few effectors, or the multifactorial activities of vesicle trafficking and bacterial replication.

2.7. Discussion.

The microarray data presented in this chapter round out the existing literature, which currently consists of data obtained from *L. pneumophila* infection in a human macrophage cell line (70) and in mouse macrophages deficient in multiple immunosurveillance pathways (67). To these reports we can now add several useful contributions: we have characterized the transcriptional response to *L. pneumophila* in wildtype mouse macrophages; excluded a role for flagellin in induction of transcriptional responses; and determined the impact of the bacterial chaperone proteins IcmS and IcmW on the host transcriptome. Furthermore, by dissecting the contributions of known host sensing pathways, we have identified a cluster of "orphan" gene targets, which are not classical targets of any known pathway that recognizes *L. pneumophila*. These genes are Dot/Icm-dependent and require secretion of bacterial effectors, as evidenced by their defective induction during infection with Δ*icmS*Δ*icmW*. They contain both Myd88-dependent and Myd88-independent genes, indicating a partial role for TLR signaling in their induction. From these observations, it is not clear what the bacterial "ligand" or inducer of these genes may be; however, the requirement for secretion of bacterial effectors, many of which are enzymes that extensively manipulate host cell processes, suggests the tantalizing possibility that an *L. pneumophila*-encoded activity elicits this specific transcriptional response from the host.

Chapter Three:
An Innate Response to A Pathogen-Encoded Activity

3.1. Introduction

In the last chapter, I identified a cluster of transcriptional targets that were induced in wildtype macrophages by infection with Dot/Icm$^+$ *L. pneumophila*, but not an avirulent Dot/Icm$^-$ mutant. Among these were genes whose induction could not be explained solely by known PAMP-sensing pathways. In this chapter, I elucidate the host pathways that result in this unique transcriptional response. I demonstrate induction of these targets in response to a bacterial activity: specifically, the enzymatic activity of five secreted bacterial effectors that inhibit host protein synthesis. Effector-dependent inhibition of protein synthesis synergized with PRR signaling to elicit the full transcriptional response to *L. pneumophila*. The response to the bacterial effectors could be recapitulated through the use of pharmacological agents or toxins that inhibit host translation, administered in conjunction with a PRR agonist. Thus, our results provide a striking example of a host response that is shaped not only by PAMPs but also by a complementary "effector-triggered" mechanism that represents a novel mode of immune responsiveness in metazoans. Much of the data presented here were previously published in the journal *PLoS Pathogens* (65).

3.2. Validation of "orphan" gene targets and *in vivo* relevance.

Through the microarray data presented in Chapter Two, we identified a cluster of genes that were induced by *L. pneumophila* in a Dot/Icm-dependent manner, and that were not canonical targets of the cytosolic sensing pathways already known to sense *L. pneumophila* (namely, Nod1/Nod2, Mda-5/Rig-I/Mavs, and Nlrc4/Naip5). For reasons that will become clear further on in the chapter, we referred to this subset of genes as the 'Effector-Triggered Response,' or ETR. To facilitate further characterization of the ETR, we first selected several of the most highly induced genes for validation by quantitative RT-PCR. We confirmed that *Il23a*, *Csf2* and *Gem* transcripts were induced 100 to >1000-fold more by pathogenic wildtype *L. pneumophila* as compared to the Δ*dotA* mutant (Figure 3.1A). In subsequent experiments we focused on these three genes, as they provided a sensitive readout for this entire subset of transcriptional targets.

To assess whether targets of the ETR might be important during *L. pneumophila* infection *in vivo*, we infected B6 and *Il23a*$^{-/-}$ mice intranasally with *L. pneumophila*. *Il23a*$^{-/-}$ mice displayed a significant defect in host cell recruitment to the lungs 24 hours after infection (Figure 3.1B), consistent with the known role of IL-23 in neutrophil recruitment to sites of infection (80). The phenotype of *Il23a*$^{-/-}$ mice was not due to decreased bacterial burden in these mice (Figure 3.1B). Thus at least one transcriptional target of the ETR plays a role in the host response, though numerous redundant pathways clearly recognize *L. pneumophila in vivo* (38).

3.3. Known innate immune pathways are not sufficient to induce the full ETR.

To identify the host pathway(s) responsible for induction of the ETR, we first examined pathways known to recognize *L. pneumophila*. Induction of the representative genes *Il23a*, *Csf2*, and *Gem* did not require the previously described Naip5/Nlrc4 flagellin-sensing pathway (56), as infection with a flagellin-deficient mutant (Δ*flaA*) also robustly induced these genes (Figures 3.1A, 2.1 and Appendix 2). Moreover, *Il23a, Csf2* and *Gem* were strongly (>1000-fold) induced in the absence of the Mavs/Irf3/Irf7 signaling axis shown previously to respond to *L. pneumophila* (47, 49, 81) (Figure 3.1C, and data not shown). As previously reported (67), we confirmed that *Myd88*$^{-/-}$ and *Rip2*$^{-/-}$ macrophages, which are defective in TLR and Nod1/Nod2 signaling, respectively, strongly upregulated *Il23a* and *Gem* following infection with wildtype *L. pneumophila* (Figure 3.2A). Induction of *Il23a* was abrogated in *Myd88*$^{-/-}$ *Rip2*$^{-/-}$ and *Myd88*$^{-/-}$ *Nod1*$^{-/-}$ *Nod2*$^{-/-}$ macrophages; however, these macrophages still robustly induced *Gem* (Figure 3.2A, and data not shown). These data indicate that TLR/Nod signaling is necessary for induction of some, but not all, genes in the ETR. Furthermore, the intact induction of *Gem* in *Myd88*$^{-/-}$*/Nod1*$^{-/-}$ *Nod2*$^{-/-}$ macrophages implies the existence of an additional pathway.

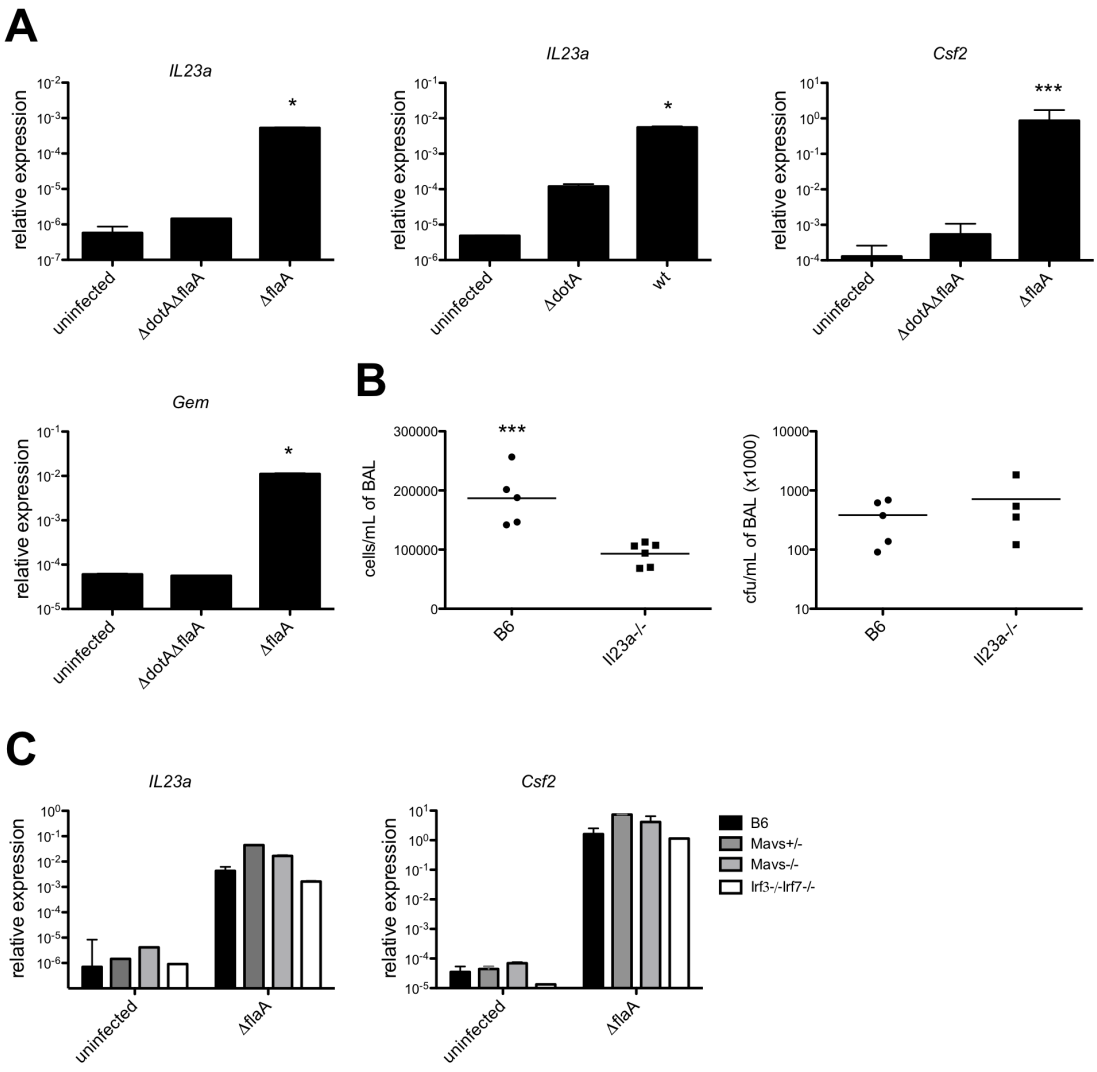

Figure 3.1. A unique transcriptional response in macrophages infected with virulent *L. pneumophila*. (A) B6 macrophages were infected for 6h with the specified strains of *L. pneumophila*. Levels of the indicated transcripts were measured by quantitative RT-PCR. (B) Mice were infected intranasally with 2 x 10^6 *L. pneumophila* and bronchoalveolar lavage was performed 24h post infection. Host cells recovered from bronchoalveolar lavage fluid (BALF) were counted with a hemocytometer (left panel). A portion of each sample was plated on BCYE plates to enumerate cfu (right panel). (C) Macrophages were infected for 6h with *L. pneumophila*. Levels of the indicated transcripts were measured by quantitative RT-PCR. *, $p < 0.05$ versus uninfected. ***, $p < 0.005$ versus uninfected.

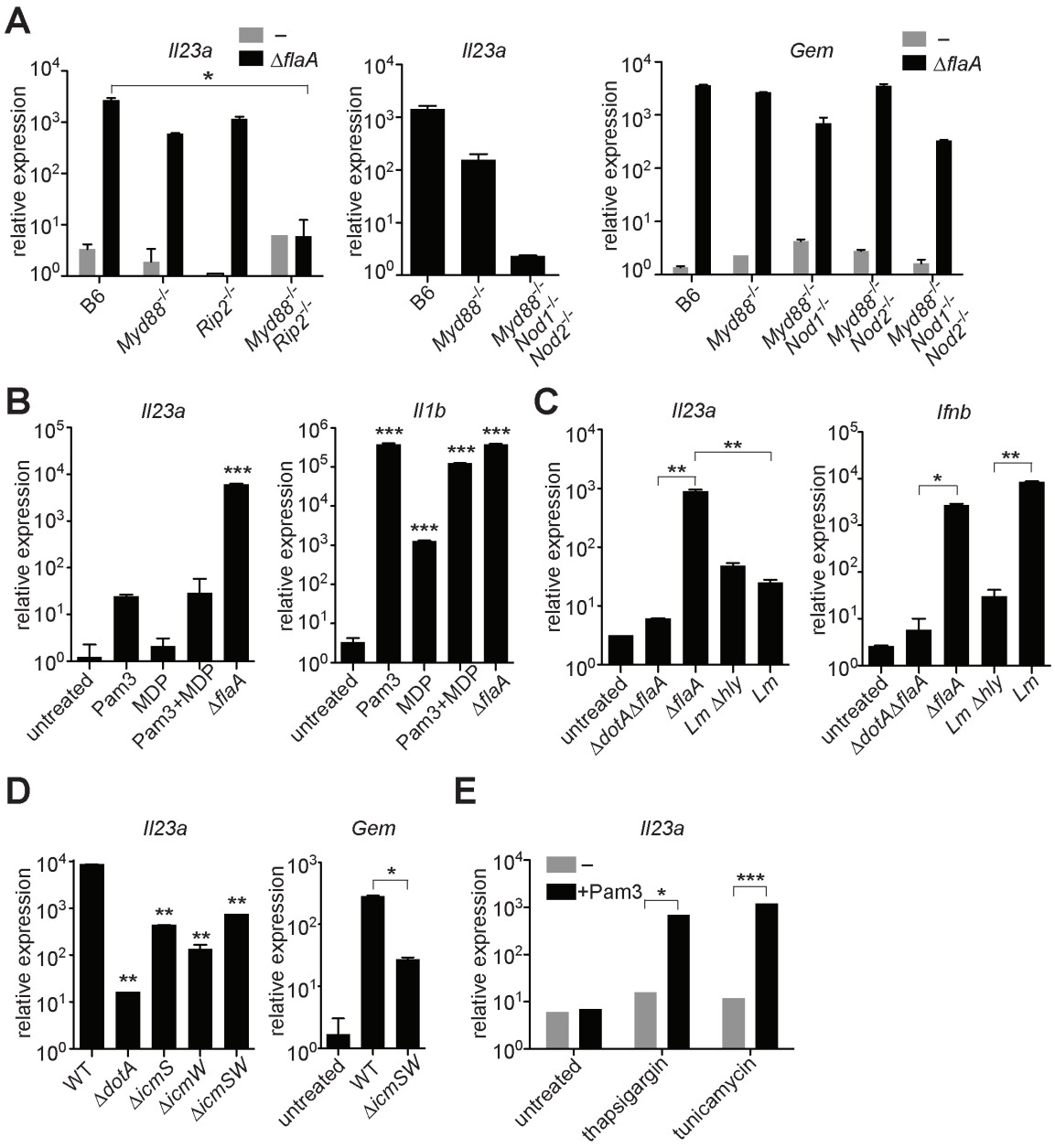

Figure 3.2. TLR and Nod signaling alone do not account for the unique response to virulent *L. pneumophila*, which can be recapitulated by ER stress inducers that also inhibit translation. In all panels, the indicated transcripts were measured by quantitative RT-PCR. (A) Macrophages were infected with Δ*flaA L. pneumophila* for 6h. (B) Macrophages were infected with *L. pneumophila* or were treated with Pam3CSK4 (10 ng/mL) and/or transfected with MDP (10μg/mL) for 6h. (C) B6 macrophages were infected with *L. pneumophila,* wildtype *L. monocytogenes* (Lm) or the avirulent *L. monocytogenes* Δ*hly* mutant for 4h. (D) B6 macrophages were infected with the indicated strains of *L. pneumophila* for 6h. **, $p < 0.01$ compared to wildtype (WT). (E) Uninfected B6 macrophages were treated with thapsigargin (500nM) or tunicamycin (5μg/mL) for 6h alone or in conjunction with Pam3CSK4 (1ng/mL). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

To address the further question of whether TLR/Nod signaling was *sufficient* for induction of the ETR, we treated uninfected macrophages with synthetic TLR2 and/or Nod2 ligands (Pam3CSK4 and MDP, respectively). These ligands did induce low levels of *Il23a*, but could not recapitulate the robust (100-1000 fold) upregulation indicative of the ETR (Figure 3.2B). The defective induction of ETR target genes was not due to inefficient delivery of the ligands, as Pam3CSK4 and MDP were able to strongly induce *Il1b* (Figure 3.2B). To confirm this result in a more physiologically relevant system, we infected macrophages with the Gram-positive intracellular bacterial pathogen *Listeria monocytogenes*, which is known to activate both TLRs and Nods (71). Infection with *L. monocytogenes* resulted only in weak *Il23a* induction (~50 fold less than wildtype *L. pneumophila* at the same initial multiplicity of infection) (Figure 3.2C). Furthermore, this weak *Il23a* induction did not represent a cytosolic sensing pathway, since the vacuole-trapped mutant Δ*hly L. monocytogenes* induced similar levels (Figure 3.2C). A failure to strongly upregulate *Il23a* did not appear to be due to poor infectivity of *L. monocytogenes*, since the cytosolically-induced gene *Ifnb* (71) was robustly transcribed (Figure 3.2C). Taken together, these results suggest that TLR/Nod signaling, while necessary for transcription of some ETR targets, is not sufficient to account for the full induction of the ETR by *L. pneumophila*.

3.4. Five *L. pneumophila* effectors that inhibit host protein translation are required to induce the full effector-triggered response.

Though PRRs do play some role in induction of the ETR, we could not identify a known PAMP-sensing pathway that fully accounted for this robust transcriptional response. Therefore we considered the hypothesis that host cells respond to an *L. pneumophila*-encoded activity in addition to PAMPs. Consistent with this hypothesis, we had previously determined that the bacterial chaperone proteins IcmS and IcmW, which are required for secretion of most *L. pneumophila* effectors, affect the host transcriptional response to *L. pneumophila* (Figure 2.5, Appendices 6 and 7). We therefore examined ETR targets in the context of infection with Δ*icmS*/Δ*icmW L. pneumophila.* Macrophages infected with this mutant strain exhibited a ~50-fold defect in induction of *Il23a* and *Gem* (Figure 3.2D). In contrast, induction of the cytosolic response gene *Ifnb* was intact during infection with Δ*icmS*/Δ*icmW* (data not shown), indicating that these bacteria did have functional secretion systems and were capable of activating cytosolic PRR pathways in general. Thus, secreted effectors (or the physiological stresses they impart) appear to participate in induction of the ETR.

To identify potential host pathways capable of inducing ETR target genes, we treated macrophages with known inducers of host cell stress responses. We found that the pharmacological agents thapsigargin and tunicamycin, which inhibit host translation indirectly via induction of endoplasmic-reticulum (ER) stress (82), synergized with a TLR2 ligand to induce high levels of *Il23a* and *Gem* (Figure 3.2E, and data not shown). To test whether *L. pneumophila* might elicit the ETR via induction of ER stress, we measured Xbp-1 splicing and transcription of classical ER stress markers in macrophages infected with *L. pneumophila.* However, we found no evidence of ER stress in these macrophages (data not shown). Instead, we considered the possibility that thapsigargin induces the ETR through inhibition of protein synthesis. In fact, the *L. pneumophila* Dot/Icm system was previously reported to translocate several effector enzymes that inhibit host translation (76, 83, 84). Therefore we hypothesized that inhibition of host protein synthesis by *L. pneumophila* (85) might be responsible for induction of the ETR.

To determine whether inhibition of host translation by *L. pneumophila* was critical for induction of the ETR, we generated a mutant strain of *L. pneumophila*, called Δ*5*, which lacks five genes encoding effectors that inhibit host translation (*lgt1*, *lgt2*, *lgt3*, *sidI*, *sidL*; Figure 3.3; Appendix 8). Three of these effectors (Lgt1, Lgt2, Lgt3), which share considerable sequence homology, are glucosyltransferases that modify the mammalian elongation factor eEF1A and block host translation both *in vitro* and in mammalian cells (83, 84). A fourth effector (SidI)

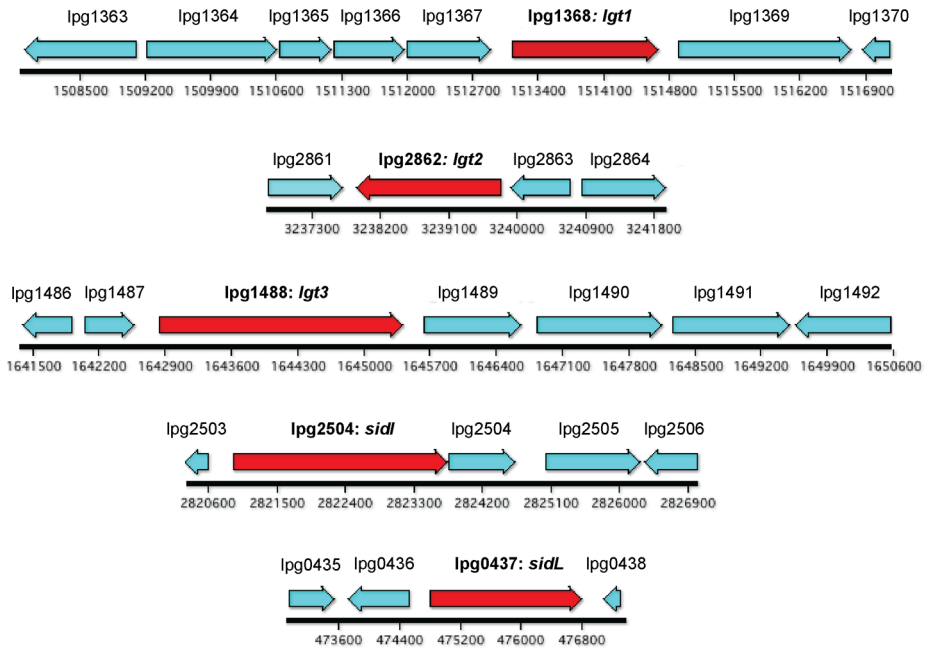

Figure 3.3. Genes deleted in Δ*5 L. pneumophila*. Numbers refer to the nucleotide position in the published *L. pneumophila* LP01 genome (GenBank Accession #AE017354).

binds both eEF1A and another host elongation factor, eEF1Bγ, and has also been shown to inhibit translation *in vitro* and in cells infected with *L. pneumophila* (76). The fifth effector, SidL, is toxic to mammalian cells and is capable of inhibiting protein translation *in vitro* via an unknown mechanism (data not shown). Moreover, its expression by *L. pneumophila* enhances global translation inhibition in infected macrophages (see below).

These 5 effectors appear to be important for survival within the *L. pneumophila*'s natural host, since the Δ*5* mutant displayed a ~10-fold growth defect in *Dictyostelium* amoebae (Figure 3.4A). By contrast, the Δ*5* mutant showed no growth defect in macrophages (Figure 3.4B), but was defective, compared to wildtype, in its ability to inhibit host protein synthesis (Figure 3.4C). Although to a lesser degree than wildtype bacteria, the Δ*5* mutant still appears to partially inhibit host protein synthesis, suggesting that *L. pneumophila* may encode additional inhibitors of host translation. Nevertheless, macrophages infected with Δ*5* exhibited striking defects in induction of the ETR, including a ~50-fold defect in induction of *Il23a, Gem, and Csf2* (Figure 3.4D and Appendix 9). Importantly, the Dot/Icm-dependent induction of *Ifnb,* which is induced via a separate pathway (47, 49, 51), remained intact (Figure 3.4D), implying that the Δ*5* mutant was competent for infection and Dot/Icm function. Individual deletion mutants of each of the five effectors showed no defect in *Il23a*, *Csf2*, or *Gem* induction, whereas a mutant lacking four of the five (Δ*lgt1*Δ*lgt2*Δ*lgt3*Δ*sidI*) had a partial defect (Figure 3.4D, and data not shown). Complementation of Δ*5* with wildtype *lgt2* or *lgt3* restored induction of *Il23a* and *Gem*, but complementation with mutant *lgt2* or *lgt3* lacking catalytic activity did not (Figure 3.4E), even though the mutant proteins were well expressed (data not shown). These results are significant because they show that macrophages make an innate response to a pathogen-encoded *activity* and that recognition of the effector molecules themselves is not likely to explain the ETR.

We then tested more directly whether the ETR was induced by translation inhibition. The defective induction of *Il23a*, *Csf2,* and *Gem* in macrophages infected with Δ*dotA* or Δ*5* was rescued by addition of the translation inhibitor cycloheximide (Figure 3.5A, and data not shown). These results support the hypothesis that induction of the ETR by *L. pneumophila* involves inhibition of translation by the five deleted effectors. Importantly, the potent induction of *Il23a, Csf2* and *Gem* by *L. pneumophila* could be recapitulated in uninfected macrophages by treatment

with the translation elongation inhibitors cycloheximide (Figure 3.5B) or puromycin (Figure 3.5C), or the initiation inhibitor bruceantin (Figure 3.5D), in conjunction with the TLR2 ligand Pam3CSK4. These three translation inhibitors possess different targets and modes of action, making it unlikely that the common host response to each of them is due to nonspecific drug effects.

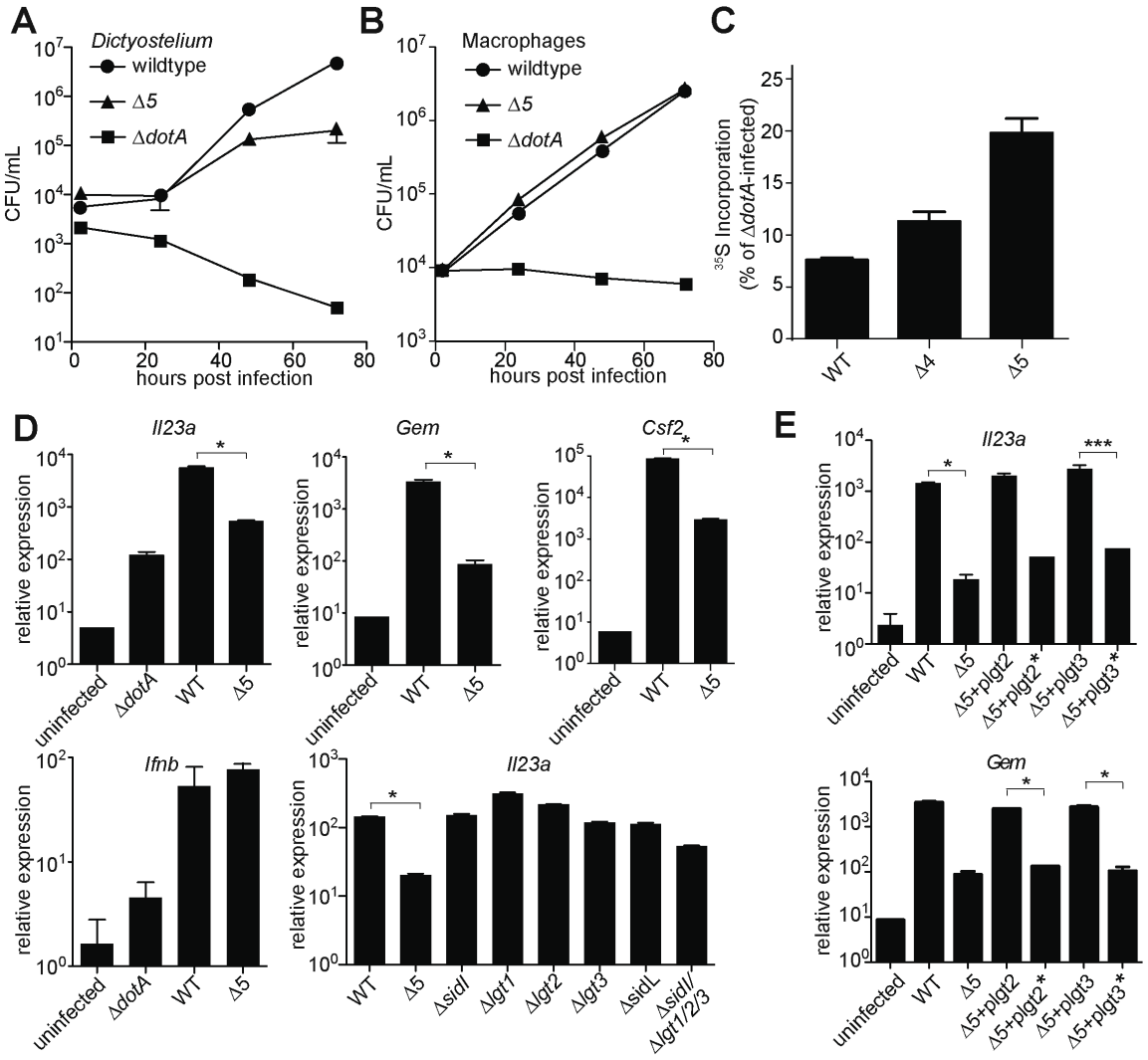

Figure 3.4. A mutant *L. pneumophila* lacking 5 bacterial effectors that inhibit host protein synthesis is defective in induction of the host 'effector-triggered response'. Growth of the indicated strains of *L. pneumophila* was measured in amoebae (A) or A/J macrophages (B). (C) Global host protein synthesis was measured by ^{35}S-methionine incorporation in macrophages infected for 2.5h with the indicated strains. (D) *Myd88*$^{-/-}$ (bottom right graph) or *Caspase1*$^{-/-}$ (all others) macrophages were infected for 6h with the specified strains. The indicated transcripts were measured by quantitative RT-PCR. (E) *Caspase1*$^{-/-}$ macrophages were infected for 6h with the specified strains. Indicated strains carried plasmids that constitutively expressed either a functional (plgt2, plgt3) or a catalytically inactive (plgt2*, plgt3*) bacterial effector. Δ5, Δ*lgt1*Δ*lgt2*Δ*lgt3*Δ*sidI*Δ*sidL*. Δ4, Δ*lgt1*Δ*lgt2*Δ*lgt3*Δ*sidI*. *, $p < 0.05$. ***, $p < 0.005$.

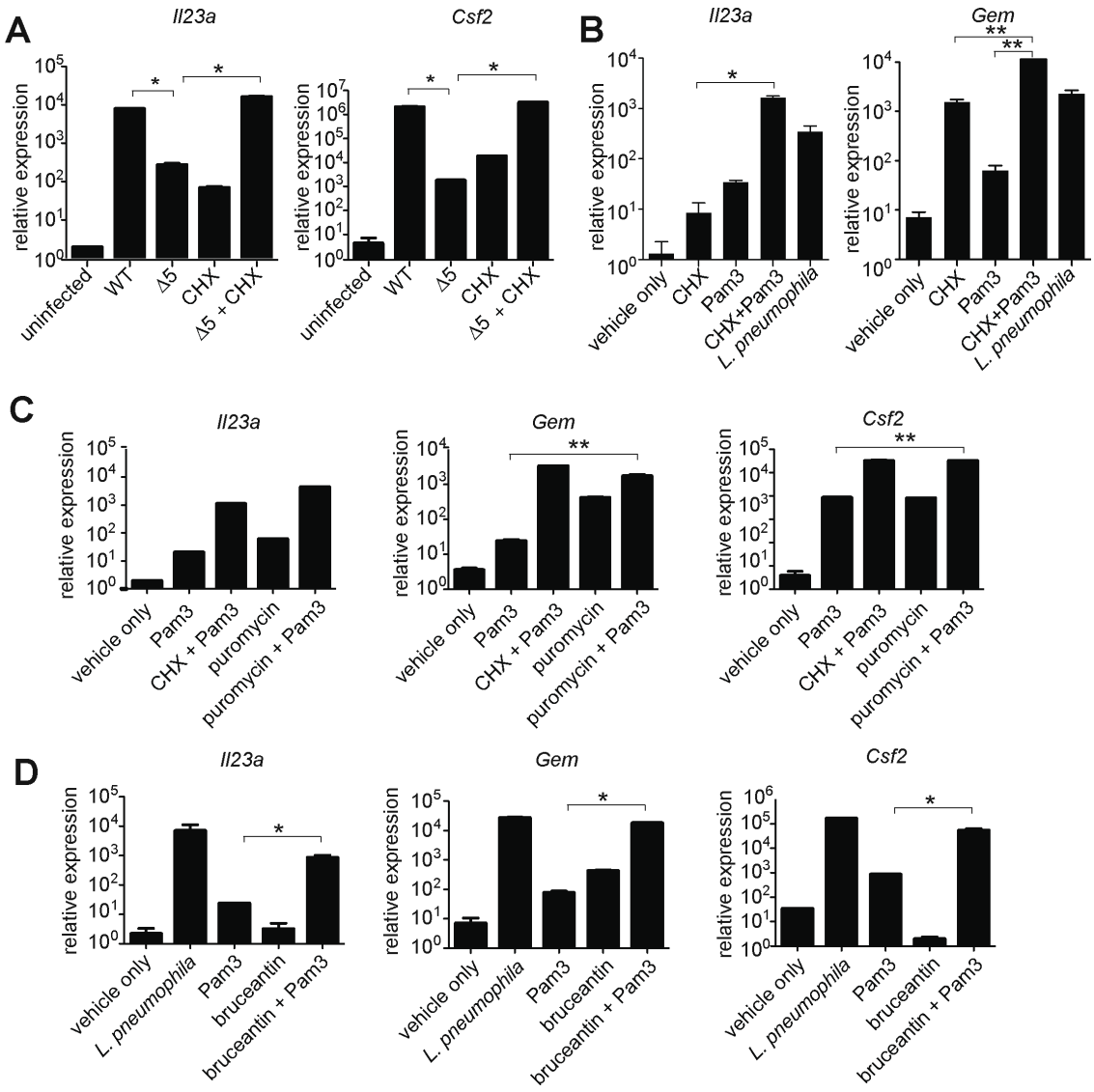

Figure 3.5. Induction of the 'effector-triggered response' can be recapitulated by pharmacological inhibitors of translation. (**A**) B6 macrophages were infected for 6h with the indicated strains, alone or with CHX (5μg/mL). (**B, C, D**) B6 macrophages were infected or were treated for 4h with CHX (10μg/mL; B), puromycin (20μg/mL; C) or bruceantin (50nM; D) alone or in conjunction with Pam3CSK4 (10ng/mL). CHX, cycloheximide. *, $p < 0.05$. **, $p < 0.01$.

Thus, translation inhibition in the context of TLR signaling provokes a specific transcriptional response. Translation inhibitors alone were capable of inducing some, but not all, effector-triggered transcriptional targets (Figure 3.5B, C, and D), supporting our model that translation inhibition acts in concert with classical PRR signaling to generate the full effector-dependent signature. Microarray analysis indicated that the five effectors accounted for induction of at least 54 (~30%) of the Dot/Icm-dependent genes (Figure 3.6 and Appendix 9).

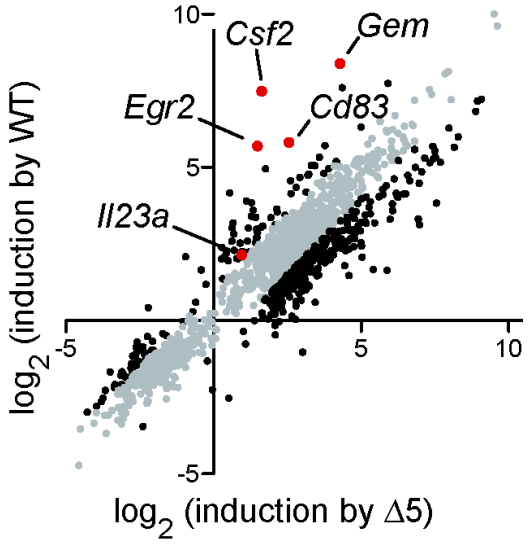

Figure 3.6. Differential transcriptional response to mutant *L. pneumophila* lacking the five effectors. *Caspase1*$^{-/-}$ macrophages were infected for 6h with the indicated strains. RNA was amplified and hybridized to MEEBO arrays. Black and red dots, genes exhibiting greater than 2-fold difference in induction between wildtype (WT) and Δ*5*. Red dots indicate labeled genes.

3.5. Inhibition of translation by *L. pneumophila* effectors results in sustained loss of IκB.

We investigated how inhibition of protein synthesis by *L. pneumophila* might elicit a host response. Although translation inhibition by cycloheximide has long been reported to induce cytokine production (86), the mechanism by which it acts remains poorly understood. Since the induction of *Il23a* and *Csf2* is NF-κB dependent ((87), and data not shown), we examined a role for this pro-inflammatory transcription factor in induction of these ETR targets. NF-κB is normally suppressed by its labile inhibitor IκB, which is ubiquitinated and degraded in response to TLR and other inflammatory stimuli. IκB is itself a target of NF-κB-dependent transcription, and resynthesis of IκB is critical for the homeostatic termination of NF-κB signaling. In the absence of protein synthesis, we hypothesized that IκB may fail to be resynthesized as it turns over, thereby permitting continued NF-κB activity. To test this hypothesis, we measured IκB levels in infected macrophages over time. We observed a prolonged decrease in levels of IκB protein in macrophages infected with wildtype *L. pneumophila*, consistent with previous observations (67) (Figure 3.7A). In contrast, infection with Δ*5* triggered only a transient loss of IκB, similar to infection with the secretion-deficient Δ*dotA* mutant (Figure 3.7A). The Δ*5* mutant could induce sustained IκB degradation when complemented with plasmid-encoded Lgt3, but not with a mutant effector lacking glucosyltransferase activity (Figure 3.7B), demonstrating that the sustained loss of IκB is due to the activity of the bacterial effector. To confirm that the prolonged loss of IκB did indeed result in sustained NF-κB activation, we measured NF-κB translocation to the nucleus in macrophages infected with wildtype, Δ*dotA,* or Δ*5 L. pneumophila*. (Nuclear localization corresponds to NF-κB activity.) While all three strains initially induced nuclear translocation of NF-κB, at later timepoints we observed decreased levels of nuclear NF-κB in macrophages infected with the Δ*dotA* or Δ*5* strains compared to those infected with wildtype *L. pneumophila* (Figure 3.7C). Thus, translation inhibition by the 5 effectors results in sustained loss of IκB and enhanced activation of NF-κB.

NF-κB signaling is also inhibited by other *de novo* expressed proteins such as A20 (88). We therefore used *A20*$^{-/-}$ macrophages, which exhibit prolonged NF-κB activation in response to TLR signaling (88), to further test the hypothesis that sustained NF-κB signaling can induce targets of the ETR. Strikingly, we found that the defective induction of *Il23a* and *Csf2* by Δ*5* was rescued in *A20*$^{-/-}$ macrophages (Figure 3.7D). Taken together, these observations suggest a model in which disrupted protein synthesis, and the subsequent failure to synthesize inhibitors of NF-κB signaling (*e.g.* IκB and A20), leads to sustained activation of NF-κB (Figure 3.8). In turn, we suggest that this prolonged activation of NF-κB results in enhanced transcription of a specific subset of genes.

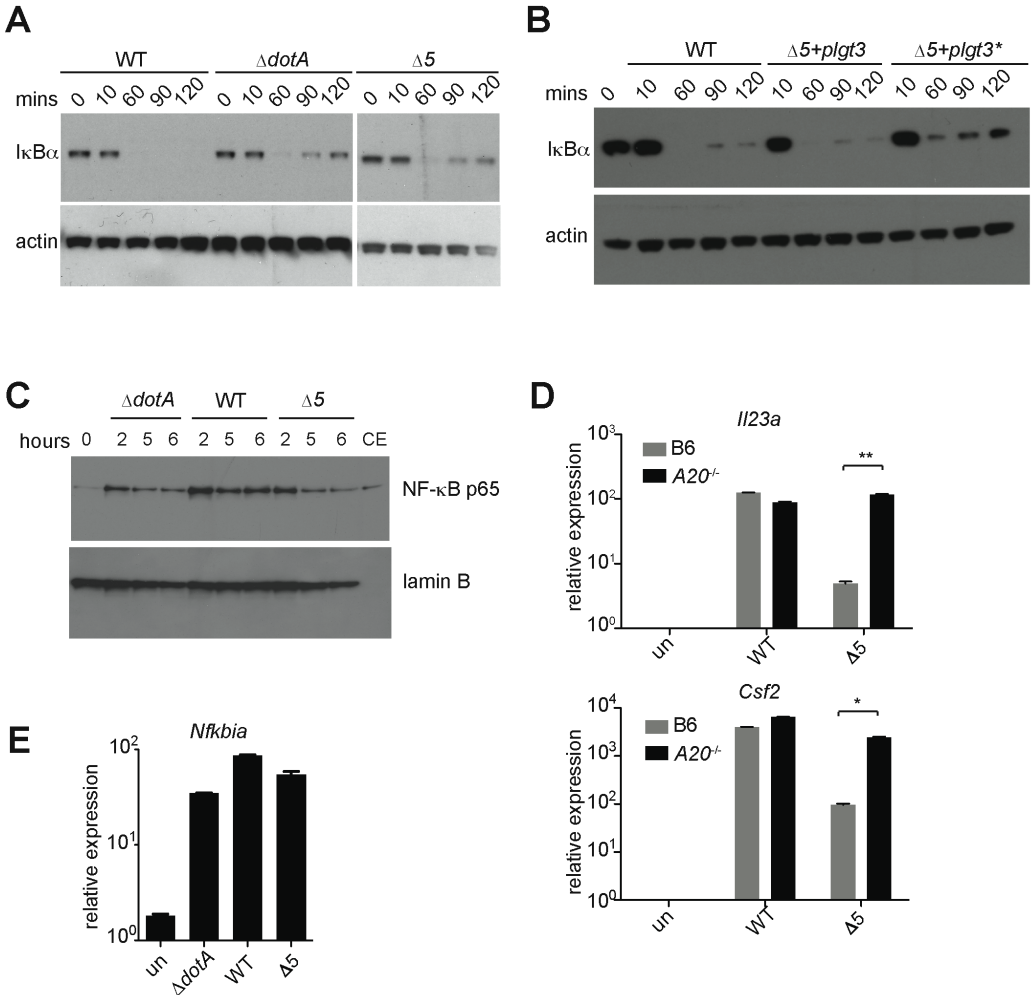

Figure 3.7. Expression of the 5 *L. pneumophila* effectors and induction of 'effector-triggered' genes correlates with sustained loss of inhibitors of the NF-κB transcription factor. (A, B) *Caspase-1*$^{-/-}$ macrophages were infected at an MOI of 2 for the times indicated. Cell lysates were analyzed by Western blotting with anti-IκBα antibody (top panels) or anti-β-actin antibody (bottom panels). (B) The indicated strains carried a plasmid encoding either a functional (plgt3) or catalytically inactive (plgt3*) effector. (C) B6 macrophages were infected at an MOI of 2 for the times indicated. Nuclear extracts were analyzed by Western blotting with anti-NF-κB antibody (top panel) or anti-lamin-B antibody (bottom panel) as a loading control. Cytoplasmic extract of untreated macrophages (CE) was included for comparison. (D, E) B6 (D, E) or *A20*$^{-/-}$ (D) macrophages were infected for 6h, and levels of the indicated transcripts were measured by quantitative RT-PCR. (E) The NF-κB target gene *Nfkbia* encodes the inhibitor IκB.

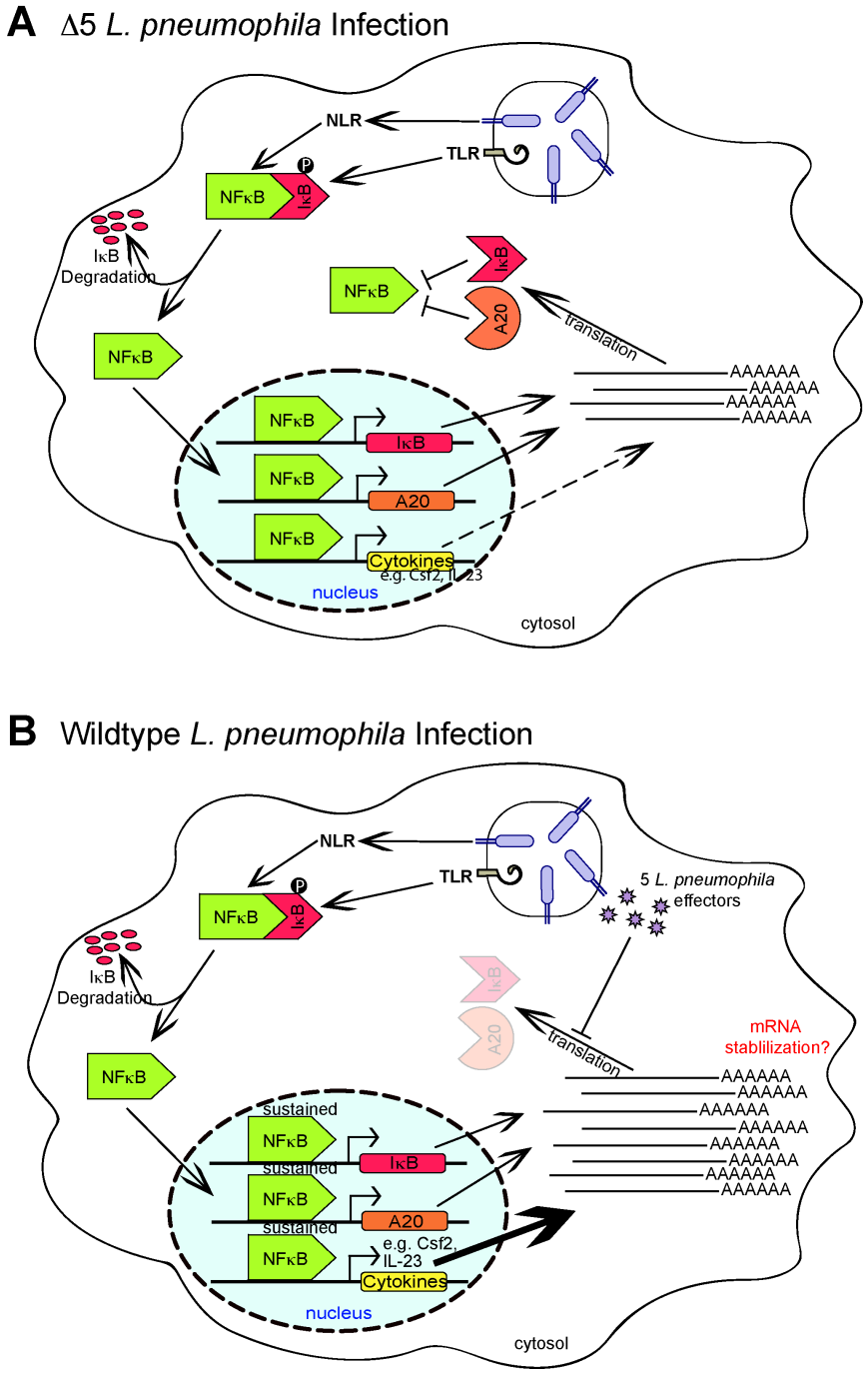

Figure 3.8. **Model of NF-κB activation and superinduction by translation inhibitors**. (A) NF-κB activation by TLR signaling, via the adaptor Myd88, or Nod signaling, via Rip2, normally leads to synthesis of inhibitory proteins, including IκB and A20, which act to shut off NF-κB signaling. (B) When translation is inhibited, IκB and A20 fail to be synthesized, allowing sustained activation of NF-κB and subsequent robust transcription of a subset of target genes.

Importantly, sustained NF-κB activation did not appear to result in transcriptional superinduction of all NF-κB-dependent target genes. Microarray analysis (Figure 3.6 and Appendix 8) suggested that only a subset of NF-κB-induced genes was preferentially induced by translation inhibition. For example, *Nfkbia* (encoding IκBα), a known NF-κB target gene, was not dramatically superinduced by wildtype compared to Δ*5 L. pneumophila* (Figure 3.7E). The molecular mechanism that results in specific superinduction of certain NF-κB-dependent target genes is not yet clear and may be complex (see Discussion). In addition to *de novo* transcription, the measured increase in ETR transcripts could be due to mRNA stabilization, which has been reported to occur during pharmacological inhibition of protein synthesis (89). In light of this possibility, we tested whether mRNA stabilization contributed to induction of the ETR by infecting macrophages in the presence of the transcription inhibitor actinomycin D and quantifying ETR target mRNAs at successive timepoints. Our results suggested that RNA stabilization does not play a major role in induction of these particular ETR targets (Figure 3.9A), though we do not rule it out as a possible mechanism for increasing some mRNA transcripts in the ETR. Instead, we confirmed that the increase in expression of ETR target genes does involve *de novo* transcription, by quantifying transcript levels using primers specific for unspliced mRNA (Figure 3.9B).

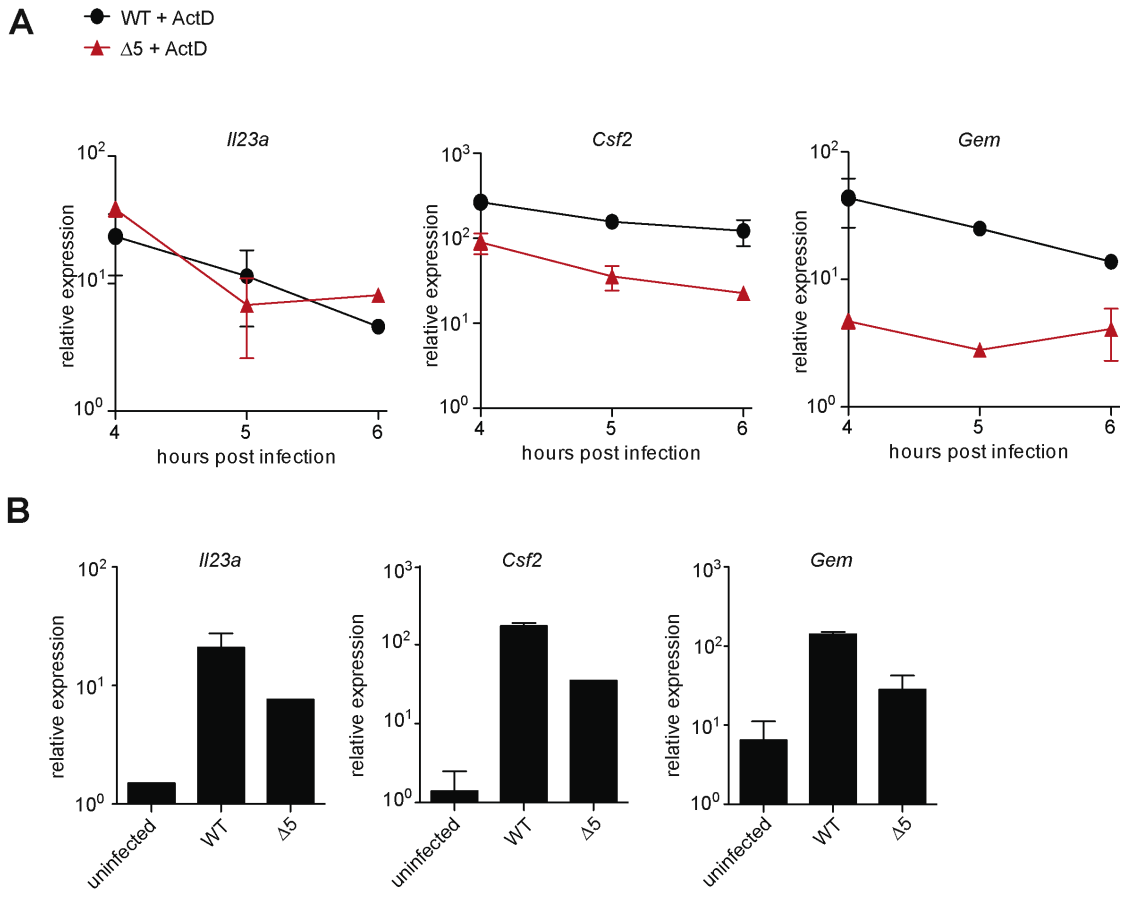

Figure 3.9. New transcription and mRNA stabilization of ETR target genes. (A) To assess RNA stability, the transcription inhibitor Actinomycin D (10μg/mL) was added to macrophages 4h post infection. RNA was collected at successive timepoints, and transcripts were measured by quantitative RT-PCR. (B) After a 6h infection in B6 macrophages, *de novo* transcription of the indicated genes was measured by quantitative RT-PCR with primers that specifically targeted the pre-spliced mRNA.

3.6. Inhibition of translation leads to activation of host MAP kinases.

Having demonstrated a mechanism by which *L. pneumophila* activates host NF-κB, we were curious as to whether other host signaling pathways might also be activated by translation inhibition. It has been previously reported that *L. pneumophila* activates host MAP kinases in a manner that is TLR and Nod-independent, and instead dependent on secretion of bacterial effectors (67). We therefore examined a role for translation inhibition in the activation of host MAP kinases by *L. pneumophila*.

Consistent with the previous report (67), we observed Dot/Icm-dependent phosphorylation of the host MAP kinases p38 and SAPK/JNK in TLR/Nod-deficient macrophages infected with *L. pneumophila* (Figure 3.10A). MAPK activation was largely abrogated upon infection with the Δ5 mutant, indicating a requirement for the five bacterial effectors that inhibit translation (Figure 3.10A). MAPK phosphorylation could be restored by complementation of the Δ5 mutant with a wildtype effector, but not by complementation with an effector harboring a point mutation that abolished catalytic activity (Figure 3.10B). Furthermore, several known inhibitors of translation were able to activate MAP kinases in uninfected macrophages (Figure 3.10C), a phenomenon that has been reported in the literature in other cell types. Taken together, these data indicate that the pathogen-encoded activity of translation inhibition—rather than direct recognition of the bacterial effectors themselves—induces activation of host MAP kinases. This effector-dependent MAPK activation has consequences for the host transcriptional response, as MAPK-dependent genes such as *Il1a*, *Dusp1*, and *c-fos* are induced in an effector-dependent manner in macrophages lacking TLR and Nod signaling (Figure 3.10D). Furthermore, MAP kinase induction results in a translational response, including production of IL-1α protein (Figure 3.10E). However, in a wildtype macrophage, TLRs and Nods also activate MAP kinases in response to *L. pneumophila*, making the effector-dependent MAP kinase activation redundant (data not shown).

3.7. Paradoxical increase in protein production under conditions where protein synthesis is inhibited.

Although inhibition of protein synthesis potently induces transcription of certain target genes, a central question is whether this transcriptional response is sufficient to overcome the translational block, and result in increased protein production. Accordingly, we measured the protein levels of GM-CSF (encoded by the *Csf2* gene) in the supernatant of infected macrophages. GM-CSF protein was preferentially produced by cells infected with wildtype *L. pneumophila* as compared to cells infected with Δ5 (Figure 3.11A). The defect in cytokine production by Δ5-infected macrophages was not due to poor bacterial growth (Figure 3.4B), increased cytotoxicity (Figure 3.11B), or defective secretion (Figure 3.11C), and could be rescued by addition of cycloheximide (Figure 3.11D). Thus translation inhibition can paradoxically lead to increased production of certain proteins, perhaps because transcriptional superinduction of specific transcripts is sufficient to overcome the partial translational block mediated by *L. pneumophila*.

We investigated whether production of GM-CSF protein was due to preferential translation of *Csf2* mRNAs or, alternatively, was simply proportional to the amount of *Csf2* mRNA present in the cellular pool. To distinguish between these two scenarios, we isolated both polysome-associated and total mRNA from macrophages infected with either wildtype or *ΔdotA L. pneumophila*. We then performed quantitative RT-PCR to determine the fraction of *Csf2* message in the total and polysome-associated mRNA pools. Values obtained from polysomal fractions were normalized to 18S rRNA to control for the total number of ribosomes assayed. We found that the proportion of *Csf2* in the polysome-associated mRNA pool was roughly equal to its proportion in the total mRNA pool, implying that these mRNAs are not selected for preferential translation (data not shown). Rather, we favor the model in which the most abundant mRNA transcripts, including *Csf2*, are also the most translated.

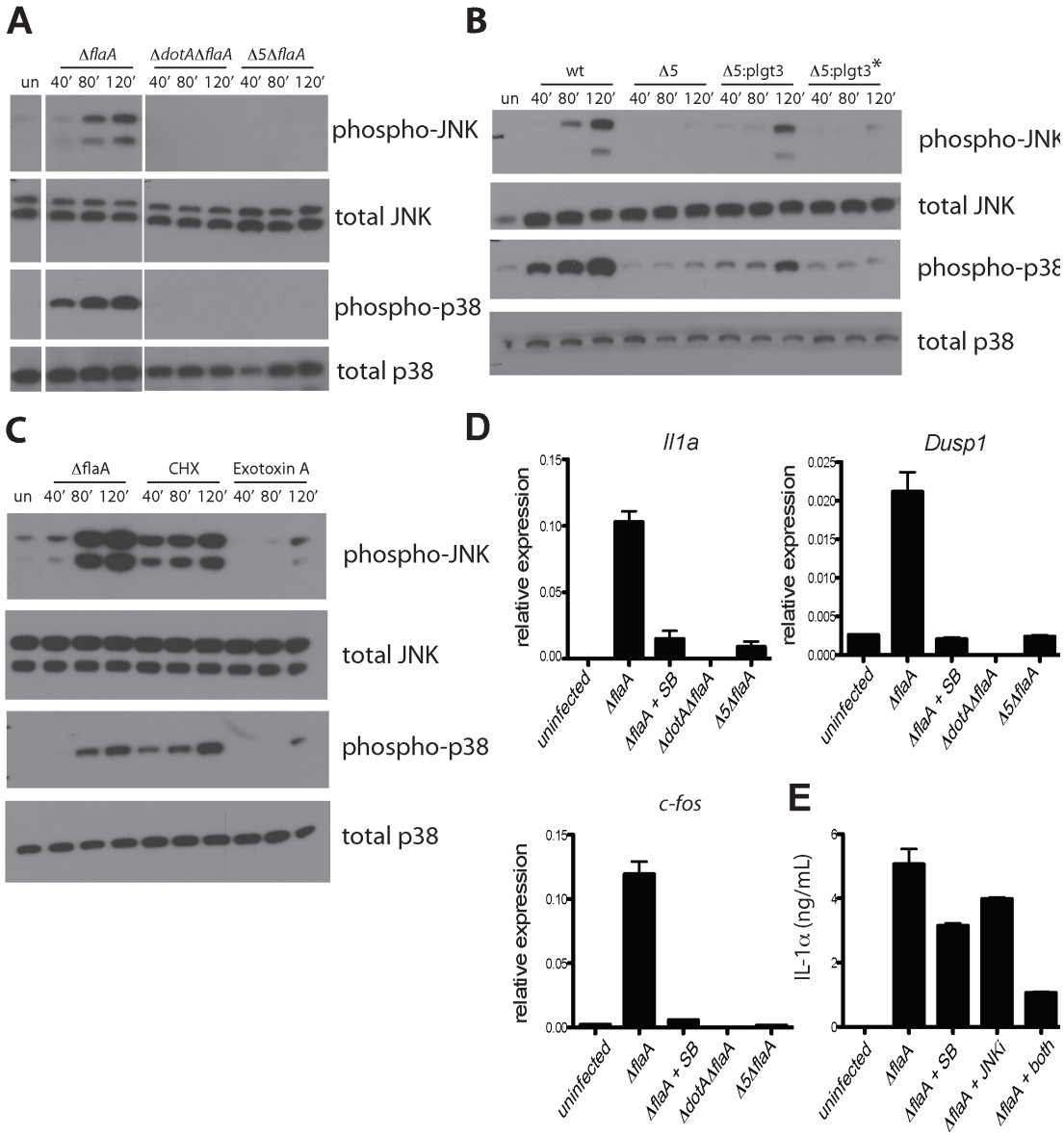

Figure 3.10. Effector-dependent activation of host MAP kinases in macrophages lacking TLR and Nod signaling. (A-C) *Myd88*$^{-/-}$*/Nod1*$^{-/-}$*/Nod2*$^{-/-}$ macrophages were infected for the indicated times with the specified strains of *L. pneumophila.* In (C) some uninfected macrophages were also treated with cycloheximide (CHX; 10μg/mL) or Exotoxin A (1μg/mL). Cell lysates were blotted for the indicated phosphorylated and total MAP kinases. (B) Indicated strains were complemented with plasmids that constitutively expressed either a functional (plgt3) or a catalytically inactive (plgt3*) bacterial effector. (D) *Myd88*$^{-/-}$*/Nod1*$^{-/-}$*/Nod2*$^{-/-}$ macrophages were infected with the indicated strains alone or in the presence of the p38 inhibitor SB203580 (SB). RNA was harvested 4h post infection and levels of the indicated transcripts were measured by quantitative RT-PCR. (E) B6 macrophages were infected for 24h with *L. pneumophila* alone or in the presence of p38 (SB) or JNK (JNKi) inhibitors, or both inhibitors. Supernatant IL-1α was measured by ELISA.

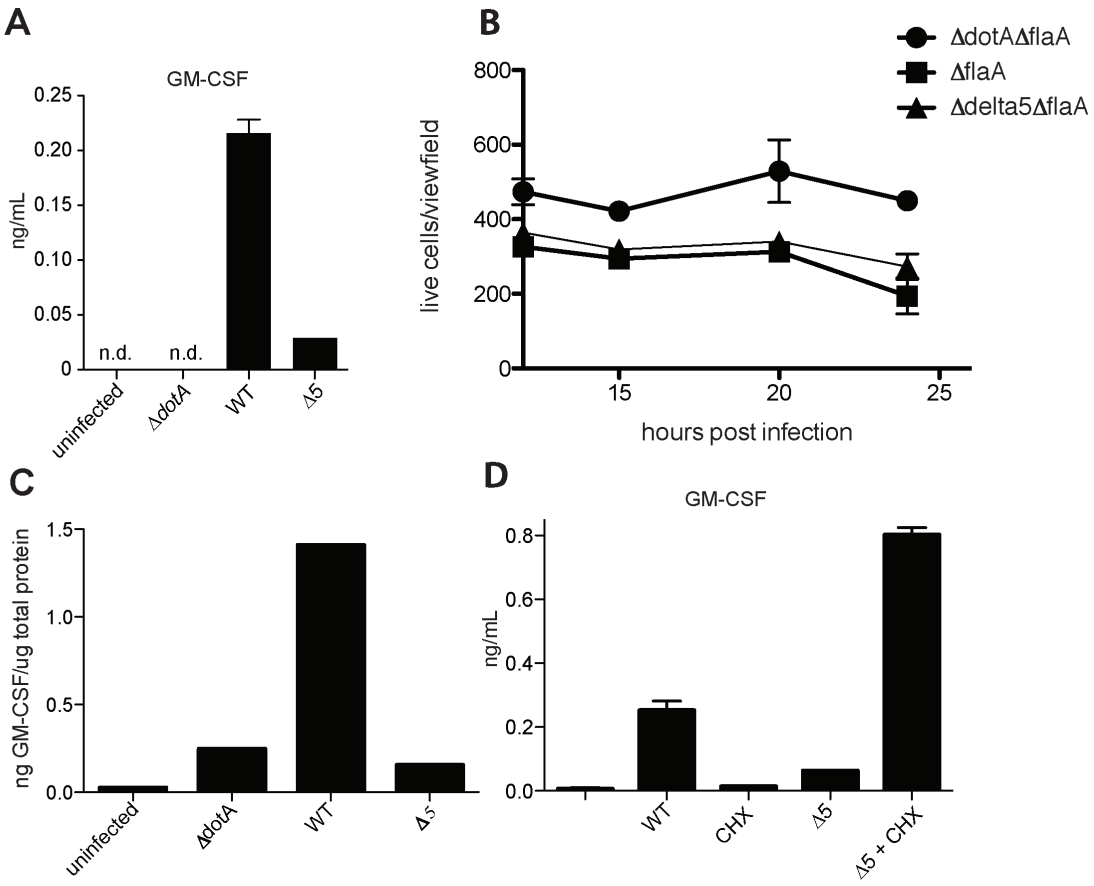

Figure 3.11. Translation inhibition by Δ*5*Δ*flaA L. pneumophila* results in production of cytokine protein. (A, C, D) B6 macrophages were infected for 6h (C) or 24h (A, D) with the indicated strains of *L. pneumophila* and/or treated with cycloheximide (5μg/mL). Protein levels in the supernatant (A, D) or cell lysate (C) were assayed by ELISA. (B) B6 macrophages were infected at an MOI of 1. At indicated timepoints, the number of surviving cells was determined by Neutral Red assay. Bacteria lacking flagellin were used to avoid Caspase-1-dependent cell death.

3.8. Host response to translation inhibition by bacterial toxins *in vitro* and *in vivo*.

We did not observe defects in cytokine induction or altered bacterial replication in B6 mice infected with the Δ*5* mutant. This is perhaps not surprising, since many redundant innate immune signaling pathways are known to recognize and restrict the growth of *L. pneumophila in vivo* (38). Hoping to remove redundant pathways, we also infected *Myd88*$^{-/-}$, *TLR2*$^{-/-}$, and *Nod1*$^{-/-}$*/Nod2*$^{-/-}$ mice with the Δ5 mutant. However, we failed to find consistent differences between mutant and wildtype bacteria even in partially immunodeficient mice (data not shown). In addition to the redundancy of multiple immunosurveillance pathways, it is possible that the presence of other cell types *in vivo* obscured a role for translation inhibition in eliciting a response from macrophages. Indeed, we found that dendritic cells infected with *L. pneumophila* upregulate ETR target genes independently of the Dot/Icm secretion system (Figure 3.12A), and hence translation inhibition appears not to be essential for their response to *L. pneumophila*.

However, many other pathogens also produce toxins that inhibit host protein synthesis (*e.g.*, Diphtheria Toxin, Shiga Toxin, *Pseudomonas* Exotoxin A). Thus, to test whether translation inhibition may be a general stimulus that acts with PRRs to elicit a host response to diverse pathogens, we treated uninfected macrophages with Diphtheria Toxin (DT) or Exotoxin A (ExoA) in conjunction with a TLR2 ligand. Importantly, both of these toxins inhibit translation by ADP-ribosylation of eEF2, a mechanism of action distinct from that employed by the five *L. pneumophila* effectors. When administered with Pam3CSK4, both toxins robustly induced *Il23a* (Figure 3.12B). DT alone was sufficient to induce *Il23a*, most likely due to the presence of TLR ligands in the recombinant protein preparation. Consistent with these findings, Shiga Toxin, which inhibits translation by yet another mechanism, has also been reported to superinduce cytokine responses in a cultured cell line (90). The existence of a common host response to diverse mechanisms of translation inhibition provides strong evidence that host cells can specifically respond to this disruption of their physiology, in addition to recognizing microbial molecules.

Finally, since *in vivo* infection with *L. pneumophila* results in multiple redundant responses that may have obscured our ability to detect an *in vivo* phenotype for the Δ*5* mutant, we turned to a simpler model to ascertain whether the ETR can be induced *in vivo*. In this model, purified Exotoxin A was administered intranasally to inhibit host protein synthesis in the lungs. Importantly, we found that translation inhibition appears to synergize with TLRs to elicit an immune response *in vivo*, as mice treated intranasally with ExoA and Pam3CSK4 produced significant amounts of the characteristic effector-triggered cytokine GM-CSF (Figure 3.12C). Consistent with our observations *in vitro* (Figure 3.12B), intranasal instillation of ExoA or Pam3CSK4 individually resulted in a much more modest response, providing further evidence that two signals—PRR activation and translation inhibition—are needed to generate the full effector-dependent signature. ExoA alone was sufficient to induce transcription of *Gem* and *Csf2* mRNA in the lung (Figure 3.12D), again in agreement with *in vitro* observations that translation inhibition alone can induce transcription of some target genes (Figure 3.5B, C, and D). Taken together, our results demonstrate that translation inhibition by multiple pathogens can lead to a common innate response in cultured cells and *in vivo*.

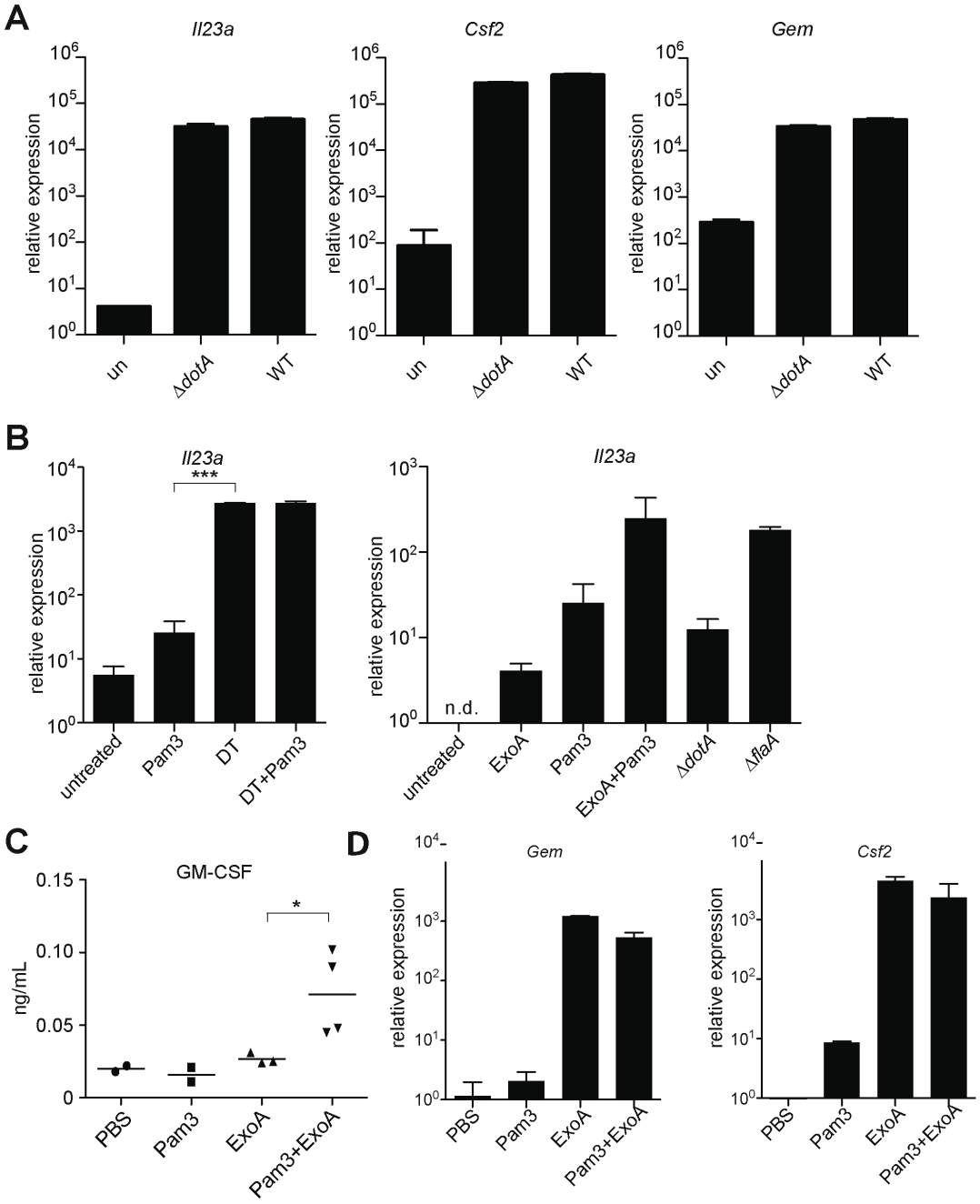

Figure 3.12. Inhibition of host translation by multiple bacterial toxins provokes an inflammatory cytokine response *in vitro* and *in vivo*. (A, B, D) The indicated transcripts were measured by quantitative RT-PCR. (A) B6 bone marrow derived dendritic cells were infected for 6h with the indicated strains at an MOI of 2. (B) B6 macrophages were treated for 5h with Diphtheria Toxin (1ng/mL; left panel) or with Exotoxin A (500ng/mL; right panel), alone or in conjunction with Pam3CSK4. n.d., not detected. (C, D) B6 mice were treated intranasally with Pam3CSK4 (10µg/mouse) or ExoA (2µg/mouse) or both in 25µL PBS. Bronchoalveolar lavage was performed 24h post infection. (C) GM-CSF levels in lavage were measured by ELISA. CHX, cycloheximide. DT, Diphtheria Toxin. ExoA, Exotoxin A. *, $p < 0.05$. ***, $p < 0.005$.

3.9. Discussion.

In this chapter, we uncovered a novel host surveillance mechanism that leads to induction of the 'orphan' gene targets identified from microarray studies in Chapter Two. In doing so, we have demonstrated that inhibition of host translation by bacterial effectors or toxins can elicit a potent response from the host. We thus provide strong evidence for a model of innate immune recognition that is complementary to, but distinct from, the classic PAMP-based model. Most notably, we show that the immune system can mount a response to a pathogen-associated *activity*, in addition to pathogen-derived molecules. In our model, it is important to emphasize that there is no need for a specific host receptor or sensor *per se*. Instead, our data support the hypothesis that a pathogen-mediated block in the synthesis of short-lived host signaling inhibitors (*e.g.* IκB, A20) results in the sustained activation of an inflammatory mediator (*e.g.* NF-κB) (Figure 6). As such, our model more closely resembles the indirect "guard" type mechanisms that plants utilize, in conjunction with PRRs, to sense pathogens (10). The labile nature of IκB makes it an effective "guard" to monitor the integrity of host translation, since the short half-life of this protein ensures that its abundance will decrease quickly during conditions where translation is inhibited.

There are growing suggestions that host responses to 'patterns of pathogenesis' (7), or harmful pathogen-associated activities, may indeed comprise a general innate immunosurveillance strategy in metazoans. For example, as noted previously in this work (Section 1.2), ion channel formation by influenza virus may activate the Nlrp3 inflammasome (30), and *Salmonella* effectors that stimulate Rho-family GTPases appear to trigger specific inflammatory responses (11). However, in these examples, both the precise host cell disruption and the mechanism by which the host responds remain unclear. Our results are significant because we have provided a mechanism by which host cells generate a unique transcriptional response to a specific pathogen-encoded activity, namely, inhibition of host protein synthesis.

An important question is whether the innate response to translation inhibition represents a host strategy for detecting and containing a pathogen, or is rather a manipulation of the host immune system by the bacterium. Given the natural history of *L. pneumophila*, we consider it unlikely that this pathogen has evolved to manipulate the innate immune system (35). *L. pneumophila* is not thought to be transmitted among mammals; instead, our data (Figure 3.4A and B) suggest that the five effectors described here probably evolved to aid survival in amoebae, the natural hosts of *L. pneumophila*. We therefore favor the hypothesis that the innate immune system has evolved to respond to disruptions in protein translation, an essential activity that is targeted by multiple viral and bacterial pathogens.

We observed that inhibition of translation in the context of PRR signaling results in the transcriptional superinduction of a specific subset of >50 genes, including *Il23a*, *Gem*, and *Csf2*, that constitute an 'effector-triggered' response. We propose that at least some of these genes are superinduced upon the sustained activation of transcription factors such as NF-κB, as well as transcription factors downstream of host MAP kinases. At the same time, it is important to emphasize that the host response to protein synthesis inhibition is complex. Interestingly, we observed that not all NF-κB-dependent target genes are superinduced by translation inhibition. For example, *Nfkbia* (encoding the IκB protein) was not superinduced in wildtype *L. pneumophila* infection (Figure 3.7E). This selective superinduction of certain target genes may be significant, since it allows the host to respond to a pathogen-dependent stress by altering not only the magnitude but also the composition of the transcriptional response. Moreover, if IκB were superinduced, this would presumably act to reverse or prevent sustained NF-κB signaling, resulting in little net gain.

The mechanism by which prolonged NF-κB signaling may preferentially enhance transcription of the specific subset of effector-triggered genes is not yet clear. However, recent studies have shown that the chromatin context for several of these genes (*e.g.*, *Il23a, Csf2*) is in a relatively 'closed' conformation (91, 92). This may render the genes refractory to strong transcriptional induction under a normal TLR stimulus, but enable them to become highly

induced upon prolonged NF-κB activation. It is interesting to note that genes such as *Il23a* and *Csf2* are classified as 'primary' response genes (91, 92) simply because they are inducible in the presence of cycloheximide. What is not often discussed is the possibility, demonstrated here, that inhibition of protein synthesis by cycloheximide is a key stimulus that induces transcription of these genes.

The consequences of the host response to translation inhibition are likely to be difficult to measure in the context of a microbial infection *in vivo*. Presumably, most pathogens that disrupt host translation derive benefit from this activity, perhaps by increasing availability of amino acid nutrients or by dampening production of the host response. These benefits may be offset by an enhanced host response to translation inhibition itself. It is possible that the robust innate immune response to translation inhibition serves primarily to compensate for the decrease in translation, resulting in little net change in the output of the immune response. Accordingly, the lack of an apparent phenotype during *in vivo* infection with Δ*5* may reflect the sum of multiple positive and negative effects that result from translation inhibition. Additionally, as suggested by our data (Figure 3.12A) the response to *L. pneumophila in vivo* may involve non-macrophage cell types in which translation inhibition does not play a crucial role.

While PRR-based sensing of microbial molecules is certainly a fundamental mode of innate immune recognition, it is not clear how PRRs alone might be able to distinguish pathogens from non-pathogens, and thereby mount responses commensurate with the potential threat. Our results demonstrate that pathogen-mediated interference with a key host process (*i.e.*, host protein synthesis), in concert with PRR signaling, results in an immune response that is qualitatively distinct from the response to an avirulent microbe. Although induction of some genes in the ETR (*e.g.*, *Gem*) occurs in response to inhibition of protein synthesis alone, much of the ETR is due to the combined effects of PAMP recognition and effector-dependent inhibition of protein synthesis. A requirement for two signals might be rationalized by the fact that the ETR includes potent inflammatory cytokines such as GM-CSF or IL-23, which can drive pathological inflammation (93) and autoimmunity (94) if expressed inappropriately. Restricting production of potentially dangerous cytokines to instances where a pathogenic microbe is present may be a strategy by which hosts avoid self-damage unless necessary for self-defense. Thus, we propose that the host response to a harmful pathogen-encoded activity may represent a general mechanism by which the immune systems of metazoans distinguish pathogens from non-pathogens.

Chapter Four:
Two novel TNF-inducible inflammasomes initiate pyroptosis in response to *L. pneumophila*

4.1. Introduction

In this chapter, I will present evidence for two novel inflammasomes that are enhanced by the inflammatory cytokine TNF and activated in response to pathogenic *L. pneumophila*. Conceptually, these inflammasomes resemble the surveillance system presented in Chapter Three of this work in that both require cooperation between microbe-sensing and pathogen-recognition pathways, resulting in a unique host response that could be harmful if directed against an inappropriate target. Preliminary data from this project have been published in *Cellular Microbiology* (40).

Inflammasomes, introduced in Chapter One, are multiprotein cytosolic platforms that activate the proteases Caspase-1 and/or Caspase-11 in response to diverse microbial and host-derived stimuli (95). Inflammasomes represent an interesting case in our consideration of how the host may distinguish pathogens from non-pathogens: while several inflammasomes recognize molecules, such as flagellin and DNA, that are common to both pathogenic and non-pathogenic microbes, these molecules must be delivered to the cytosol in order to be detected. The cytosol is a "privileged" site (96): commensal bacteria cannot usually access it, while pathogenic microbes often do, through use of secretion systems, pore-forming toxins, or escape from the phagosome and cytosolic invasion. Thus, although several specific inflammasomes recognize PAMPs, inflammasome activation implies the presence of a pathogen-associated activity that is capable of delivering the molecules to the cytosolic receptor.

The Naip5/Nlrc4 inflammasome, which recognizes *L. pneumophila* flagellin and leads to pyroptosis and processing of pro-inflammatory cytokines, has been well characterized (48, 50, 56, 97). However, other host factors also contribute to restriction of *L. pneumophila* growth. One important such determinant is production of the cytokine Tumor Necrosis Factor-α (TNF). Indeed, humans receiving anti-TNF treatments are at high risk of infection with a number of intracellular pathogens, including *L. pneumophila* (98, 99). Here we show that macrophages deficient in TNF signaling are permissive to *L. pneumophila*, despite the presence of a functional Naip5/Nlrc4 inflammasome. Addition of exogenous TNF rescues bacterial restriction. Interestingly, however, there appears to be some functional interaction between TNF-dependent restriction and the Naip5/Nlrc4 inflammasome, since TNF is less effective at restricting the growth of flagellin-deficient bacteria. This potential crosstalk between TNF signaling and inflammasome activation was of great interest to us, since it suggests the integration of information both about the presence of a microbe (*i.e.*, through TLR-dependent recognition of a PAMP leading to TNF induction) and the identification of that microbe as a pathogen (based on its access to the cytosolic inflammasome).

Macrophages begin to produce detectable levels of TNF within 20 hours after infection with *L. pneumophila*, with maximal TNF levels reached at around 30 hours post infection (40). This TNF is entirely Myd88-dependent and largely TLR2-dependent (40, 43); therefore, it is induced by pathogenic and non-pathogenic *L. pneumophila* alike (67), since both secretion-competent and secretion-deficient bacteria can interact with the extracellular recognition domain of membrane-bound TLR2.

TNF is known to exert a variety of antimicrobial effects. It can cause autophagy (100); increase production of reactive oxygen and nitrogen species (101); activate the NF-κB transcription factor and downstream transcription and survival programs (102); or lead to either a Caspase-8-dependent apoptotic cell death (102), or a caspase-independent death called programmed necrosis (103, 104). We examined these possibilities in the context of *L. pneumophila* infection, but could not find a clear role for them in restriction of bacterial growth (data not shown). Instead, we discovered that TNF upregulates two distinct, novel forms of host cell death in macrophages infected with *L. pneumophila*: a Caspase-1/11-dependent death that is independent of known inflammasome initiators, and a Caspase-1/11-independent cell death

downstream of the flagellin sensors Naip5 and Nlrc4. We propose that cytokine-dependent upregulation of a cytosolic inflammasome, accessible only to pathogens, represents another mode by which the innate immune system can generate a tailored response to threats that it encounters.

4.2. TNF restricts second-round *L. pneumophila* growth by both flagellin-dependent and flagellin-independent mechanisms.

To examine the role of TNF in restriction of *L. pneumophila*, we infected macrophages lacking all known ligands of the TNF receptor 1 (TNFR1), namely TNF, lymphotoxin-α and lymphotoxin-β. We call these macrophages $TLT^{-/-}$, for **T**nf/**L**ympho**T**oxin$^{-/-}$. We performed these infections with bacteria expressing a luciferase/luciferin operon that allows us to monitor growth by measuring luminescence (40). Using this system, we observed that $TLT^{-/-}$ macrophages permit increased growth of *L. pneumophila* compared to wildtype B6 macrophages (Figure 4.1A). This permissiveness is less visible during infection with flagellin-deficient bacteria that evade Naip5/Nlrc4-dependent restriction, perhaps because these macrophages already permit near-maximal growth (Figure 4.1A). However, increased permissiveness of the $TLT^{-/-}$ macrophages even to flagellin-deficient bacteria can be seen in some experiments (40). Addition of exogenous TNF to infected macrophages rescues the phenotype, indicating that lymphotoxin is not necessary for restriction of *L. pneumophila*, and TNF is sufficient (Figure 4.1B).

L. pneumophila takes 16-24 hours to complete an infection cycle—that is, to infect a host cell, replicate to high titers, lyse or escape from that cell and go on to infect new hosts (105). The enhanced bacterial growth in $TLT^{-/-}$ macrophages is not evident until approximately 24 hours post infection (Figure 4.1A), around which time the bacteria begin their second cycle of infection. Thus, we infer that TNF does not play a role in the first round of infection, but exerts

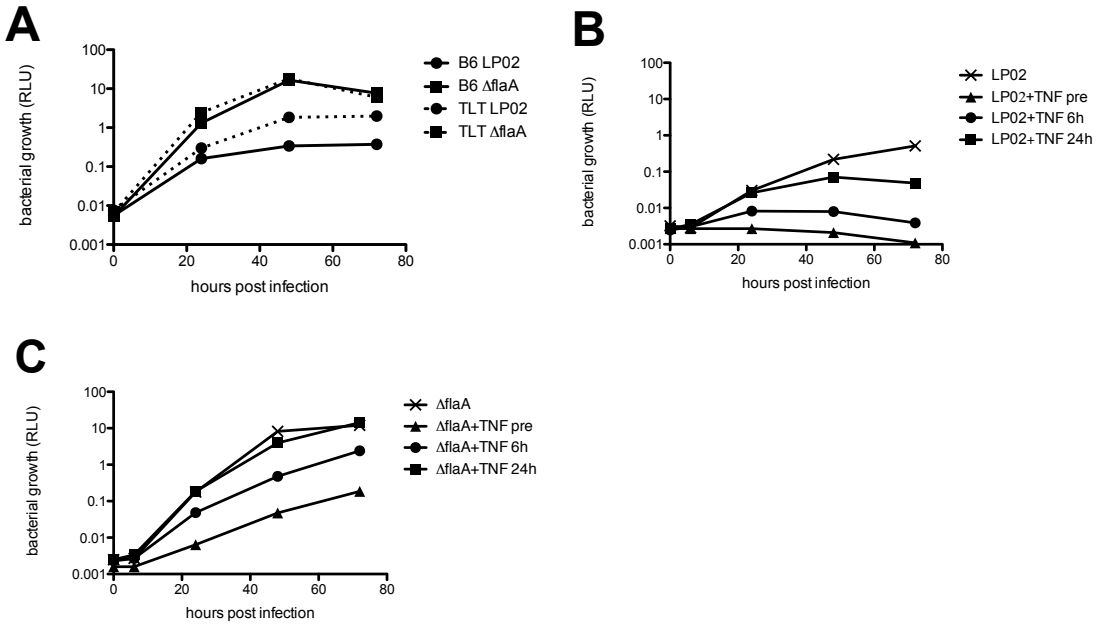

Figure 4.1. TNF restricts the growth of *L. pneumophila* in macrophages. B6 (A) or $TLT^{-/-}$ (A, B, C) macrophages were infected with luminescent wildtype *L. pneumophila* (LP02) or a flagellin-deficient mutant (Δ*flaA*) in macrophages. Bacterial growth was measured in Relative Luminescence Units (RLU). (B, C) TNF was added to $TLT^{-/-}$ macrophages at the indicated timepoints. Pre, macrophages were pretreated with TNF overnight before infection. 6h and 24h, TNF was added 6 and 24 hours post infection.

its anti-replicative effects during the second infection cycle and beyond, when new sets of macrophages are infected. This model is consistent with measurements of the kinetics of TNF production by infected macrophages (40).

To ascertain whether TNF could affect *L. pneumophila* growth during the first round of infection if it were present in sufficient concentrations, we added exogenous TNF to infected $TLT^{-/-}$ macrophages at various timepoints and measured bacterial replication by the luminescence assay. We observed that the earlier TNF was added, the more effective it was in restricting growth of *L. pneumophila* (Figures 4.1B, C). Interestingly, however, exogenous TNF was notably more effective at restricting the growth of bacteria that expressed flagellin; for example, while treatment with TNF at 6 hours post infection resulted in restriction of both the wildtype LP02 strain and the isogenic flagellin-deficient bacteria, adding TNF at 24 hours post infection effectively curtailed growth of LP02 but not *ΔflaA* (Figures 4.1B, C). Taken together, these data suggest that TNF restricts *L. pneumophila* by two distinct mechanisms, one of which is also dependent on bacterial expression of flagellin. Both of these TNF-dependent pathways appear to be necessary for full restriction of bacterial growth.

4.3. Macrophages pretreated with TNF exhibit increases in two distinct forms of lytic death when infected with *L. pneumophila*.

We wished to further examine the mechanisms by which TNF restricts growth of *L. pneumophila*. TNF elicits several potentially antibacterial programs in macrophages, including production of reactive oxygen and nitrogen species, Caspase-8-dependent apoptosis, and autophagy (100-104). However, we could not find a clear role for any of these in restriction of *L. pneumophila* (data not shown). We therefore considered what is currently the best-characterized host mechanism for restriction of *L. pneumophila* growth: namely, the Naip5/Nlrc4 inflammasome. In response to bacterial flagellin, this host pathway elicits a lytic host cell death called pyroptosis, which presumably inhibits bacterial growth by depriving the bacteria of their replicative niche (50) and, *in vivo*, delivering the bacteria to neutrophils for killing (106).

Since host cell death has been linked to restriction of *L. pneumophila*, we examined a role for TNF in *L. pneumophila*-induced macrophage death. To do this, we pretreated macrophages with TNF and then infected with *L. pneumophila*, measuring death by lactate dehydrogenase release (LDH) assay. By pretreating macrophages with TNF, we hoped to approximate the conditions of the "second round" of *L. pneumophila* infection, when the bacteria infect macrophages that were not infected initially but have been exposed to the TNF produced by infected macrophages for several or many hours. Interestingly, we found that pretreatment of macrophages with TNF resulted in enhanced macrophage death upon infection with *L. pneumophila* (Figure 4.2A). The increase in cell death was proportional to the length of TNF pretreatment, reaching a maximum with 16-24 hours of pretreatment. Thus, both macrophage death and bacterial restriction correlate positively to length of TNF pretreatment (Figures 4.1B and 4.2A), consistent with the hypothesis that restriction is effected through host cell death.

To determine whether TNF interacted functionally with flagellin to induce host cell death, as suggested by our growth curves, we infected TNF-pretreated macrophages with either wildtype *L. pneumophila*, or flagellin-deficient bacteria that do not induce cell death through the Naip5/Nlrc4 pathway. Both strains induced increased death in TNF-pretreated macrophages (Figure 4.2B). We then tested whether this increased death was Caspase-1-dependent, as is true for the canonical Naip5/Nlrc4 pathway (56). Surprisingly, we found that wildtype *L. pneumophila* still killed TNF-pretreated macrophages that lacked Caspase-1 (Figure 4.2B). This remaining death was entirely flagellin-dependent, as flagellin-deficient bacteria induced no death in Caspase-1-deficient macrophages regardless of TNF treatment (Figure 4.2B). These results allowed us to define two distinct TNF-upregulated cell death pathways (Figure 4.3): a flagellin-dependent death that is Caspase-1-independent, and a flagellin-independent death that is Caspase-1-dependent (or Caspase-11-dependent; see Section 4.4).

We were curious as to whether other factors could also upregulate these two death pathways. The cytokine Interferon-β (IFN) has been shown to restrict *L. pneumophila* growth in macrophages by an unknown mechanism (40, 49). IFN is historically considered an antiviral cytokine, and its role in bacterial infections is poorly understood (107). Macrophages pretreated with IFN exhibited increased cell death in response to both wildtype and flagellin-deficient *L. pneumophila*, indicating that multiple cytokines can elicit this host response and suggesting a possible mechanism by which IFN restricts *L. pneumophila* growth (Figure 4.2C).

4.4. Involvement of Caspase-1 versus Caspase-11 in flagellin-independent cell death.

Caspase1$^{-/-}$ macrophages are also deficient in Caspase-11, due to a null allele in the 129 mouse strain in which the knockouts were generated (108, 109). Thus, abrogation of flagellin-independent TNF-enhanced macrophage death in *Caspase1*$^{-/-}$ macrophages could be due to the deficiency of either Caspase-1 or Caspase-11. Indeed, we detected the cleaved forms of both Caspase-1 and Caspase-11 in supernatants of macrophages pretreated with TNF and infected with flagellin-deficient *L. pneumophila* (Figure 4.4A, B), indicating that both of these caspases are activated during infection.

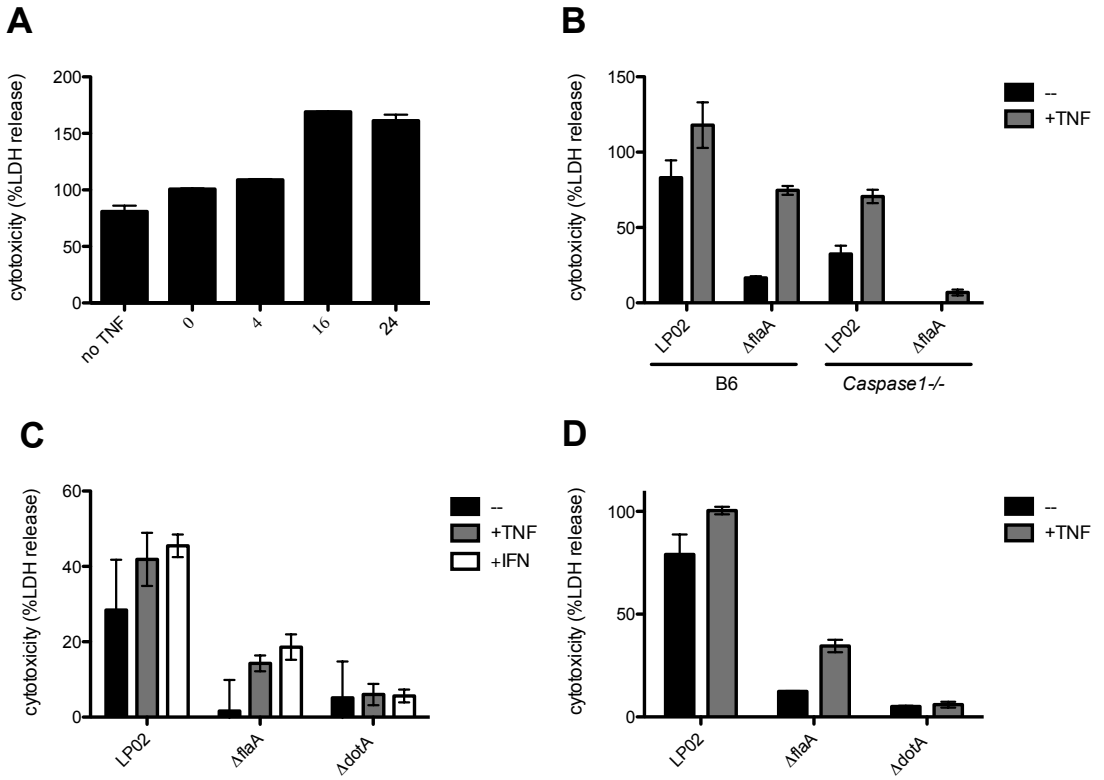

Figure 4.2. TNF primes macrophages for two distinct forms of pyroptosis in response to Dot/Icm^{+} *L. pneumophila*. (A-D) Death of infected macrophages was measured after 4 hours by LDH release assay. (A) B6 macrophages were pretreated for the indicated number of hours with TNF and infected with LP02. (B) B6 or *Caspase-1*$^{-/-}$ macrophages were pretreated overnight with TNF as indicated and infected with LP02 or DflaA. (C) B6 macrophages were pretreated with either TNF or Interferon-β (IFN). (D) B6 macrophages were pretreated overnight with TNF and infected with the indicated strains.

Because we do not currently have access to *Caspase11*$^{-/-}$ mice, we infected macrophages derived from a wildtype (*Caspase11*^{null}) 129 mouse to test the involvement of this protease. 129-derived macrophages were resistant to death upon TNF treatment and infection, despite the presence of functional Caspase-1 (Figure 4.4C). This result suggests that Caspase-11, rather than Caspase-1, is responsible for flagellin-independent death, although there are many other strain-specific differences that could also potentially affect host death in response to *L. pneumophila*. To more directly test Caspase-1 and Caspase-11 for a role in this inflammasome pathway, we expressed either Caspase-1 or Caspase-11 in *Caspase1*$^{-/-}$ (*Caspase11*^{null}) macrophages via retroviral transduction, then infected the transduced macrophages with various strains of *L. pneumophila*. Macrophages complemented with Caspase-11, but not Caspase-1, underwent pyroptosis upon infection in response to bacteria that activate the flagellin-independent inflammasome (discussed in greater detail below), indicating that Caspase-11 is likely the relevant 'death effector' for this inflammasome (Fig 4.3D).

4.5. TNF-enhanced cell death is dependent on bacterial secretion.

Ultimately, we are interested in uncovering mechanisms by which the host can respond specifically to pathogenic bacteria. TNF, which is robustly induced by surface-localized TLRs, is made in response to both wildtype *L. pneumophila* and an avirulent *ΔdotA* mutant (67). However, we found that *ΔdotA* bacteria do not kill macrophages even if they have been pretreated with TNF (Figure 4.2C), indicating that bacterial secretion is required to induce this host response. This is significant for several reasons. First, it suggests that the host integrates multiple cues—*i.e.,* TLR signaling and a secretion-dependent signal—to generate a unique response to pathogenic *L. pneumophila*. Second, the nature of the response is drastic, a lytic death that not only destroys a host cell but also releases potential inflammatory mediators. Making such a response to an evidently harmless microbe would be wasteful and potentially damaging to the host; thus, we suggest that the requirement for multiple signals serves to limit this response to occasions when it is necessary (110).

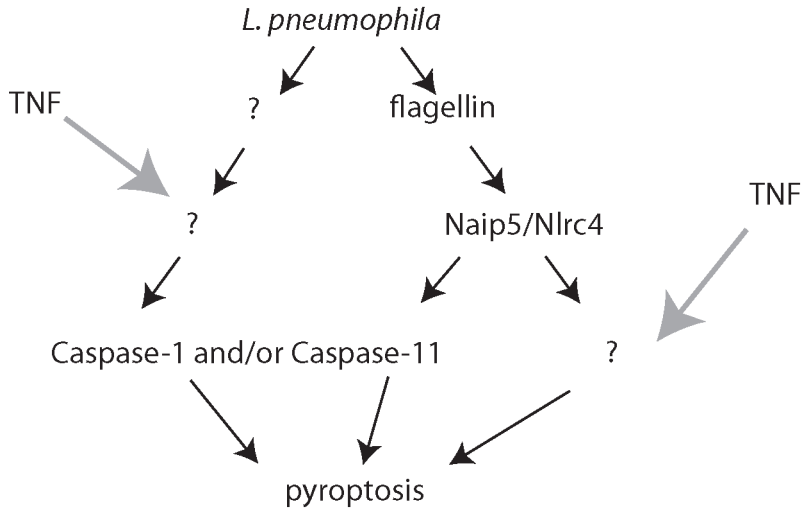

Figure 4.3. Preliminary model: two novel TNF-inducible inflammasomes that respond to *L. pneumophila*. In addition to the known Naip5/Nlrc4 inflammasome that activates Caspase-1-dependent pyroptosis in response to flagellin, we have revealed two additional death mechanisms upregulated by TNF treatment (grey arrows). One is Caspase-1 or Caspase-11-dependent, but the bacterial "ligand" and host "sensor" are unknown (question marks). The other involves flagellin, Naip5 and Nlrc4, is independent of Caspase-1 and -11.

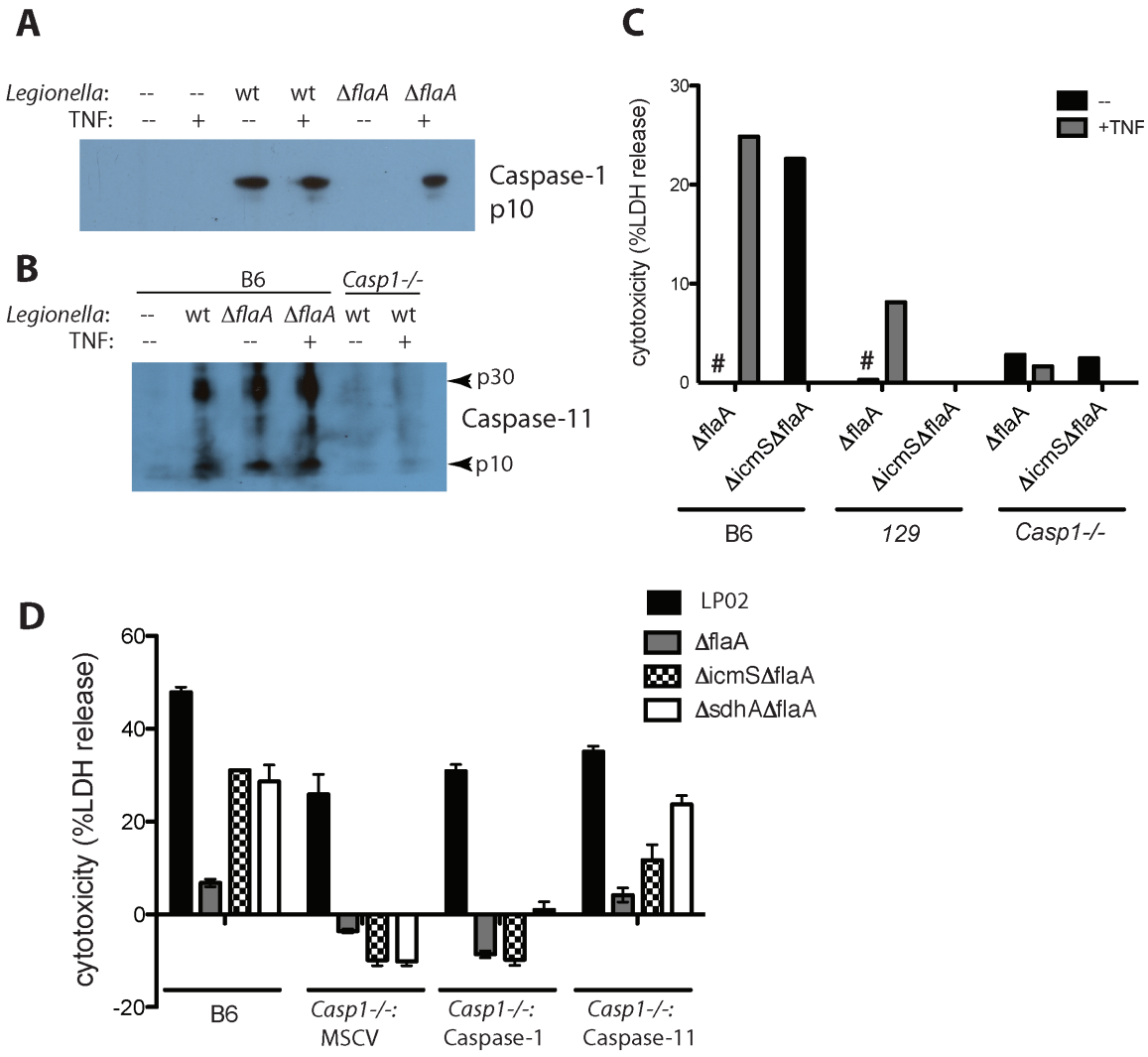

Figure 4.4. Death of TNF-treated macrophages infected with flagellin-deficient *L. pneumophila* is dependent on Caspase-11. (A, B) Supernatants of macrophages infected for 4h as indicated were precipitated with TCA and blotted for Caspase-1 (A) and Caspase-11 (B). In B, samples were pretreated for 4h with Pam3CSK4 (500 ng/mL). (C, D) Macrophages were pretreated with TNF and infected as indicated. Death was measured 4 hours post infection by LDH release assay. #, none detected. TNF treatment with *ΔicmSΔflaA* infection was not done in (B). In (D) *Caspase1*$^{-/-}$ macrophages (which also lack Caspase-11) were complemented with Caspase-1 or Caspase-11 using a retroviral MSCV vector.

4.6. Previously described inflammasomes cannot account for either TNF-enhanced cell death pathway.

The requirement for bacterial secretion to induce TNF-enhanced cell death (here abbreviated TECD) parallels what is already known about inflammasome activation in response to *L. pneumophila* and other stimuli: it requires delivery of microbial ligands to the cytosol (95, 96). To further dissect the mechanisms of the two TECD pathways, we tested whether known inflammasome components were required for either form of death. We infected macrophages individually deficient in the sensors Aim2 and Nlrp3, or the inflammasome adaptor Asc, with both wildtype and flagellin-deficient bacteria. Interestingly, both forms of TECD remained intact in these macrophages, indicating that the death is independent of these known inflammasomes (Figures 4.5A, B, C)

We also tested whether Naip5 and Nlrc4 were involved in TNF-enhanced cell death. The flagellin-dependent TECD was indeed dependent on both these host proteins (Figure 4.5D and data not shown), which is perhaps not surprising, given the known role for Naip5 and Nlrc4 in activation of the flagellin-responsive inflammasome. However, this TNF-induced inflammasome activation is distinct from the canonical Naip5/Nlrc4 pathway in that it is Caspase-1/11-independent (Figure 4.2B). This observation complicates the question of whether the flagellin-dependent cell death should indeed be considered "inflammasome-dependent"; although it requires the inflammasome components Naip5 and Nlrc4, the usual definition of inflammasomes includes activation of Caspase-1/11. However, for simplicity, we will continue to use the term "inflammasome" to refer to the initiating complex of this cell death.

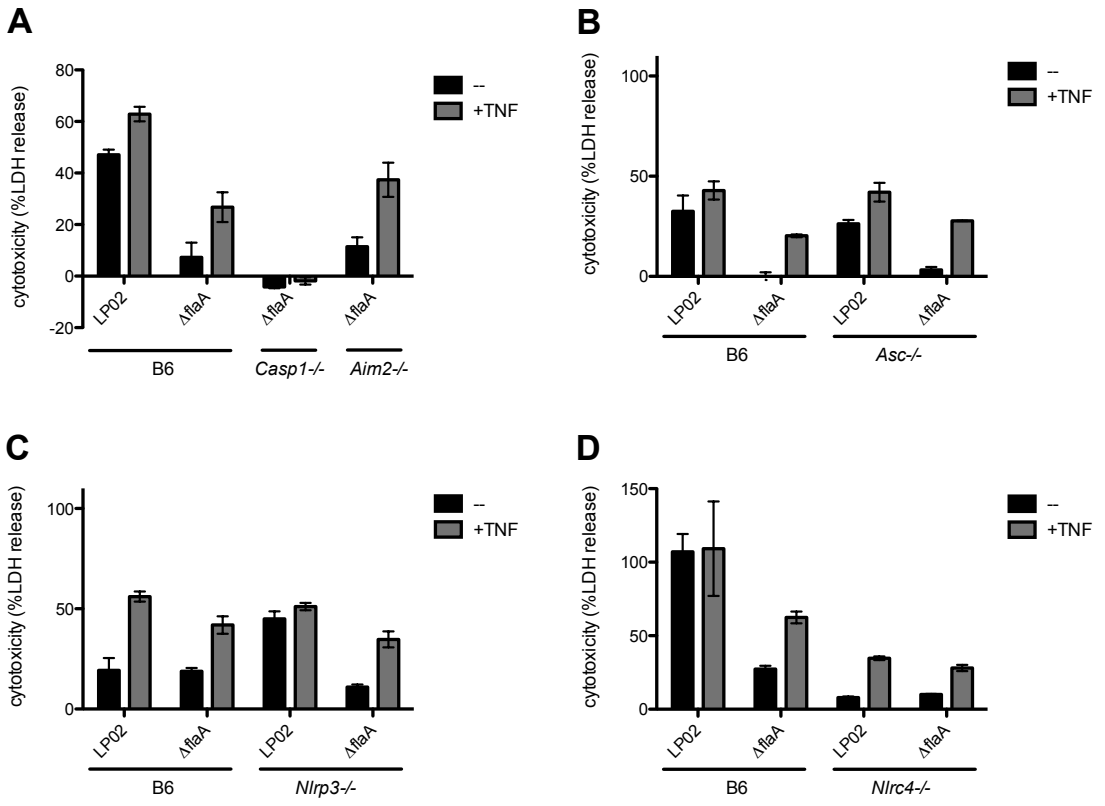

Figure 4.5. TNF-enhanced pyroptosis is independent of known inflammasomes. Macrophages deficient in Aim2 (A), Asc (B), Nalp3 (C) or Nlrc4 (D) were pretreated with TNF overnight as indicated and infected with wildtype or flagellin-deficient *L. pneumophila* for 4 hours. Macrophage death was measured by LDH release assay.

No role for Naip5 or Nlrc4 was observed during infection with flagellin-deficient bacteria, which is consistent with Naip5's established role as a flagellin sensor (56, 97) but which also rules out Nlrc4 as a possible adaptor for sensors of other microbial components, as has been reported (97, 111).

4.7. Flagellin-independent, Caspase-1/11-dependent TECD requires cIAP1.

We wished to identify the host protein(s) responsible for initiating TECD in response to flagellin-deficient bacteria. Previous work has established that in order to recruit and activate Caspase-1, an NLR must either contain a CARD (Caspase Activation and Recruitment Domain), or must interact with one of the CARD-containing adaptor proteins, Asc or Nlrc4 (109). Since the TNF-enhanced, flagellin-independent activation of Caspase-1/11 is Asc- and Nlrc4-independent (Figure 4.5B), we reasoned that the responsible host NLR must itself contain a CARD. We also hypothesized that the NLR might be induced transcriptionally by TNF. We therefore searched for TNF-inducible proteins, expressed in macrophages, that contained a CARD. We identified five proteins that fit these criteria: Nod1, Nod2, cIAP1, cIAP2, and Caspase-11 itself. The NLRs Nod1 and Nod2, extensively characterized as cytosolic sensors of bacterial PGN (17, 18), did not appear to play a role in Caspase-1/11-dependent TECD (Figure 4.6A). We then tested the CARD-containing proteins cIAP1 and cIAP2 (encoded by the genes *Birc2* and *Birc3*, respectively). These highly similar proteins play known roles in NF-κB activation downstream of the TNF receptor, and possibly in other pathways as well (112), by ubiquitinating and causing the degradation of pathway components (113, 114). To our surprise, macrophages deficient in cIAP1 were resistant to flagellin-independent TECD (Figure 4.6B), indicating involvement of this protein in the TNF-enhanced pyroptosis induced by *L. pneumophila*. In contrast, *Birc3*$^{-/-}$ macrophages exhibited no defect in TECD (Figure 4.6B). Consistent with previous reports (114), the lack of death in *Birc2*$^{-/-}$ macrophages was not due to poor responsiveness to TNF overall, since we observed intact upregulation of several gene targets in response to TNF (Figures 4.6C, D). Furthermore, *Birc2*$^{-/-}$ macrophages pretreated with IFN were also resistant to *L. pneumophila*-induced death (Figure 4.6E). Taken together, these results indicate that cIAP1 acts in the TNF-enhanced cell death pathway at the level of death itself, rather than in the upstream signaling events that prime the cells for death. Finally, we found that cIAP1 was required for cleavage of Caspase-1 in TNF-pretreated macrophages infected with flagellin-deficient *L. pneumophila* (Figure 4.6F), indicating that cIAP1 acts upstream of Caspase-1. These data are consistent with a model in which cIAP1 acts as an NLR that recruits and activates Caspase-1 in response to an unidentified, but secretion-dependent signal from *L. pneumophila*. This result does not rule out the involvement of Caspase-11 in cIAP1-dependent cell death, as Caspase-11 activation is reported to be upstream of Caspase-1 cleavage under certain circumstances (108).

The implication of cIAP1 in a cell death pathway is somewhat surprising, as the IAPs are generally considered to be anti-apoptotic (115, 116) (indeed, the name stands for **I**nhibitor of **A**poptosis **P**rotein). However, several observations are consistent with a role for cIAP1 in promoting cell death. First, much characterization of cIAP1 and 2 as anti-apoptotic factors has been done in overexpression systems or tumor cell lines that may not reflect their physiological activities (113, 117). Second, it has been reported that caspase-dependent cleavage may convert cIAP1 from an anti-apoptotic factor to a promoter of cell death (118). Third, and most intriguingly, the cIAPs share domain architecture with several NLRs known to activate the inflammasome. Both cIAP1 and 2 have three N-terminal BIR (Baculoviral Inhibitor of apoptosis Repeat) sequences, a central CARD, and a C-terminal RING domain that encodes an E3 ligase (115, 116). N-terminal BIR domains are also found in the NLR Naip5, which itself was originally named as an anti-apoptotic factor (Neuronal Apoptosis Inhibitor Protein) (119) but has since been established as a crucial component of the inflammasome that initiates Caspase-1-dependent cell death in response to flagellin. As previously noted, CARD domains are a common feature of inflammasome activators. Thus, from a structure-function perspective, it is plausible that cIAP1 may act as an NLR in activation of a novel inflammasome.

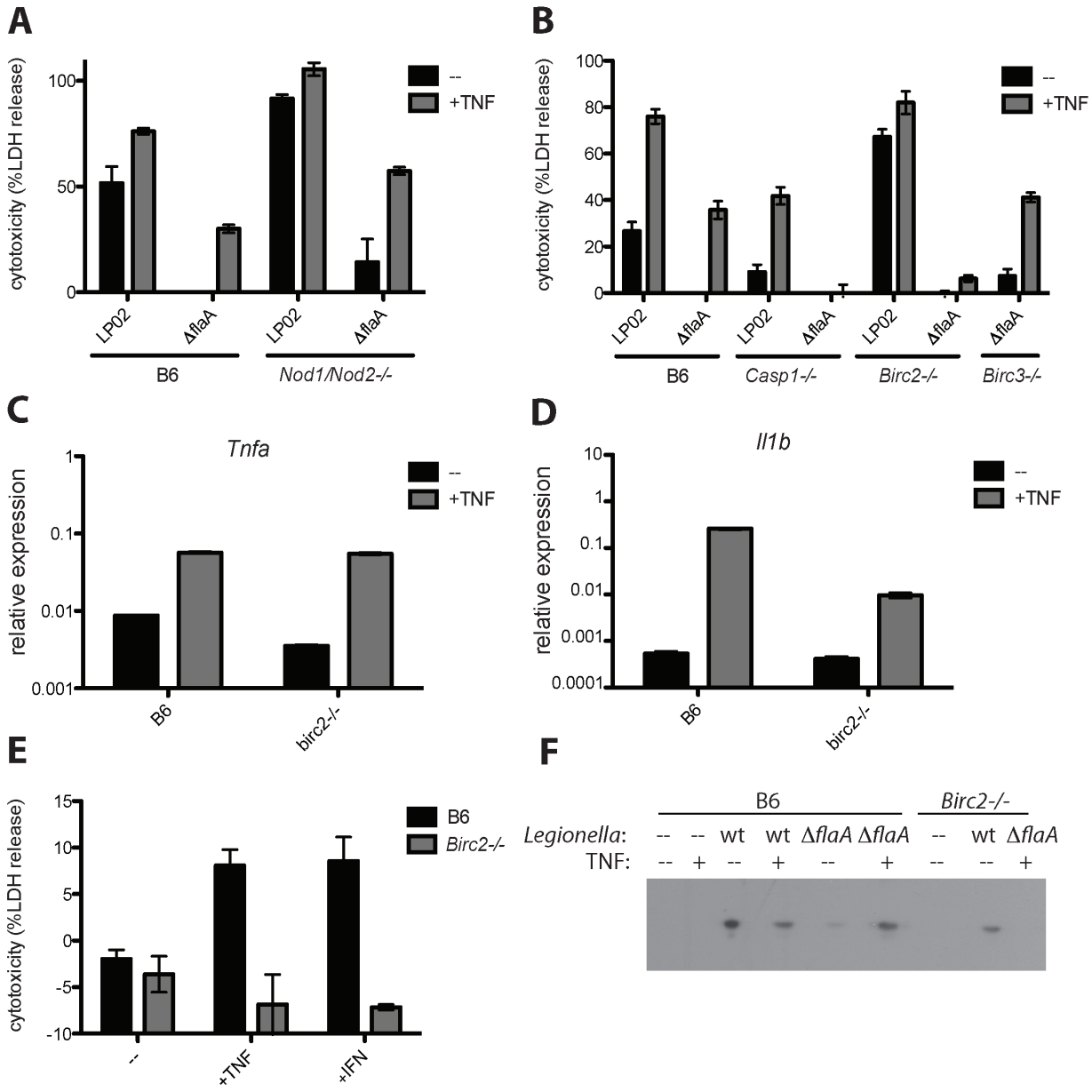

Figure 4.6. Flagellin-independent TNF-enhanced death requires cIAP1. (A, B, E) The specified macrophages were pretreated overnight with IFN or TNF and infected as indicated, and macrophage death was measured 4 hours post infection by LDH release assay. (C, D) RNA was harvested from B6 or *Birc2*$^{-/-}$ macrophages treated overnight with TNF. Levels of the indicated mRNA transcripts were measured by quantitative RT-PCR. (F) B6 or *Birc2*$^{-/-}$ macrophages were treated with TNF overnight where indicated and infected. After 4h, supernatants were harvested, TCA-precipitated, and blotted for cleaved Caspase-1.

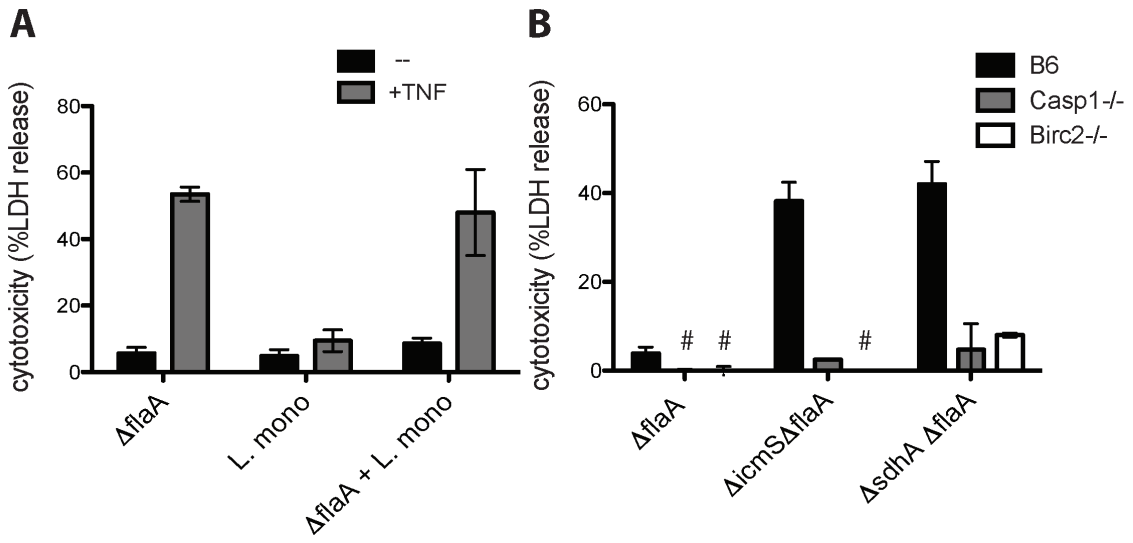

Figure 4.7. Bacterial determinants of flagellin-independent cell death. (A, B) B6 (A) or otherwise indicated (B) macrophages were infected with the indicated strains of *L. pneumophila* or *L. monocytogenes* (L. mono) with or without TNF pretreatment, as specified. Host cell death was measured 4 hours post infection by LDH assay.

4.8. Bacterial factors in cIAP1-dependent cell death.

We wished to investigate the nature of the bacterially derived signal that induces cIAP1-dependent Caspase-1 and -11 activation and cell death. First, we tested whether this was a common response to intracellular bacteria in macrophages pretreated with TNF. To this end, we infected TNF-pretreated macrophages with the gram-positive intracellular pathogen *Listeria monocytogenes*, which escapes into the cytosol upon infection (120). *L. monocytogenes* induced negligible amounts of cell death even in TNF-pretreated macrophages (Figure 4.7A). *L. monocytogenes* did not appear to actively suppress death, since macrophages coinfected with *ΔflaA L. pneumophila* and *L. monocytogenes* still died (Figure 4.7A). These results indicate that death is not a common response to intracellular bacteria in TNF-exposed macrophages, and suggest that the bacterial ligand is not simply a PAMP shared between *L. pneumophila* and *L. monocytogenes*.

Because death was dependent on bacterial secretion (Figure 4.2D), we wondered if one or more secreted bacterial effectors might induce or influence this host cell death. We therefore infected macrophages with flagellin-deficient *L. pneumophila* lacking the chaperone protein IcmS, described in Chapter Two. Briefly, this mutant strain has a functional Type IV secretion system but fails to secrete most effectors (77, 78). To our surprise, the *ΔicmSΔflaA* strain was hypercytotoxic to macrophages even without TNF pretreatment (Figure 4.7B). This death was entirely dependent on cIAP1 (Figure 4.7B), and was abrogated in both *Caspase1*$^{-/-}$ macrophages (Figure 4.7B) and Caspase-11-null 129 macrophages (Figure 4.4C).

We hypothesized, then, that wildtype bacteria secrete an IcmS-dependent effector that suppresses cIAP1-dependent cell death. Indeed, one potential such protein has been described in the literature: bacteria lacking the translocated effector SdhA kill macrophages in a flagellin-independent manner (49, 121). The function of SdhA is not known, and the effector possesses no homology to known proteins (49, 121). We infected B6 and *Birc2*$^{-/-}$ macrophages with a flagellin-deficient *ΔsdhA* mutant strain. Interestingly, these bacteria also induced high levels of macrophage death that were abrogated in *Birc2*$^{-/-}$ macrophages (Figure 4.7B). As with the *ΔicmS* strain, cytotoxicity did not require TNF pretreatment. We presume that the *ΔicmS* mutant

induces death because of a failure to secrete SdhA. While it has not been formally demonstrated that icmS is required for secretion of SdhA, the vast majority of tested *L. pneumophila* effectors are IcmS-dependent (77, 78).

4.9. Model of flagellin-independent TNF-enhanced macrophage death.

Initially, we explored the hypothesis that TNF enhanced macrophage death in response to *L. pneumophila* by increasing the expression of an NLR or other inflammasome component. Precedent for this model comes from both the Nlrp3 and Aim2 inflammasomes, which require TLR and IFN signaling, respectively, for their upregulation (122-125). Additionally, the length of TNF pretreatment required for maximal cell death (16-20 hours) would seem to rule out the more rapid and transient effector functions downstream of TNF, such as induction of ROS/RNI.

However, this model of increased NLR expression is undermined by the observation that a mutant strain lacking a single effector, SdhA, hyperinduces a TNF-independent cell death that is genetically indistinguishable from that enhanced by TNF treatment. This result suggests that SdhA normally functions to suppress host cell death in response to a bacterial ligand or signal produced by wildtype *L. pneumophila*. Delivery of this "death" ligand must be independent of the bacterial chaperone IcmS, since *ΔicmS* mutants induce cIAP1-dependent pyroptosis; thus, the ligand could be an IcmS-independent effector protein (of which, however, only one has been described (78, 126)), or a non-protein ligand such as nucleic acid or fragments of cell wall. If the ligand is a PAMP, however, it appears not to be simply a molecule common to (or identical in) both *L. pneumophila* and *L. monocytogenes*, as only the former pathogen kills TNF-pretreated macrophages (Figure 4.7A).

It is not yet clear how cIAP1 might actually sense *L. pneumophila*. While the N-terminal BIR domains of cIAP1 superficially resemble the N-terminal domain of the NLR Naip5, at least one of cIAP1's BIR domains is known to mediate association with Traf2, a signaling component downstream of the TNF receptor, rather than an inflammasome component (127). However, the three BIR domains of cIAP1 are distinct, and it is possible that one or both of the other BIR domains interact with a bacterial ligand. The BIR domains of cIAP1 have also been shown to interact with caspases (128), although specific interactions with Caspase-1 or Caspase-11 have not been reported. We have not examined whether the E3 ligase activity of cIAP1 is required for inflammasome activation; this is an intriguing question for further study.

Another pressing question addresses the connection between TNF and the bacterial effector SdhA: cIAP1-dependent death is promoted by the former and suppressed by the latter. Therefore, one possibility is that TNF may promote a cell process that generates a ligand for cIAP1 or translocates a ligand to the cytosol, and that SdhA antagonizes this process. One potential cell process could be phagosome maturation, which TNF appears to enhance in macrophages (129, 130). In fact, it has been suggested that SdhA helps maintain the integrity of the *Legionella*-containing vacuole (L. Creasey and R. Isberg, manuscript submitted). A previous report has demonstrated that macrophages pretreated with Interferon-γ, then infected with bacteria, translocate fragments of degraded bacteria to the cytosol where they are sensed by cytosolic receptors (131). TNF may similarly prime phagosomes to activate the cIAP1 inflammasome (Figure 4.7).

Our two models—that TNF upregulates a protein required for cell death, or that TNF promotes phagosome maturation and ligand generation—need not be mutually exclusive. Indeed, TNF may promote phagosome maturation by upregulation of some host factor, such as a vacuolar ATPase. In any case, TNF is a pleiotropic cytokine and is likely to restrict growth of *L. pneumophila* via multiple mechanisms.

4.10. Genetic components of flagellin-dependent death.

In contrast to the cIAP1-dependent cell death discussed extensively above, a second type of TNF-enhanced cell death is independent of host Caspase-1 and Caspase-11 (Figure 4.2B) and dependent on bacterial flagellin (Figure 4.2B) and the host NLRs Naip5 and Nlrc4 (Figure 4.5D, and data not shown). LDH release assays suggested that this cell death was rapid and lytic, properties typically associated with the Caspase-1/11-dependent cell death called pyroptosis,

rather than the slower and more contained apoptosis (132). Therefore we wondered whether this Caspase-1/11-independent TECD resembled pyroptosis or apoptosis in its morphology. We used time-lapse microscopy to observe death of *Caspase1*$^{-/-}$ macrophages infected with wildtype *L. pneumophila* in the presence or absence of TNF. Apoptotic cells, identified by classic membrane blebbing and shrinkage of nuclei, were observed under both conditions. Additionally, some infected TNF-pretreated macrophages rapidly lost membrane integrity and leaked their cellular contents with morphology resembling pyroptosis (see Methods). No loss of membrane integrity was observed in infected macrophages that were not pretreated with TNF. Thus, flagellin-dependent TECD can be classified morphologically as pyroptosis. This finding is significant, because pyroptosis has been previously considered a Caspase-1/11-dependent phenomenon (132). These results suggest the need to redefine this important innate immunological response, which has already been implicated in restriction of bacterial growth *in vivo* (106).

We sought to further characterize Caspase-1/11-independent pyroptosis by identifying the host effector protein(s) downstream of Naip5 and Nlrc4. Death of *Caspase1*$^{-/-}$ macrophages could be inhibited by the pan-caspase inhibitor ZVAD-FMK, indicating that one or more other caspases are involved in this death (Figure 4.8A). In contrast, cell death was intact in the presence of necrostatin, ruling out programmed necrosis (103, 104) as the mechanism of this death (Figure 4.8A).

Mice express a variety of different caspases. We have ruled out Caspase-1 and Caspase-11 (Figure 4.2B). To test a role for Caspase-2, we generated mice doubly deficient in Caspase-1 and Caspase-2. However, we found no role for Caspase-2 in either pyroptosis (Figure 4.8B) or restriction of bacterial growth (data not shown). We are currently generating *Caspase1*$^{-/-}$ *Caspase7*$^{-/-}$ mice to test a role for Caspase-7 in TNF-enhanced pyroptosis, though preliminary experiments suggest that Caspase-7 is not involved (data not shown).

We also took an unbiased approach to identify caspases activated downstream of Naip5 and Nlrc4. We infected TNF-pretreated *Caspase1*$^{-/-}$ macrophages with *L. pneumophila* in the presence of a fluorescent warhead, AWP28, that binds covalently to activated caspases (A. Puri and M. Bogyo, unpublished data). SDS-PAGE analysis of labeled caspases indicated that Caspase-3 and Caspase-7 are robustly activated in infected *Caspase1*$^{-/-}$ macrophages (Figure 4.8C). However, we observed this activation in non-pretreated macrophages as well as TNF-pretreated macrophages, making the significance of this finding unclear. Further research is needed to determine whether Caspase-3 and/or -7 are in fact responsible for lytic death downstream of flagellin—a result that would entail a novel activator of the apoptotic caspases as well as a novel outcome.

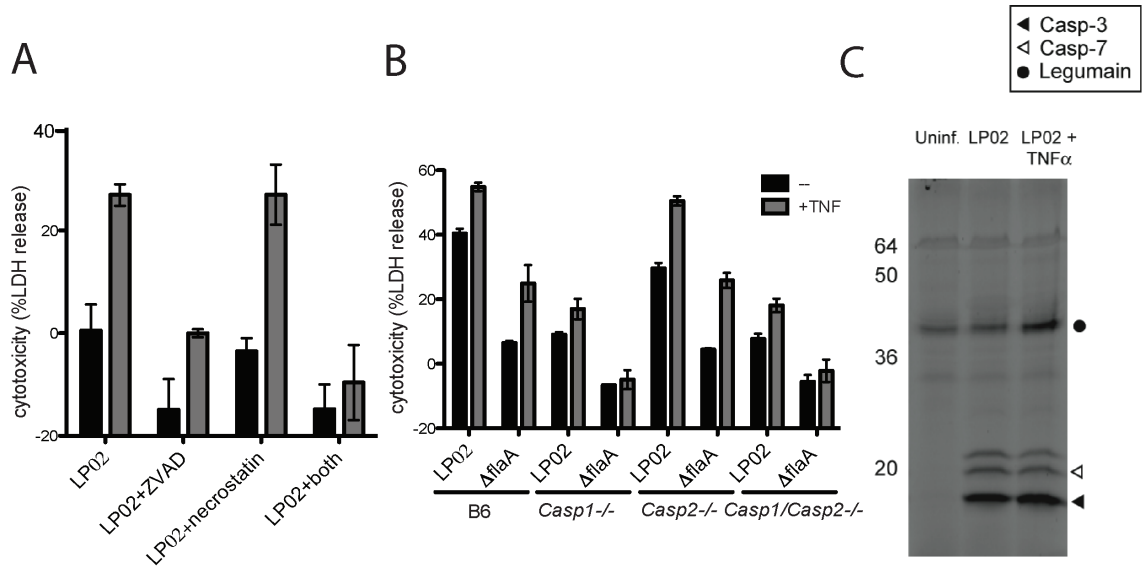

Figure 4.8. Involvement of other caspases in flagellin-dependent TNF-enhanced cell death. (A, B) Macrophages were pretreated with TNF overnight and infected as indicated. Necrostatin (30μM) and ZVAD (30μM) were added 30 min before infection. Death was measured by LDH release assay 4 hours post infection. (C) *Caspase1*$^{-/-}$ macrophages were pretreated with TNF overnight, then infected with wildtype *L. pneumophila* for 2h. Active caspases were labeled with the fluorescent probe AWP28 (1μM) and fluorescence was visualized on a gel using a Typhoon scanner.

4.11. Discussion.

Here we have demonstrated two distinct and novel mechanisms by which macrophages respond to *L. pneumophila* (Figure 9). Because of the common involvement of specific host proteins and the shared outcome of lytic host cell death, we have termed these mechanisms "inflammasomes"; however, further research is needed to understand whether the molecular interactions in these pathways are indeed analogous to previously described inflammasomes. Nevertheless, this work establishes several novel facets of cytosolic immunosurveillance that merit further study.

First, the observation of a lytic cell death that is activated by known inflammasome initiators (Naip5 and Nlrc4), but that is Caspase-1/11-independent, calls for a broader definition of pyroptosis, which has previously been considered a Caspase-1/11-dependent phenomenon. Semantics aside, this work establishes an alternate pathway for activating pyroptotic programs in the absence of Caspase-1 and Caspase-11. This may be important in situations when a pathogen purposefully interferes with caspase activation, as in multiple viral infections (102). Furthermore, our laboratory has observed that activation of a Naip5/Nlrc4-dependent inflammasome *in vivo* has consequences that are independent of Caspase-1/11 (J. von Moltke and R.E. Vance, upublished data). These consequences may be explained by the alternative cell death pathway demonstrated here.

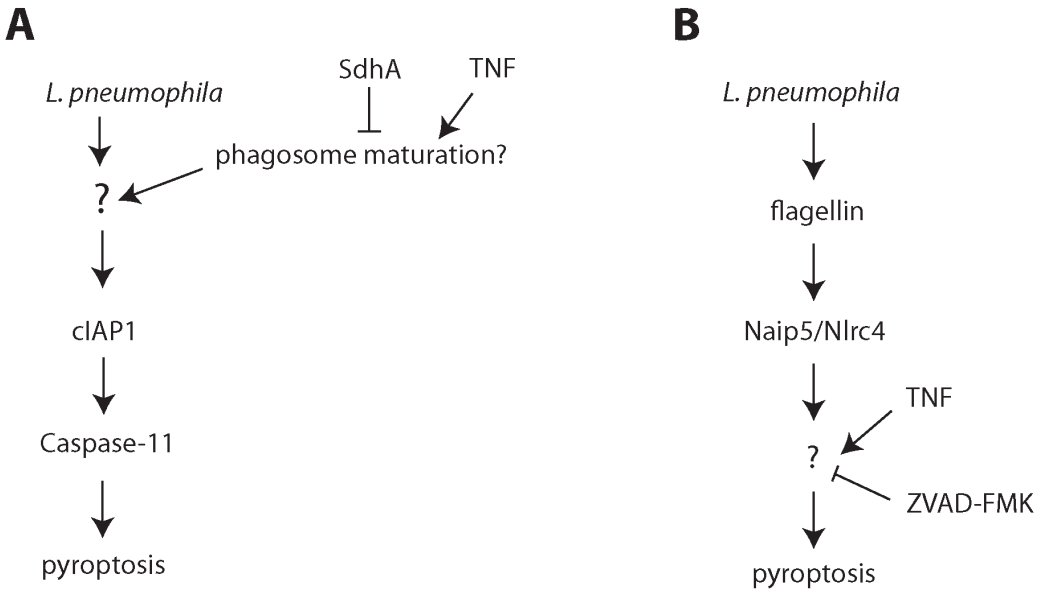

Figure 4.9. Models of TNF-inducible inflammasome activation. (A) An unidentified (but secretion-dependent) signal from *L. pneumophila* activates Caspase-11-dependent pyroptosis via cIAP1 in TNF-treated macrophages. The bacterial effector SdhA blocks this death, perhaps by antagonizing TNF-enhanced phagosome maturation. (B) *L. pneumophila* flagellin activates Naip5 and Nlrc4, which activate an unidentified protease that is sensitive to ZVAD-FMK to induce pyroptosis, independently of Caspase-1 and -11.

Another important finding of this work is a novel role for cIAP1 in eliciting pyroptosis in response to a bacterial pathogen. Many questions remain about this pathway, including the nature of the secretion-dependent bacterial signal and the ability of cIAP1, but not its close homolog cIAP2, to activate Caspase-1 and Caspase-11. However, considering the structural similarities between cIAP1 and Naip5, we suggest that BIR-domain containing proteins may represent a previously unappreciated class of inflammasome-activating proteins that elicit pyroptosis under certain conditions. These conditions may be cell type or stimulus specific, or they may be the result of cytokine pretreatment in the case of cIAP1. In any case, this is certainly not the first example of a protein that can promote cell survival in some contexts and cell death in others (133).

The functional interactions between host and bacterial factors in induction of cIAP1-dependent pyroptosis also provide a rich avenue for further studies. Loss of a single bacterial effector, SdhA, results in a drastic host response indistinguishable to that elicited by TNF pretreatment. It will be informative to study the mechanism of SdhA action in host cells, to determine whether SdhA specifically antagonizes a host cell death pathway or whether it modulates a bacterial activity or phagosomal property that indirectly leads to pyroptosis.

The protein SdhA is required for growth in mammalian macrophages, but dispensable for growth in amoebae (121). This is puzzling, since *L. pneumophila* is not thought to have evolved in mammals. However, it may reflect a specific role for SdhA in macrophages to counteract the innate immune system, which does not exist in amoebae. It is tempting to speculate that *L. pneumophila* may indeed have a naturally occurring vertebrate host, perhaps a fish or other aquatic animal, in which SdhA may undergo positive selection.

It was our initial investigation into the mechanism by which TNF restricts growth of *L. pneumophila* that led us to the discovery of the two novel TNF-upregulated inflammasomes. We have not directly demonstrated that these inflammasomes are in fact responsible for TNF-dependent restriction of bacterial growth. Furthermore, TNF may aid in bacterial restriction via multiple mechanisms. However, our data are consistent with a model in which TNF-upregulated inflammasomes indeed help control *L. pneumophila* replication by enabling infected macrophages to die rapidly, depriving the bacteria of their replicative niche and releasing inflammatory signals that may arm neighboring cells to better fight infection. Several observations support this model: first, there is a direct correlation between time of TNF addition, level of host cell death upon infection, and restriction of bacterial growth. Second, TNF appears to exert both flagellin-dependent and flagellin-independent effects on bacterial growth, which could be explained by the activity of the two distinct novel inflammasomes. Third, bacterial replication in the initial infection cycle (0-24 hours post infection) is identical between wildtype and TNF-deficient macrophages; it is only in subsequent rounds of infection that TNF-deficient macrophages show increased permissiveness. Thus, TNF exerts its effects in secondarily infected macrophages that are presumably exposed to TNF before they become infected—essentially a "pretreatment" that parallels the conditions in our cell death assays. Finally, comparisons to the Naip5/Nlrc4 inflammasome would suggest that host cell death can effectively curtail the growth of *L. pneumophila*. Thus, we favor the hypothesis that TNF restricts growth of *L. pneumophila* by enhancing the activity of two distinct novel inflammasomes that cause infected cells to undergo pyroptosis.

Chapter Five:
Conclusions, Questions, and Perspectives

5.1. Review of findings.

In this dissertation, I have elucidated several novel innate surveillance pathways that contribute to the immune response to our model intracellular bacterial pathogen, *L. pneumophila*. In addition to providing mechanistic insight into the host response to one particular pathogen, these studies also have interesting implications for our understanding of the "wiring" of the innate immune system—in particular, how pathogenic microbes are distinguished from non-pathogens and how the immune system is able to appropriately scale its response according to the threat at hand.

In Chapter Three, I described a novel mode of innate immune sensing in mammals—one based on recognition not only of microbial molecules, but also of a pathogen-associated activity. In this immunosurveillance pathway, which was termed an 'Effector-Triggered Response' after the nomenclature used in plants (10), TLR and Nod1/2 signaling synergized with translation inhibition to selectively superinduce a subset of host transcriptional targets, including stress-related genes and pro-inflammatory cytokines. Translation inhibition in the context of PRR signaling could also elicit an immune response *in vivo,* although the role of this pathway in microbial infection remains to be determined.

Chapter Four focused on a distinct set of immunosurveillance systems, in which cytosolic TNF-inducible inflammasomes detected *L. pneumophila* in a manner dependent on bacterial secretion, leading to rapid and lytic host cell death (pyroptosis). The novel inflammasomes consist of some known inflammasome proteins (Naip5, Nlrc4, Caspase-11) as well as others that have not previously been linked to inflammasome activation (cIAP1; possibly Caspase-3 and Caspase-7). These inflammasomes are not observable in resting macrophages infected with wildtype *L. pneumophila*, but they can be activated by pretreatment with TNF or other inducers, perhaps mimicking the second round of macrophage infection in tissue culture. While we have not assessed the activity of these inflammasomes *in vivo*, mouse infection studies with other intracellular bacterial pathogens would suggest that any mechanism that enhances host cell death upon infection with *L. pneumophila* could aid in restriction of the bacteria (106, 134).

5.2. Common themes in innate immune surveillance.

While distinct in their particulars, the pathways described in Chapters Three and Four share some common features that may offer insight into general innate immune strategies. First, both the 'Effector-Triggered Response' and the TNF-inducible inflammasomes require the integration of multiple signals. In both cases, one signal emanates from a host receptor located at the cell membrane (*i.e.*, a TLR) that can distinguish between self-molecules and microbes based on pattern recognition, but that does not itself distinguish between pathogens and non-pathogens. Specifically, both wildtype and secretion-deficient bacteria trigger TLR-dependent NF-κB activation and transcription of target genes, including TNF, which can then act in an autocrine or paracrine fashion to upregulate additional targets and activate the macropahge.

A second signal, however, is transmitted only by virulent bacteria that are competent for Type IV secretion. In the Effector-Triggered Response, this signal is derived from five secreted effectors that inhibit host translation, leading to loss of anti-inflammatory inhibitors, sustained NF-κB and MAP kinase activation, and superinduction of a subset of target genes. In the case of the TNF-inducible inflammasomes, only infection with secretion-competent bacteria leads to inflammasome activation and pyroptosis, most likely due to the cytosolic delivery of bacterial ligands (including flagellin, as has previously been demonstrated (57)) via the Type IV secretion system. The localization of both of these second signals to the cytosol, as well as the disruption to host physiology that translation inhibition represents, both suggest the presence of a harmful agent. Taken together, "signal one" and "signal two" can be interpreted by the immune system as evidence of a microbe that causes harm—that is, of a pathogen.

A second common feature of both of these immunosurveillance pathways is the relatively dramatic nature of the response that each elicits. The Effector-Triggered Response results in a striking, hundred- to thousand-fold induction of certain transcriptional targets, including markers of DNA damage and cell stress and pro-inflammatory cytokines that can be damaging to the host when expressed inappropriately. (Indeed, the cytokine IL-23, robustly induced by the Effector-Triggered Response, has been linked to pathology in several models of autoimmune diseases.) Such impressive induction of these stress- and inflammation-related targets is not observed in infection with avirulent Dot/Icm$^-$ *L. pneumophila*, even though this bacterium does stimulate TLRs. Thus, detection of a pathogen-encoded activity switches on a distinct host transcriptional program that indicates cell stress and includes production of potentially harmful cytokines.

The TNF-inducible inflammasomes induce a similarly (or even more) drastic response: lytic host cell death, which is presumably accompanied by processing and release of the pro-inflammatory cytokines IL-1β and IL-18. This response is irreversible, and results in loss of host cells and in inflammation that could also be harmful to the host if unleashed inappropriately. As antigens, pyroptotic cells may contain costimulatory signals that could conceivably lead to generation of immune responses against self molecules—bad news for the host. In contrast, TNF alone may either stimulate cell survival or an apoptotic cell death that is immunologically silent (102, 135). (TNF alone may also direct cells to commit programmed necrosis, but only in cases where caspases are inhibited—often indicating the presence of an infectious virus (101, 103).)

Thus, in both of these cases, the addition of a pathogen-specific, cytosolically localized signal converts the host response from a relatively benign and reversible one to a much more significant, potentially damaging, and (in the case of inflammasome activation) irreversible response. Conceptually, this makes sense: the more drastic responses require more input from multiple innate immune signaling pathways, in particular those that selectively indicate the presence of a pathogen.

5.3. Two-signal models in innate immunity.

The pathways we describe here might be considered innate parallels to the "two-signal models" that govern activation of the B and T cells of the adaptive immune system. These two-signal models, in a variety of forms, have helped shape our understanding of the adaptive immune response for over 40 years (136). In its simplest form, the current two-signal model of lymphocyte activation states that stimulation of B and T cells requires cooperation between two distinct signals: an antigen-specific signal that engages the B or T cell receptor, and a second receptor-mediated "co-stimulatory" signal that licenses the recipient lymphocyte to respond to antigen. In the case of T cells, this second signal is provided by an antigen-presenting cell that has been activated by contact with microbes. Activated T cells can then present a co-stimulatory signal to B cells. The two-signal model for B and T cell activation provides a molecular mechanism for ensuring that the adaptive responses of lymphocytes are directed toward foreign microbial antigens. The requirement for two signals is thought to act as a safeguard that regulates the powerful and potentially harmful immune reaction, and prevents the accidental triggering of responses against the host's own tissues.

In the same way, the innate immune system may itself be regulated by two-signal frameworks, such as the ones described in this work. While certainly not all innate immune responses fall neatly into a two-signal model, many do seem to—particularly when considering the immune response to virulent microbes, which both trigger and manipulate multiple innate pathways.

Within two-signal models of innate immune sensing, we have herein set forth several examples of cooperation between PRRs and pathways that detect pathogen-associated activities. Conceptually, the latter are intriguing because they offer a way to selectively respond to pathogenic bacteria (whereas both pathogens and non-pathogens have the potential to trigger pathways that sense conserved microbial ligands). As more examples of effector-triggered immunity are characterized in metazoans, we hope to better understand how the innate immune

system integrates multiple signals in order to contain infection and elicit an appropriate adaptive response.

5.4. Remaining questions.

5.4.1. The role of translation inhibition recognition in vivo.

In this study, we did not observe differences in the *in vivo* immune responses to wildtype *L. pneumophila* and a mutant strain, Δ5, lacking five effectors that inhibit host translation. As mentioned previously, this may be due both to redundancy among innate immune pathways that recognize *L. pneumophila*, and also to the sum of positive and negative effects of translation inhibition on the bacteria themselves. While the mutant strain exhibits no growth defect in cultured macrophages, the environment in the mouse lung may more closely resemble natural growth in amoebae, where these effectors *are* required for growth. Thus, a less robust immune response in mice infected with Δ5 may be offset—and therefore masked—by a growth defect in the mutant bacteria themselves.

In future experiments, the role of detection of translation inhibition may perhaps be revealed *in vivo* by removal of redundant immunosurveillance pathways, for example in gene-targeted mice lacking the common TLR adaptor Myd88 and/or the Nod adaptor Rip2. Some attempts were made in this direction already, but these experiments were probably confounded by a dual requirement for Myd88 in both TLR signaling (which itself is probably redundant with sensing of translation inhibition *in vivo*) and in signaling downstream of the IL-1 receptor, which is known to be critical for neutrophil recruitment, one of the main hallmarks of *L. pneumophila* infection and a major determinant of bacterial clearance. It is possible that sensing of translation inhibition might be crucial for neutrophil recruitment during lung infection of chimeric mice, made by transferring *Myd88*$^{-/-}$*/Nod1*$^{-/-}$*/Nod2*$^{-/-}$ bone marrow into wildtype B6 recipients. These mice would lack TLR and Nod signaling, but IL-1R signaling to stromal cells (which themselves make chemokines that recruit neutrophils (42)) would be intact.

Additionally, there is evidence that *L. pneumophila* possesses still more secreted effectors that inhibit host translation, particularly at late timepoints (Figure 3.4C and K. Barry, unpublished data). Discovery and deletion of these genes in the Δ5 background might create a larger *in vivo* phenotype for this mutant, allowing resolution of the effects of translation inhibition.

Finally, it remains an open question as to whether detection of translation inhibition might have any impact on the adaptive immune response. Immunization studies, for example using a translation-inhibiting toxin such as *Pseudomonas* Exotoxin A as an adjuvant, could assess the capabilities of the sensing pathway to elicit adaptive responses. It will be of particular interest to determine what type of adaptive response is induced (if any); identification of IL-23 as a major component of the Effector-Triggered Response suggests that this pathway may skew development of the adaptive response toward Th17 cells (80, 94).

5.4.2. Comprehensive mechanisms of TNF-inducible inflammasomes.

We have discovered two novel cytokine-inducible inflammasomes: one that is cIAP1- and Caspase-11-dependent, and another that requires flagellin, Naip5 and Nlrc4. In both cases, part of the inflammasome activation pathway remains mysterious: in the former pathway, we do not understand how *L. pneumophila* is sensed by cIAP1 or how cIAP1 interacts with downstream inflammasome components, and in the latter example, the "death effectors" downstream of inflammasome activation remain unidentified.

While we did observe activation of Caspase-3 and Caspase-7 in macrophages infected with wildtype *L. pneumophila*, it still remains to be seen whether this activation is dependent on flagellin, Naip5 and Nlrc4, and also whether these caspases are responsible for death. This seems somewhat unlikely, since we observed activated Caspase-3 and -7 in both TNF-pretreated macrophages, which do undergo pyroptosis, and in untreated cells, which do not. Several alternative hypotheses also exist. TNF may somehow convert Caspase-3 and -7 into pyroptotic, rather than apoptotic, caspases; or the "death effector" could be a non-caspase protease that is inhibited by ZVAD-FMK, such as a cathepsin. In the latter hypothesis, TNF might be required

not for expression or activation of the protease, but for phagosomal events that might lead to leakage or rupture of lysosomal proteases into the cytosol, as has been described for another inflammasome (137). We are currently breeding *Caspase1*$^{-/-}$*/Caspase7*$^{-/-}$ mice in order to categorically rule out a requirement for Caspase-7 in this inflammasome. While others have implicated Caspase-7 in inflammasome activation by *L. pneumophila* infection (138), our preliminary experiments in a heterologous 293T system do not appear to support a role for Caspase-7 in flagellin-dependent inflammasome activation (data not shown).

Perhaps the most interesting outstanding questions have to do with the mechanism and extent of the novel cIAP1-dependent inflammasome we have discovered. How does cIAP1 detect *L. pneumophila*? What role do TNF and other cytokines play in this process, and why is the requirement for cytokine treatment circumvented in macrophages infected with *ΔsdhA L. pneumophila*? How does cIAP1 interact—directly or indirectly—with caspase-11, caspase-1, and/or other inflammasome components? Perhaps most importantly, is this a general strategy to detect intracellular pathogens? If so, what specifically is the pathogen-derived signal? These questions may be answered both by biochemical characterization of the interactions between cIAP1 and (*e.g.*) Caspase-11, and by cell biological experiments examining localization of cIAP1 and dynamics of phagosomal maturation in infected macrophages.

5.5. Last words.

In the last few decades, several autoimmune diseases have been linked to dysregulation at the level of the innate immune response. For example, hyperactive TLR7 signaling may promote lupus (139, 140), while mutations in Nod2 are associated with Crohn's Disease (141), which itself appears to involve dysregulated interactions between the host and the gut flora (142, 143). These examples underscore the critical need for the innate immune system to make accurate "assessments" of infectious threats: while hyporesponsiveness to pathogens could result in susceptibility to infections, innate responses toward inappropriate targets, such as self nucleic acid or commensal flora, can also cause great bodily harm.

By elucidating several novel modes of pathogen recognition in the context of PRR signaling, this work provides a mechanistic understanding of how the innate immune system can integrate multiple pieces of information about a microbe, resulting in a response specifically tailored to the threat at hand. In turn, this specificity of the innate response translates into a similarly tuned adaptive response, appropriate not only for the class of microbe detected but also for the severity of the infectious threat that the microbe poses.

Since innate immune receptors are relatively few in number and are hardwired in the germline, they must target a relatively low number of somewhat slow-to-evolve microbial features. Unlike many microbial molecules, pathogen-associated activities cannot easily be altered without negative consequences for the pathogen. Furthermore, many diverse pathogens—including both bacteria and viruses—perturb a relatively small set of host physiological processes, such as plasma membrane integrity, vesicle trafficking, host translation, and cytoskeletal dynamics. Thus, pathogen-associated activities are equivalent to a set of highly conserved, difficult-to-modify features that make conceptually attractive targets for innate immune recognition. While recognition of pathogenic activities may not provide information about the *class* of pathogen present, this information may be obtained by simultaneous detection of microbial molecules, chosen in turn for their high conservation and slow evolvability. Recognition of pathogenic activities therefore provides an important complement to the long-established recognition of microbial molecules that serves as the cornerstone of innate immunity.

Materials and Methods

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee at the University of California, Berkeley (Protocol number R301-0311BCR).

Mice and cell culture. All experiments with mice and mouse-derived cells were performed on the B6 background unless otherwise indicated. Macrophages were derived from the bone marrow of the following mouse strains: C57BL/6J, 129, *Caspase-2*$^{-/-}$ and *TNF/Lymphotoxin-α/β* (TLT)$^{-/-}$ (all Jackson Labs); *A20*$^{-/-}$ (A. Ma, UCSF), *Nlrp3*$^{-/-}$ (R. Flavell, Yale University) *Caspase-1*$^{-/-}$ (M. Starnbach, Harvard Medical School), *Nlrc4*$^{-/-}$ and *Asc*$^{-/-}$ (V. Dixit, Genentech), *Mavs*$^{-/-}$ (Z. Chen, University of Texas SW), *Irf3/Irf7*$^{-/-}$ and *Aim2*$^{-/-}$ (K. Fitzgerald, U. Mass Medical School), *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ (M. Saleh, McGill University), *Myd88*$^{-/-}$ (G. Barton, UC Berkeley), *Rip2*$^{-/-}$ (M. Kelliher, U. Mass Medical School), *Myd88*$^{-/-}$ *Rip2*$^{-/-}$ (C. Roy, Yale University), and *Myd88*$^{-/-}$ *Nod1*$^{-/-}$ *Nod2*$^{-/-}$ (generated from crosses at UC Berkeley). *Il23a*$^{-/-}$ mice were from N. Ghilardi (Genentech). Macrophages were derived from bone marrow by 8d culture in RPMI supplemented with 10% serum, 100μM streptomycin, 100 U/mL penicillin, 2mM L-glutamine, and 10% supernatant from 3T3-M-CSF cells, with feeding on day 5. Dendritic cells were derived from B6 bone marrow by 6d culture in RPMI supplemented with 10% serum, 100μM streptomycin, 100 U/mL penicillin, 2mM glutamine, and recombinant GM-CSF (1:1000, PeproTech). *Dictyostelium discoideum* amoebae were cultured at 21°C in HL-5 medium (0.056M glucose, 0.5% yeast extract, 0.5% proteose peptone, 0.5% thiotone, 2.5mM Na_2HPO_4, 2.5 mM KH_2PO_4, pH 6.9).

Bacterial Strains. The *L. pneumophila* wildtype strain LP02 is a streptomycin-resistant thymidine auxotroph derived from *L. pneumophila* LP01. The Δ*dotA*, Δ*flaA*, Δ*icmS*, Δ*icmW,* Δ*icmS*Δ*icmW* and Δ*sdhA*Δ*flaA* mutants have been described (40, 49-51, 121, 144), as have the luminescent "lux" strains of LP02 and ΔflaA (40). The Δ*icmS,* Δ*icmW,* and Δ*icmS*Δ*icmW* strains are in the LP01 background. Additionally, an Δ*icmS* strain on the LP02 background was obtained from R. Isberg, and a Δ*icmS*Δ*flaA* strain was generated using a previously described deletion plasmid for flagellin (50). Mutants lacking one or more effectors were generated from LP02 by sequential in-frame deletion using the suicide plasmid pSR47S as described (76). Sequences of primers used for constructing deletion plasmids are listed in Table S3. Mutants were complemented with the indicated effectors expressed from the *L. pneumophila sidF* promoter in the plasmid pJB908, which encodes thymidine synthetase as a selectable marker. *L. monocytogenes* strain 10403S and the isogenic Δ*hly* mutant have been described (145).

Microarrays. Macrophage RNA from 1.5 x 10^6 cells (6 well dishes) was isolated using the Ambion RNAqueous Kit (Applied Biosystems) and amplified with the Ambion Amino Allyl MessageAmp II aRNA Amplification Kit (Applied Biosystems) according to the manufacturer's protocol. Microarrays were performed as described (71). Briefly, spotted microarrays utilizing the MEEBO 70-mer oligonucleotide set (Illumina) were printed at the UCSF Center for Advanced Technology. Microarray probes were generated by coupling amplified RNA to Cy dyes. After hybridization, arrays were washed, scanned on a GenePix 4000B Scanner (Molecular Devices), and gridded using SpotReader software (Niles Scientific). Analysis was performed using the GenePix Pro 6 and Acuity 4 software packages (Molecular Devices). Two independent experiments were performed. Some microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE26491.

Infections and reagents. Macrophages were plated in 6 well dishes at a density of 1.5 x 10^6 cells per well and infected at an MOI of 1 by centrifugation for 10min at 400 x *g*, or were treated

with puromycin, thapsigargin, tunicamycin, cycloheximide, necrostatin (all Sigma), SB203580 and JNK inhibitor II (Calbiochem), ZVAD-FMK (Alexis), recombinant TNF (eBioscience) or Interferon-β (R&D Systems), Exotoxin A (List Biological Labs), transfected synthetic muramyl-dipeptide (MDP) (CalBiochem), or a synthetic bacterial lipopeptide (Pam3CSK4) (Invivogen). Dendritic cells were plated at a density of 10^6 cells per well and infected at an MOI of 2 as described above. Lipofectamine 2000 (Invitrogen) was used for transfections. Bruceantin was the kind gift of S. Starck and N. Shastri (UC Berkeley), who obtained it from the National Cancer Institute, NIH (Open Repository NSC165563). A fusion of diphtheria toxin to the lethal factor translocation signal (LFn-DT) was the gift of B. Krantz (UC Berkeley) and was delivered to cells via the pore formed by anthrax protective antigen (PA) as described (146).

Quantitative RT-PCR. Macrophage RNA was harvested 4-6 hours post infection, as indicated, and isolated with the RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA samples were treated with RQ1 DNase (Promega) prior to reverse transcription with Superscript III (Invitrogen). cDNA reactions were primed with poly dT for measurement of mature transcripts, and with random hexamers (Invitrogen) for measurement of unspliced transcripts. Quantitative PCR was performed as described (49) using the Step One Plus RT PCR System (Applied Biosystems) with Platinum Taq DNA polymerase (Invitrogen) and EvaGreen (Biotium). Transcript levels were normalized to *Rps17*. Primer sequences are listed in Appendix 10.

mRNA stabilization assay. Macrophages were infected in 6-well dishes at an MOI of 1, as described above. The transcription inhibitor Actinomycin D (10μg/mL, Sigma) was added 4 hours post infection. RNA was harvested at successive timepoints and levels of indicated transcripts were assessed by quantitative RT-PCR.

***In vivo* experiments.** Age- and sex-matched mice were anesthetized with ketamine and infected intranasally with 2 x 10^6 LP01, or with LP02 or Δ5 strains complemented with plasmid pJB908, which encodes thymidine synthetase, in 20μL PBS essentially as described (49), or were treated with ExoA or Pam3CSK4 in 25μL PBS. Bronchoalveolar lavage was performed 24 hours post infection by introducing 800μL PBS into the trachea with a catheter (BD Angiocath 18g, 1.3 x 48mm). Lavage fluid was analyzed by ELISA. Total host cells in the lavage were counted on a hemocytometer. For RT-PCR experiments, all lavage samples receiving identical treatments were pooled, and RNA was isolated from the pooled cells using the RNeasy Kit as described above. FACS analysis of lavage samples labeled with anti-GR-1-PeCy7 and anti-Ly6G-PE (eBioscience) indicated that most cells in lavage were neutrophils. CFU were enumerated by hypotonic lysis of host cells in the lavage followed by plating on CBYE plates.

Western blots. Macrophages were plated in 6 well dishes at a density of 2 x 10^6 cells per well and infected at an MOI of 2. For whole cell extract, cells were lysed in RIPA buffer supplemented with 2 mM $NaVO_3$, 1 mM PMSF, 1mM DTT, and 1 X Complete Protease Inhibitor Cocktail (Roche). For nuclear translocation experiments, nuclear and cytosolic fractions were obtained using the NE-PER kit (Pierce) according to the manufacturer's protocol. For phospho-specific MAP kinase blots, cells were serum-starved for 4-8h before infection, then harvested in RIPA buffer supplemented with 2 mM $NaVO_3$, 50mM NaF, 1 mM PMSF, 1mM EDTA, and 1X Complete Protease Inhibitor Cocktail (Roche). Protein levels were normalized using the micro-BCA kit (Pierce) and then separated on 10% NuPAGE bis-tris gels (Invitrogen). Proteins were transferred to PVDF membranes and immunoblotted with antibodies to IκBα, NF-κB p65, lamin-B, β-actin (all Santa Cruz), phospho- and total SAPK/JNK, or phospho- and total p38 (all Cell Signaling). For phospho-blots, 2mM $NaVO_3$ and 20mM NaF were added to blocking and antibody solutions.

Caspase cleavage blots. Macrophages were infected at an MOI of 2. Media was removed 1h post infection and replaced with 1mL of serum-free RPMI per well. Supernatants were harvested 4h post infection. Proteins were precipitated in 10% trichloroacetic acid, pelleted, washed in acetone, and resuspended in SDS-page sample buffer. Samples were run on NuPage 4-12% Bis-Tris gels (Invitrogen), transferred to PVDF membranes, and blotted for Caspase-1 p10 (Santa Cruz) or Caspase-11 (Novus Biologicals).

ELISA. Macrophages were plated in 24 well dishes at a density of 5 x 10^5 cells per well and infected at an MOI of 1. After 24h, supernatants were collected, cleared by centrifugation, and analyzed by ELISA using paired GM-CSF or IL-1α antibodies (eBioscience). For quantification of intracellular GM-CSF, ELISAs were performed using cytoplasmic extract of macrophages infected for 6h with the indicated strains. Levels of GM-CSF were normalized to total protein concentration. Recombinant GM-CSF (eBioscience) was used as a standard.

Growth in bone marrow derived macrophages. Intracellular bacterial growth of wildtype and mutant *L. pneumophila* in A/J macrophages was evaluated by plating as described (76). Growth of luminescent bacteria was measured in a luminometer as described (40).

Growth in amoebae. *D. discoideum* (strain AX3K, gift of D. Knecht to R. Isberg) was plated into 24-well plates at a density of 5 x 10^5 cells per well in MB medium (modified HL-5 medium, without glucose and with 20 mM MES buffer) three hours before infection with the indicated *L. pneumophila* strains at an MOI of 0.05. The plates were spun at 1000 rpm for 5 minutes and incubated at 25°C. After two hours, wells were washed 3X with PBS to synchronize the infection. At successive time points, infected cells were lysed with 0.2% saponin and bacterial growth was determined by plating on growth medium.

Protein synthesis assay. 2 x 10^6 macrophages were seeded in 6-well plates and infected with bacterial strains at an MOI of 2. After 2.5h, the infected cells were incubated with 1μCi ^{35}S-methionine (Perkin Elmer) in RPMI-met (Invitrogen). After chase-labeling for an hour, the cells were washed 3x with PBS, lysed with 0.1% SDS and precipitated with TCA (76). The protein precipitates were filtered onto 0.45mm Millipore membranes and washed twice with PBS. Retained ^{35}S was determined by a liquid scintillation counter.

LDH release assay. Macrophages were plated in 96-well dishes at a density of 10^5 cells per well and infected at an MOI of 1. Some wells were treated with 1% Triton-X (final concentration) to lyse all cells. 4 hours post infection, LDH activity in the supernatant was measured by SkyTox as described (56).

Long-term cytotoxicity assay. Macrophages were plated in 96 well dishes at a density of 5 x 10^4 cells per well and infected at an MOI of 1. At successive timepoints, Neutral Red (Sigma) was added to a final concentration of 1% and incubated for 1h. Cells were then washed with PBS, photographed, and counted (50).

Polysome fractionation. 3 x 10^7 B6 macrophages per sample were infected with ΔflaA or ΔdotA *L. pneumophila* at an MOI of 2. 3h post infection, cells were harvested in polysome extraction buffer (100mM Tris-Cl, 200mM $MgCl_2$, 200mM KCl, 5mM EGTA, 1mM DTT, 1% Triton X-100, 100μg/mL cycloheximide, 1X Protease Inhibitor Cocktail, pH 8). Lysates were cleared by centrifugation, snap-frozen in liquid nitrogen, and kept at -80 until analysis by sucrose gradient. For this step, solutions of 15%, 30%, 45%, and 60% sucrose in Sucrose Gradient Buffer (40mM Tris-Cl, 10mM $MgCl_2$, 20mM KCl, 100μg/mL cycloheximide, pH 7.4) were layered in SW41 11mL polyethylene tubes with the most dense solution at the bottom. Lysates were applied to the gradient and centrifuged at 60,000RPM for 90 min. Sequential 1mL

fractions were collected from the gradient as described (147), and RNA was isolated from the fractions corresponding to polysomes using Trizol reagent (Invitrogen). *Csf2* and β-*actin* transcripts in polysomes were measured by quantitative RT-PCR and compared to levels of those same transcripts in the total cellular mRNA pool. As a control for the total amount of polysomes recovered, *Csf2* and actin transcripts were normalized to levels of ribosomal 18S RNA. All primers are listed in Appendix 10.

Assays for ER stress. To look for Xbp-1 splicing, Xbp-1 transcript was amplified from cDNA made from macrophages either infected with various strains of *L. pneumophila* or treated with the known ER stress inducer, thapsigargin (1μM). Full-length (473 bp) and smaller, spliced transcripts were resolved on a 3% agarose gel. Additionally, transcript levels of two ER stress-induced genes, *Bip* and *Chop*, were measured by quantitative RT-PCR. Primers for all three assays are listed in Appendix 10.

Time-lapse microscopy. *Caspase1*$^{-/-}$ macrophages, some pretreated overnight with TNF (10 ng/mL), were infected with GFP^{+} LP02 at an MOI of 1. Media was changed 1h post infection, and ethidium homodimer (EthD; 2 μM; Invitrogen) was added to the fresh media. Photographs were taken on a live cell inverted microscope every 5 min from 1-3h post infection. Cells were scored for apoptosis based on membrane blebbing and nuclear condensation, or for pyroptosis by loss of membrane integrity and cytosolic contents, and by acquisition of red fluorescence (indicating uptake of EthD).

Labeling of active caspases. *Caspase1*$^{-/-}$ macrophages, pretreated overnight with TNF (10 ng/mL) where indicated, were infected with LP02 at an MOI of 1. Media was removed 1h post infection, and 1mL of plain RPMI containing the caspase-specific fluorescent probe AWP28 (1mM; kind gift of A. Puri and M. Bogyo) was added. 2h post infection, cells were harvested in 50 μL sample buffer per well and run on an SDS-page gel. Fluorescence was visualized on a Typhoon imager.

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Appendix One. Genes induced twofold or more in *Caspase1*$^{-/-}$ macrophages infected with wildtype or *ΔdotA L. pneumophila*. Data are expressed as $\log_2$(induction) normalized to expression in uninfected macrophages. Results from one representative microarray experiment are shown.

gene name	*ΔdotA*	wildtype
interleukin 6	2.84	5.193
plasminogen activator, tissue	1.118	4.251
early growth response 1	0.97	4.184
colony stimulating factor 2 (granulocyte-macrophage)	-0.365	4.16
chemokine (C-X-C motif) ligand 5	2.518	4.147
chemokine (C-C motif) ligand 20	1.676	4.062
lysyl oxidase	2.842	3.994
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	2.428	3.901
solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	2.9	3.728
sprouty homolog 1 (Drosophila)	1.798	3.671
RIKEN cDNA 4930412F09 gene	0.357	3.527
carbonic anhydrase 2	1.572	3.493
selenocysteine lyase	0.154	3.432
colony stimulating factor 3 (granulocyte)	0.809	3.367
interleukin 1 family, member 6	2.323	3.313
GTP binding protein (gene overexpressed in skeletal muscle)	0.235	3.308
hypoxia inducible factor 1, alpha subunit	1.988	3.3
defensin beta 12	2.336	3.283
interleukin 23, alpha subunit p19	0.204	3.264
zinc finger and BTB domain containing 10	2.925	3.227
mucosa associated lymphoid tissue lymphoma translocation gene 1	1.637	3.186
GA repeat binding protein, beta 2	0.872	3.165
B-cell translocation gene 2, anti-proliferative	1.097	3.142
interleukin 1 family, member 9	2.225	3.098
CD83 antigen	-0.985	3.064
developmental pluripotency associated 2	1.944	3.064
TSC22 domain family, member 1	1.459	3.058
dual specificity phosphatase 16	2.045	3.024
homeo box A9	0.716	2.997
Tnf receptor-associated factor 1	1.233	2.987
adrenomedullin	0.605	2.978
amphiregulin	0.63	2.976
interleukin 12b	3.005	2.974
AXIN1 up-regulated 1	0.479	2.97
arginase type II	0.836	2.969
predicted gene, OTTMUSG00000007655	2.321	2.963
complement factor B	2.715	2.937
CD69 antigen	2.823	2.928
cathelicidin antimicrobial peptide	2.418	2.893
coagulation factor III	1.061	2.874
chemokine (C-C motif) ligand 4	2.371	2.87
protein kinase, AMP-activated, alpha 2 catalytic subunit	1.698	2.856
SLAM family member 7	2.418	2.855

olfactory receptor 214	1.896	2.837
zinc finger CCCH type containing 12C	1.866	2.83
adenosine A2b receptor	1.568	2.83
protein tyrosine phosphatase, receptor type, G	0.433	2.829
ankyrin repeat domain 57	1.45	2.819
sorbin and SH3 domain containing 2	0.748	2.81
solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	2.109	2.784
G protein-coupled receptor 109A	2.329	2.783
mucolipin 1	1.038	2.783
colony stimulating factor 1 (macrophage)	0.796	2.781
matrix metallopeptidase 13	1.486	2.757
lipocalin 2	2.26	2.735
chemokine (C-C motif) ligand 7	1.133	2.731
tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase	0.291	2.729
RIKEN cDNA 2010107E04 gene	1.579	2.724
guanylate nucleotide binding protein 5	2.145	2.68
formyl peptide receptor 1	2.668	2.664
selectin, endothelial cell	0.253	2.653
fibrinogen-like protein 2	2.497	2.638
preimplantation protein 3	1.049	2.624
interleukin 1 alpha	2.044	2.623
frizzled homolog 1 (Drosophila)	1.861	2.621
predicted gene, EG434025	-1.149	2.611
pleckstrin homology-like domain, family A, member 1	1.281	2.61
predicted gene, OTTMUSG00000015529	0.871	2.602
coagulation factor X	2.122	2.585
endothelin 1	1.702	2.575
RIKEN cDNA 4631424J17 gene	0.137	2.561
aryl-hydrocarbon receptor	1.471	2.558
RIKEN cDNA 5430405N12 gene	-0.007	2.555
muted	0.045	2.553
RAB11 family interacting protein 1 (class I)	1.582	2.542
RIKEN cDNA 2810004A10 gene	1.438	2.522
tumor necrosis factor, alpha-induced protein 3	0.869	2.516
EMI domain containing 1	1.837	2.512
serum amyloid A 1	2.064	2.501
HLA-B-associated transcript 3	1.726	2.477
actin-binding LIM protein 2	1.233	2.458
Tnf receptor associated factor 4	1.757	2.423
proviral integration site 1	1.106	2.42
ras homolog gene family, member B	-0.25	2.419
RIKEN cDNA 4930589L23 gene	0.229	2.415
leukotriene B4 receptor 2	0.299	2.41
acid phosphatase 1, soluble	0.448	2.404
DNA binding protein with his-thr domain	1.65	2.404
tetratricopeptide repeat domain 17	0.534	2.397
zinc finger protein 688	0.737	2.386
RIKEN cDNA 4930583C14 gene	0.473	2.377

mannoside acetylglucosaminyltransferase 5, isoenzyme B	2.086	2.36
human immunodeficiency virus type I enhancer binding protein 3	1.098	2.347
cholesterol 25-hydroxylase	1.757	2.336
RIKEN cDNA 1810009N23 gene	2.92	2.328
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	0.57	2.315
RIKEN cDNA C230073O14 gene	2.078	2.301
immediate early response 3	0.692	2.285
tripartite motif protein 13	1.958	2.281
RIKEN cDNA 1700027A07 gene	0.555	2.278
interferon beta 1, fibroblast	0.324	2.267
caspase recruitment domain family, member 15	1.35	2.261
RIKEN cDNA 4833411O04 gene	0.485	2.251
ubiquitin interaction motif containing 1	0.545	2.244
Jun dimerization protein 2	0.864	2.238
phosphatidylinositol 3-kinase, C2 domain containing, alpha polypeptide	0.761	2.238
complement component 4B (Childo blood group)	1.39	2.238
nudix (nucleoside diphosphate linked moiety X)-type motif 11	0.215	2.221
guanylate cyclase 2g	0.667	2.211
B-cell translocation gene 3	1.085	2.203
small nuclear RNA activating complex, polypeptide 3	1.402	2.201
interleukin-1 receptor-associated kinase 3	1.74	2.195
heat shock protein 1A	0.988	2.194
GTPase activating protein and VPS9 domains 1	1.701	2.193
BCL2-associated transcription factor 1	1.95	2.192
prominin 2	1.492	2.177
histone cluster 1, H1b	0.173	2.171
RIKEN cDNA 2210037E17 gene	0.982	2.171
solute carrier family 2 (facilitated glucose transporter), member 2	-0.181	2.168
RasGEF domain family, member 1B	0.63	2.159
zinc finger protein 289	1.531	2.15
PHD finger protein 20-like 1	0.458	2.149
endothelin receptor type B	1.515	2.148
dual specificity phosphatase 1	0.499	2.139
Cdc42 binding protein kinase alpha	1.116	2.137
RIKEN cDNA D930030K17 gene	0.973	2.134
dual specificity phosphatase 2	0.914	2.129
CDC42 effector protein (Rho GTPase binding) 1	0.963	2.12
RIKEN cDNA 1700029G01 gene	0.907	2.107
insulin-like growth factor 2 mRNA binding protein 2	1.326	2.099
expressed sequence AA536749	1.64	2.097
aldehyde dehydrogenase family 1, subfamily A1	-0.158	2.095
RIKEN cDNA 5430433G21 gene	1.466	2.087
serum amyloid A 3	2.175	2.086
RIKEN cDNA D930007M16 gene	0.908	2.082
growth arrest and DNA-damage-inducible 45 alpha	-0.387	2.078
protein kinase, cAMP dependent regulatory, type II beta	1.545	2.078
expressed sequence AI504432	1.662	2.076
SAM domain, SH3 domain and nuclear localization signals, 1	1.296	2.076

RIKEN cDNA E130201H02 gene	1.702	2.075
paired immunoglobin-like type 2 receptor alpha	1.414	2.074
metallothionein 2	0.91	2.043
RIKEN cDNA A630077B13 gene	2.13	2.042
apolipoprotein C-III	0.957	2.038
regulator of G-protein signaling 1	-0.429	2.031
jumonji domain containing 2A	1.31	2.028
caspase 4, apoptosis-related cysteine peptidase	1.054	2.027
RIKEN cDNA A430027H14 gene	1.703	2.018
nuclear RNA export factor 1 homolog (S. cerevisiae)	0.747	2.017
RIKEN cDNA 6720422M22 gene	1.304	2.012
interleukin enhancer binding factor 3	0.585	2.012
interleukin 1 receptor antagonist	1.318	2.009
protein phosphatase 1, regulatory (inhibitor) subunit 13B	1.093	2.009
procollagen, type XXVII, alpha 1	-0.138	2.006
cadherin 22	2.386	2.004
CDC-like kinase 1	-0.409	1.998
hect domain and RLD 5	0.568	1.974
AMP deaminase 3	0.931	1.966
RIKEN cDNA A930009K04 gene	1.761	1.964
filamin, beta	1.145	1.954
haptoglobin	2.111	1.929
RIKEN cDNA 1810005K13 gene	2.298	1.909
Rho GTPase activating protein 22	-0.3	1.893
G protein-coupled receptor 18	2.105	1.892
chemokine (C-C motif) ligand 5	1.387	1.891
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 7	0.512	1.878
Fas (TNF receptor superfamily member)	1.788	1.876
RIKEN cDNA 1110059G02 gene	1.103	1.875
histone cluster 1, H4i	-0.007	1.871
tumor necrosis factor (ligand) superfamily, member 9	0.805	1.866
nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	1.54	1.836
chemokine (C-C motif) ligand 12	1.41	1.832
NADPH oxidase 1	1.159	1.832
interferon regulatory factor 4	-0.218	1.82
rad and gem related GTP binding protein 2	0.58	1.82
MAX dimerization protein 1	1.125	1.805
G protein-coupled receptor 149	2.331	1.805
small proline-rich protein 2I	2.134	1.789
FYVE, RhoGEF and PH domain containing 3	2.417	1.778
secretory leukocyte peptidase inhibitor	1.731	1.77
RIKEN cDNA 9030617O03 gene	-0.318	1.768
serine (or cysteine) peptidase inhibitor, clade B, member 2	0.916	1.762
interferon-induced protein with tetratricopeptide repeats 1	1.324	1.76
Rous sarcoma oncogene	1.318	1.76
B-cell translocation gene 1, anti-proliferative	0.165	1.742
RIKEN cDNA 9530006C21 gene	0.896	1.718
poliovirus receptor	0.376	1.716

pleckstrin	1.13	1.711
C-type lectin domain family 4, member e	1.227	1.701
solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member	0.688	1.696
Rap guanine nucleotide exchange factor (GEF) 2	1.148	1.684
inhibitor of DNA binding 3	1.254	1.668
proline-rich nuclear receptor coactivator 1	1.009	1.667
ring finger and FYVE like domain containing protein	1.176	1.666
guanylate nucleotide binding protein 3	1.891	1.662
RIKEN cDNA 4930524B15 gene	0.76	1.66
transcription factor EC	1.157	1.658
uridine phosphorylase 1	0.686	1.644
carbonic anhydrase 6	2.003	1.643
RIKEN cDNA 3110027N22 gene	1.064	1.641
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	0.261	1.637
RIKEN cDNA B830007D08 gene	1.111	1.629
serine (or cysteine) peptidase inhibitor, clade E, member 1	-0.533	1.626
tumor necrosis factor (ligand) superfamily, member 4	0.29	1.626
cDNA sequence BC006779	0.56	1.625
testis expressed gene 10	2.233	1.625
otoraplin	0.366	1.624
RIKEN cDNA D330017J20 gene	0.072	1.617
RAS guanyl releasing protein 1	1.286	1.605
HtrA serine peptidase 1	1.182	1.605
histone cluster 1, H4c	0.618	1.603
suppressor of cytokine signaling 1	0.802	1.594
coiled-coil domain containing 63	0.286	1.591
jumonji domain containing 3	0.309	1.587
cyclin-dependent kinase 5, regulatory subunit (p35) 1	-0.304	1.572
RIKEN cDNA A130086G11 gene	0.876	1.567
coiled-coil domain containing 80	0.828	1.567
baculoviral IAP repeat-containing 3	1.153	1.564
bactericidal/permeability-increasing protein-like 2	0.84	1.564
translocase of inner mitochondrial membrane 23 homolog (yeast)	2.392	1.562
cyclin L1	0.402	1.554
myeloid differentiation primary response gene 116	0.606	1.553
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.51	1.547
ankyrin repeat and BTB (POZ) domain containing 2	0.824	1.545
dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	0.593	1.517
plectin 1	1.088	1.504
Janus kinase 2	1.043	1.495
inhibin beta-A	0.745	1.482
pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4	1.123	1.476
chemokine (C-X-C motif) ligand 2	1.165	1.474
RAS guanyl releasing protein 1	0.85	1.471
PR domain containing 1, with ZNF domain	0.618	1.466
SKI-like	0.926	1.466
protocadherin 8	0.401	1.452

RIKEN cDNA A630081D01 gene	0.433	1.447
wingless-related MMTV integration site 2	2.42	1.442
jagged 1	0.684	1.439
protein phosphatase, EF hand calcium-binding domain 2	0.6	1.435
transmembrane protein 39a	0.296	1.434
RIKEN cDNA 2810433D01 gene	2.246	1.432
coiled-coil domain containing 11	0.229	1.425
RIKEN cDNA 4732474O15 gene	0.338	1.417
nuclear protein 1	0.948	1.414
Mediterranean fever	1.122	1.387
schlafen 4	1.451	1.382
ganglioside-induced differentiation-associated-protein 10	1.321	1.372
RIKEN cDNA 1200009I06 gene	1.388	1.364
inhibitor of kappaB kinase beta	0.248	1.362
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.318	1.358
IKAROS family zinc finger 1	1.104	1.348
C-type lectin domain family 2, member d	1.393	1.347
RIKEN cDNA 3021401C12 gene	0.693	1.344
nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta	0.626	1.343
syntaxin 11	1.085	1.336
receptor (TNFRSF)-interacting serine-threonine kinase 2	0.725	1.334
choline kinase alpha	0.231	1.331
immediate early response 2	0.43	1.33
RIKEN cDNA D130040H23 gene	0.416	1.328
plasminogen activator, urokinase receptor	0.054	1.324
tumor necrosis factor, alpha-induced protein 2	0.327	1.32
MARCKS-like 1	0.589	1.318
histone cluster 1, H1a	2.48	1.316
G protein-coupled receptor 132	0.788	1.313
interleukin 1 alpha	-0.047	1.308
interleukin 15	0.691	1.308
kinesin light chain 2	0.142	1.306
histone cluster 1, H4i	-0.365	1.3
erythroid differentiation regulator 1	2.084	1.299
interleukin 10	0.278	1.296
IKAROS family zinc finger 4	0.365	1.295
myeloid cell nuclear differentiation antigen	0.203	1.29
thymidylate kinase family LPS-inducible member	1.469	1.288
solute carrier organic anion transporter family, member 3a1	0.919	1.285
suppressor of cytokine signaling 3	1.565	1.284
protocadherin 10	2.156	1.277
growth arrest and DNA-damage-inducible 45 beta	0.835	1.276
interferon regulatory factor 1	1.206	1.276
Kruppel-like factor 6	0.391	1.275
histone cluster 1, H1e	0.697	1.266
beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	2.176	1.254
interleukin 31	2.194	1.253
RIKEN cDNA 2310002B14 gene	2.454	1.239

polo-like kinase 2 (Drosophila)	-0.405	1.233
chemokine (C-X-C motif) ligand 10	1.05	1.227
RIKEN cDNA B430201A12 gene	1.248	1.221
guanylate binding protein 6	1.734	1.217
nucleoporin 54	0.63	1.213
RIKEN cDNA 4930439A04 gene	0.649	1.205
RIKEN cDNA 1110061O04 gene	0.419	1.203
nuclear receptor subfamily 1, group D, member 1	0.06	1.187
interferon-induced protein with tetratricopeptide repeats 2	1.616	1.186
RIKEN cDNA 0610037M15 gene	0.125	1.185
cDNA sequence BC031441	0.214	1.184
Cnksr family member 3	0.065	1.177
camello-like 5	1.049	1.176
ring finger and FYVE like domain containing protein	0.276	1.172
cDNA sequence BC011487	2	1.171
myxovirus (influenza virus) resistance 1	1.517	1.17
potassium voltage-gated channel, shaker-related subfamily, member 3	0.933	1.16
chloride intracellular channel 4 (mitochondrial)	0.978	1.155
TRAF family member-associated Nf-kappa B activator	0.807	1.153
EST AA407452	1.024	1.146
phorbol-12-myristate-13-acetate-induced protein 1	0.406	1.139
fem-1 homolog c (C.elegans)	0.447	1.136
RIKEN cDNA 6030465E24 gene	2.048	1.127
RIKEN cDNA 5730557B15 gene	0.869	1.122
SH3-domain kinase binding protein 1	2.107	1.118
RIKEN cDNA 9630026M06 gene	0.401	1.116
interleukin 27	0.806	1.113
RIKEN cDNA 2010109K09 gene	1.191	1.11
three prime repair exonuclease 1	0.138	1.098
RIKEN cDNA 2600010E01 gene	0.192	1.096
cyclin L1	0.726	1.095
G protein-coupled receptor 87	2.366	1.092
Janus kinase 2	2.306	1.09
pleckstrin homology domain containing, family G (with RhoGef domain) member 1	0.99	1.082
melanoma antigen	2.421	1.077
N-acylsphingosine amidohydrolase 3-like	0.101	1.075
SCO-spondin	0.283	1.07
retinoblastoma binding protein 8	0.246	1.064
kinectin 1	0.192	1.064
cDNA sequence BC065085	0.803	1.06
inositol polyphosphate-5-phosphatase A	2.313	1.041
mitogen-activated protein kinase kinase kinase 6	0.391	1.036
chemokine (C-C motif) receptor-like 1	-0.041	1.036
Down syndrome critical region homolog 1 (human)	0.219	1.035
proviral integration site 3	0.223	1.028
ATPase, H+ transporting, lysosomal V0 subunit C	0.73	1.023
RIKEN cDNA 5230400M03 gene	0.748	1.014
interferon gamma induced GTPase	0.894	1.013

metallothionein 1	1.02	1.011
tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	0.63	1.009
GTPase, IMAP family member 6	0.33	1.006

Appendix Two. Genes induced twofold or more in *Caspase1*$^{-/-}$ macrophages infected with wildtype or *ΔflaA L. pneumophila*. Data are expressed as $\log_2$(induction) normalized to expression in uninfected macrophages. Results from one representative microarray experiment are shown.

gene name	**wildtype**	***ΔflaA***
interleukin 6	5.193	4.876
plasminogen activator, tissue	4.251	5.711
early growth response 1	4.184	5.258
colony stimulating factor 2 (granulocyte-macrophage)	4.16	4.783
chemokine (C-X-C motif) ligand 5	4.147	3.746
chemokine (C-C motif) ligand 20	4.062	4.489
lysyl oxidase	3.994	3.193
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	3.901	4.21
solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	3.728	2.779
sprouty homolog 1 (Drosophila)	3.671	4.83
RIKEN cDNA 4930412F09 gene	3.527	4.274
carbonic anhydrase 2	3.493	4.41
selenocysteine lyase	3.432	0.397
colony stimulating factor 3 (granulocyte)	3.367	2.942
interleukin 1 family, member 6	3.313	2.842
GTP binding protein (gene overexpressed in skeletal muscle)	3.308	4.973
hypoxia inducible factor 1, alpha subunit	3.3	3.635
defensin beta 12	3.283	2.631
interleukin 23, alpha subunit p19	3.264	3.966
zinc finger and BTB domain containing 10	3.227	3.724
mucosa associated lymphoid tissue lymphoma translocation gene 1	3.186	3.93
GA repeat binding protein, beta 2	3.165	0.939
B-cell translocation gene 2, anti-proliferative	3.142	3.804
interleukin 1 family, member 9	3.098	2.488
CD83 antigen	3.064	4.016
developmental pluripotency associated 2	3.064	2.349
TSC22 domain family, member 1	3.058	3.864
dual specificity phosphatase 16	3.024	3.349
homeo box A9	2.997	0.301
Tnf receptor-associated factor 1	2.987	3.359
adrenomedullin	2.978	4.205
amphiregulin	2.976	3.591
interleukin 12b	2.974	3.69
AXIN1 up-regulated 1	2.97	4.448
arginase type II	2.969	3.918
predicted gene, OTTMUSG00000007655	2.963	1.574
complement factor B	2.937	3.371
CD69 antigen	2.928	3.508

cathelicidin antimicrobial peptide	2.893	2.609
coagulation factor III	2.874	3.018
chemokine (C-C motif) ligand 4	2.87	3.414
protein kinase, AMP-activated, alpha 2 catalytic subunit	2.856	0.085
SLAM family member 7	2.855	2.625
olfactory receptor 214	2.837	2.877
adenosine A2b receptor	2.83	3.247
zinc finger CCCH type containing 12C	2.83	3.067
protein tyrosine phosphatase, receptor type, G	2.829	3.488
ankyrin repeat domain 57	2.819	3.75
sorbin and SH3 domain containing 2	2.81	2.728
solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	2.784	2.144
G protein-coupled receptor 109A	2.783	2.376
mucolipin 1	2.783	3.421
colony stimulating factor 1 (macrophage)	2.781	3.635
matrix metallopeptidase 13	2.757	3.166
lipocalin 2	2.735	2.211
chemokine (C-C motif) ligand 7	2.731	3.396
tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase	2.729	1.947
RIKEN cDNA 2010107E04 gene	2.724	2.022
guanylate nucleotide binding protein 5	2.68	3.85
formyl peptide receptor 1	2.664	2.467
selectin, endothelial cell	2.653	3.808
fibrinogen-like protein 2	2.638	2.587
preimplantation protein 3	2.624	2.363
interleukin 1 alpha	2.623	3.237
frizzled homolog 1 (Drosophila)	2.621	2.592
predicted gene, EG434025	2.611	1.74
pleckstrin homology-like domain, family A, member 1	2.61	3.023
predicted gene, OTTMUSG00000015529	2.602	2.606
coagulation factor X	2.585	2.543
endothelin 1	2.575	4.07
RIKEN cDNA 4631424J17 gene	2.561	1.939
aryl-hydrocarbon receptor	2.558	2.584
RIKEN cDNA 5430405N12 gene	2.555	3.039
muted	2.553	2.184
RAB11 family interacting protein 1 (class I)	2.542	2.976
RIKEN cDNA 2810004A10 gene	2.522	2.87
tumor necrosis factor, alpha-induced protein 3	2.516	3.721
EMI domain containing 1	2.512	0.854
serum amyloid A 1	2.501	1.895
HLA-B-associated transcript 3	2.477	1.974
actin-binding LIM protein 2	2.458	1.723
Tnf receptor associated factor 4	2.423	2.424

proviral integration site 1	2.42	3.612
ras homolog gene family, member B	2.419	3.482
RIKEN cDNA 4930589L23 gene	2.415	0.549
leukotriene B4 receptor 2	2.41	2.263
acid phosphatase 1, soluble	2.404	1.377
DNA binding protein with his-thr domain	2.404	1.951
tetratricopeptide repeat domain 17	2.397	1.75
zinc finger protein 688	2.386	2.031
RIKEN cDNA 4930583C14 gene	2.377	2.837
mannoside acetylglucosaminyltransferase 5, isoenzyme B	2.36	1.537
human immunodeficiency virus type I enhancer binding protein 3	2.347	3.249
cholesterol 25-hydroxylase	2.336	2.384
RIKEN cDNA 1810009N23 gene	2.328	2.133
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	2.315	3.062
RIKEN cDNA C230073O14 gene	2.301	2.847
immediate early response 3	2.285	2.58
tripartite motif protein 13	2.281	2.183
RIKEN cDNA 1700027A07 gene	2.278	0.055
interferon beta 1, fibroblast	2.267	4.729
caspase recruitment domain family, member 15	2.261	3.276
RIKEN cDNA 4833411O04 gene	2.251	3.847
ubiquitin interaction motif containing 1	2.244	1.875
complement component 4B (Childo blood group)	2.238	1.582
Jun dimerization protein 2	2.238	1.931
phosphatidylinositol 3-kinase, C2 domain containing, alpha polypeptide	2.238	2.361
nudix (nucleoside diphosphate linked moiety X)-type motif 11	2.221	0.284
guanylate cyclase 2g	2.211	2.798
B-cell translocation gene 3	2.203	1.544
small nuclear RNA activating complex, polypeptide 3	2.201	1.726
interleukin-1 receptor-associated kinase 3	2.195	2.3
heat shock protein 1A	2.194	2.74
GTPase activating protein and VPS9 domains 1	2.193	1.743
BCL2-associated transcription factor 1	2.192	2.358
prominin 2	2.177	0.413
histone cluster 1, H1b	2.171	2.194
RIKEN cDNA 2210037E17 gene	2.171	2.697
solute carrier family 2 (facilitated glucose transporter), member 2	2.168	1.945
RasGEF domain family, member 1B	2.159	2.811
zinc finger protein 289	2.15	1.663
PHD finger protein 20-like 1	2.149	3.126
endothelin receptor type B	2.148	2.524
dual specificity phosphatase 1	2.139	2.323
Cdc42 binding protein kinase alpha	2.137	1.758

RIKEN cDNA D930030K17 gene	2.134	0.269
dual specificity phosphatase 2	2.129	2.397
CDC42 effector protein (Rho GTPase binding) 1	2.12	3.168
RIKEN cDNA 1700029G01 gene	2.107	3.299
insulin-like growth factor 2 mRNA binding protein 2	2.099	2.141
expressed sequence AA536749	2.097	1.539
aldehyde dehydrogenase family 1, subfamily A1	2.095	0.447
RIKEN cDNA 5430433G21 gene	2.087	1.861
serum amyloid A 3	2.086	2.856
RIKEN cDNA D930007M16 gene	2.082	0.736
growth arrest and DNA-damage-inducible 45 alpha	2.078	2.592
protein kinase, cAMP dependent regulatory, type II beta	2.078	0.104
expressed sequence AI504432	2.076	1.879
SAM domain, SH3 domain and nuclear localization signals, 1	2.076	2.972
RIKEN cDNA E130201H02 gene	2.075	1.803
paired immunoglobin-like type 2 receptor alpha	2.074	2.174
metallothionein 2	2.043	2.187
RIKEN cDNA A630077B13 gene	2.042	2.984
apolipoprotein C-III	2.038	1.048
regulator of G-protein signaling 1	2.031	3.211
jumonji domain containing 2A	2.028	1.286
caspase 4, apoptosis-related cysteine peptidase	2.027	3.976
RIKEN cDNA A430027H14 gene	2.018	0.034
nuclear RNA export factor 1 homolog (S. cerevisiae)	2.017	0.935
interleukin enhancer binding factor 3	2.012	1.72
RIKEN cDNA 6720422M22 gene	2.012	0.692
interleukin 1 receptor antagonist	2.009	1.882
protein phosphatase 1, regulatory (inhibitor) subunit 13B	2.009	1.255
procollagen, type XXVII, alpha 1	2.006	3.177
cadherin 22	2.004	0.481
CDC-like kinase 1	1.998	2.591
hect domain and RLD 5	1.974	2.605
AMP deaminase 3	1.966	3.296
RIKEN cDNA A930009K04 gene	1.964	1.484
filamin, beta	1.954	1.757
haptoglobin	1.929	1.458
RIKEN cDNA 1810005K13 gene	1.909	1.77
Rho GTPase activating protein 22	1.893	3.138
G protein-coupled receptor 18	1.892	1.58
chemokine (C-C motif) ligand 5	1.891	1.71
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 7	1.878	2.435
Fas (TNF receptor superfamily member)	1.876	2.404
RIKEN cDNA 1110059G02 gene	1.875	2.708
histone cluster 1, H4i	1.871	2.543

tumor necrosis factor (ligand) superfamily, member 9	1.866	2.474
nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	1.836	2.597
chemokine (C-C motif) ligand 12	1.832	2.558
NADPH oxidase 1	1.832	2.268
interferon regulatory factor 4	1.82	2.992
rad and gem related GTP binding protein 2	1.82	2.775
G protein-coupled receptor 149	1.805	0.729
MAX dimerization protein 1	1.805	2.501
small proline-rich protein 2I	1.789	2.089
FYVE, RhoGEF and PH domain containing 3	1.778	1.852
secretory leukocyte peptidase inhibitor	1.77	2.033
RIKEN cDNA 9030617O03 gene	1.768	2.807
serine (or cysteine) peptidase inhibitor, clade B, member 2	1.762	1.459
interferon-induced protein with tetratricopeptide repeats 1	1.76	1.997
Rous sarcoma oncogene	1.76	2.132
B-cell translocation gene 1, anti-proliferative	1.742	2.456
RIKEN cDNA 9530006C21 gene	1.718	2.248
poliovirus receptor	1.716	2.996
pleckstrin	1.711	2.221
C-type lectin domain family 4, member e	1.701	1.705
solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), membe	1.696	1.655
Rap guanine nucleotide exchange factor (GEF) 2	1.684	2.532
inhibitor of DNA binding 3	1.668	2.189
proline-rich nuclear receptor coactivator 1	1.667	2.911
ring finger and FYVE like domain containing protein	1.666	2.321
guanylate nucleotide binding protein 3	1.662	3.427
RIKEN cDNA 4930524B15 gene	1.66	2.77
transcription factor EC	1.658	2.462
uridine phosphorylase 1	1.644	2.157
carbonic anhydrase 6	1.643	1.085
RIKEN cDNA 3110027N22 gene	1.641	2.816
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	1.637	3.005
RIKEN cDNA B830007D08 gene	1.629	2.477
serine (or cysteine) peptidase inhibitor, clade E, member 1	1.626	1.593
tumor necrosis factor (ligand) superfamily, member 4	1.626	2.796
cDNA sequence BC006779	1.625	2.483
testis expressed gene 10	1.625	0.806
otoraplin	1.624	2.252
RIKEN cDNA D330017J20 gene	1.617	2.33
HtrA serine peptidase 1	1.605	2.388
RAS guanyl releasing protein 1	1.605	2.003
histone cluster 1, H4c	1.603	1.983
suppressor of cytokine signaling 1	1.594	1.957
coiled-coil domain containing 63	1.591	2.697

jumonji domain containing 3	1.587	2.147
cyclin-dependent kinase 5, regulatory subunit (p35) 1	1.572	2.67
coiled-coil domain containing 80	1.567	2.187
RIKEN cDNA A130086G11 gene	1.567	2.308
bactericidal/permeability-increasing protein-like 2	1.564	2.406
baculoviral IAP repeat-containing 3	1.564	2.058
translocase of inner mitochondrial membrane 23 homolog (yeast)	1.562	1.227
cyclin L1	1.554	2.262
myeloid differentiation primary response gene 116	1.553	1.708
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.547	1.92
ankyrin repeat and BTB (POZ) domain containing 2	1.545	2.545
dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	1.517	2.177
plectin 1	1.504	1.116
Janus kinase 2	1.495	1.528
inhibin beta-A	1.482	1.628
pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4	1.476	2.113
chemokine (C-X-C motif) ligand 2	1.474	1.739
RAS guanyl releasing protein 1	1.471	2.076
PR domain containing 1, with ZNF domain	1.466	1.857
SKI-like	1.466	2.648
protocadherin 8	1.452	2.149
RIKEN cDNA A630081D01 gene	1.447	2.578
wingless-related MMTV integration site 2	1.442	0.228
jagged 1	1.439	2.647
protein phosphatase, EF hand calcium-binding domain 2	1.435	1.848
transmembrane protein 39a	1.434	2.345
RIKEN cDNA 2810433D01 gene	1.432	2.212
coiled-coil domain containing 11	1.425	2.564
RIKEN cDNA 4732474O15 gene	1.417	2.076
nuclear protein 1	1.414	2.376
Mediterranean fever	1.387	1.58
schlafen 4	1.382	2.169
ganglioside-induced differentiation-associated-protein 10	1.372	2.013
RIKEN cDNA 1200009I06 gene	1.364	2.215
inhibitor of kappaB kinase beta	1.362	2.11
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	1.358	2.12
IKAROS family zinc finger 1	1.348	1.877
C-type lectin domain family 2, member d	1.347	1.645
RIKEN cDNA 3021401C12 gene	1.344	2.136
nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta	1.343	1.671
syntaxin 11	1.336	2.042
receptor (TNFRSF)-interacting serine-threonine kinase 2	1.334	1.941

choline kinase alpha	1.331	1.63
immediate early response 2	1.33	1.231
RIKEN cDNA D130040H23 gene	1.328	3.26
plasminogen activator, urokinase receptor	1.324	1.05
tumor necrosis factor, alpha-induced protein 2	1.32	1.815
MARCKS-like 1	1.318	2.041
histone cluster 1, H1a	1.316	1.611
G protein-coupled receptor 132	1.313	2.02
interleukin 1 alpha	1.308	1.972
interleukin 15	1.308	2.72
kinesin light chain 2	1.306	1.626
histone cluster 1, H4i	1.3	1.442
erythroid differentiation regulator 1	1.299	2.51
interleukin 10	1.296	1.471
IKAROS family zinc finger 4	1.295	2.626
myeloid cell nuclear differentiation antigen	1.29	0.963
thymidylate kinase family LPS-inducible member	1.288	2.37
solute carrier organic anion transporter family, member 3a1	1.285	0.996
suppressor of cytokine signaling 3	1.284	1.902
protocadherin 10	1.277	1.332
growth arrest and DNA-damage-inducible 45 beta	1.276	1.934
interferon regulatory factor 1	1.276	2.393
Kruppel-like factor 6	1.275	2.55
histone cluster 1, H1e	1.266	2.147
beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	1.254	0.688
interleukin 31	1.253	0.911
RIKEN cDNA 2310002B14 gene	1.239	0.204
polo-like kinase 2 (Drosophila)	1.233	1.864
chemokine (C-X-C motif) ligand 10	1.227	1.51
RIKEN cDNA B430201A12 gene	1.221	0.966
guanylate binding protein 6	1.217	2.661
nucleoporin 54	1.213	2.084
RIKEN cDNA 4930439A04 gene	1.205	2.102
RIKEN cDNA 1110061O04 gene	1.203	2.027
nuclear receptor subfamily 1, group D, member 1	1.187	2.55
interferon-induced protein with tetratricopeptide repeats 2	1.186	2.559
RIKEN cDNA 0610037M15 gene	1.185	2.917
cDNA sequence BC031441	1.184	2.089
Cnksr family member 3	1.177	2.312
camello-like 5	1.176	2.031
ring finger and FYVE like domain containing protein	1.172	1.43
cDNA sequence BC011487	1.171	0.953
myxovirus (influenza virus) resistance 1	1.17	2.168
potassium voltage-gated channel, shaker-related subfamily, member 3	1.16	0.974
chloride intracellular channel 4 (mitochondrial)	1.155	1.857

TRAF family member-associated Nf-kappa B activator	1.153	2.124
EST AA407452	1.146	1.588
phorbol-12-myristate-13-acetate-induced protein 1	1.139	1.527
fem-1 homolog c (C.elegans)	1.136	0.912
RIKEN cDNA 6030465E24 gene	1.127	2.103
RIKEN cDNA 5730557B15 gene	1.122	1.294
SH3-domain kinase binding protein 1	1.118	0.947
RIKEN cDNA 9630026M06 gene	1.116	1.817
interleukin 27	1.113	1.376
RIKEN cDNA 2010109K09 gene	1.11	1.625
three prime repair exonuclease 1	1.098	1.821
RIKEN cDNA 2600010E01 gene	1.096	1.609
cyclin L1	1.095	1.65
G protein-coupled receptor 87	1.092	0.933
Janus kinase 2	1.09	0.909
pleckstrin homology domain containing, family G (with RhoGef domain) member 1	1.082	1.262
melanoma antigen	1.077	0.026
N-acylsphingosine amidohydrolase 3-like	1.075	1.256
SCO-spondin	1.07	0.723
kinectin 1	1.064	2.278
retinoblastoma binding protein 8	1.064	2.02
cDNA sequence BC065085	1.06	1.024
inositol polyphosphate-5-phosphatase A	1.041	0.788
chemokine (C-C motif) receptor-like 1	1.036	2.955
mitogen-activated protein kinase kinase kinase 6	1.036	1.296
Down syndrome critical region homolog 1 (human)	1.035	1.529
proviral integration site 3	1.028	1.057
ATPase, H+ transporting, lysosomal V0 subunit C	1.023	2.108
RIKEN cDNA 5230400M03 gene	1.014	2
interferon gamma induced GTPase	1.013	1.389
metallothionein 1	1.011	2.061
tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	1.009	1.455
GTPase, IMAP family member 6	1.006	1.429

Appendix Three. Dot/Icm-dependent genes in *Caspase1*$^{-/-}$ macrophages. Genes with a twofold or greater difference in induction between wildtype *L. pneumophila* and *ΔdotA* are listed. Data are expressed as $\log_2$(induction) normalized to expression in uninfected macrophages. The values shown are from one representative experiment.

gene name	***ΔdotA***	**wildtype**
protease, serine, 3	-4.221	0.842
colony stimulating factor 2 (granulocyte-macrophage)	-0.365	4.16
CD83 antigen	-0.985	3.064
predicted gene, EG434025	-1.149	2.611
selenocysteine lyase	0.154	3.432
early growth response 1	0.97	4.184
RIKEN cDNA 4930412F09 gene	0.357	3.527
plasminogen activator, tissue	1.118	4.251
purine rich element binding protein B	-2.929	0.166
GTP binding protein (gene overexpressed in skeletal muscle)	0.235	3.308
interleukin 23, alpha subunit p19	0.204	3.264
topoisomerase (DNA) II beta	-2.562	0.157
ras homolog gene family, member B	-0.25	2.419
RIKEN cDNA 5430405N12 gene	-0.007	2.555
colony stimulating factor 3 (granulocyte)	0.809	3.367
muted	0.045	2.553
AXIN1 up-regulated 1	0.479	2.97
zinc finger, CCHC domain containing 6	-2.353	0.127
growth arrest and DNA-damage-inducible 45 alpha	-0.387	2.078
regulator of G-protein signaling 1	-0.429	2.031
tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase	0.291	2.729
RIKEN cDNA 4631424J17 gene	0.137	2.561
CDC-like kinase 1	-0.409	1.998
selectin, endothelial cell	0.253	2.653
protein tyrosine phosphatase, receptor type, G	0.433	2.829
chemokine (C-C motif) ligand 20	1.676	4.062
adrenomedullin	0.605	2.978
interleukin 6	2.84	5.193
solute carrier family 2 (facilitated glucose transporter), member 2	-0.181	2.168
amphiregulin	0.63	2.976
GA repeat binding protein, beta 2	0.872	3.165
homeo box A9	0.716	2.997
aldehyde dehydrogenase family 1, subfamily A1	-0.158	2.095
Rho GTPase activating protein 22	-0.3	1.893
RIKEN cDNA 4930589L23 gene	0.229	2.415
serine (or cysteine) peptidase inhibitor, clade E, member 1	-0.533	1.626
procollagen, type XXVII, alpha 1	-0.138	2.006
arginase type II	0.836	2.969
leukotriene B4 receptor 2	0.299	2.41

RIKEN cDNA 9030617O03 gene	-0.318	1.768
sorbin and SH3 domain containing 2	0.748	2.81
B-cell translocation gene 2, anti-proliferative	1.097	3.142
interferon regulatory factor 4	-0.218	1.82
nudix (nucleoside diphosphate linked moiety X)-type motif 11	0.215	2.221
histone cluster 1, H1b	0.173	2.171
colony stimulating factor 1 (macrophage)	0.796	2.781
acid phosphatase 1, soluble	0.448	2.404
interferon beta 1, fibroblast	0.324	2.267
carbonic anhydrase 2	1.572	3.493
RIKEN cDNA 4930583C14 gene	0.473	2.377
histone cluster 1, H4i	-0.007	1.871
cyclin-dependent kinase 5, regulatory subunit (p35) 1	-0.304	1.572
sprouty homolog 1 (Drosophila)	1.798	3.671
tetratricopeptide repeat domain 17	0.534	2.397
coagulation factor III	1.061	2.874
RIKEN cDNA 4833411O04 gene	0.485	2.251
Tnf receptor-associated factor 1	1.233	2.987
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	0.57	2.315
mucolipin 1	1.038	2.783
predicted gene, OTTMUSG00000015529	0.871	2.602
RIKEN cDNA 1700027A07 gene	0.555	2.278
ubiquitin interaction motif containing 1	0.545	2.244
PHD finger protein 20-like 1	0.458	2.149
histone cluster 1, H4i	-0.365	1.3
zinc finger protein 688	0.737	2.386
tumor necrosis factor, alpha-induced protein 3	0.869	2.516
dual specificity phosphatase 1	0.499	2.139
polo-like kinase 2 (Drosophila)	-0.405	1.233
chemokine (C-X-C motif) ligand 5	2.518	4.147
transformed mouse 3T3 cell double minute 2	-0.837	0.772
TSC22 domain family, member 1	1.459	3.058
chemokine (C-C motif) ligand 7	1.133	2.731
immediate early response 3	0.692	2.285
antigen identified by monoclonal antibody Ki 67	-0.631	0.946
B-cell translocation gene 1, anti-proliferative	0.165	1.742
preimplantation protein 3	1.049	2.624
TG interacting factor	-1.032	0.523
mucosa associated lymphoid tissue lymphoma translocation gene 1	1.637	3.186
RIKEN cDNA D330017J20 gene	0.072	1.617
guanylate cyclase 2g	0.667	2.211
RasGEF domain family, member 1B	0.63	2.159
phosphatidylinositol 3-kinase, C2 domain containing, alpha polypeptide	0.761	2.238

nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	2.428	3.901
interleukin enhancer binding factor 3	0.585	2.012
hect domain and RLD 5	0.568	1.974
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	0.261	1.637
Jun dimerization protein 2	0.864	2.238
ankyrin repeat domain 57	1.45	2.819
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 7	0.512	1.878
caveolin 2	-3.041	-1.678
interleukin 1 alpha	-0.047	1.308
poliovirus receptor	0.376	1.716
tumor necrosis factor (ligand) superfamily, member 4	0.29	1.626
pleckstrin homology-like domain, family A, member 1	1.281	2.61
proviral integration site 1	1.106	2.42
hypoxia inducible factor 1, alpha subunit	1.988	3.3
histone cluster 1, H4d	-0.49	0.819
coiled-coil domain containing 63	0.286	1.591
jumonji domain containing 3	0.309	1.587
matrix metallopeptidase 13	1.486	2.757
plasminogen activator, urokinase receptor	0.054	1.324
nuclear RNA export factor 1 homolog (S. cerevisiae)	0.747	2.017
adenosine A2b receptor	1.568	2.83
interleukin 1 beta	-0.596	0.663
otoraplin	0.366	1.624
human immunodeficiency virus type I enhancer binding protein 3	1.098	2.347
Jun oncogene	-0.28	0.962
rad and gem related GTP binding protein 2	0.58	1.82
actin-binding LIM protein 2	1.233	2.458
dual specificity phosphatase 2	0.914	2.129
PDZ and LIM domain 7	-0.285	0.927
kelch-like 25 (Drosophila)	-0.223	0.987
heat shock protein 1A	0.988	2.194
RIKEN cDNA 1700029G01 gene	0.907	2.107
coiled-coil domain containing 11	0.229	1.425
RIKEN cDNA 2210037E17 gene	0.982	2.171
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	-0.597	0.577
RIKEN cDNA D930007M16 gene	0.908	2.082
kinesin light chain 2	0.142	1.306
RIKEN cDNA D930030K17 gene	0.973	2.134
protein kinase, AMP-activated, alpha 2 catalytic subunit	1.698	2.856
CDC42 effector protein (Rho GTPase binding) 1	0.963	2.12
cyclin L1	0.402	1.554
lysyl oxidase	2.842	3.994
vomeronasal 1 receptor, C6	-0.856	0.293

RIKEN cDNA 2010107E04 gene	1.579	2.724
transmembrane protein 39a	0.296	1.434
basic helix-loop-helix domain containing, class B2	-0.906	0.23
metallothionein 2	0.91	2.043
nuclear receptor subfamily 1, group D, member 1	0.06	1.187
developmental pluripotency associated 2	1.944	3.064
B-cell translocation gene 3	1.085	2.203
inhibitor of kappaB kinase beta	0.248	1.362
Cnksr family member 3	0.065	1.177
choline kinase alpha	0.231	1.331
myeloid cell nuclear differentiation antigen	0.203	1.29
aryl-hydrocarbon receptor	1.471	2.558
RIKEN cDNA 2810004A10 gene	1.438	2.522
apolipoprotein C-III	0.957	2.038
RIKEN cDNA 4732474O15 gene	0.338	1.417
chemokine (C-C motif) receptor-like 1	-0.041	1.036
cDNA sequence BC006779	0.56	1.625
tumor necrosis factor (ligand) superfamily, member 9	0.805	1.866
RIKEN cDNA 0610037M15 gene	0.125	1.185
protocadherin 8	0.401	1.452
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.318	1.358
AMP deaminase 3	0.931	1.966
Cdc42 binding protein kinase alpha	1.116	2.137
interleukin 10	0.278	1.296
RIKEN cDNA A630081D01 gene	0.433	1.447
solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), membe	0.688	1.696

Appendix Four. Genes induced twofold or more in B6 and TLR-deficient (*Myd88*$^{-/-}$) macrophages infected with *ΔflaA or ΔdotA L. pneumophila*. Data are expressed as $\log_2$ (induction) normalized to expression in uninfected macrophages. Results from one microarray experiment are shown.

Gene name	B6 ΔdotA	B6 *ΔflaA*	*Myd88-/-* ΔdotA	*Myd88-/-* *ΔflaA*
early growth response 1	1.343	7.325	-0.635	6.207
chemokine (C-X-C motif) ligand 1	3.365	6.376	0.421	6.096
interleukin 1 alpha	2.681	5.92	0.437	7.743
chemokine (C-X-C motif) ligand 2	3.068	5.739	1.764	n.d.
colony stimulating factor 2 (granulocyte-macrophage)	2.089	5.729	-0.387	5.531
GTP binding protein (gene overexpressed in skeletal muscle)	0.054	5.297	-0.408	6.381
early growth response 2	0.988	5.072	0.285	4.301
cysteine-serine-rich nuclear protein 1	0.198	4.569	-0.019	4.055
CD83 antigen	0.606	4.417	0.462	n.d.
interleukin 1 beta	3.539	4.403	0.669	6.23
colony stimulating factor 1 (macrophage)	-0.336	4.39	0.048	6.648
interferon beta 1, fibroblast	0.487	4.39	0.438	4.28
lymphotoxin A	2.117	4.326	1.349	6.399
prostaglandin-endoperoxide synthase 2	3.061	4.088	-1.479	5.446
tumor necrosis factor, alpha-induced protein 3	1.595	3.977	1.07	n.d.
antigen identified by monoclonal antibody Ki 67	0.463	3.824	0.38	1.413
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	1.304	3.758	0.207	4.504
growth arrest and DNA-damage-inducible 45 beta	0.393	3.462	-0.036	4.967
chemokine (C-C motif) receptor-like 2	0.733	3.39	0.347	4.643
zinc finger protein 36	0.567	3.321	-0.148	2.254
protein phosphatase 1, regulatory (inhibitor) subunit 15A	0.418	3.292	0.42	4.034
mucosa associated lymphoid tissue lymphoma translocation gene 1	1.607	3.252	0.25	3.97
chemokine (C-X-C motif) ligand 10	0.884	3.244	4.043	8.25
interleukin 6	3.012	3.18	n.d.	n.d.
immediate early response 3	1.879	3.115	0.105	3.469
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	1.68	3.089	-0.003	2.783
reticuloendotheliosis oncogene	0.918	3.067	0.023	3.806
regulator of G-protein signaling 1	0.162	3.065	0.247	4.125
colony stimulating factor 3 (granulocyte)	2.64	3.023	n.d.	n.d.
dual specificity phosphatase 1	1.032	2.997	0.272	n.d.
chemokine (C-C motif) ligand 7	1.141	2.824	-0.37	4.779

TSC22 domain family, member 1	1.371	2.754	0.156	4.675
arginase type II	0.363	2.732	0.695	3.74
vascular cell adhesion molecule 1	0.7	2.687	1.864	6.568
histone cluster 1, H4i	0.397	2.672	-0.307	1.587
interleukin 10	1.444	2.636	-0.072	0.698
chemokine (C-C motif) ligand 6	1.516	2.627	0.347	n.d.
tumor necrosis factor (ligand) superfamily, member 9	0.484	2.611	0.082	4.387
pleckstrin homology-like domain, family A, member 1	1.369	2.574	-0.224	3.532
growth arrest and DNA-damage-inducible 45 alpha	0.917	2.544	0.671	5.931
cell division cycle 6 homolog (S. cerevisiae)	-0.308	2.528	-0.081	1.023
interferon regulatory factor 1	0.546	2.487	0.491	3.343
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	0.497	2.483	0.287	3.74
suppressor of cytokine signaling 3	1.383	2.443	0.49	6.243
solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	1.339	2.402	0.42	n.d.
chemokine (C-C motif) ligand 3	2.157	2.371	0.123	4.022
dual specificity phosphatase 2	1.145	2.362	0.215	2.65
PR domain containing 1, with ZNF domain	0.564	2.36	0.613	3.393
ERBB receptor feedback inhibitor 1	0.169	2.359	0.183	3.589
tumor necrosis factor, alpha-induced protein 2	1.53	2.356	0.985	3.701
cyclin L1	-0.109	2.351	0.352	4.568
histone cluster 1, H4m	0.136	2.347	0.099	1.837
CD40 antigen	1.58	2.347	1.827	6.07
B-cell leukemia/lymphoma 2 related protein A1d	2.347	2.302	0.36	n.d.
nucleotide-binding oligomerization domain containing 2	1.027	2.276	1.067	3.89
chemokine (C-C motif) ligand 2	0.132	2.242	-0.156	n.d.
chemokine (C-X-C motif) ligand 3	1.894	2.157	0.216	5.284
ankyrin repeat domain 57	0.281	2.147	-0.505	0.178
choline kinase alpha	-0.378	2.126	0.434	3.635
oxidized low density lipoprotein (lectin-like) receptor 1	0.438	2.108	1.009	4.225
histone cluster 1, H2bh	0.437	2.105	-0.472	1.188
RasGEF domain family, member 1B	0.772	2.101	0.332	3.395
intercellular adhesion molecule 1	1.03	2.09	2.014	n.d.
RIKEN cDNA A630081D01 gene	0.462	2.053	0.469	1.655
zinc finger CCCH type containing 12C	0.686	2.045	-0.088	4.675
cytochrome P450, family 2, subfamily d, polypeptide 32, pseudogene	1.157	2.037	0.564	n.d.
Jun-B oncogene	0.987	2.028	0.433	3.14

B-cell leukemia/lymphoma 2 related protein A1a	2.001	2.015	0.303	2.366
RIKEN cDNA 2410039M03 gene	1.192	1.942	0.746	3.824
solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	0.669	1.9	0.194	3.339
cDNA sequence BC006779	0.879	1.877	0.539	2.93
adenosine A2b receptor	1.41	1.831	0.766	4.309
ADP-ribosylation factor-like 5C	0.696	1.753	1.658	4.082
polo-like kinase 2 (Drosophila)	0.3	1.745	0.07	2.325
BCL6 interacting corepressor	0.178	1.707	0.372	3.373
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.454	1.694	0.126	2.727
TNF receptor-associated factor 1	1.191	1.676	1.855	5.649
phorbol-12-myristate-13-acetate-induced protein 1	-0.474	1.66	0.34	2.322
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.743	1.656	1.234	4.424
phosphodiesterase 4B, cAMP specific	1.235	1.655	1.169	4.676
SAM domain, SH3 domain and nuclear localization signals, 1	0.855	1.648	0.565	2.66
transformed mouse 3T3 cell double minute 2	0.547	1.636	0.295	2.156
ankyrin repeat domain 33B	0.824	1.634	0.191	2.793
zinc finger, SWIM domain containing 4	0.408	1.594	0.215	2.006
interferon-related developmental regulator 1	-0.478	1.586	0.297	3.891
C-type lectin domain family 4, member e	1.505	1.577	2.624	5.061
interferon activated gene 205	1.198	1.569	1.566	3.299
plasminogen activator, urokinase receptor	0.455	1.567	0.449	4.023
Jun dimerization protein 2	0.546	1.561	1.072	4.91
Fc receptor, IgG, low affinity IIb	1.65	1.561	1.104	2.126
endothelin 1	2.343	1.55	n.d.	n.d.
tet oncogene family member 2	0.525	1.542	0.256	2.101
GTP cyclohydrolase 1	0.921	1.531	0.647	2.366
dual adaptor for phosphotyrosine and 3-phosphoinositides 1	-0.303	1.51	0.235	2.85
Kruppel-like factor 6	0.282	1.496	-0.172	2.405
TNFAIP3 interacting protein 1	1.04	1.491	0.749	2.661
MARCKS-like 1	0.672	1.481	2.073	5.269
heat shock protein 1B	0.751	1.47	0.323	2.88
NLR family, pyrin domain containing 3	0.952	1.462	0.711	2.82
Jun oncogene	-0.132	1.454	-0.071	2.941
Rho guanine nucleotide exchange factor (GEF) 3	1.102	1.454	0.285	2.308
icos ligand	0.057	1.436	0.189	2.498
matrix metallopeptidase 13	1.092	1.431	0.855	3.845
CD69 antigen	1.486	1.427	2.369	6.923

ring finger protein 19B	0.998	1.423	0.88	2.921
PDZ and LIM domain 7	0.042	1.395	0.146	2.041
dual specificity phosphatase 16	0.861	1.379	0.735	4.201
proviral integration site 1	0.981	1.379	0.12	3.741
ORAI calcium release-activated calcium modulator 2	0.413	1.372	0.865	2.457
serine/threonine kinase 40	0.883	1.365	0.29	2.423
cholesterol 25-hydroxylase	1.494	1.357	0.906	3.483
chemokine (C-C motif) ligand 5	0.955	1.341	1.834	4.24
dual specificity phosphatase 4	0.451	1.335	0.453	3.033
proline rich 5 like	-0.233	1.333	0.269	2.131
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	0.447	1.329	0.485	2.688
caspase 4, apoptosis-related cysteine peptidase	0.77	1.324	1.408	3.495
syntaxin 11	0.803	1.317	0.238	2.928
three prime repair exonuclease 1	0.729	1.308	0.703	2.527
jagged 1	0.348	1.277	0.259	2.42
syndecan 4	0.602	1.268	0.715	2.81
interleukin 27	1.027	1.255	0.669	2.414
ISG15 ubiquitin-like modifier	0.63	1.247	0.689	2.171
TGFB-induced factor homeobox 1	-0.082	1.244	0.516	2.039
zinc finger protein 800	0.788	1.243	0.433	2.308
poliovirus receptor	0.577	1.242	0.304	2.056
Janus kinase 2	0.947	1.235	0.399	2.165
solute carrier family 2 (facilitated glucose transporter), member 6	1.061	1.224	0.97	2.648
RAB20, member RAS oncogene family	0.822	1.22	0.438	3.366
myxovirus (influenza virus) resistance 2	0.469	1.206	0.799	3.704
WD repeat and SOCS box-containing 1	0.225	1.178	0.37	2.05
G protein-coupled receptor 84	1.034	1.161	2.412	5.12
latent transforming growth factor beta binding protein 1	0.122	1.138	n.d.	2.945
myeloid cell nuclear differentiation antigen	0.53	1.138	1.598	3.602
KDM1 lysine (K)-specific demethylase 6B	0.138	1.131	-0.435	3.017
RIKEN cDNA 2810474O19 gene	0.656	1.129	-0.124	2.189
chemokine (C-C motif) ligand 12	0.536	1.125	0.747	4.603
solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	1.492	1.113	1.692	2.318
pleckstrin homology-like domain, family B, member 1	0.144	1.107	0.911	3.283
phospholipid scramblase 1	-0.067	1.096	0.59	3.218
ubiquitin-conjugating enzyme E2F (putative)	0.326	1.077	0.653	2.365
TRAF family member-associated Nf-kappa B activator	0.312	1.067	1.287	4.641
RIKEN cDNA 2500002B13 gene	0.87	1.05	1.295	3.063

platelet derived growth factor, B polypeptide	0.066	1.006	0.861	3.789
phosphodiesterase 4A, cAMP specific	1.155	1.005	0.558	2.964
ankyrin repeat and BTB (POZ) domain containing 2	0.054	1.002	0.714	4.65

Appendix Five. Dot/Icm-dependent genes in B6 and *Myd88*$^{-/-}$ macrophages. Genes induced fourfold or more and with a twofold or greater difference between *ΔflaA* and *ΔdotA* are depicted. Data are expressed as $\log_2$(induction) normalized to uninfected macrophages. Results from one microarray experiment are shown.

Gene name	B6 ΔdotA	B6 *ΔflaA*	*Myd88-/-* ΔdotA	*Myd88-/-* *ΔflaA*
early growth response 1	1.343	7.325	-0.635	6.207
GTP binding protein (gene overexpressed in skeletal muscle)	0.054	5.297	-0.408	6.381
colony stimulating factor 1 (macrophage)	-0.336	4.39	0.048	6.648
cysteine-serine-rich nuclear protein 1	0.198	4.569	-0.019	4.055
early growth response 2	0.988	5.072	0.285	4.301
interferon beta 1, fibroblast	0.487	4.39	0.438	4.28
CD83 antigen	0.606	4.417	0.462	n.d.
colony stimulating factor 2 (granulocyte-macrophage)	2.089	5.729	-0.387	5.531
antigen identified by monoclonal antibody Ki 67	0.463	3.824	0.38	1.413
interleukin 1 alpha	2.681	5.92	0.437	7.743
growth arrest and DNA-damage-inducible 45 beta	0.393	3.462	-0.036	4.967
chemokine (C-X-C motif) ligand 1	3.365	6.376	0.421	6.096
regulator of G-protein signaling 1	0.162	3.065	0.247	4.125
protein phosphatase 1, regulatory (inhibitor) subunit 15A	0.418	3.292	0.42	4.034
cell division cycle 6 homolog (S. cerevisiae)	-0.308	2.528	-0.081	1.023
zinc finger protein 36	0.567	3.321	-0.148	2.254
chemokine (C-X-C motif) ligand 2	3.068	5.739	1.764	n.d.
chemokine (C-C motif) receptor-like 2	0.733	3.39	0.347	4.643
choline kinase alpha	-0.378	2.126	0.434	3.635
cyclin L1	-0.109	2.351	0.352	4.568
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	1.304	3.758	0.207	4.504
tumor necrosis factor, alpha-induced protein 3	1.595	3.977	1.07	n.d.
arginase type II	0.363	2.732	0.695	3.74
chemokine (C-X-C motif) ligand 10	0.884	3.244	4.043	8.25
histone cluster 1, H4i	0.397	2.672	-0.307	1.587
histone cluster 1, H4m	0.136	2.347	0.099	1.837
lymphotoxin A	2.117	4.326	1.349	6.399
ERBB receptor feedback inhibitor 1	0.169	2.359	0.183	3.589
reticuloendotheliosis oncogene	0.918	3.067	0.023	3.806
phorbol-12-myristate-13-acetate-induced protein 1	-0.474	1.66	0.34	2.322
tumor necrosis factor (ligand) superfamily, member 9	0.484	2.611	0.082	4.387
chemokine (C-C motif) ligand 2	0.132	2.242	-0.156	n.d.
interferon-related developmental regulator 1	-0.478	1.586	0.297	3.891

vascular cell adhesion molecule 1	0.7	2.687	1.864	6.568
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	0.497	2.483	0.287	3.74
dual specificity phosphatase 1	1.032	2.997	0.272	n.d.
interferon regulatory factor 1	0.546	2.487	0.491	3.343
dual adaptor for phosphotyrosine and 3-phosphoinositides 1	-0.303	1.51	0.235	2.85
PR domain containing 1, with ZNF domain	0.564	2.36	0.613	3.393
growth arrest and DNA-damage-inducible 45 gamma	-0.73	0.992	0.11	2.285
chemokine (C-C motif) ligand 7	1.141	2.824	-0.37	4.779
oxidized low density lipoprotein (lectin-like) receptor 1	0.438	2.108	1.009	4.225
histone cluster 1, H2bh	0.437	2.105	-0.472	1.188
mucosa associated lymphoid tissue lymphoma translocation gene 1	1.607	3.252	0.25	3.97
growth arrest and DNA-damage-inducible 45 alpha	0.917	2.544	0.671	5.931
RIKEN cDNA A630081D01 gene	0.462	2.053	0.469	1.655
Jun oncogene	-0.132	1.454	-0.071	2.941
proline rich 5 like	-0.233	1.333	0.269	2.131
BCL6 interacting corepressor	0.178	1.707	0.372	3.373
polo-like kinase 2 (Drosophila)	0.3	1.745	0.07	2.325
RIKEN cDNA 5430416N02 gene	-0.654	0.762	-0.379	2.194
ras homolog gene family, member B	-0.712	0.704	-0.025	2.142
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	1.68	3.089	-0.003	2.783
TSC22 domain family, member 1	1.371	2.754	0.156	4.675
icos ligand	0.057	1.436	0.189	2.498
zinc finger CCCH type containing 12C	0.686	2.045	-0.088	4.675
PDZ and LIM domain 7	0.042	1.395	0.146	2.041
RasGEF domain family, member 1B	0.772	2.101	0.332	3.395
TGFB-induced factor homeobox 1	-0.082	1.244	0.516	2.039
nucleotide-binding oligomerization domain containing 2	1.027	2.276	1.067	3.89
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.454	1.694	0.126	2.727
immediate early response 3	1.879	3.115	0.105	3.469
solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	0.669	1.9	0.194	3.339
dual specificity phosphatase 2	1.145	2.362	0.215	2.65
Kruppel-like factor 6	0.282	1.496	-0.172	2.405
pleckstrin homology-like domain, family A, member 1	1.369	2.574	-0.224	3.532
zinc finger, SWIM domain containing 4	0.408	1.594	0.215	2.006

polypyrimidine tract binding protein 2	-0.589	0.577	0.276	2.217
phospholipid scramblase 1	-0.067	1.096	0.59	3.218
DnaJ (Hsp40) homolog, subfamily B, member 2	-0.535	0.593	0.57	2.114
enhancer of polycomb homolog 1 (Drosophila)	-0.173	0.954	0.211	2.055
plasminogen activator, urokinase receptor	0.455	1.567	0.449	4.023
chemokine (C-C motif) ligand 6	1.516	2.627	0.347	n.d.
transformed mouse 3T3 cell double minute 2	0.547	1.636	0.295	2.156
solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	1.339	2.402	0.42	n.d.
suppressor of cytokine signaling 3	1.383	2.443	0.49	6.243
intercellular adhesion molecule 1	1.03	2.09	2.014	n.d.
ADP-ribosylation factor-like 5C	0.696	1.753	1.658	4.082
arginine/serine-rich coiled-coil 2	-0.056	0.99	0.289	2.351
Jun-B oncogene	0.987	2.028	0.433	3.14
RIKEN cDNA 3110043O21 gene	-0.087	0.948	0.435	2.833
prostaglandin-endoperoxide synthase 2	3.061	4.088	-1.479	5.446
tet oncogene family member 2	0.525	1.542	0.256	2.101
latent transforming growth factor beta binding protein 1	0.122	1.138	n.d.	2.945
Jun dimerization protein 2	0.546	1.561	1.072	4.91

Appendix 6. Transcriptional response of B6 and *Myd88*$^{-/-}$*/Trif*$^{-/-}$ (MyT) macrophages infected with wildtype LP01 or *ΔicmSΔicmW* (icmSW) *L. pneumophila*. Data are expressed as $\log_2$(induction) normalized to expression in uninfected macrophages. Results from one representative microarray experiment are shown.

Gene name	B6 LP01	B6 icmSW	MyT LP01	MyT icmSW
1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, delta)	2.072	2.081	1.646	0.987
2'-5' oligoadenylate synthetase-like 1	2.773	2.627	3.64	3.232
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	3.993	4.327	3.129	0.455
acid phosphatase 5, tartrate resistant	4.24	4.375	n.d.	n.d.
acyl-CoA synthetase long-chain family member 1	3.855	2.728	2.771	1.264
acyloxyacyl hydrolase	3.209	3.093	2.148	1.552
adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1	2.528	1.652	1.594	0.935
adenosine A2b receptor	6.249	6.109	6.786	3.163
adenosine monophosphate deaminase 3	3.195	2.493	2.521	1.576
adenylate kinase 4	3.979	4.327	1.947	2.337
ADP-ribosylation factor-like 5C	3.221	2.892	4.152	3.319
adrenomedullin	7.306	4.117	n.d.	n.d.
AE binding protein 2	2.859	1.269	2.556	1.175
agrin	2.153	1.951	1.426	0.732
alkB, alkylation repair homolog 1 (E. coli)	2.061	1.033	1.209	0.509
aminolevulinic acid synthase 1	3.98	4.019	n.d.	0.904
ankyrin repeat and BTB (POZ) domain containing 2	5.349	4.382	5.404	2.144
ankyrin repeat domain 33B	2.644	0.989	2.188	0.507
aquaporin 9	2.171	2.445	n.d.	n.d.
ArfGAP with FG repeats 1	2.095	1.359	1.27	0.282
arginase type II	2.133	1.915	0.97	-1.094
arginase, liver	3.362	4.03	1.556	0.326
argininosuccinate synthetase 1	3.595	3.811	2.154	2.215
ATP-binding cassette, sub-family C (CFTR/MRP), member 1	2.193	1.8	1.893	1.738
avian reticuloendotheliosis viral (v-rel) oncogene related B	2.438	1.638	3.668	2.211
B-cell leukemia/lymphoma 2 related protein A1a	5.158	5.552	n.d.	n.d.
B-cell leukemia/lymphoma 2 related protein A1c	5.199	5.157	3.385	2.106
B-cell leukemia/lymphoma 2 related protein A1d	4.793	5.755	3.521	2.059
B-cell leukemia/lymphoma 3	2.337	2.144	2.892	1.306
B-cell translocation gene 2, anti-proliferative	2.592	1.106	1.832	-0.291
baculoviral IAP repeat-containing 3	3.645	2.634	3.579	2.491
basic helix-loop-helix family, member e40	2.423	0.868	3.377	1.122
basic leucine zipper transcription factor, ATF-like	3.905	4.752	1.745	1.116

basic leucine zipper transcription factor, ATF-like 2	2.157	2.831	2.035	2.102
BCL6 interacting corepressor	3.393	0.346	3.317	0.116
bromodomain adjacent to zinc finger domain 1A	2.293	0.943	2.595	0.432
butyrophilin-like 2	2.907	3.459	1.762	2.201
C-type lectin domain family 4, member e	6.98	6.401	6.585	5.002
C-type lectin domain family 5, member a	4.102	3.466	4.385	3.167
calcium channel, voltage-dependent, T type, alpha 1G subunit	5.023	5.513	3.84	2.418
carbohydrate (N-acetylglucosamino) sulfotransferase 7	2.938	2.697	2.684	2.509
carbohydrate sulfotransferase 11	2.226	2.555	n.d.	n.d.
carbonic anhydrase 13	2.941	2.892	1.423	0.599
carboxypeptidase D	2.036	1.524	1.312	0.72
caspase 4, apoptosis-related cysteine peptidase	2.89	2.719	3.017	2.724
caveolin 1, caveolae protein	2.58	2.268	1.541	1.788
CCAAT/enhancer binding protein (C/EBP), beta	2.291	2.536	2.805	1.286
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	2.622	0.547	2.966	0.755
CD14 antigen	2.402	2.965	1.654	0.844
CD274 antigen	5.284	5.146	4.533	2.516
CD300e antigen	2.118	2.632	-0.253	0.101
CD4 antigen	2.036	-0.03	0.588	-0.086
CD40 antigen	7.51	6.821	6.733	4.188
CD69 antigen	7.541	7.679	5.566	4.427
CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associate	2.364	2.282	n.d.	n.d.
CD82 antigen	2.016	1.822	2.316	1.636
CD83 antigen	6.387	3.121	n.d.	2.633
CDC42 effector protein (Rho GTPase binding) 2	5.588	5.494	5.113	3.487
cDNA sequence BC006779	3.353	2.38	3.815	1.957
ceroid-lipofuscinosis, neuronal 5	2.043	2.123	1.799	0.7
chemokine (C-C motif) ligand 12	4.706	5.771	4.14	3.175
chemokine (C-C motif) ligand 17	3.822	4.986	8.034	5.206
chemokine (C-C motif) ligand 2	5.313	5.558	4.91	1.467
chemokine (C-C motif) ligand 3	5.888	7.333	n.d.	n.d.
chemokine (C-C motif) ligand 5	6.501	8.227	n.d.	n.d.
chemokine (C-C motif) ligand 6	4.46	5.421	0.297	-1.537
chemokine (C-C motif) ligand 7	6.319	6.653	5.494	1.923
chemokine (C-C motif) receptor-like 2	5.463	4.288	4.044	2.053
chemokine (C-X-C motif) ligand 10	5.834	5.578	6.128	5.665
chemokine (C-X-C motif) ligand 16	2.178	1.543	2.066	1.089
chemokine (C-X-C motif) ligand 3	9.504	9.476	8.924	6.174
chemokine (C-X-C motif) ligand 9	2.874	2.54	1.907	2.454
cholesterol 25-hydroxylase	6.122	4.856	3.683	2.534

choline kinase alpha	2.222	-0.591	2.239	-0.103
coagulation factor III	6.447	5.9	4.029	-0.166
coagulation factor X	4.706	5.213	1.683	2.108
coenzyme Q10 homolog B (S. cerevisiae)	2.541	1.74	1.256	0.761
coiled-coil domain containing 50	2.224	1.294	1.833	1.094
coiled-coil domain containing 86	2.29	2.391	1.492	0.676
coiled-coil domain containing 88B	2.444	1.81	1.647	1.459
collagen, type IV, alpha 2	2.324	1.923	1.17	0.605
colony stimulating factor 1 (macrophage)	8.524	4.714	8.439	4.18
colony stimulating factor 2 (granulocyte-macrophage)	9.253	7.089	n.d.	n.d.
colony stimulating factor 3 (granulocyte)	6.394	7.71	2.639	1.546
complement component 3	4.253	4.046	3.76	3.567
complement factor B	5.155	5.167	n.d.	n.d.
cyclin L1	3.335	1.132	2.938	0.631
cyclin-dependent kinase inhibitor 1A (P21)	2.347	1.757	n.d.	0.556
cysteine-serine-rich nuclear protein 1	5.184	2.735	4.913	2.454
cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	2.848	2.497	4.564	4.248
cytochrome b-245, beta polypeptide	2.268	1.618	1.478	1.321
cytochrome P450, family 2, subfamily d, polypeptide 32, pseudogene	5.191	3.972	n.d.	n.d.
cytokine induced apoptosis inhibitor 1	2.455	1.312	2.053	0.808
cytokine inducible SH2-containing protein	7.389	5.356	6.995	1.246
cytoplasmic polyadenylation element binding protein 4	2.151	0.814	1.878	0.919
DCN1, defective in cullin neddylation 1, domain containing 3 (S. cerevisiae)	2.371	2.045	1.174	0.026
DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	2.24	1.765	1.75	1.247
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	2.711	1.878	2.107	1.591
dedicator of cytokinesis 10	2.197	1.379	1.837	0.65
density-regulated protein	3.549	3.37	1.23	0.808
DNA segment, Chr 16, ERATO Doi 472, expressed	2.684	3.413	0.762	0.433
DNA-damage regulated autophagy modulator 1	2.609	3.102	1.921	1.508
DnaJ (Hsp40) homolog, subfamily B, member 2	2.312	0.531	1.563	-0.359
dual specificity phosphatase 1	2.455	2.064	2.401	1.327
dual specificity phosphatase 16	5.168	5.115	3.551	0.681
dual specificity phosphatase 2	5.321	4.288	4.558	2.401
dual specificity phosphatase 4	2.086	1.594	2.645	1.187
dual specificity phosphatase 6	2.06	1.557	1.868	0.411
dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	2.039	0.995	1.707	0.472
E26 avian leukemia oncogene 2, 3' domain	5.078	5.083	n.d.	n.d.
early growth response 1	5.189	2.325	5.774	2.138
EH-domain containing 1	4.559	4.486	4.283	2.303

elongation factor RNA polymerase II 2	2.183	1.174	1.427	1.027
endothelin receptor type B	2.989	2.622	2.725	1.419
Epstein-Barr virus induced gene 3	2.626	2.772	2.214	0.905
ERBB receptor feedback inhibitor 1	4.62	2.646	3.361	0.307
ERO1-like (S. cerevisiae)	2.976	2.419	1.644	0.673
ets variant gene 6 (TEL oncogene)	2.463	2.263	2.515	1.475
exostoses (multiple) 1	3.06	2.937	2.13	1.194
ezrin	2.079	1.628	1.954	1.675
family with sequence similarity 108, member C	2.846	1.783	0.353	0.211
family with sequence similarity 133, member B	2.85	0.656	n.d.	n.d.
Fas (TNF receptor superfamily member 6)	3.631	3.854	3.372	2.629
Fc receptor, IgG, low affinity IIb	4.867	5.139	2.134	1.527
FCH and double SH3 domains 2	2.852	1.579	1.9	0.572
FERM domain containing 6	2.057	0.111	n.d.	n.d.
fibrinogen-like protein 2	2.494	2.359	0.081	0.117
fibronectin leucine rich transmembrane protein 3	5.109	5.135	6.165	3.597
filamin A interacting protein 1-like	2.573	1.661	1.933	0.973
filamin, beta	3.332	2.53	1.091	0.104
formin binding protein 1-like	2.787	2.653	2.735	1.464
formin-like 2	2.111	1.825	2.364	1.575
formyl peptide receptor 1	7.391	7.727	2.059	1.805
formyl peptide receptor 2	6.739	7.806	2.267	2.598
fos-like antigen 2	2.413	2.878	2.179	-0.454
G protein-coupled receptor 18	3.826	4.474	1.953	2.322
G protein-coupled receptor 84	4.457	5.168	3.403	1.756
G protein-coupled receptor 85	3.68	2.79	2.184	0.69
gap junction protein, alpha 1	3.683	4.108	2.698	2.253
glutathione reductase	2.333	2.433	0.257	0.675
glycerol kinase	3.247	2.854	0.89	0.567
glycerol phosphate dehydrogenase 2, mitochondrial	2.375	2.698	1.061	0.765
glycogen synthase 1, muscle	2.132	2.4	0.638	0.313
GRAM domain containing 1A	2.243	1.877	1.503	-0.247
GRAM domain containing 1B	2.296	1.221	1.503	0.054
growth arrest and DNA-damage-inducible 45 alpha	4.663	2.796	4.198	1.531
growth arrest and DNA-damage-inducible 45 beta	5.84	3.416	5.195	1.847
growth arrest specific 7	2.08	2.568	1.055	1.039
GTP binding protein (gene overexpressed in skeletal muscle)	7.644	3.076	n.d.	n.d.
GTP binding protein 4	2.341	2.118	1.509	0.681
GTP cyclohydrolase 1	3.401	2.736	2.737	1.674
GTPase, very large interferon inducible 1	2.504	1.398	2.002	1.422
guanylate binding protein 3	3.877	3.561	3.434	3.355
guanylate binding protein 5	6.269	5.604	n.d.	n.d.
guanylate binding protein 7	3.734	3.404	3.492	2.497
haptoglobin	2.794	3.7	1.494	2.142

heat shock protein 1B	4.13	2.441	5.178	2.952
hemopoietic cell kinase	2.939	2.466	-0.149	-0.361
heterogeneous nuclear ribonucleoprotein L-like	2.493	1.11	1.759	0.205
hexokinase 2	2.044	2.033	0.206	0.011
histocompatibility 2, class II antigen A, beta 1	3.758	4.989	2.167	2.065
histocompatibility 2, Q region locus 7	2.983	1.683	2.705	1.852
homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.003	2.061	1.209	0.541
hyaluronan synthase1	5.013	5.615	0.817	1.562
hypoxia inducible factor 1, alpha subunit	2.659	1.838	n.d.	n.d.
icos ligand	2.977	1.977	4.088	2.318
IKAROS family zinc finger 1	2.044	1.967	0.772	0.156
immediate early response 3	5.053	5.488	2.962	1.389
immunoglobulin superfamily, member 6	2.912	2.207	2.346	1.744
insulin-like growth factor 2 mRNA binding protein 2	2.865	2.661	0.847	0.078
insulin-like growth factor binding protein 7	3.635	4.175	2.45	1.208
integrin alpha L	2.314	1.771	0.655	0.209
interferon activated gene 203	2.575	2.168	2.051	1.844
interferon activated gene 204	3.044	2.713	1.774	1.559
interferon activated gene 205	6.844	6.458	8.115	7.296
interferon gamma inducible protein 47	2.546	2.512	2.293	2.222
interferon regulatory factor 1	3.351	2.131	3.953	2.236
interferon-induced protein with tetratricopeptide repeats 1	3.514	2.523	3.91	3.827
interferon-induced protein with tetratricopeptide repeats 2	2.896	2.338	4.231	4.163
interferon-induced protein with tetratricopeptide repeats 3	2.445	1.987	4.106	4.409
interferon-related developmental regulator 1	3.141	1.998	3.269	1.099
interleukin 1 alpha	7.36	6.181	3.238	-0.19
interleukin 1 beta	9.699	10.525	n.d.	n.d.
interleukin 1 receptor antagonist	5.672	6.205	2.533	1.321
interleukin 10	4.497	2.636	-0.135	0.233
interleukin 13 receptor, alpha 1	3.15	2.664	2.48	1.89
interleukin 27	3.623	3.053	n.d.	0.954
interleukin 4 induced 1	6.47	6.221	3.796	3.307
interleukin 6	8.399	8.785	1.904	1.775
interleukin-1 receptor-associated kinase 2	2.209	2.145	1.03	0.198
interleukin-1 receptor-associated kinase 3	3.39	3.671	0.527	0.307
intraflagellar transport 57 homolog (Chlamydomonas)	2.475	2.178	0.882	0.599
jagged 1	2.684	1.493	3.491	2.208
Janus kinase 2	4.779	3.544	2.357	0.787
Jun dimerization protein 2	4.904	4.492	4.82	2.341

Jun-B oncogene	2.746	1.927	3.317	1.682
karyopherin (importin) alpha 3	2.561	1.589	1.877	0.521
kinectin 1	2.667	0.77	2.151	0.15
Kruppel-like factor 7 (ubiquitous)	4.053	4.488	3.049	2.293
La ribonucleoprotein domain family, member 1	2.278	1.765	1.243	1.205
La ribonucleoprotein domain family, member 4B	2.076	1.346	1.315	0.348
LAG1 homolog, ceramide synthase 6	3.036	2.195	2.613	1.496
leucine rich repeat containing 8 family, member C	2.401	1.844	2.107	0.174
leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	2.108	1.891	0.627	0.354
lipocalin 2	8.056	9.644	-0.09	-1.033
lymphocyte cytosolic protein 2	3.929	3.511	2.937	1.851
lymphotoxin A	6.943	5.669	2.908	0.444
lysine (K)-specific demethylase 4A	2.956	2.708	1.748	1.172
lysophosphatidic acid receptor 1	3.928	3.23	4.734	3.636
macrophage receptor with collagenous structure	6.781	7.221	1.549	1.783
major facilitator superfamily domain containing 7A	2.255	2.218	0.488	0.727
MAP kinase-activated protein kinase 2	2.897	2.944	2.833	2.004
MARCKS-like 1	5.174	4.389	n.d.	n.d.
matrix metallopeptidase 13	5.998	5.672	4.416	1.901
matrix metallopeptidase 14 (membrane-inserted)	7.027	6.932	6.448	5.75
matrix metallopeptidase 9	4.844	4.863	1.687	1.335
membrane-spanning 4-domains, subfamily A, member 4C	2.266	2.149	2.457	2.91
metadherin	2.99	2.638	2.316	1.499
mitochondrial ribosomal protein L27	2.009	2.056	0.853	0.624
mucosa associated lymphoid tissue lymphoma translocation gene 1	5.701	4.389	6.415	3.358
myelocytomatosis oncogene	2.068	1.027	2.871	1.335
myeloid cell nuclear differentiation antigen	3.767	3.707	3.847	3.257
myeloid nuclear differentiation antigen like	2.461	2.277	1.576	1.036
myeloid-associated differentiation marker	2.198	2.651	0.63	0.624
myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 6	2.198	1.948	2.142	1.842
myotubularin related protein 12	2.411	-0.139	2.034	0.514
myotubularin related protein 14	2.451	1.433	1.505	0.382
myxovirus (influenza virus) resistance 1	3.944	3.464	6.965	6.703
myxovirus (influenza virus) resistance 2	3.72	3.414	4.594	4.142
NADPH oxidase 1	4.748	3.811	7.21	5.02
NEDD4 binding protein 1	3.168	3.258	2.878	1.833
neutrophil cytosolic factor 1	2.004	0.671	0.421	-0.127
niacin receptor 1	7.33	7.518	2.961	1.446
nicotinamide phosphoribosyltransferase	2.444	1.912	0.639	0.78
NLR family, pyrin domain containing 3	3.594	3.141	1.959	0.047
NOP58 ribonucleoprotein homolog (yeast)	2.05	1.621	0.882	0.531

nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	3.065	2.67	2.708	1.175
nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	4.085	3.043	1.849	0.298
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.804	3.205	3.938	2.838
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	3.115	2.103	1.91	0.367
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta	2.405	1.493	1.567	0.84
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	4.537	4.433	5.476	3.928
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	6.282	4.774	2.744	1.375
nuclear factor, interleukin 3, regulated	2.53	2.719	0.567	-0.518
nuclear receptor coactivator 5	2.021	0.885	0.918	0.061
nuclear receptor subfamily 4, group A, member 1	2.929	0.933	0.647	-0.605
nucleolar and coiled-body phosphoprotein 1	2.186	1.872	1.42	0.93
nucleoporin 54	2.951	0.568	2.046	0.276
nucleoporin 54	2.326	0.641	1.493	-0.095
nucleotide-binding oligomerization domain containing 1	2.224	2.385	1.885	1.595
nucleotide-binding oligomerization domain containing 2	4.651	3.421	5.354	2.441
oncostatin M	3.687	3.158	1.386	-0.766
ORAI calcium release-activated calcium modulator 2	3.327	2.511	2.661	1.585
ornithine decarboxylase, structural 1	2.141	1.537	n.d.	n.d.
oxidized low density lipoprotein (lectin-like) receptor 1	6.332	5.372	3.922	2.049
paired immunoglobin-like type 2 receptor alpha	6.017	5.945	2.267	1.43
paired immunoglobin-like type 2 receptor beta 1	2.281	3.002	0.654	0.762
PAP associated domain containing 7	2.02	1.663	2.252	1.86
PDZ and LIM domain 7	2.046	0.288	2.556	0.664
phosphatidic acid phosphatase type 2A	3.251	3.607	-0.025	0.461
phosphatidylglycerophosphate synthase 1	2.945	2.693	n.d.	0.993
phosphodiesterase 4A, cAMP specific	3.987	3.993	n.d.	n.d.
phosphodiesterase 4B, cAMP specific	5.024	4.184	4.799	2.885
phospholipase A2, group IVA (cytosolic, calcium-dependent)	3.687	3.091	2.187	1.992
phospholipid scramblase 1	3.355	0.774	2.703	0.091
phosphotyrosine interaction domain containing 1	2.062	1.838	2.149	1.944
plasminogen activator, tissue	7.587	4.946	4.99	1.736
plasminogen activator, urokinase receptor	4.342	4.22	2.782	0.339
platelet-activating factor receptor	2.602	2.262	1.793	1.246

pleckstrin	3.137	3.021	n.d.	n.d.
pleckstrin homology-like domain, family B, member 1	2.293	0.718	2.979	1.234
podoplanin	2.294	2.284	0.486	0.87
poliovirus receptor	4.305	2.023	4.701	1.465
polo-like kinase 3 (Drosophila)	2.721	2.783	1.706	0.627
poly (ADP-ribose) polymerase family, member 14	2.038	1.966	2.385	2.288
polymerase (RNA) III (DNA directed) polypeptide D	2.267	1.739	1.503	0.785
potassium channel tetramerisation domain containing 12	2.072	1.784	0.542	0.501
predicted gene 5424	2.509	2.921	2.322	2.487
predicted gene 6501	2.211	1.043	1.365	0.705
predicted pseudogene 6206	2.306	1.652	0.544	0.004
predicted pseudogene 6532	2.125	1.738	0.061	0.41
pro-platelet basic protein	2.919	3.568	0.782	1.539
proline-serine-threonine phosphatase-interacting protein 2	3.477	3.378	1.546	0.959
prolyl-tRNA synthetase (mitochondrial)(putative)	2.181	1.623	1.558	0.581
prostaglandin E synthase	5.295	5.948	4.54	3.475
prostaglandin I receptor (IP)	2.026	2.132	2.622	2.675
prostaglandin-endoperoxide synthase 2	8.73	7.747	n.d.	n.d.
proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)	3.377	4.443	2.044	2.157
protein kinase C, delta	2.339	1.953	1.075	0.237
protein kinase, cAMP dependent regulatory, type II beta	2.117	1.502	0.127	0.33
protein phosphatase 1, regulatory (inhibitor) subunit 15A	4.206	2.223	2.994	0.451
protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform	2.293	1.293	1.174	0.436
protein tyrosine phosphatase, non-receptor type 12	2.513	1.939	2.175	0.759
proviral integration site 1	4.342	3.153	5.411	2.477
PTPRF interacting protein, binding protein 1 (liprin beta 1)	2.312	0.899	1.876	1.063
purinergic receptor P2Y, G-protein coupled 2	3.244	2.711	0.847	0.334
RAB20, member RAS oncogene family	3.525	3.591	3.717	1.834
radical S-adenosyl methionine domain containing 2	2.9	3.384	n.d.	n.d.
ral guanine nucleotide dissociation stimulator	3.136	3.125	2.345	1.299
Rap guanine nucleotide exchange factor (GEF) 2	3.163	2.495	2.374	1.088
Ras association (RalGDS/AF-6) domain family member 4	2.309	2.327	1.581	1.097
RAS guanyl releasing protein 1	4.985	4.746	6.34	4.017
ras homolog gene family, member B	2.972	0.321	2.399	-0.595
RAS p21 protein activator 2	3.419	2.02	1.979	0.169

RasGEF domain family, member 1B	2.842	1.214	2.913	1.191
receptor (TNFRSF)-interacting serine-threonine kinase 2	3.833	2.073	2.3	0.696
recombination signal binding protein for immunoglobulin kappa J region	3.425	2.511	n.d.	n.d.
regulator of calcineurin 1	2.126	0.526	1.568	0.032
regulator of G-protein signaling 1	3.553	-0.392	2.179	-0.207
regulator of G-protein signaling 16	2.028	0.567	3.609	2.145
related RAS viral (r-ras) oncogene homolog 2	3.564	2.164	2.768	0.623
reticuloendotheliosis oncogene	4.681	2.288	3.984	1.196
reticulon 1	2.941	2.68	0.533	0.894
Rho guanine nucleotide exchange factor (GEF) 3	2.705	2.082	2.015	-0.053
rhomboid 5 homolog 2 (Drosophila)	2.603	2.43	2.01	0.679
RIKEN cDNA 1200003I10 gene	3.998	3.109	n.d.	n.d.
RIKEN cDNA 1200009I06 gene	3.429	3.641	3.654	2.542
RIKEN cDNA 1200015M12 gene	4.275	3.657	n.d.	n.d.
RIKEN cDNA 2410039M03 gene	5.491	4.638	2.515	0.416
RIKEN cDNA 2810474O19 gene	2.772	-0.012	2.152	0.282
RIKEN cDNA 3110043O21 gene	2.153	1.002	2.098	0.397
RIKEN cDNA 4930431L21 gene	4.782	3.986	2.773	1.367
RIKEN cDNA 5730508B09 gene	2.065	1.652	0.835	0.741
RIKEN cDNA 5730528L13 gene	3.124	3.31	2.2	2.343
RIKEN cDNA 6330409N04 gene	2.599	2.03	2.168	1.35
RIKEN cDNA 9030625A04 gene	2.922	1.957	0.733	-0.095
RIKEN cDNA A930033M14 gene	3.03	3.565	2.932	3.088
ring finger and FYVE like domain containing protein	3.103	2.581	3.003	0.472
ring finger protein 19B	2.649	1.635	4.229	2.763
RIO kinase 3 (yeast)	2.313	1.536	0.914	0.361
RNA terminal phosphate cyclase-like 1	2.297	2.207	1.144	1.138
Rous sarcoma oncogene	5.29	4.856	3.523	2.334
RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)	3.313	2.467	2.205	1.378
SAM domain, SH3 domain and nuclear localization signals, 1	3.178	2.76	2.282	-0.267
schlafen 1	2.127	2.367	3.27	2.748
schlafen 2	2.256	1.821	2.025	1.973
schlafen 4	3.289	2.951	2.501	1.999
schlafen 5	2.805	2.095	2.008	1.945
selectin, endothelial cell	7.855	2.508	6.841	1.501
sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (s	2.178	1.997	1.527	0.782
septin 11	2.006	1.571	1.697	0.957
serglycin	2.399	2.543	1.685	1.023

serine (or cysteine) peptidase inhibitor, clade B, member 2	5.456	5.844	-0.289	-2.411
serine (or cysteine) peptidase inhibitor, clade E, member 1	5.017	2.591	3.36	0.713
serine/threonine kinase 40	3.62	3.365	2.639	0.47
serum amyloid A 3	7.481	10.073	n.d.	n.d.
SET domain containing (lysine methyltransferase) 8	2.089	2.016	0.744	0.825
SH3 domain binding glutamic acid-rich protein like 2	2.106	1.477	n.d.	n.d.
SLAM family member 7	2.292	2.531	-0.159	0.255
SLAM family member 8	2.751	3.394	n.d.	n.d.
solute carrier family 15, member 3	3.43	3.051	0.99	0.503
solute carrier family 2 (facilitated glucose transporter), member 1	4.046	3.741	1.752	0.755
solute carrier family 2 (facilitated glucose transporter), member 6	3.312	2.734	3.367	2.569
solute carrier family 20, member 1	2.707	2.312	1.224	0.218
solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	2.667	1.805	3.203	1.424
solute carrier family 28 (sodium-coupled nucleoside transporter), member 2	2.462	1.734	1.148	-0.008
solute carrier family 31, member 2	3.089	3.082	1.041	1.167
solute carrier family 39 (zinc transporter), member 14	4.293	3.954	2.941	1.756
solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	5.035	5.538	n.d.	n.d.
solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	5.313	4.443	4.156	1.735
solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	3.144	2.391	2.591	1.664
solute carrier organic anion transporter family, member 3a1	5.402	4.897	2.305	1.849
sorting nexin 10	2.044	1.561	0.861	0.163
sorting nexin 18	2.434	2.188	1.89	-0.35
spermatogenesis associated 13	2.688	3.002	2.902	2.14
Spi-C transcription factor (Spi-1/PU.1 related)	2.46	3.778	2.599	3.362
spleen tyrosine kinase	2.505	2.219	n.d.	n.d.
sprouty homolog 1 (Drosophila)	4.885	5.604	2.518	0.513
sprouty protein with EVH-1 domain 1, related sequence	2.313	1.796	2.533	1.168
src-like adaptor	3.641	4.098	1.339	0.684
ST3 beta-galactoside alpha-2,3-sialyltransferase 1	3.395	2.826	2.993	2.085
ST3 beta-galactoside alpha-2,3-sialyltransferase 3	2.929	2.836	1.863	1.56
START domain containing 7	2.924	2.197	1.389	0.06
stress-induced phosphoprotein 1	2.037	1.364	0.622	-0.799

suppression of tumorigenicity 7	4.801	4.125	2.471	1.638
suppressor of cytokine signaling 1	4.519	5.193	2.417	1.608
synaptotagmin binding, cytoplasmic RNA interacting protein	2.398	1.468	1.427	0.272
syndecan 4	3.074	3.521	4.629	3.054
syntaxin 11	2.478	1.491	2.867	1.16
syntaxin 6	2.745	2.038	1.542	0.303
T cell-interacting, activating receptor on myeloid cells 1	5.597	6.072	4.82	5.231
tet oncogene family member 2	3.13	1.832	1.806	0.21
thioredoxin reductase 1	2.855	2.338	1.694	1.61
threonine aldolase 1	2.668	2.247	n.d.	n.d.
TNF receptor-associated factor 1	6.734	5.909	6.818	4.599
TNFAIP3 interacting protein 1	4.292	3.871	3.51	2.039
toll-like receptor 1	2.203	2.47	1.594	1.139
toll-like receptor 2	3.688	n.d.	4.531	3.574
topoisomerase (DNA) I	2.148	1.07	n.d.	n.d.
torsin family 3, member A	2.148	2.343	1.995	1.906
TRAF family member-associated Nf-kappa B activator	3.999	2.705	3.721	1.734
transcription factor EC	4.005	3.484	2.964	2.226
transient receptor potential cation channel, subfamily M, member 2	2.33	2.299	0.906	1.042
transmembrane protein 2	3.611	3.628	1.8	1.344
transmembrane protein 38B	2.084	2.291	0.133	-0.118
transmembrane protein 39a	4.036	1.465	2.525	0.195
tripartite motif-containing 21	2.112	1.363	1.136	1.283
tripartite motif-containing 30	2.043	1.803	1.314	0.202
tripartite motif-containing 36	3.344	1.728	1.589	-0.054
tropomyosin 4	2.941	2.676	0.899	0.269
TSC22 domain family, member 1	3.8	1.305	3.655	0.903
TSR1, 20S rRNA accumulation, homolog (yeast)	2.049	1.647	n.d.	n.d.
tumor necrosis factor (ligand) superfamily, member 9	5.29	4.352	5.878	3.381
tumor necrosis factor receptor superfamily, member 1b	3.708	3.319	0.823	0.097
tumor necrosis factor, alpha-induced protein 2	5.118	3.845	5.474	2.891
tumor necrosis factor, alpha-induced protein 3	6.287	4.908	7.209	3.979
ubiquitin specific peptidase 18	3.765	3.897	3.459	3.25
UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	2.326	1.995	1.75	0.889
uridine phosphorylase 1	3.849	4.865	0.609	-0.296
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	5.387	3.62	6.441	3.421
v-myc myelocytomatosis viral oncogene homolog 1,	3.425	2.378	3.372	2.256

lung carcinoma derived (avian)				
vascular cell adhesion molecule 1	7.586	5.749	n.d.	n.d.
vasodilator-stimulated phosphoprotein	3.248	3.365	2.617	2.022
WD repeat domain 43	2.171	1.757	1.118	0.391
Z-DNA binding protein 1	3.033	3.107	n.d.	n.d.
zinc finger CCCH type containing 12A	3.325	3.311	n.d.	n.d.
zinc finger CCCH type containing 12C	4.681	2.81	5.049	1.776
zinc finger protein 263	2.93	1.789	1.992	0.77
zinc finger protein 36	2.253	1.905	2.286	0.355
zinc finger protein 800	2.081	2.025	1.698	0.682

Appendix 7. IcmS/IcmW-dependent genes in *Myd88*$^{-/-}$/*Trif*$^{-/-}$ macrophages. The listed genes were induced fourfold or more by LP01 in B6 macrophages, and had a twofold or greater difference between LP01 and *ΔicmSΔicmW* in *Myd88*$^{-/-}$/*Trif*$^{-/-}$ (MyT) macrophages. Data are expressed as $\log_2$(induction) normalized to expression in uninfected macrophages. Results from one representative microarray experiment are shown.

Gene name	B6 LP01	B6 icmSW	MyT LP01	MyT icmSW
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	3.993	4.327	3.129	0.455
acyl-CoA synthetase long-chain family member 1	3.855	2.728	2.771	1.264
adenosine A2b receptor	6.249	6.109	6.786	3.163
AE binding protein 2	2.859	1.269	2.556	1.175
ankyrin repeat and BTB (POZ) domain containing 2	5.349	4.382	5.404	2.144
ankyrin repeat domain 33B	2.644	0.989	2.188	0.507
arginase type II	2.133	1.915	0.97	-1.094
arginase, liver	3.362	4.03	1.556	0.326
avian reticuloendotheliosis viral (v-rel) oncogene related B	2.438	1.638	3.668	2.211
B-cell leukemia/lymphoma 2 related protein A1c	5.199	5.157	3.385	2.106
B-cell leukemia/lymphoma 2 related protein A1d	4.793	5.755	3.521	2.059
B-cell leukemia/lymphoma 3	2.337	2.144	2.892	1.306
B-cell translocation gene 2, anti-proliferative	2.592	1.106	1.832	-0.291
baculoviral IAP repeat-containing 3	3.645	2.634	3.579	2.491
basic helix-loop-helix family, member e40	2.423	0.868	3.377	1.122
BCL6 interacting corepressor	3.393	0.346	3.317	0.116
bromodomain adjacent to zinc finger domain 1A	2.293	0.943	2.595	0.432
C-type lectin domain family 4, member e	6.98	6.401	6.585	5.002
C-type lectin domain family 5, member a	4.102	3.466	4.385	3.167
calcium channel, voltage-dependent, T type, alpha 1G subunit	5.023	5.513	3.84	2.418
CCAAT/enhancer binding protein (C/EBP), beta	2.291	2.536	2.805	1.286
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	2.622	0.547	2.966	0.755
CD274 antigen	5.284	5.146	4.533	2.516
CD40 antigen	7.51	6.821	6.733	4.188
CD69 antigen	7.541	7.679	5.566	4.427
CDC42 effector protein (Rho GTPase binding) 2	5.588	5.494	5.113	3.487
cDNA sequence BC006779	3.353	2.38	3.815	1.957
ceroid-lipofuscinosis, neuronal 5	2.043	2.123	1.799	0.7
chemokine (C-C motif) ligand 17	3.822	4.986	8.034	5.206
chemokine (C-C motif) ligand 2	5.313	5.558	4.91	1.467
chemokine (C-C motif) ligand 6	4.46	5.421	0.297	-1.537
chemokine (C-C motif) ligand 7	6.319	6.653	5.494	1.923
chemokine (C-C motif) receptor-like 2	5.463	4.288	4.044	2.053
chemokine (C-X-C motif) ligand 3	9.504	9.476	8.924	6.174
cholesterol 25-hydroxylase	6.122	4.856	3.683	2.534
choline kinase alpha	2.222	-0.591	2.239	-0.103

coagulation factor III	6.447	5.9	4.029	-0.166
colony stimulating factor 1 (macrophage)	8.524	4.714	8.439	4.18
colony stimulating factor 3 (granulocyte)	6.394	7.71	2.639	1.546
cyclin L1	3.335	1.132	2.938	0.631
cysteine-serine-rich nuclear protein 1	5.184	2.735	4.913	2.454
cytokine induced apoptosis inhibitor 1	2.455	1.312	2.053	0.808
cytokine inducible SH2-containing protein	7.389	5.356	6.995	1.246
DCN1, defective in cullin neddylation 1, domain containing 3 (S. cerevisiae)	2.371	2.045	1.174	0.026
dedicator of cytokinesis 10	2.197	1.379	1.837	0.65
DnaJ (Hsp40) homolog, subfamily B, member 2	2.312	0.531	1.563	-0.359
dual specificity phosphatase 1	2.455	2.064	2.401	1.327
dual specificity phosphatase 16	5.168	5.115	3.551	0.681
dual specificity phosphatase 2	5.321	4.288	4.558	2.401
dual specificity phosphatase 4	2.086	1.594	2.645	1.187
dual specificity phosphatase 6	2.06	1.557	1.868	0.411
dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	2.039	0.995	1.707	0.472
early growth response 1	5.189	2.325	5.774	2.138
EH-domain containing 1	4.559	4.486	4.283	2.303
endothelin receptor type B	2.989	2.622	2.725	1.419
Epstein-Barr virus induced gene 3	2.626	2.772	2.214	0.905
ERBB receptor feedback inhibitor 1	4.62	2.646	3.361	0.307
ets variant gene 6 (TEL oncogene)	2.463	2.263	2.515	1.475
FCH and double SH3 domains 2	2.852	1.579	1.9	0.572
fibronectin leucine rich transmembrane protein 3	5.109	5.135	6.165	3.597
formin binding protein 1-like	2.787	2.653	2.735	1.464
fos-like antigen 2	2.413	2.878	2.179	-0.454
G protein-coupled receptor 84	4.457	5.168	3.403	1.756
G protein-coupled receptor 85	3.68	2.79	2.184	0.69
GRAM domain containing 1A	2.243	1.877	1.503	-0.247
GRAM domain containing 1B	2.296	1.221	1.503	0.054
growth arrest and DNA-damage-inducible 45 alpha	4.663	2.796	4.198	1.531
growth arrest and DNA-damage-inducible 45 beta	5.84	3.416	5.195	1.847
GTP cyclohydrolase 1	3.401	2.736	2.737	1.674
heat shock protein 1B	4.13	2.441	5.178	2.952
heterogeneous nuclear ribonucleoprotein L-like	2.493	1.11	1.759	0.205
icos ligand	2.977	1.977	4.088	2.318
immediate early response 3	5.053	5.488	2.962	1.389
insulin-like growth factor binding protein 7	3.635	4.175	2.45	1.208
interferon regulatory factor 1	3.351	2.131	3.953	2.236
interferon-related developmental regulator 1	3.141	1.998	3.269	1.099
interleukin 1 alpha	7.36	6.181	3.238	-0.19
interleukin 1 receptor antagonist	5.672	6.205	2.533	1.321
jagged 1	2.684	1.493	3.491	2.208

Janus kinase 2	4.779	3.544	2.357	0.787
Jun dimerization protein 2	4.904	4.492	4.82	2.341
Jun-B oncogene	2.746	1.927	3.317	1.682
karyopherin (importin) alpha 3	2.561	1.589	1.877	0.521
kinectin 1	2.667	0.77	2.151	0.15
LAG1 homolog, ceramide synthase 6	3.036	2.195	2.613	1.496
leucine rich repeat containing 8 family, member C	2.401	1.844	2.107	0.174
lymphocyte cytosolic protein 2	3.929	3.511	2.937	1.851
lymphotoxin A	6.943	5.669	2.908	0.444
lysophosphatidic acid receptor 1	3.928	3.23	4.734	3.636
matrix metallopeptidase 13	5.998	5.672	4.416	1.901
mucosa associated lymphoid tissue lymphoma translocation gene 1	5.701	4.389	6.415	3.358
myelocytomatosis oncogene	2.068	1.027	2.871	1.335
myotubularin related protein 12	2.411	-0.139	2.034	0.514
myotubularin related protein 14	2.451	1.433	1.505	0.382
NADPH oxidase 1	4.748	3.811	7.21	5.02
NEDD4 binding protein 1	3.168	3.258	2.878	1.833
niacin receptor 1	7.33	7.518	2.961	1.446
NLR family, pyrin domain containing 3	3.594	3.141	1.959	0.047
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	3.065	2.67	2.708	1.175
nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	4.085	3.043	1.849	0.298
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.804	3.205	3.938	2.838
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	3.115	2.103	1.91	0.367
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	4.537	4.433	5.476	3.928
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	6.282	4.774	2.744	1.375
nuclear factor, interleukin 3, regulated	2.53	2.719	0.567	-0.518
nuclear receptor subfamily 4, group A, member 1	2.929	0.933	0.647	-0.605
nucleoporin 54	2.951	0.568	2.046	0.276
nucleoporin 54	2.326	0.641	1.493	-0.095
nucleotide-binding oligomerization domain containing 2	4.651	3.421	5.354	2.441
oncostatin M	3.687	3.158	1.386	-0.766
ORAI calcium release-activated calcium modulator 2	3.327	2.511	2.661	1.585
oxidized low density lipoprotein (lectin-like) receptor 1	6.332	5.372	3.922	2.049
PDZ and LIM domain 7	2.046	0.288	2.556	0.664
phosphodiesterase 4B, cAMP specific	5.024	4.184	4.799	2.885
phospholipid scramblase 1	3.355	0.774	2.703	0.091
plasminogen activator, tissue	7.587	4.946	4.99	1.736
plasminogen activator, urokinase receptor	4.342	4.22	2.782	0.339

pleckstrin homology-like domain, family B, member 1	2.293	0.718	2.979	1.234
poliovirus receptor	4.305	2.023	4.701	1.465
polo-like kinase 3 (Drosophila)	2.721	2.783	1.706	0.627
prostaglandin E synthase	5.295	5.948	4.54	3.475
protein phosphatase 1, regulatory (inhibitor) subunit 15A	4.206	2.223	2.994	0.451
protein tyrosine phosphatase, non-receptor type 12	2.513	1.939	2.175	0.759
proviral integration site 1	4.342	3.153	5.411	2.477
RAB20, member RAS oncogene family	3.525	3.591	3.717	1.834
ral guanine nucleotide dissociation stimulator	3.136	3.125	2.345	1.299
Rap guanine nucleotide exchange factor (GEF) 2	3.163	2.495	2.374	1.088
RAS guanyl releasing protein 1	4.985	4.746	6.34	4.017
ras homolog gene family, member B	2.972	0.321	2.399	-0.595
RAS p21 protein activator 2	3.419	2.02	1.979	0.169
RasGEF domain family, member 1B	2.842	1.214	2.913	1.191
receptor (TNFRSF)-interacting serine-threonine kinase 2	3.833	2.073	2.3	0.696
regulator of calcineurin 1	2.126	0.526	1.568	0.032
regulator of G-protein signaling 1	3.553	-0.392	2.179	-0.207
regulator of G-protein signaling 16	2.028	0.567	3.609	2.145
related RAS viral (r-ras) oncogene homolog 2	3.564	2.164	2.768	0.623
reticuloendotheliosis oncogene	4.681	2.288	3.984	1.196
Rho guanine nucleotide exchange factor (GEF) 3	2.705	2.082	2.015	-0.053
rhomboid 5 homolog 2 (Drosophila)	2.603	2.43	2.01	0.679
RIKEN cDNA 1200009I06 gene	3.429	3.641	3.654	2.542
RIKEN cDNA 2410039M03 gene	5.491	4.638	2.515	0.416
RIKEN cDNA 2810474O19 gene	2.772	-0.012	2.152	0.282
RIKEN cDNA 3110043O21 gene	2.153	1.002	2.098	0.397
RIKEN cDNA 4930431L21 gene	4.782	3.986	2.773	1.367
ring finger and FYVE like domain containing protein	3.103	2.581	3.003	0.472
ring finger protein 19B	2.649	1.635	4.229	2.763
Rous sarcoma oncogene	5.29	4.856	3.523	2.334
SAM domain, SH3 domain and nuclear localization signals, 1	3.178	2.76	2.282	-0.267
selectin, endothelial cell	7.855	2.508	6.841	1.501
serine (or cysteine) peptidase inhibitor, clade B, member 2	5.456	5.844	-0.289	-2.411
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tumor necrosis factor, alpha-induced protein 2	5.118	3.845	5.474	2.891
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v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	5.387	3.62	6.441	3.421
v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	3.425	2.378	3.372	2.256
zinc finger CCCH type containing 12C	4.681	2.81	5.049	1.776
zinc finger protein 263	2.93	1.789	1.992	0.77
zinc finger protein 36	2.253	1.905	2.286	0.355
zinc finger protein 800	2.081	2.025	1.698	0.682

Appendix 8. Gene information and primers for the genes deleted in *Δ5 L. pneumophila*. Annotations refer to the published *L. pneumophila* LP01 genome (Genbank Accession # AE017354).

Effector	Gene Annotation	Deletion Primers	Reference
Lgt1	Lpg1368	Upstream F: CCTGGTCGACAATACAGCAGTTTAATCC, Upstream R: CCCGGATCCTAGAGAGTTGTTGATCCC, Downstream F: CCCGGATCCGAGGGTTTGAGTTGGTTG, Downstream R: CCAGAGCTCTGCTTATAGTCTTCTTCG	Belyi et al. (2006) PNAS 103: 16953–16958
Lgt2	Lpg2862	Upstream F: AAAGAGCTCACTCGAATAGTACGGGGG Upstream R: AAAGGATCCTTACATCCACCCTCAGTC Downstream F: AAAGGATCCCTGCGAACGGATGGCTTG Downstream R: AAAGTCGACCGTAGCAATGCCTAGCAG	Belyi et al. (2006) J Bacteriol 190: 3026–3035
Lgt3	Lpg1488	Upstream F: AAAGTCGACTCTCTTTGTTTTTCAAGAG Upstream R: AACGGATCCTGTCGATTTGATATTCTC Downstream F: AAAGGATCCCAATCTCAAAATCTTGAAG Downstream R: AAAGAGCTCGTGATGTAGGAGAACTTG	Belyi et al. (2006) J Bacteriol 190: 3026–3035
SidI	Lpg2504	See reference	Shen et al. (2009) Cell Microbiol 11: 911–926
SidL	Lpg0437	Upstream F: GGGGTCGACGAGAGTTATATATGAGAG Upstream R: CTCGGATCCAGATCTTTTCTCAGTTTC Downstream F: CCAGGATCCACGACCATACGCCAAAAG Downstream R: GCGGAGCTCTGATGACCCAAGTCTATG	Fontana et al. (2011) PLoS Pathogens 7(2): e1001289

Appendix 9. Transcriptional response of *Caspase1*$^{-/-}$ macrophages to wildtype LP02 or Δ5 mutant *L. pneumophila*. Data are expressed as $\log_2$(induction) normalized to expression in uninfected macrophages. Results from one representative microarray experiment are shown.

gene name	wildtype	Δ5
colony stimulating factor 3 (granulocyte)	10.004	9.514
cold autoinflammatory syndrome 1 homolog (human)	9.619	9.612
GTP binding protein (gene overexpressed in skeletal muscle)	8.389	4.286
prostaglandin E synthase	8.139	7.961
gene model 1960, (NCBI)	8.059	7.846
colony stimulating factor 1 (macrophage)	7.747	5.91
early growth response 1	7.61	4.36
plasminogen activator, tissue	7.555	7.322
colony stimulating factor 2 (granulocyte-macrophage)	7.488	1.632
interferon activated gene 205	7.23	9.075
predicted gene, OTTMUSG00000007655	7.216	8.06
vascular cell adhesion molecule 1	7.194	6.814
interferon activated gene 205	7.164	8.946
ectonucleotide pyrophosphatase/phosphodiesterase 4	6.828	8.884
ring finger and FYVE like domain containing protein	6.698	6.145
colony stimulating factor 3 (granulocyte)	6.5	5.652
Fas (TNF receptor superfamily member)	6.415	6.723
RIKEN cDNA 1110001A07 gene	6.375	6.765
Mediterranean fever	6.343	6.472
RIKEN cDNA 1110015O18 gene	6.323	5.011
macrophage activation 2 like	6.285	7.986
colony stimulating factor 1 (macrophage)	6.241	6.077
C-type lectin domain family 4, member e	6.207	5.861
tumor necrosis factor, alpha-induced protein 3	6.167	5.887
formyl peptide receptor 1	6.12	6.251
guanylate nucleotide binding protein 2	5.999	8.297
lipocalin 2	5.997	6.42
lysyl oxidase	5.943	5.379
dual specificity phosphatase 2	5.911	6.451
caspase 4, apoptosis-related cysteine peptidase	5.867	6.032
chemokine (C-C motif) receptor-like 2	5.854	6.818
CD83 antigen	5.815	2.556
expressed sequence AI553587	5.762	5.236
arginase type II	5.718	3.781
solute carrier organic anion transporter family, member 3a1	5.71	6.57
early growth response 2	5.699	1.496
CD274 antigen	5.692	7.437
Fas (TNF receptor superfamily member)	5.639	6.031
myxovirus (influenza virus) resistance 2	5.594	6.82
histocompatibility 2, M region locus 2	5.561	5.576
SLAM family member 8	5.546	6.425
endothelin 1	5.542	7.496
reticuloendotheliosis oncogene	5.521	6.066

B-cell leukemia/lymphoma 2 related protein A1a	5.507	5.75
caspase 4, apoptosis-related cysteine peptidase	5.443	5.424
B-cell leukemia/lymphoma 2 related protein A1d	5.37	5.52
cDNA sequence BC052046	5.359	5.247
solute carrier organic anion transporter family, member 3a1	5.357	6.15
CD40 antigen	5.342	5.986
cyclin L1	5.33	3.59
Fc receptor, IgG, low affinity IIb	5.317	5.576
guanylate nucleotide binding protein 5	5.311	7.155
myxovirus (influenza virus) resistance 1	5.29	7.455
triggering receptor expressed on myeloid cells-like 4	5.289	4.994
tumor necrosis factor, alpha-induced protein 2	5.278	5.191
expressed sequence AI553587	5.276	4.904
arrestin domain containing 4	5.235	4.823
frizzled homolog 1 (Drosophila)	5.226	6.269
longevity assurance homolog 6 (S. cerevisiae)	5.222	6.05
potassium channel tetramerisation domain containing 12	5.198	5.023
ADP-ribosylation factor-like 5C	5.196	4.155
Fc receptor, IgG, low affinity IIb	5.192	5.37
solute carrier family 4, sodium bicarbonate cotransporter, member 7	5.189	4.824
immediate early response 3	5.182	4.173
interleukin 15 receptor, alpha chain	5.172	7.685
myxovirus (influenza virus) resistance 1	5.161	7.328
interleukin 1 family, member 6	5.119	5.481
G protein-coupled receptor 109A	5.11	5.794
phosphatidic acid phosphatase 2a	5.087	4.763
transmembrane protein 142B	5.074	3.318
TSC22 domain family, member 1	5.061	3.442
immediate early response 3	5.057	4.898
mucosa associated lymphoid tissue lymphoma translocation gene 1	5.054	4.086
histocompatibility 2, class II antigen A, beta 1	5.049	5.281
RIKEN cDNA 4930431L21 gene	5.04	4.188
phosphodiesterase 4A, cAMP specific	5.034	4.874
MARCKS-like 1	5.034	5.234
AXIN1 up-regulated 1	5.02	4.291
guanylate binding protein 6	5.008	6.842
cytokine inducible SH2-containing protein	4.984	4.501
RIKEN cDNA A030007L17 gene	4.984	5.567
growth arrest and DNA-damage-inducible 45 alpha	4.964	1.768
immediate early response 3	4.957	3.904
CD40 antigen	4.877	5.054
proviral integration site 1	4.872	5.303
protein phosphatase 1K (PP2C domain containing)	4.862	7.055
adenosine A2a receptor	4.845	5.031
formyl peptide receptor, related sequence 2	4.838	5.352
Tnf receptor-associated factor 1	4.831	4.594
pleckstrin homology-like domain, family A, member 1	4.811	3.74

growth arrest and DNA-damage-inducible 45 beta	4.777	3.227
phosphatidic acid phosphatase 2a	4.767	4.194
tumor necrosis factor (ligand) superfamily, member 9	4.752	3.203
matrix metallopeptidase 13	4.738	6.587
B-cell leukemia/lymphoma 2 related protein A1a	4.736	4.917
longevity assurance homolog 6 (S. cerevisiae)	4.724	5.537
solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	4.69	3.964
delta-like 1 homolog (Drosophila)	4.684	4.533
PR domain containing 1, with ZNF domain	4.674	2.929
RIKEN cDNA A730010A20 gene	4.672	5.59
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	4.651	4.897
solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	4.633	4.316
interleukin 10	4.628	6.119
protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein 2	4.616	4.417
SAM domain, SH3 domain and nuclear localization signals, 1	4.573	4.632
TSC22 domain family, member 1	4.566	2.618
interleukin 15	4.565	6.653
interferon regulatory factor 1	4.561	5.003
CDC42 effector protein (Rho GTPase binding) 2	4.543	3.698
solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	4.535	4.198
histocompatibility 2, class II antigen A, beta 1	4.497	4.646
zinc finger protein 719	4.495	3.523
predicted gene, EG622976	4.489	4.726
toll-like receptor 3	4.488	6.974
interleukin-1 receptor-associated kinase 3	4.457	4.257
phosphodiesterase 4B, cAMP specific	4.425	3.946
solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	4.421	4.017
interferon activated gene 205	4.415	5.852
basonuclin 2	4.408	4.408
triggering receptor expressed on myeloid cells 1	4.401	3.901
limb expression 1 homolog (chicken)	4.4	4.04
DNA segment, Chr 16, ERATO Doi 472, expressed	4.393	5.03
human immunodeficiency virus type I enhancer binding protein 3	4.375	3.712
ankyrin repeat domain 57	4.372	4.199
zinc finger CCCH type containing 12C	4.366	3.663
inhibitor of DNA binding 3	4.353	2.803
RIKEN cDNA E130014J05 gene	4.349	4.657
Suppression of tumorigenicity 7	4.335	4.103
RAS guanyl releasing protein 1	4.328	5.064
matrix metallopeptidase 14 (membrane-inserted)	4.325	4.396
serine (or cysteine) peptidase inhibitor, clade B, member 2	4.322	4.204
interferon regulatory factor 1	4.305	4.475

B-cell leukemia/lymphoma 3	4.3	4.119
olfactory receptor 1248	4.282	5.738
carbonic anhydrase 2	4.204	3.135
MARCKS-like 1	4.195	4.345
interleukin 4 induced 1	4.186	4.951
Rap guanine nucleotide exchange factor (GEF) 2	4.182	4.905
olfactory receptor 862	4.182	4.915
cyclin-dependent kinase 5, regulatory subunit (p35) 1	4.176	0.547
protein phosphatase 1K (PP2C domain containing)	4.161	6.199
MAS-related GPR, member A4	4.156	4.008
RIKEN cDNA A930033M14 gene	4.152	4.176
myeloid differentiation primary response gene 116	4.147	2.63
interferon-stimulated protein	4.139	6.518
icos ligand	4.129	3.753
T-cell specific GTPase	4.127	6.434
basic leucine zipper transcription factor, ATF-like 2	4.125	5.747
baculoviral IAP repeat-containing 3	4.089	4.2
solute carrier family 2 (facilitated glucose transporter), member 6	4.088	4.621
chemokine (C-C motif) ligand 5	4.072	6.221
2'-5' oligoadenylate synthetase 2	4.049	4.402
ADP-ribosylation factor-like 13B	4.047	5.608
fibronectin leucine rich transmembrane protein 3	4.045	3.819
WD repeat domain 37	4.041	5.178
expressed sequence C79127	4.035	3.648
RIKEN cDNA 1190003J15 gene	4.031	3.709
RIKEN cDNA E430029J22 gene	4.026	6.24
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	3.992	3.791
ral guanine nucleotide dissociation stimulator	3.975	3.93
syntaxin 11	3.972	5.004
glycerol phosphate dehydrogenase 2, mitochondrial	3.962	4.233
RIKEN cDNA B230207M22 gene	3.943	3.265
RIKEN cDNA 1200002N14 gene	3.921	4.431
chemokine (C-X3-C motif) ligand 1	3.908	3.978
TNFAIP3 interacting protein 1	3.896	3.558
interferon alpha 5	3.893	4.606
MAX dimerization protein 1	3.88	5.314
Jun-B oncogene	3.877	3.423
MARCKS-like 1	3.875	4.114
syndecan 4	3.862	3.516
interferon regulatory factor 1	3.861	4.168
selectin, endothelial cell	3.86	1.283
ankyrin repeat and BTB (POZ) domain containing 2	3.854	4.679
RIKEN cDNA A630077B13 gene	3.839	7.554
dedicator of cytokinesis 10	3.834	3.446
RIKEN cDNA E130014J05 gene	3.832	4.093
interferon-induced protein with tetratricopeptide repeats 3	3.814	6.067
tumor necrosis factor receptor superfamily, member 1b	3.794	4.377

mitogen activated protein kinase kinase kinase 8	3.778	4.652
v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	3.778	4.457
RIKEN cDNA 2810409K11 gene	3.759	3.166
zinc finger CCCH type containing 12A	3.744	3.61
spermatogenesis associated 13	3.733	3.692
threonine aldolase 1	3.723	3.333
TRAF family member-associated Nf-kappa B activator	3.723	4.028
SPT2, Suppressor of Ty, domain containing 1 (S. cerevisiae)	3.713	3.95
interferon activated gene 205	3.707	5.273
trichorhinophalangeal syndrome I (human)	3.673	3.638
SAM domain, SH3 domain and nuclear localization signals, 1	3.672	3.88
integrin alpha 5 (fibronectin receptor alpha)	3.663	3.116
RIKEN cDNA 4930583C14 gene	3.647	2.146
expressed sequence AI504432	3.643	3.793
sprouty homolog 1 (Drosophila)	3.628	3.676
proviral integration site 2	3.626	3.117
oxidized low density lipoprotein (lectin-like) receptor 1	3.619	3.044
regulator of G-protein signaling 3	3.616	4.498
hepsin	3.615	3.087
solute carrier organic anion transporter family, member 3a1	3.612	4.446
interferon regulatory factor 1	3.609	3.907
coiled-coil domain containing 63	3.605	1.339
suppressor of cytokine signaling 1	3.588	6.072
Janus kinase 2	3.586	4.692
poly (ADP-ribose) polymerase family, member 8	3.566	4.837
Fc receptor, IgG, low affinity IIb	3.555	4.145
uridine phosphorylase 1	3.543	4.435
RAB20, member RAS oncogene family	3.539	3.192
tumor necrosis factor receptor superfamily, member 1b	3.535	4.18
protein tyrosine phosphatase, receptor type, J	3.521	3.909
transcription factor EC	3.52	3.477
bromodomain adjacent to zinc finger domain 1A	3.518	2.993
regulator of G-protein signaling 16	3.518	3.382
2'-5' oligoadenylate synthetase 1B	3.516	4.133
caspase recruitment domain family, member 15	3.514	3.146
cDNA sequence BC006779	3.512	3.77
formin binding protein 1-like	3.512	3.484
predicted gene, EG628870	3.508	3.762
transmembrane protein 176B	3.501	3.137
TNFAIP3 interacting protein 1	3.495	2.863
complement factor B	3.487	5.321
LIM domain only 4	3.482	3.145
GTP cyclohydrolase 1	3.47	3.938
AT rich interactive domain 5B (Mrf1 like)	3.466	3.925
potassium channel tetramerisation domain containing 12	3.463	3.112
NLR family, pyrin domain containing 4E	3.46	3.827
interferon gamma induced GTPase	3.454	5.138

nuclear receptor coactivator 2	3.45	3.254
matrix metallopeptidase 9	3.448	2.428
signal peptide, CUB domain, EGF-like 2	3.447	3.457
IKAROS family zinc finger 1	3.446	4.728
limb expression 1 homolog (chicken)	3.428	3.315
AE binding protein 2	3.427	3.174
Ras association (RalGDS/AF-6) domain family 4	3.427	3.576
RIKEN cDNA D130040H23 gene	3.413	1.284
mesoderm induction early response 1 homolog (Xenopus laevis	3.395	4
ERBB receptor feedback inhibitor 1	3.372	1.44
histone cluster 1, H4a	3.366	1.948
DNA segment, Chr 3, University of California at Los Angeles 1	3.357	3.657
Metadherin	3.355	3.33
tripartite motif protein 13	3.354	2.476
B-cell leukemia/lymphoma 2 related protein A1c	3.341	3.6
SLAM family member 7	3.341	4.773
interferon activated gene 205	3.335	4.571
Kruppel-like factor 7 (ubiquitous)	3.319	2.955
interferon inducible GTPase 1	3.318	6.939
mitogen-activated protein kinase 11	3.314	3.319
membrane-spanning 4-domains, subfamily A, member 4C	3.312	5.986
C-type lectin domain family 5, member a	3.306	2.683
BCL2-like 11 (apoptosis facilitator)	3.303	2.431
receptor (TNFRSF)-interacting serine-threonine kinase 2	3.301	4.29
dual specificity phosphatase 16	3.296	3.46
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	3.296	4.146
CD300e antigen	3.295	3.351
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4	3.286	4.025
zinc finger CCCH type containing 12C	3.284	2.475
RIKEN cDNA 1200009I06 gene	3.283	3.497
Spi-C transcription factor (Spi-1/PU.1 related)	3.267	2.77
carbohydrate (N-acetylglucosamino) sulfotransferase 7	3.267	3.924
transformed mouse 3T3 cell double minute 2	3.259	2.915
Janus kinase 2	3.252	4.308
RIKEN cDNA 1200003I10 gene	3.247	2.814
acyl-CoA synthetase long-chain family member 1	3.245	2.795
transmembrane protein 49	3.245	3.109
MAX dimerization protein 1	3.238	5.145
wingless-related MMTV integration site 6	3.237	3.081
transmembrane protein 2	3.235	4.018
dual specificity phosphatase 1	3.234	3.337
RIKEN cDNA C330006P03 gene	3.23	3.881
Kruppel-like factor 6	3.222	2.998
dual adaptor for phosphotyrosine and 3-phosphoinositides 1	3.221	2.698
interleukin 1 receptor antagonist	3.219	5.005
RAB11 family interacting protein 1 (class I)	3.217	2.853
RIKEN cDNA A930009K04 gene	3.217	3.395

RNA binding protein gene with multiple splicing	3.214	3.523
solute carrier family 31, member 2	3.207	2.91
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	3.206	4.093
avian reticuloendotheliosis viral (v-rel) oncogene related B	3.205	2.671
RNA binding protein gene with multiple splicing	3.198	3.626
grancalcin	3.191	5.457
fibrillin 1	3.189	4.965
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	3.188	0.661
kinectin 1	3.188	2.682
ring finger and FYVE like domain containing protein	3.188	2.647
RIKEN cDNA 9330158H04 gene	3.184	3.159
cytochrome b-245, beta polypeptide	3.172	3.208
vascular endothelial growth factor C	3.168	3.201
StAR-related lipid transfer (START) domain containing 5	3.158	3.417
RIKEN cDNA 9030607L20 gene	3.154	3.516
cyclin L1	3.151	2.202
cDNA sequence BC013672	3.148	4.545
fibromodulin	3.147	3.03
RIKEN cDNA 9030625A04 gene	3.137	3.931
chemokine (C-X-C motif) ligand 9	3.134	6.984
solute carrier family 25, member 33	3.127	2.089
human immunodeficiency virus type I enhancer binding protein 1	3.116	2.845
forkhead box P4	3.113	2.885
RIKEN cDNA 3110003A17 gene	3.107	2.964
centrosomal protein 68	3.106	3.548
nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	3.103	3.295
nucleoporin 54	3.099	3.081
RIKEN cDNA 4631422O05 gene	3.095	4.011
interferon-induced protein with tetratricopeptide repeats 1	3.095	4.901
Jun dimerization protein 2	3.087	1.668
chymotrypsin-like	3.082	3.957
cDNA sequence BC004022	3.078	3.973
gap junction membrane channel protein alpha 1	3.072	2.366
filamin, beta	3.069	3.565
lymphocyte cytosolic protein 2	3.066	3.286
Down syndrome critical region homolog 1 (human)	3.065	2.324
regulator of G-protein signaling 1	3.057	1.614
RIKEN cDNA 2010109I03 gene	3.053	2.891
MORN repeat containing 3	3.05	3.272
interferon, alpha 14	3.049	3.799
Metadherin	3.037	3.112
RIKEN cDNA 9030617O03 gene	3.036	0.638
RIKEN cDNA 5730557B15 gene	3.036	2.84
POU domain, class 2, transcription factor 2	3.03	3.07
forkhead box A2	3.03	3.892
RIKEN cDNA 5530400B01 gene	3.028	1.183

poliovirus receptor	3.021	2.209
immunoglobulin superfamily, member 6	3.015	2.557
carbonic anhydrase 13	3.009	3.797
expressed sequence AI586015	3.007	2.92
nuclear protein 1	3.003	2.944
LIM domain only 4	3.003	2.803
intraflagellar transport 57 homolog (Chlamydomonas)	2.999	2.918
PR domain containing 2, with ZNF domain	2.994	1.998
histone cluster 1, H4f	2.99	1.467
deltex 2 homolog (Drosophila)	2.984	3.231
interleukin 27	2.977	4.911
syndecan 1	2.975	2.547
immunity-related GTPase family, M	2.974	4.691
solute carrier family 15, member 3	2.971	3.3
src-like adaptor	2.97	2.698
DNA (cytosine-5-)-methyltransferase 3-like	2.969	2.393
RIKEN cDNA 5730557B15 gene	2.968	2.194
C-type lectin domain family 5, member a	2.954	2.658
actin-like 6B	2.95	3.034
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	2.942	2.279
RIKEN cDNA 4932438A13 gene	2.942	4.397
beta-carotene 9', 10'-dioxygenase 2	2.941	4.658
interleukin 15	2.933	4.974
cathelicidin antimicrobial peptide	2.932	2.351
predicted gene, EG627782	2.931	3.416
zinc finger homeobox 1a	2.929	4.077
inositol 1,4,5-trisphosphate 3-kinase B	2.926	3.075
RIKEN cDNA 2700019D07 gene	2.924	4.282
dual specificity phosphatase 16	2.922	3.167
phosphatidylglycerophosphate synthase 1	2.918	3.077
leucyl/cystinyl aminopeptidase	2.914	3.98
thioredoxin reductase 1	2.911	2.426
tumor necrosis factor (ligand) superfamily, member 4	2.91	3.923
RIKEN cDNA 5730557L09 gene	2.909	3.125
chemokine (C-X-C motif) ligand 16	2.902	2.519
solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	2.901	2.37
SKI-like	2.896	2.87
vesicle-associated membrane protein 8	2.894	3.016
MBD2-interacting zinc finger	2.886	3.829
dual specificity phosphatase 16	2.883	3.254
endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	2.883	3.752
caveolin, caveolae protein 1	2.879	2.72
RIKEN cDNA 1700021N21 gene	2.879	3.595
major facilitator superfamily domain containing 7	2.876	3.438
interferon-induced protein with tetratricopeptide repeats 3	2.871	4.81
RIKEN cDNA A930003A15 gene	2.865	2.68
proline-serine-threonine phosphatase-interacting protein 2	2.859	3.224

MOB1, Mps One Binder kinase activator-like 1A (yeast)	2.858	3.408
phospholipid scramblase 1	2.857	2.373
pleckstrin	2.857	2.903
RIKEN cDNA A630001G21 gene	2.85	3.273
PHD finger protein 20-like 1	2.849	3.121
phosphatidic acid phosphatase type 2B	2.845	3.359
histocompatibility 2, Q region locus 7	2.841	2.761
ankyrin repeat domain 57	2.838	2.785
interferon-related developmental regulator 1	2.835	0.69
trophoblast glycoprotein	2.833	3.574
RIKEN cDNA 3021401C12 gene	2.832	2.3
GATA binding protein 1	2.832	2.964
basic leucine zipper transcription factor, ATF-like	2.825	3.08
ring finger protein 24	2.821	3.513
phosphatidic acid phosphatase 2a	2.816	2.459
CD82 antigen	2.815	2.551
zinc finger protein 131	2.81	1.99
tripartite motif-containing 36	2.809	2.787
TAP binding protein-like	2.809	3.707
syntaxin 6	2.807	2.865
heat shock protein 1A	2.799	2.825
RIKEN cDNA 2810474O19 gene	2.797	3.321
RIKEN cDNA 2900084C01 gene	2.792	2.654
Metadherin	2.791	2.947
N-myc downstream regulated-like	2.79	3.14
RIKEN cDNA 5430413K10 gene	2.789	3.102
zinc finger protein 281	2.788	3.561
RNA binding protein gene with multiple s	2.784	3.064
RIKEN cDNA 2010012C16 gene	2.784	3.502
poliovirus receptor-related 2	2.78	3.203
RIKEN cDNA 4833411O04 gene	2.777	2.938
cold autoinflammatory syndrome 1 homolog (human)	2.769	2.457
integrin alpha V	2.763	2.631
protein kinase C, mu	2.757	3.038
RIKEN cDNA 2410127E16 gene	2.746	2.408
RasGEF domain family, member 1B	2.734	1.87
TSC22 domain family 2	2.733	2.618
RIKEN cDNA 2810487A22 gene	2.73	2.671
solute carrier family 39 (zinc transporter), member 14	2.727	2.614
exostoses (multiple) 1	2.724	3.434
inositol polyphosphate-5-phosphatase B	2.724	3.595
splicing factor, arginine/serine-rich 3 (SRp20)	2.723	1.47
jagged 1	2.72	2.369
solute carrier family 15, member 3	2.72	3.107
vesicle transport through interaction with t-SNAREs homolog 1A (yeast)	2.713	2.171
IBR domain containing 3	2.713	2.933
Rho guanine nucleotide exchange factor (GEF) 3	2.71	3.126

wingless-related MMTV integration site 6	2.707	2.099
RIKEN cDNA 3110043O21 gene	2.704	2.14
ST3 beta-galactoside alpha-2,3-sialyltransferase 1	2.702	3.308
toll-like receptor 1	2.697	2.793
centromere protein J	2.697	4.183
AMP deaminase 3	2.693	1.457
leucine rich repeat containing 8 family, member C	2.69	3.846
spleen tyrosine kinase	2.689	2.662
F-box and leucine-rich repeat protein 21	2.689	2.975
RIKEN cDNA 1110028C15 gene	2.688	2.261
serine/threonine kinase 40	2.686	2.441
RIKEN cDNA 1700021J08 gene	2.681	2.989
interferon-induced protein with tetratricopeptide repeats 3	2.677	4.793
olfactory receptor 18	2.676	3.722
glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	2.675	4.047
trichorhinophalangeal syndrome I (human)	2.671	2.63
2'-5' oligoadenylate synthetase-like 1	2.668	4.639
RIKEN cDNA 4631422O05 gene	2.667	3.435
2'-5' oligoadenylate synthetase-like 1	2.666	4.338
translocation associated membrane protein 1-like 1	2.663	3.341
RIKEN cDNA 2810439L12 gene	2.662	2.653
oncostatin M	2.661	2.39
insulin-like growth factor binding protein 2	2.653	2.491
bromodomain adjacent to zinc finger domain 1A	2.648	2.003
membrane-associated ring finger (C3HC4) 1	2.647	3.674
RIKEN cDNA 3110027N22 gene	2.644	2.885
Notch gene homolog 1 (Drosophila)	2.643	3.093
cDNA sequence BC020489	2.642	4.318
calcium/calmodulin-dependent protein kinase II, beta	2.641	2.856
ring finger protein 14	2.641	3.011
cDNA sequence BC033915	2.637	2.973
torsin family 3, member A	2.634	3.812
grainyhead-like 1 (Drosophila)	2.632	3.319
RIKEN cDNA 5730508B09 gene	2.632	3.84
glycoprotein 49 A	2.631	2.498
DCN1, defective in cullin neddylation 1, domain containing 3 (S. cerevisiae)	2.63	3.045
membrane-spanning 4-domains, subfamily A, member 6C	2.627	3.201
T-box 10	2.624	2.425
StAR-related lipid transfer (START) domain containing 5	2.623	2.627
nuclear transcription factor, X-box binding-like 1	2.622	4.291
ST3 beta-galactoside alpha-2,3-sialyltransferase 1	2.621	2.972
caspase 1	2.618	3.342
lymphocyte cytosolic protein 2	2.612	2.922
phospholipase C, delta 3	2.611	2.143
histone cluster 2, H4	2.61	1.156
chondroitin sulfate proteoglycan 2	2.61	4.571
caspase recruitment domain 4	2.599	4.346

regulator of G-protein signaling 1	2.597	0.628
AT rich interactive domain 1B (Swi1 like	2.596	2.651
cDNA sequence BC027231	2.595	2.584
RIKEN cDNA 1700061D14 gene	2.578	2.819
proteasome (prosome, macropain) subunit, alpha type 6	2.576	2.583
UBX domain containing 5	2.573	3.22
RIKEN cDNA 1700093C20 gene	2.571	2.974
eukaryotic translation elongation factor 1 epsilon 1	2.566	2.278
baculoviral IAP repeat-containing 2	2.559	3.24
predicted gene, EG634650	2.557	3.136
GNAS (guanine nucleotide binding protein	2.556	2.605
jumonji domain containing 2A	2.556	2.806
guanylate nucleotide binding protein 5	2.555	4.478
WAS protein homology region 2 domain con	2.551	4.131
G protein-coupled receptor 31, D17Leh66c	2.549	2.871
TRAF family member-associated Nf-kappa B activator	2.548	2.974
interferon alpha B	2.545	3.19
RIKEN cDNA 4921517N04 gene	2.54	2.519
RAS and EF hand domain containing	2.54	4.997
headcase homolog (Drosophila)	2.539	2.371
hook homolog 3 (Drosophila)	2.539	2.757
ATG9 autophagy related 9 homolog B (S. c	2.539	2.831
histone cluster 1, H4m	2.537	0.905
zinc finger protein 655	2.537	2.601
expressed sequence AA407659	2.536	2.451
transmembrane protein 2	2.535	3.324
RIKEN cDNA A430093F15 gene	2.532	2.097
RIKEN cDNA 5730528L13 gene	2.53	1.92
desmoglein 2	2.53	2.94
adenylate kinase 3 alpha-like 1	2.528	2.119
sialophorin	2.527	2.206
GRB2-related adaptor protein	2.526	2.044
grancalcin	2.513	3.805
GRAM domain containing 1A	2.511	2.63
RIKEN cDNA D330045A20 gene	2.51	1.906
receptor (TNFRSF)-interacting serine-threonine kinase 2	2.507	3.702
RIKEN cDNA 2810408M09 gene	2.503	1.764
thiosulfate sulfurtransferase, mitochondrial	2.5	2.672
RIKEN cDNA A530088I07 gene	2.498	2.113
YME1-like 1 (S. cerevisiae)	2.497	2.995
ubiquitin domain containing 2	2.495	2.894
sideroflexin 4	2.494	2.718
GRAM domain containing 1A	2.493	2.317
RNA binding motif protein 39	2.491	2.052
Erbb2 interacting protein	2.49	2.776
predicted gene, EG434179	2.488	2.918
leucine rich repeat containing 14	2.484	1.551
sialophorin	2.481	1.899

killer cell lectin-like receptor, subfam	2.479	2.882
nuclear receptor subfamily 1, group H, member 3	2.478	2.114
RIKEN cDNA 2310004N24 gene	2.478	2.72
sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (se	2.478	3.237
expressed sequence AI451617	2.477	3.336
proteoglycan 1, secretory granule	2.463	2.266
DnaJ (Hsp40) homolog, subfamily B, member 6	2.463	3.069
proteosome (prosome, macropain) 26S subunit, non-ATPase, 10	2.461	2.322
RIKEN cDNA 1500012F01 gene	2.461	2.263
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	2.46	1.711
interferon induced with helicase C domain 1	2.457	4.226
Suppression of tumorigenicity 7	2.451	2.178
formin-like 2	2.451	3.243
5'-nucleotidase, cytosolic III	2.451	4.208
RIKEN cDNA 1110061O04 gene	2.45	3.086
Kruppel-like factor 7 (ubiquitous)	2.449	2.1
Z-DNA binding protein 1	2.445	3.755
tubulin tyrosine ligase-like family, mem	2.44	2.659
Bcl6 interacting corepressor	2.439	1.553
tyrosyl-tRNA synthetase 2 (mitochondrial)	2.437	2.584
RIKEN cDNA 1110061O04 gene	2.436	3.26
schlafen 1	2.434	3.437
schlafen 4	2.433	4.289
RIKEN cDNA 1700019C18 gene	2.432	2.501
hemopoietic cell kinase	2.431	2.542
interleukin 15 receptor, alpha chain	2.43	5.013
CD86 antigen	2.428	4.337
enhancer of polycomb homolog 1 (Drosophila)	2.426	1.861
zinc finger protein 263	2.424	1.513
interleukin 18	2.421	4.426
histocompatibility 2, class II antigen E beta	2.419	2.415
ATP binding domain 3	2.416	1.863
RIKEN cDNA 2500002B13 gene	2.414	2.337
sprouty homolog 4 (Drosophila)	2.414	4.76
sodium channel, voltage-gated, type VIII	2.412	2.78
guanine nucleotide binding protein (G protein), beta polypeptide 1-like	2.412	2.715
periphilin 1	2.406	3.158
histone cluster 1, H4i	2.399	0.283
interleukin-1 receptor-associated kinase 2	2.398	2.64
MTERF domain containing 1	2.398	2.346
Traf2 binding protein	2.392	3.237
RIKEN cDNA 5031415C07 gene	2.391	3.503
plasminogen activator, urokinase receptor	2.39	2.139
RING CCCH (C3H) domains 1	2.39	2.66
Gardner-Rasheed feline sarcoma viral (Fgr) oncogene homolog	2.388	1.874

RIKEN cDNA 2810474O19 gene	2.388	2.729
myeloid/lymphoid or mixed lineage-leukemia translocation to 6 homolog (Drosophila)	2.386	1.715
non-catalytic region of tyrosine kinase adaptor protein 1	2.386	2.723
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	2.386	3.102
nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta	2.384	1.895
RIKEN cDNA 2610027C15 gene	2.377	2.734
B-cell translocation gene 1, anti-proliferative	2.374	1.762
RIKEN cDNA 4933421O10 gene	2.365	2.544
DNA segment, Chr 12, ERATO Doi 553, expr	2.362	2.356
PHD finger protein 11	2.362	4.349
protein phosphatase 1, regulatory (inhibitor) subunit 12A	2.358	2.645
nuclear receptor subfamily 1, group H, member 3	2.354	1.949
vasodilator-stimulated phosphoprotein	2.353	2.364
GA repeat binding protein, beta 1	2.352	2.488
RIKEN cDNA 5830454D03 gene	2.351	1.863
ATPase, class V, type 10A	2.347	4.118
adhesion regulating molecule 1	2.345	2.298
cholinergic receptor, nicotinic, beta polypeptide 4	2.345	4.592
argininosuccinate synthetase 1	2.343	2.998
interferon activated gene 203	2.343	3.408
Epstein-Barr virus induced gene 3	2.342	2.483
intraflagellar transport 57 homolog (Chlamydomonas)	2.339	2.227
chromogranin B	2.336	3.122
adrenergic receptor, beta 1	2.328	2.493
RAS protein-specific guanine nucleotide-releasing factor 2	2.319	2.849
ubiquitin-conjugating enzyme E2F (putative)	2.317	2.046
ubiquitin C	2.316	2.348
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	2.313	2.926
retinoic acid induced 12	2.312	2.285
RAS p21 protein activator 4	2.309	2.643
MYB binding protein (P160) 1a	2.303	2.169
ribosomal protein S9	2.298	1.925
DNA segment, Chr 12, ERATO Doi 553, expressed	2.298	2.373
EH-domain containing 1	2.297	1.774
AMP deaminase 3	2.294	0.696
RIKEN cDNA 1110001D16 gene	2.29	3.99
Tnf receptor-associated factor 6	2.286	1.633
protein tyrosine phosphatase, non-receptor type 12	2.286	2.414
DNA segment, Chr 15, ERATO Doi 682, expressed	2.286	2.702
C-type lectin domain family 4, member n	2.285	1.736
sodium channel, voltage-gated, type I, beta	2.285	1.813
dedicator of cytokinesis 10	2.285	2.586
salvador homolog 1 (Drosophila)	2.285	3.096
cholesterol 25-hydroxylase	2.284	4.479
RIKEN cDNA 4833438C02 gene	2.28	1.852
RIKEN cDNA 2610101N10 gene	2.279	2.498

insulin-like growth factor binding protein 7	2.278	2.77
neuropilin 2	2.276	2.486
Ras association (RalGDS/AF-6) domain family 4	2.27	3.021
solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	2.266	1.323
plasma membrane proteolipid	2.266	3.03
Tnf receptor-associated factor 6	2.264	2.019
glycerol kinase	2.264	2.563
interleukin 17 receptor A	2.26	1.465
IKAROS family zinc finger 1	2.26	3.373
ethanolamine kinase 1	2.26	3.701
GTPase, IMAP family member 6	2.259	0.967
acyloxyacyl hydrolase	2.259	2.312
solute carrier family 44, member 1	2.246	2.383
cDNA sequence AY078069	2.243	1.188
spermatogenesis and oogenesis specific b	2.243	2.553
acyl-CoA synthetase medium-chain family member 2	2.242	2.138
limb expression 1 homolog (chicken)	2.239	1.913
hematopoietic cell specific Lyn substrate 1	2.237	1.988
calcitonin receptor-like	2.237	2.306
homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.235	1.089
golgi SNAP receptor complex member 2	2.234	2.173
2'-5' oligoadenylate synthetase 1A	2.232	2.941
interferon induced transmembrane protein 1	2.231	2.622
density-regulated protein	2.231	2.376
glycerophosphodiester phosphodiesterase domain containing 1	2.225	1.871
paired immunoglobin-like type 2 receptor alpha	2.224	1.622
keratin 40	2.224	2.572
pleiomorphic adenoma gene-like 2	2.22	2.731
sin3 associated polypeptide	2.22	3.071
peroxisome biogenesis factor 16	2.217	1.486
B-cell translocation gene 1, anti-proliferative	2.216	1.358
DNA segment, Chr 14, ERATO Doi 668, expressed	2.216	3.796
solute carrier family 31, member 1	2.215	2.062
regulator of G-protein signaling 1	2.214	0.432
caspase recruitment domain family, member 12	2.211	1.502
ubiquitin-conjugating enzyme E2F (putative)	2.207	2.316
RIKEN cDNA 6330409N04 gene	2.207	2.641
ubiquitin specific peptidase 25	2.206	2.908
DNA segment, Chr 11, ERATO Doi 759, expressed	2.204	2.948
transmembrane protein 49	2.202	1.836
olfactory receptor 304	2.201	2.235
oxidative-stress responsive 1	2.196	2.693
SET domain containing (lysine methyltransferase) 8	2.194	1.813
polycystic kidney disease 1 homolog	2.193	2.027
phosphatidic acid phosphatase type 2B	2.19	2.637
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	2.189	3.189

vinculin	2.188	2.072
nuclear factor, interleukin 3, regulated	2.183	3.842
WD repeat domain 37	2.181	3.215
DNA segment, Chr 10, Brigham & Women's Genetics 1379 expressed	2.179	2.444
solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	2.178	1.901
TRAF family member-associated Nf-kappa B activator	2.178	2.512
eukaryotic translation initiation factor 2-alpha kinase 2	2.178	3.079
chemokine (C-X-C motif) ligand 7	2.176	2.113
carboxypeptidase D	2.173	2.501
dishevelled associated activator of morphogenesis 1	2.172	2.504
platelet-activating factor receptor	2.17	1.794
REX1, RNA exonuclease 1 homolog (S. cerevisiae)	2.17	1.998
Wdr45 like	2.168	2.101
RIKEN cDNA 5830484A20 gene	2.165	3.482
AF4/FMR2 family, member 1	2.161	3.978
midline 1	2.16	2.156
nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	2.159	2.142
cAMP responsive element binding protein 5	2.158	2.788
hexamthylene bis-acetamide inducible 2	2.156	1.322
B-cell translocation gene 1, anti-proliferative	2.155	1.316
integrin beta 4 binding protein	2.154	2.028
interleukin 23, alpha subunit p19	2.153	0.957
SH2B adaptor protein 2	2.153	1.392
ST3 beta-galactoside alpha-2,3-sialyltransferase 3	2.152	2.155
abhydrolase domain containing 9	2.151	3.164
cDNA sequence BC013712	2.148	4.064
SLIT-ROBO Rho GTPase activating protein 2	2.147	2.15
myeloid differentiation primary response gene 88	2.146	2.895
nuclear import 7 homolog (S. cerevisiae)	2.143	1.535
reticulon 2 (Z-band associated protein)	2.142	1.935
myristoylated alanine rich protein kinase C substrate	2.141	2.086
leukocyte immunoglobulin-like receptor, subfamily B, member 4	2.141	2.202
expressed sequence AA960436	2.14	3.454
interferon-induced protein 44	2.139	3.599
ubiquitin C	2.138	2.221
GTPase, very large interferon inducible 1	2.138	3.178
Josephin domain containing 3	2.135	0.938
regulator of G-protein signaling 8	2.134	2.25
MIT, microtubule interacting and transport, domain containing 1	2.134	3.102
RIKEN cDNA 2810474O19 gene	2.133	2.599
transmembrane protein 176A	2.131	1.923
RIKEN cDNA D230002A01 gene	2.13	1.948
retrotransposon gag domain containing 4	2.125	1.527
G protein-coupled receptor 126	2.124	2.319

niban protein	2.122	2.081
limb expression 1 homolog (chicken)	2.122	2.076
PR domain containing 2, with ZNF domain	2.12	1.239
RIKEN cDNA 2610024E20 gene	2.12	2.025
protein tyrosine phosphatase, receptor type, C	2.12	2.513
eukaryotic translation initiation factor 4, gamma 2	2.118	2.076
Rous sarcoma oncogene	2.118	2.312
cDNA sequence BC023892	2.118	3.208
expressed in non-metastatic cells 6, protein	2.115	1.831
LYR motif containing 1	2.115	2.415
START domain containing 7	2.112	2.218
RIKEN cDNA 2410004B18 gene	2.112	2.185
prostaglandin I receptor (IP)	2.107	1.325
oxoglutarate (alpha-ketoglutarate) recep	2.106	2.752
histocompatibility 2, class II antigen A, beta 1	2.104	2.075
RIKEN cDNA E230013L22 gene	2.103	1.297
S100 calcium binding protein A8 (calgranulin A)	2.103	1.85
ankyrin repeat domain 17	2.102	2.749
interleukin 17 receptor A	2.099	1.392
interferon activated gene 203	2.098	3.018
receptor transporter protein 4	2.095	2.438
Rho GTPase activating protein 22	2.087	-0.942
aminopeptidase puromycin sensitive	2.086	2.218
RIKEN cDNA 6720420G18 gene	2.085	2.224
RIKEN cDNA B430119L13 gene	2.083	2.151
tachykinin receptor 1	2.082	2.088
RIKEN cDNA 2610027H17 gene	2.082	2.467
H3 histone, family 3B	2.082	2.351
haptoglobin	2.081	1.859
phospholipase A2, group IVA (cytosolic, calcium-dependent)	2.081	2.266
centaurin, alpha 1	2.079	1.847
ataxin 7	2.078	1.202
tumor necrosis factor (ligand) superfamily, member 10	2.078	5.039
cDNA sequence BC003324	2.077	1.822
N-myc downstream regulated gene 1	2.076	2.276
RIKEN cDNA D530037H12 gene	2.075	1.531
protein phosphatase 1, regulatory subunit 10	2.075	1.982
PHD finger protein 20-like 1	2.075	2.173
olfactory receptor 426	2.075	2.435
absent in melanoma 2	2.074	3.486
RAB32, member RAS oncogene family	2.072	1.714
RNA binding motif protein 7	2.072	1.923
claudin domain containing 1	2.072	2.452
RIKEN cDNA 2610028A01 gene	2.068	1.575
RIKEN cDNA 1810054D07 gene	2.067	2.661
pericentriolar material 1	2.066	2.105
RIKEN cDNA 1700027J05 gene	2.064	3.806
interferon gamma receptor 1	2.063	0.939

T-cell activation GTPase activating protein 1	2.06	3.528
protein phosphatase 4, regulatory subunit 2	2.059	1.78
eukaryotic translation initiation factor 3, subunit 9 (eta)	2.059	2.244
neutrophil cytosolic factor 4	2.058	1.976
phospholipase C, delta 1	2.058	2.333
Luc7 homolog (S. cerevisiae)-like	2.057	1.747
solute carrier family 44, member 1	2.056	1.946
phospholipase A2, group IVC (cytosolic,	2.056	2.315
ring finger protein 135	2.056	3.195
nucleoporin 54	2.055	1.9
EGF-like module containing, mucin-like, hormone receptor-like sequence 1	2.055	2.2
predicted gene, OTTMUSG00000000712	2.054	2.276
CD302 antigen	2.053	1.707
RIKEN cDNA 2400003C14 gene	2.053	2.355
topoisomerase (DNA) I	2.052	1.621
polypyrimidine tract binding protein 1	2.051	1.806
PRP38 pre-mRNA processing factor 38 (yeast) domain containing A	2.051	3.52
jumonji domain containing 2A	2.05	2.384
solute carrier family 39 (metal ion transporter), member 13	2.049	1.452
spindle assembly 6 homolog (C. elegans)	2.045	2.658
interleukin 1 receptor antagonist	2.044	3.511
Rho family GTPase 3	2.04	3.035
selenophosphate synthetase 2	2.037	1.606
CREB binding protein	2.036	1.883
brix domain containing 1	2.036	1.894
leucine-rich repeats and calponin homology (CH) domain containing 1	2.035	3.298
pellino 1	2.034	3.962
THAP domain containing, apoptosis associated protein 1	2.033	1.888
discs, large homolog 1 (Drosophila)	2.033	2.065
RIKEN cDNA 9130017C17 gene	2.033	2.069
leucine rich repeat containing 38	2.032	1.939
immediate early response 5	2.03	1.134
mitogen-activated protein kinase kinase kinase 6	2.028	0.79
agrin	2.028	1.785
adrenomedullin	2.027	1.051
glycerol kinase	2.025	2.428
RIKEN cDNA 2900057D21 gene	2.024	2.116
RIKEN cDNA 2310005L22 gene	2.023	0.271
ATP-binding cassette, sub-family A (ABC1), member 1	2.023	1.802
ceramide kinase-like	2.023	2.23
immunity-related GTPase family, M	2.023	3.428
RIKEN cDNA 1810026B05 gene	2.02	0.831
GTP cyclohydrolase 1	2.02	2.543
presenilin 1	2.017	2.649
CWF19-like 2, cell cycle control (S. pombe)	2.016	2.624
sterile alpha motif domain containing 9-like	2.015	3.161

BMP and activin membrane-bound inhibitor, pseudogene (Xenopus laevis)	2.015	3.325
dysferlin	2.014	2.222
formin binding protein 4	2.014	2.74
TNFAIP3 interacting protein 3	2.013	2.369
nucleolar protein family A, member 2	2.013	1.974
aryl-hydrocarbon receptor	2.012	1.286
mortality factor 4 like 2	2.012	1.722
interferon (alpha and beta) receptor 2	2.012	1.956
inhibitor of kappaB kinase epsilon	2.011	1.733
malic enzyme, supernatant	2.011	1.759
lung-inducible neuralized-related C3HC4 RING domain protein	2.011	1.702
RIKEN cDNA 1810013L24 gene	2.01	1.646
proteoglycan 1, secretory granule	2.01	1.901
neuronal pentraxin receptor	2.008	2.192
thrombospondin 1	2.007	2.196
ATP-binding cassette, sub-family F (GCN20), member 1	2.004	2.123
schlafen 2	2.003	2.575
RIKEN cDNA 4930529M08 gene	2.003	3.081
RIKEN cDNA 2210037E17 gene	2.001	1.811
UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)	2.001	1.858
colony stimulating factor 3 receptor (granulocyte)	2.001	2.263
spermatogenesis associated 2	2	1.883

Appendix 10. PCR primers used in this study.

Gene	Sense Primer	Antisense Primer
Rps17	CGCCATTATCCCCAGCAAG	TGTCGGGATCCACCTCAATG
IL23a	CTAAAAATAATGTGCCCCGTATCC	GCTCCCCTTTGAAGATGTCAGAG
Gem	ACTGTGAGGTCTTGGGAGAAGA	AGAGTAGACGATCAGATAGGCAT
Csf2	GAAGAGGTAGAAGTCGTCTCTA	TATGTCTGGTAGTAGCTGGC
Ifnb	AAACTCATGAGCAGTCTGCA	AGGAGATCTTCAGTTTCGGAGG
Il1b	GACCTGTTCTTTGAAGTTGACGG	TGTCGTTGCTTGGTTCTCCTTG
Nfkbia	TGACTTTGGGTGCTGATGTC	AAGCTGGTAGGGGGAGTAGC
prespliced *IL23a*	CTAAAAATAATGTGCCCCGTATCC	AGCTAAATGGCCATGGACGCT
prespliced *Gem*	CAGTTTTCTTGTTTTCTCTCACTTG	AGAGTAGACGATCAGATAGGCAT
prespliced *Csf2*	GAAGAGGTAGAAGTCGTCTCTA	ACTGGCAGGGAAGAGTGTA
Dusp1	ACG GGG CTC AGC CTC CC	GTC AAG CAT ATC CTT CCG AGA A
Il1a	ATGACCTGCAACAGGAAGTAAAA	TGTGATGAGTTTTGGTGTTTCTG
c-fos	GAA GGG GCA AAG TAG AGC AG	CAA CGC AGA CTT CTC ATC TTC A
B-actin	TGG CAT TGT TAC CAA CTG GGA CG	GCT TCT CTT TGA TGT CAC GCA CG
18S	CTC CTC TCC TAC TTG GAT AAC	AGC CGG GAG GGA GCT CA
Il12b	ATG TAC CAG ACA GAG TTC CAG GCC A	TAT GAT TCA GAG ACT GCA TCA GCT
Chop	ACG AAG AGG AAG AAT CAA AAA CCT TCA	CTG ACT GGA ATC TGG AGA GCG A
Bip	TCA AGT TCT TGC CAT TCA AGG TGG TT	GTG AGA ACC ATG GCA GAA ATT TCT TCT
Birc2	AGC ACA GAC AGT TCT ATC CCA	ACA GGT TGG AGT GAA TGC CA
Birc3	TGC AGA AGA CGA GAT GAG AGA	AGT GAT GGC CCT TGC ACT TA
Xbp-1	A AAC AGA GTA GCA GCG CAG ACT GC	TC CTT CTG GGT AGA CCT CTG GGA G
Tnf	ATC CAT TCT CTA CCC AGC CCC CAC	CCA GGT CAC TGT CCC AGC ATC TTG